CHEMICAL ANALYSIS AND BIOLOGICAL ACTIVITY OF SCHIZOGYNE SERICEA AND LONICERA CAERULEA

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ABSTRACT

*Schizogyne sericea* is a halophytic species widespread throughout all Canary Islands. It has been traditionally used as an analgesic, astringent, anti-inflammatory and wound-healing agent. It is well-known that the chemical composition of a single species may differ with the harvesting region, possibly influencing the biological properties.

*Lonicera caerulea* is a long-living shrub native to the Northern Hemisphere whose berries are a rich source of vitamin C, anthocyanins and flavanols. Therefore, the fruits are health-promoting phytochemicals with a broad range of potential applications.

In the present work, aerial parts of *S. sericea* harvested from three different regions in Tenerife, were hydrodistilled and extracted by Naviglio® for the first time. The volatile composition of the essential oils obtained was analyzed by means of GC-MS, while specific phenolic compounds occurring in water and ethanolic extracts of both plants were quantified by HPLC-DAD. *In vitro* biological tests were performed to investigate the antioxidant, antidiabetic, antimicrobial, anti-quorum sensing and cytotoxic activity.

GC-MS analysis of *S. sericea* essential oils yielded a similar chemical composition for the samples from the different geographic areas. *p*-Cymene was the main component. High levels of phenolic compounds such as dicaffeoylquinic acids were detected by HPLC in all extracts and also confirmed by the Folin Ciocalteu method.

The obtained results in DPPH, ABTS and FRAP assays showed a good antioxidant power, with TEAC values in the range of 72,5-596 and 121-960 μmol TE/g for *L. caerulea* and *S. sericea*, respectively. No inhibitory effect on DPPIV activity was observed. Both extracts from *S. sericea* and *L. caerulea* did not exert notable antimicrobial effects against all strains tested.

Regarding the anti-quorum sensing assay, a decrease in pyocyanin production was noticed with *S. sericea* ethanol extract from Palm Mar, while not effecting growth of *P. aeruginosa*. However, further experiments are needed to optimize the experimental conditions.

The MTT assay demonstrated that the aforementioned extract induced toxic effects even stronger than the positive control cisplatin, with IC₅₀ values of 0.74, 0.32 and 0.52 μg/mL for the A375, MDA-MB 231 and HCT-116 human tumour cell lines, respectively. According to the NCI plant screening program, our findings confirm the potential of *Schizogyne* extracts as a source of anticancer drugs. Therefore, future experiments are in progress in order to determine their mode of action and effects on non-tumour cell lines.
SAMENVATTING

Schizogyne sericea is een halofyt waarvan het verspreidingsgebied zich uitstrekt over de Canarische Eilanden. Het wordt traditioneel gebruikt als analgetisch, adstringent, anti-inflammatoir en wondhelend agens. Het is bekend dat de chemische compositie van een enkel species kan variëren met de oogstregio, waardoor de biologische eigenschappen kunnen beïnvloed worden.

Lonicera caerulea is een winterharte struik die van nature voorkomt op het noordelijk halfrond. De bessen vormen een rijke bron van vitamine C, anthocyaninen en flavanolen. Hierdoor hebben de vruchten, die een positief effect op de gezondheid uitoefenen, een breed spectrum van mogelijke toepassingen.

In het huidige werk werden bovengrondse plantendelen van S. sericea, geoogst in 3 verschillende regio’s op Tenerife, aan waterdistillatie onderworpen en voor de eerste keer geëxtraheerd met Naviglio®. De vluchtige compositie van de verkregen essentiële oliën werd geanalyseerd met behulp van GC-MS. Specifieke fenol bestanddelen van de water en ethanol extracten van beide planten werden gekwantificeerd met HPLC-DAD. In vitro biologische testen werden uitgevoerd om de antioxidant, antidiabetische, antimicrobiologische, anti-quorum sensing en cytotoxische activiteit te onderzoeken.

GC-MS analyse van S. sericea leverde een gelijkaardige chemische samenstelling van de stalen van de verschillende geografische gebieden op. p-Cymene was het hoofdbestanddeel. Grote hoeveelheden fenol bestanddelen zoals dicaffeoylkinine zuren werden m.b.v. HPLC gedetecteerd in alle extracten en bevestigd met de Folin Ciocalteu methode.

De resultaten van de DPPH, ABTS en FRAP testen wezen op goede antioxidant eigenschappen, met TEAC waarden in de range van 72,5-596 en 121-960 µmol TE/g voor L. caerulea and S. sericea, respectievelijk. Er werd geen inhiberend effect op de DPPIV activiteit waargenomen. Beide extracten van S. sericea en L. caerulea vertoonden geen noemenswaardig antimicrobiologisch effect tegen de geteste stammen. Wat betreft de anti-quorum sensing test, werd er een afname in pyocyanine productie opgemerkt bij S. sericea ethanol extract van Palm Mar zonder daarbij de groei van P. aeruginosa te beïnvloeden. Echter, verdere experimenten zijn noodzakelijk om de experimentele condities te optimaliseren.
De MTT test demonstreerde dat *S. sericea* ethanol extract van Palm Mar toxische effecten induceerde die zelfs sterker bleken dan die van de positieve controle cisplatine, met IC\textsubscript{50} waarden van 0,74, 0,32 en 0,52 μg/mL voor de A375, MDA-MB 231 en HCT-116 humane tumor celllijnen, respectievelijk. Volgens het NCI screeningsprogramma voor plant extracten bevestigen onze bevindingen het potentieel van *Schizogyne* extracten als een bron voor antikanker geneesmiddelen. Hieruit blijkt de noodzaak voor de nieuwe lopende onderzoeken naar het werkingsmechanisme en het effect op niet-tumorigene celllijnen.
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ABBREVIATIONS

3,5-di-CQA: 3,5-di-O-caffeoylquinic acid
3-CQA: 3-O-caffeoylquinic acid
5-CQA: 5-O-caffeoylquinic acid
Abs: Absorbance
ABTS: 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AI: Van den Dool Index
Calc.: Calculated
CLSI: Clinical and Laboratory Standards Institute
DMAPP: Dimethylallylpyrophosphate
DMEM: Dulbecco’s Modified Eagle’s Medium
DMSO: Dimethylsulfoxide
DPPH: 2,2-diphenyl-1-picrylhydrazyl
DPPIV: Dipeptidyl Peptidase IV
EDTA: Ethylenediaminetetraacetic acid
EO(s): Essential Oil(s)
ESI: Electrospray Ionization
eV: electron volt
FRAP: Ferric Reducing Antioxidant Power
GAE: Gallic Acid Equivalent
GLP: Glucagon-Like Peptide
Gly-Pro-pNA: Glycyl-prolyl-para-nitroanilide
GPP: Geranyl pyrophosphate
HI-FBS: Heat-Inactivated Fetal Bovine Serum
HPLC-DAD: High-Performance Liquid Chromatography-Diode Array Detector
IC_{50}: 50% Inhibition Concentration
ID: Identification
IOFI: International Organization Of The Flavour Industry
IPP: Isopentenyl pyrophosphate
IZD: Inhibition Zone Diameter
LB: Luria Bertani
*L. caerulea: Lonicera caerulea*
LD$_{50}$: Lethal Dose, 50%
Lit.: Literature
LOD: Limit Of Detection
$m/z$: mass-to-charge (ratio)
GC-MS: Gas Chromatography-Mass Spectrometry
MTT: Thiazolyl Blue Tetrazolium Bromide
NCI: National Cancer Institute
NMR: Nuclear Magnetic Resonance Spectroscopy
OD$_{600}$: Optical Density at 600 nm
Others: Aldehydes, ketones and alcohols
PBS: Phosphate-Buffered Saline
RPMI: Roswell Park Memorial Institute
*S. sericea: Schizogyne sericea*
Std.: Standard
TE: Trolox Equivalent
TEAC: Trolox Equivalent Antioxidant Capacity
TPTZ: 2,4,6-tripyridyl-s-triazine
TROLOX: 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
UV-Vis: Ultraviolet-Visible light
VOC(s): Volatile Organic Compound(s)
1 INTRODUCTION

1.1 ESSENTIAL OILS

Essential oils (EOs) are secondary plant metabolites with a wide range of biological properties and applications. They consist of mixtures of volatile organic compounds (VOCs) of low molecular weight, including esters, alcohols, aldehydes, hydrocarbons, ketones, monoterpenes, sesquiterpenes, phenols, acids, etc. (1, 2) EOs are liquid when stored at room temperature and have boiling points between 150-300 °C. Usually EO constituents are fairly hydrophobic, therefore they are highly soluble in organic solvents and only slightly soluble in water. They have to be protected against light, oxygen and moisture since they oxidize and resinify quickly. EOs are characterized by their specific optical rotation by which they can be distinguished. They possess high refractive indices and characteristic odours. (3, 4)

1.1.1 History and use of essential oils

The word ‘essential’ finds its origin in ‘quintessence’, meaning the component of a substance in its most concentrated and purest form. In the 15th century Paracelsus started to use ‘essences’ for medical purposes in Europe and during the next century EOs were sold in apothecaries for the treatment of many kinds of ailments. EOs have found their purpose in folk medicine for ages, since they display a wide range of biological properties. Nowadays studies have proven these properties, including anticancer, antioxidant, antimicrobial and antiphlogistic activities. Skin penetration enhancement for transdermal drug delivery is demonstrated, next to antinociceptive, sedative and psychoactive effects. (1, 5)

Throughout the last century, there has been a reduction in the use of plant-derived products in the medicinal industry due to the introduction of synthetic drugs. However, side-effects of the latter and increasing interest in high quality natural products have recently led to a boost of the consumption of medicinal and aromatic plants. (6) The use of EOs has been of great importance in the food industry on the one hand due to antioxidant and antimicrobial properties, on the other hand because they are natural, biodegradable and of low toxicity towards mammals. (7) The antioxidant capacity preserves food from the toxic effects of oxidants. Furthermore, these natural plant products are employed in perfume, cosmetic, and beverage industries. (1, 8)
More than 3000 different kinds of EOs have already been identified out of more than 2000 plant species. Around 300 of them are of commercial importance. About 40000-60000 tons are produced each year, worth 700 million USD. (9) According to Corley, this value increases with 8% each year. EOs are mainly produced in developing countries, amounting to 55% of the world production. (7) Every plant has the ability to produce traces of essential oils, serving as internal messengers, to attract insects for pollinisation or as a natural defence against herbivores. These functions are still hypotheses. EOs are also thought to be responsible for allelopathic communication between plants. (8)

The first article about EO constituents was published in 1833 by the French chemist M. J. Dumas. However, the most important discovery is attributed to O. Wallah, as he envisaged that several chemical structures from botanical terpenes were actually identical, but only differed in nomenclature. He performed isolation of essential oil constituents by employing fractional distillation and studied their basic properties. He characterized terpenes, related to the already-known camphor. Wallah received the Nobel Prize for Chemistry for his research. Major improvements to structure elucidation have been made by using dehydrogenation of sesqui- and diterpenes with sulfur and later selenium, next to the introduction of the isoprene (L. Ruzicka, 1953) and Woodward rules in the field of terpene chemistry. Nevertheless, the most important improvement of structure elucidation to be mentioned is the application of chromatography, nuclear magnetic resonance (NMR) and mass spectrometry during the last half century. (1)

1.1.2 Chemical nature
EO components are synthesized mainly by three different pathways: most of mono- and sesquiterpenes are derived from the mevalonate pathway, some mono- and diterpenes from the methyl-erythritol pathway and phenylpropenes from the shikimic acid pathway. Sesquiterpenes consist of three isoprene units (15 carbon atoms). Isoprene units do not act as intermediates in the biosynthesis but they can be recognized easily in the molecular structure of EOs. Head-to-tail-coupling of isopentenyl pyrophosphate (IPP) and dimethylallylpyrophosphate (DMAPP) leads to geranyl pyrophosphate (GPP), the precursor of monoterpenoids (Figure 1.2).
Further addition of isopentenyl pyrophosphate gives sesquiterpenoids. Higher terpenoids also do exist but they are not volatile and consequently not present in EOs, besides their degradation products. Hemiterpenoids are minor constituents of EOs, these include alcohols, esters, and aldehydes with an isoprene skeleton. Monoterpenoid hydrocarbons are formed as follows: addition of water to the geranyl carbocation [1] gives geraniol [2]. Oxidation of the latter leads to citral [3]. Subtraction of a proton from the geranyl carbocation gives myrcene [4], a compound frequently present in EOs. Intramolecular electrophilic addition of the geranyl carbocation leads to the formation of a monocyclic carbocation [5], which can be turned into limonene [6] by loss of a proton or to α-terpineol [7] by adding water. (+)-Limonene finds its use in the food and fragrance industry and studies have demonstrated its chemo-preventive activities. The pinyl carbocation [8] can be formed by another intramolecular addition. Subsequently this compound can lose a proton to give α-pinene [9] and its isomer β-pinene [10]. Limonene and β-pinene can be used as industrial solvents. The pinyl carbocation gives also rise to the bornyl carbocation [11] and to the fenchyl carbocation [14] by the Meerheim-Wagner rearrangement. When water is added to former, the EO component borneol [12] is formed, which can lead to camphor [13] by means of oxidation. Finally, fenchone [15] is derived from the fenchyl carbocation.

Terpenoids, shikimates and polyketides are the most important secondary metabolites concerning EOs. Either the epidermic layer of the petals can produce and immediately release essential oil, either the volatiles are synthesized and stored in secretory idioblasts, cavities or glandular trichomes. The composition of the mixture of VOCs in different parts of the same plant can differ. In addition, the chemical profile of the EO depends not only on the enzymes present in the plant, but also on the extraction method used. The yield varies along with the climate, soil type, water stress, drought, plant age, vegetative cycle stage etc., ranging from 0,5-6% by weight. Clove buds can even contain an amount of 11-15%. Generally speaking aromatic plants growing in Mediterranean and tropical regions give the highest yield of EOs. (1, 10, 11)

The essential oil composition as reported may not be the ‘true’ composition, because of the formation of artifacts during the hydrodistillation process, gas chromatography analysis, or even during the preparation of the plant material. In particular, grinding leads to the tissue disruption, whereby enzymatic reactions may be stimulated producing volatile artifacts. (1)
Figure 1.2: Synthesis of monoterpenoids
1.1.3 Hydrodistillation

Essential oils (EOs) can be obtained from the plant material only by physical or mechanical processes such as distillation or expression (Citrus fruits). Maceration, solvent extraction or super/subcritical fluid extraction are methods to obtain the volatile fraction, which is not equivalent to EOs. Steam distillation is the most popular method used to produce EOs on a large scale. (1, 5, 11)

Hydrodistillation is the oldest and simplest method to obtain essential oils. (4) The origin of the art of distillation is still a point of argue. Some claim that the procedure was invented in China, while others believe India or Persia to be the cradle of distillation. The first distillation apparatus dates from 3600 B.C. and was found in north-eastern Mesopotamia. EOs distilled from various plants were used in Ancient Egypt to embalm the dead. The term ‘essential oil’ has to be treated with caution in this context, it’s more accurate to speak about ‘fragrant alcohols’ or ‘aromatic waters’. The distillation technique was improved by the Arabs in the 9th century A.D. (12)

Briefly, the chopped plant material is put into a round bottom flask together with a certain amount of water to completely immerse the plant mass. By applying high temperature, components are liberated while the plant structure breaks down. The closed-circuit system operates under atmospheric pressure. (11) The molecular weight of the components needs to be lower than 300 Daltons to make distillation possible. Since a higher temperature increases molecular movements in the apparatus molecules from the sample diffuse into the air above. EO components evaporate subsequently, according to their degree of solubility in water. The produced steam acts as a carrier for the volatile oils to the vertical condenser. Since a negative azeotropic mixture is formed, the boiling point of the mixture will be lower than that of the individual constituents themselves. The distillate consists of two non-miscible layers. The essential oil layer can be separated from the water underneath by using the stopcock. Thereafter the collected oil is frequently redistilled for further purification. (13)

![Hydrodistillation Clevenger apparatus](image-url)
1.2 OTHER SECONDARY METABOLITES: PHENOLIC COMPOUNDS

Polyphenols are secondary plant metabolites with several hydroxyl groups attached to aromatic rings. Phenolic compounds are frequently present as glycosides. They are involved in the growth and reproduction of plants. In addition, production is stimulated as a response to aggression by pathogens. With more than 8000 different structures identified, phenols represent a widely distributed group of natural substances. Harborne has divided plant phenolics into 10 major classes according to their chemical structure. The most important classes are the phenolic acids and the flavonoids. Ferulic acid and caffeic acid (together with its esters) are the major phenolic acids found in plant foods. Together with cinnamic, sinapic, salicylic and vanillic acid they exhibit a strong antioxidant activity. Flavonoids all have a tricyclic skeleton, which can be substituted with several functional groups, e.g. with hydroxyl groups (flavanols). (+)-Catechin hydrate and (-)-epicatechin are examples of flavan-3-ols belonging to the former group. Another important class are the hydroxycinnamic acids, which for example include p-coumaric acid, 3-CQA and 5-CQA. (14)

Phenolic compounds have a beneficial effect on health due to many reasons: firstly polyphenols are able to inhibit enzymes such as xanthine oxidase and aldose reductase, targets in the treatment of brain oedema, hepatitis and cataract, respectively. (15) Secondly, phenolic compounds are proven to have cancer-preventive and neuroprotective activity. (16). Finally, plant polyphenols are able to protect digestive enzymes and intestinal epithelial cells from oxidation by free radicals formed in the stomach. This can be explained by their action as reducing agents, hydrogen donors and single oxygen quenchers. The gained electron can be delocalized over the aromatic ring and due to the resonance effect the chain reaction is interrupted. (14, 17)

1.3 PROPERTIES OF ESSENTIAL OILS AND PHENOLIC COMPOUNDS

**1.3.1. Antimicrobial activity**

EOs are well-known for their broad spectrum antimicrobial activity against Gram-positive and Gram-negative bacteria. Studies have demonstrated the effectiveness of EOs against foodborne pathogens e.g. *Listeria monocytogenes*, *E. coli O157:H7* and *Salmonella spp*. (8) Moreover, some oils are effective in eradicating biofilms resistant to standard antimicrobial treatment. Since antibiotic resistance and toxicity of long-term use of antibacterial drugs is a
growing problem, alternative approaches are necessary. Phenols show the highest antimicrobial activity of all EO constituents, followed by aldehydes, ketones, alcohols, ethers and hydrocarbons in descending order. A first mechanism that lipophilic EOs exhibit is membrane destabilization. They can pass easily through the cell wall and cell membrane, where they interact with polysaccharides, fatty acids and phospholipids making the membrane more permeable. The bacterium dies because of the loss of ions and content of the cell. A second mechanism through which antibacterial activity is exhibited is via depletion of the proton motive force. Furthermore bacteria lose their viability via binding of EO components to enzymes through which they lose their functionality. (9)

1.3.2 Anti-quorum sensing activity

When bacteria reach a certain number, they start sensing chemical signal molecules produced by the bacteria themselves (autoinducers). This cell-to-cell communication mechanism is referred to as ‘quorum sensing’ and used by bacteria to regulate their group behavior. (18) N-acylhomoserine lactones are known autoinducers produced by Gram-negative bacteria. Binding on their receptor leads to transcription regulation of genes. They start expressing tissue-damaging virulence factors like proteases, exopolysaccharides and redox-active compounds. When N-butanoyl-L-homoserine lactone binds to the RhlR transcription factor, it results in an increased production of the pigment pyocyanin. This is a quorum-sensing regulated virulence factor which can be detected easily. (19)

_Pseudomonas aeruginosa_ is an opportunistic pathogen responsible for a number of acute and chronic infections in immunocompromised patients. Autoinducers are found in biologically significant concentrations in the sputum of patients who suffer from the fatal pulmonary infection of cystic fibrosis. Various extracts from higher plant species were shown to exhibit anti-quorum sensing activities. According to previous studies, it is believed that flavonoids (such as catechin) are responsible for the anti-quorum sensing effect. They are thought to act synergistically. The likely mechanism through which pyocyanin production is inhibited by catechin is interference of the latter with the interactions of _N_-butanoyl-L-homoserine lactone and the RhlR. Inhibition of quorum sensing activity, leading to reduced virulence of pathogenic bacteria, is a possible strategy in development of new antimicrobial drugs. (19, 20)
1.3.3 Cytotoxic activity
Cancer or malignant neoplasm is the uncontrolled proliferation of human cells, which leads to invasion and metastasis. Studies have demonstrated that EOs are effective in the treatment of various types of malignant cell growth, such as gastric cancer, glioma, colon cancer, liver and pulmonary tumours, breast cancer and leukaemia. The reduction of metabolic events typical for malignant tumour growth such as permanent oxidative stress and increased cellular metabolism is due to the interference of EOs with mitochondria. Some terpenoid and polyphenolic constituents induce apoptosis and are responsible for necrosis of cancer cell lines. (9) In particular, monoterpenes are effective in chemoprevention (initiation phase) as well as in chemotherapy (promotion phase). The first mechanism is based on the prevention of chemical carcinogens interacting with DNA by detoxification of the carcinogen. The latter is due to induction of phase I and phase II enzymes. The second working mechanism is effective on the inhibition of tumour cell growth, increases the rate of tumour cell death and/or induces re-differentiation of the tumour. (21) Among polyphenols, phenolic acids, hydrolysable tannins and flavonoids have shown to possess various pharmacological effects which can be used in anti-cancer-strategies. (14)

1.3.4 Antioxidant activity
Essential oils are also known on account of their antioxidant activity. Beneficial effects of anti-oxidants include reduction of the risk for cardiovascular diseases and cancer by protecting the human cells against oxidant damage. Free radicals together with reactive oxygen species are by-products of the normal metabolism and cause this oxidative damage. (22) Next to the natural ageing process, a lot of diseases are related to this phenomenon, including diabetes, Alzheimer, Parkinson, asthma and arteriosclerosis.

Three important groups of compounds contribute to the protective antioxidant effect: the lipophilic carotenoids and the hydrophilic phenolics and vitamins. Carvacrol, thymol and eugenol are a few examples of phenolic compounds that contribute to the antioxidant effect. Flavonoids exert the strongest antioxidant activity, which increases with the number of hydroxylgroups present. The antioxidant power is absent in glycosides. Also thiols, some minerals as well as enzymes can function as anti-oxidants by neutralizing free radicals, preventing lipid peroxidation and in some cases chelating metal ions. (8, 14)
1.4 BIOLOGICAL TESTS

1.4.1 Antioxidant experiments

Various *in vitro* tests can be used to assess the antioxidant potential of plant extracts. They are based on different features of the antioxidant activity, for instance the ability to reduce ferric or to scavenge free radicals. The use of one single method to evaluate the antioxidant potential of plant extracts is not recommended because of their complex composition. Better is to combine two or more assays to obtain relevant data. (23)

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) and the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays are both indirect methods since the scavenging radicals used are not part of the real oxidative degradation. In principle, a single electron transfer reaction from the sample to the radical or metal (FRAP assay) occurs. Representing the radical by \( X^\cdot \), the metal by M(III) and the antioxidant by AH, the reactions occurring are the following:

\[
X^\cdot + AH \rightarrow X^- + AH^{\\cdot +}
\]  \[1.1\]

\[
M(III) + AH \rightarrow AH^\cdot + M(II)
\]  \[1.2\]

Where M(II) and \( X^- \) are the reduced forms of the metal and radical, respectively. The new radical formed will undergo further reactions according to the reaction stoichiometry.

According to the redox status, the colour of the free radicals changes. Hence, the only equipment needed to determine the antioxidant capacity is a UV-Vis spectrophotometer. (8, 17) The results are expressed as IC\(_{50}\) values. The half maximal inhibitory concentration corresponds with the substrate concentration giving 50 % inhibition of the absorbance. All of the assays mentioned above conform to Beer’s law over a defined concentration region:

\[
A = \varepsilon c L
\]  \[1.3\]

where \( A \) is the absorbance, \( \varepsilon \) the extinction coefficient (M\(^{-1}\) cm\(^{-1}\)), \( c \) the concentration (mol L\(^{-1}\)) and \( L \) the light path length (cm). (24) The Trolox equivalent antioxidant capacity (TEAC) expresses the antioxidant capacity of a sample, as compared to the standard 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox). Since plant extracts do not have a defined molecular weight, the TEAC values may be presented in equivalences of Trolox (TE). Trolox is a water-soluble derivative of the natural antioxidant vitamin E, therefore it serves as a positive control (Figure 1.4). (25)
1.4.2 Dipeptidyl peptidase IV inhibition

Dipeptidyl peptidase IV (DPPIV, DPP4) is a serine endonuclease that cleaves N-terminal dipeptides. One of the substrates is the incretin glucagon-like peptide-1 (GLP-1), a gastrointestinal hormone responsible for the stimulation of insulin release upon food ingestion. Since DPPIV is produced at the site of incretin secretion, GLP-1 is rapidly degraded giving GLP-1-(9-36)amide, which has a very short half life of 1-2 min. Degradation occurs by cleavage of 2 N-terminal amino acids, making the interaction with the receptor impossible. Inhibition of this process prolongs the half life with a few minutes, until they are degraded by other proteases.

As a result, inhibitors of DPPIV are potent antihyperglycemic agents. They represent a validated treatment for type 2 diabetes as they improve the control of blood glucose levels. Therapeutically used inhibitors currently on the market are sitagliptin, vildagliptin, saxagliptin, alogliptin and linagliptin. Next to its role in glucose homeostasis, DPPIV is also an interesting target in the treatment of high blood pressure. Moreover, studies with mice suggest that chronic inhibition of DPPIV has a beneficial effect in prevention of obesity. (26) DPPIV inhibition is also applied to treat fibrotic skin diseases, immune disorders, ischemia-reperfusion injuries, neurodegenerative diseases, cancer and heart failure. It even may find use in stem cell transplantation. (27, 28)

To measure DPPIV activity of samples, different approaches are possible. The DPPIV-Glo Protease luminescent kit is the most sensitive method, followed by fluorometric assays exhibiting a tenfold higher sensitivity than colourimetric tests. However, the latter are most widely used since nor calibration nor a fluorimeter is needed. (27)
1.5 EXTRACTION METHODS

1.5.1 Naviglio® Extractor

This solid-liquid extraction technique was invented by Daniele Naviglio in 2000 in Napoli. Naviglio’s principle is based on pressure and depressure. First a pressure of 5-7 bar is generated into the solid matrix during the static phase. Subsequently the active principles or essential oils are extracted mechanically during the dynamic phase, where the equilibrium is restored rapidly by the movement of the piston. A negative pressure gradient formed from the inside towards the outside of the solid matrix forces the chemically unbound substances to leave the matrix. No heating is required, which allows extraction of temperature sensitive compounds from plant material. The liquid phase used can be water or ethanol, depending on the solubility of the compounds to be extracted. Pressure on the surface of the extraction solvent containing the solid plant material is applied by the movement of two cylinders and pistons. At the bottom of each extraction chamber there is a porous set that blocks the solid matrix while passing the extraction liquid containing the soluble compounds. The extraction cycle is repeated 10-30 times to achieve an exhaustive extraction of the sample. Advantages of the Naviglio® extractor include low costs, rapid extraction, no production of by-products and the possibility to use aqueous solvents. (29-32)

![Diagram of Naviglio Extractor](image)

Figure 1.5: Static phase (left); dynamic phase (right). The circles represent the solid matrix, the other geometric figures represent the active substances extracted. The arrows indicate the pressure in bar. °

1.5.2 Solvent extraction

This simple extraction technique is used for fragile plant material that is sensitive to heat. The crude biomass is soaked in solvent (e.g. methanol, ethanol, water...) into a closed vessel.
The solvent penetrates through the cell wall and soluble components from the plant material diffuse to the solvent down the concentration gradient until equilibrium is reached. As a result, extraction is not complete and the procedure needs to be repeated several times in order to obtain maximal extraction. The plant material to solvent ratio is important considering that the amount of extracted matter increases with a bigger volume of extraction solvent. Extraction is facilitated by frequent agitation (magnetic stirrer) and is performed at room temperature. The solvent is collected and filtration/centrifugation is carried out. (4)

1.6 SCHIZOGYNE SERICEA (L. fil.) DC.

1.6.1 Plant description

*Schizogyne* is a Macaronesian endemic genus that belongs to the Asteraceae family. A sister relationship between *Limbarda chrithmoides* and *Schizogyne* has been shown. Both genera comprise woody species. (33) The yellow-flowered *S. sericea* is a halophytic species widely distributed throughout all the Canary Islands. (34) This mountainous vegetation can grow up to 1 m high on coastal rocks. The leaves are 3-5 cm long and their surface is clothed in silvery hairs. (35)

1.6.2 Phytochemical aspects

The phytochemistry is characterized by thymol derivates. Acetyl-fasniol and acetyl-schizogynol have been isolated from the aerial parts of *S. sericea*, next to vainillin, 4-formyl-benamide, 10-acetoxy-8,9-epoxy-2-methoxy-thymol isobutyrate and 8-hydroxy-9,10-bis-isobutyryloxy-thymol. Furthermore 2-methyl-2-[3-[(acetylxy)methyl]-2-oxiranyl]-5-methylphenyl isobutyrate has been found, a compound also present in *Arnica sachalinensis* (Asteraceae). Three dicaffeoylquinic acid isomers have been identified by NMR/ESI-MS; these include 1,3-di-O-caffeoylquinic acid, 3,4-di-O-caffeoylquinic acid and 3,5-di-O-caffeoylquinic acid. (36, 37)

1.6.3 Traditional uses and pharmacological properties

*S. sericea* has analgesic, astringent, as well as healing properties. A balm for external use of *S. sericea* is used in folk medicine for application on wounds. (35) It also serves as animal feed for goats. (38) 10-Acetoxy-8-hydroxy-9-isobutyryloxy-6-methoxythymol isolated from *S. sericea* exhibits anti-inflammatory activity on carrageenin-induced oedema in rats.
Studies demonstrated that this component also induces a mild reduction of spontaneous motility in mice. No antimicrobial properties can be ascribed to this component and it is not highly toxic (LD$_{50}$> 800 mg/kg). (39) Bis[helenalinyl]adipate isolated from the leaves displays anti-inflammatory properties in rats at 50 mg/kg intraperitoneal dose. (40)

1.7 LONICERA CAERULEA L.

1.7.1 Plant description

Lonicera caerulea also called blue honeysuckle is a traditional crop belonging to the Caprifoliaceae family. This long-lived shrub is one of the 180 species of the genus Lonicera and native to the Northern Hemisphere. The deciduous plants are up to 2 m in height and can grow up to 25-30 years old. Older branches have a yellowish-brown to reddish flaking bark. Bracteoles are fused into a tubular cupule enclosing the ovaries, which form a succulent fruit.

Its edible fruits are commercially produced in Russia and Japan but were little known in North America and Europe until the beginning of this century. They show an outstanding frost resistance, since they are able to survive temperatures of ~46 °C without being damaged. Cold acclimation is associated with the presence of specific kinds of proteins and carbohydrates. Blue honeysuckle fruits are dark blue, oval few-seeded berries with a bitter to sweet taste. When cultivated, they can measure 2 cm in length and weigh more than 1,5 g. They become ripe in May-June and the yield varies between 2 and 3 kg/plant. (41, 42)

1.7.2 Phytochemical aspects

The berries contain high levels of vitamin C, anthocyanins and flavanols. The major constituents are saccharides, lipids, proteins, organic acids and polyphenols. Vitamin B, magnesium, phosphorus, calcium and potassium form the minor compounds. (42)

1.7.3 Traditional uses and pharmacological properties

L. caerulea is used in folk medicine since ancient times because of its health benefits. Therefore some cultivars are selected on the base of research for commercial production. Studies have supported the use of honeysuckle berries in the treatment of atherosclerosis, hypertension, gastrointestinal disorders, diabetes, cardiovascular diseases and bacterial infections. (43, 44) Vostálová et al. have shown a preventive effect of dietary L. caerulea against UV-caused skin damage. (45)
2 OBJECTIVES

The chemical composition of the same plant species depends on the geographic region where the plant is harvested. These composition changes can influence biological properties such as antimicrobial and antioxidant power. Biological assays were already performed on the essential oil of S. sericea, the present work focuses on the aqueous and ethanol extracts of the aerial plant parts collected at three different locations in Tenerife. An innovative solid-liquid extraction technique, called Naviglio extraction, will be used to obtain these extracts.

The berries of L. caerulea are a well-known source of phenolic compounds, to which diverse health-promoting properties can be ascribed. In this work, bioassays on the extracts will be performed to find new commercial applications of the fruits. Particular phenolic constituents will be determined in the extracts of both S. sericea and L. caerulea.

The aim of this thesis is threefold: first to compare the essential oil composition of S. sericea harvested at Palm Mar, Fasnia and La Barranquera. Second to investigate the presence of certain phenolic compounds in S. sericea and L. caerulea extracts. Third, to perform biological assays to assess antioxidant, antidiabetic, antimicrobial, anti-quorum sensing and cytotoxic activity.

The volatile compounds present in the essential oil isolated by hydrodistillation will be identified by GC-MS. Aqueous and ethanol extracts will be obtained by using the Naviglio® extractor as well as simple solvent extraction and individual phenolic compounds will be characterized by HPLC–DAD analysis. The antioxidant activity will be determined by using DPPH, ABTS and FRAP assays and total phenolic content will be estimated with the Folin Ciocalteu method. Antidiabetic potential will be assayed with a colourimetric assay using Gly-Pro-pNA as a chromogenic substrate for the DPPIV enzyme. The antibacterial activity will be tested by the disk diffusion test against two Gram-positive bacteria, Staphylococcus aureus and Enterococcus faecalis as well as against two Gram-negative bacteria, E. coli and Pseudomonas aeruginosa. Antifungal properties will be tested against Candida albicans. Pyocyanin production will be measured to evaluate anti-quorum sensing activities. Finally, cytotoxic activity on three different human tumour cell lines (A375, MDA-MB 231 and HCT-116) will be evaluated by the MTT assay.
3 MATERIALS AND METHODS

3.1 PLANT MATERIAL

3.1.1 Schizogynse sericea

Plant material from three different collection sites of Tenerife was used, all collected on 25th of August 2014 during blooming. These locations include Palm Mar (Arona) (Figure 3.2 A, 28° 1'24.00"N, 16°42'8.00"W), Fasnia (B, 28°14'11.43"N, 16°26'24.79"W) and La Barranquera (Valle de Guerra) (C, 28°32'12.72"N, 16°23'46.34"W). The plant material was confirmed by Prof. dr. Maggi using available literature. (41) The aerial parts were oven-dried, cut into small pieces and stored in a dark box at room temperature. A strong aromatic odour was noticed.

3.1.2 Lonicera caerulea

The fruits were collected in the taiga near Khabarovsk (Russia, about 80 m above sea level) close to China borders in June 2014. Their identification was confirmed by Prof. dr. Maggi using available literature. (41) Berries were conserved in a falcon tube at -20 °C.
3.2 METHODS

3.2.1 Hydrodistillation
120 g of the dried plant material was weighed and put into a round bottom flask of 6 L in volume. 3,5 L of distilled water was added and the mixture was heated after shaking the flask shortly to immerse the plant mass. Distillation was achieved by a Clevenger-type apparatus, where the ascending part was covered with cotton to prevent loss of heat. This to avoid early condensation. Distillation time was prolonged to 3 h. After cooling down for 30 min, the light-yellow oil was collected into a 4 mL vial and stored in the refrigerator at -20 °C to prevent degradation. The oil yield was expressed in % (g/100 g dried plant material) and calculated with the following formula (46):

\[
\text{oil yield} = \frac{\text{amount of essential oil recovered (g)}}{\text{amount of plant material distilled (g)}} \times 100
\]  

[3.1]

3.2.2 Naviglio® extraction
Two different solvents were used for the plant extractions because of their ability to extract various compounds: water will extract only the polar constituents, while ethanol also will extract slightly non-polar constituents. 50 g of dried plant material was crushed into a mortar and put into a socket in the cylinder of the Naviglio Extractor®, Mod. 1000 cc. (Atlas Filtri Engineering, Limena, Italy). Next, 500 mL of distilled water was added. During 2,5 h, 12 extraction cycles were performed. The static phase lasted for 10 min, followed by 3 min dynamic phase. The same method was applied for the ethanol extract, using 500 mL of pure ethanol (Sigma Aldrich, Milano, Italy) instead of 500 mL water. The extracts were evaporated to dryness using the rotavapor (Buchı rotavapor R-200) at 40 °C. Next, lyophilisation was performed under vacuum (Edwards Pirani 1001, West Sussex, England).

3.2.3 Solvent extraction
5 g of *L. caerulea* berries was weighed and put into 50 mL of distilled water. After 24 h extraction, the extract was centrifuged (Sorvall® RC-5B Refrigerated Superspeed Centrifuge) for 15 min at 9000 rpm (9681 g force) at 8°C. For the ethanol extract the same method was used, except for the solvent which was 50 mL of pure ethanol. The supernatant of the ethanol extract was dried with the rotavapor at 40°C. The supernatant of the water extract was freeze dried under vacuum.
3.2.4 Chromatography

Chromatography is a laboratory technique used to separate components within liquid or gaseous mixtures. Components are distributed between a mobile phase and a stationary phase, both contained in the column. A small aliquot of the sample is injected at the top of this column. The mobile phase carries the sample through the stationary phase. Depending on their partition coefficient the analytes will elute at different points in time, called retention times. The partition coefficient $K$ is the ratio of the concentration of each component in both phases:

$$K = \frac{C_s}{C_m}$$  \hspace{1cm} [3.2]

The mobile phase flowing through the column, can either be liquid (high performance liquid chromatography) or gaseous (gas chromatography). If the stationary phase is polar, it is called normal phase chromatography, while in reversed chromatography a non-polar or weakly polar mobile phase is used to elute the molecules from the column. (47)

3.2.4.1 Mass detector

The mass spectrometer is a device developed to separate ions according to their mass-to-charge ratio ($m/z$). Both qualitative (structure) and quantitative (molecular weight) information are obtained. First the analytes in the gas phase need to be ionized, in this case this occurs by electron impact ionization, an appropriate technique for volatile molecules. When analytes enter into the ion source, they are bombarded with electrons whereby other electrons from the molecules are removed. The free electrons emitted are generated by heating of a filament and accelerated to 70 eV. Positively charged ions are produced, which can remain intact (molecular ion) or break further into smaller pieces (fragments). The generated ions travel through an analyzer (quadrupole) under high vacuum where the ions are separated by means of electrical and magnetic fields. When the detector is reached, a signal is produced which is multiplied by an electron multiplier. A mass spectrum is obtained by converting the signals graphically by computer software. It displays the $m/z$ ratios against the relative abundance of the ions. The ion that gives the highest signal is called the base peak and is taken as 100 % relative abundance. All the other signals are expressed as a percentage of this base peak. (48, 49)
3.2.4.2 Photodiode array detector

The photodiode array detector (DAD) is a UV-detector that operates under Lambert-beer law, which means that the analyte gives a response proportional to its concentration. Polychromatic light in the UV range is emitted and crosses the measuring cell comprised in the capillary. Analytes passing by absorb UV radiation, which causes a reduced light intensity. The polychromatic beam is separated by a diffraction grating into different wavelengths, which are detected by an array of photodiodes. Consequently all wavelengths are measured, giving a spectrum as well as providing spectral information. (47)

3.2.5 GC-MS

Capillary gas chromatography is the preferred technique to analyze essential oils, which are characterized by volatile constituents of various polarities: stationary phases with different polarities can be combined. (8) To characterize the chemical profile of S. sericea oil obtained by hydrodistillation, the sample was introduced into an Agilent 6890N gas chromatograph coupled to a 5973N mass spectrometer (Santa Clara (CA), USA). The column used was a HP-5 MS capillary column (5% phenylmethylpolysiloxane, 30 m, 0,25 mm i.d., 0,1 mm film thickness; J&W Scientific, Folsom). The following temperature program was used: first 5 min at 60 °C, subsequently 4 °C/min up to 220 °C, then 11 °C/min up to 280 °C, held for 15 min, for a total run of 65 min. The temperature of the injector and transfer line was set on 280°C. As carrier gas Helium was used at a flow rate of 1 mL/min with a split ratio of 1:50. The spectrum was measured from 29 to 400 m/z. Ionization occurred by electron-impact (70 eV). A 1:100 dilution was made by adding 594 µL n-hexane to 6 µL oil. n-Hexane was purchased from Carlo Erba (Milan, Italy) and was distilled by a Vigreux column before use. The injection volume was 2 µL. For peak assignment a series of aliphatic carbons (C8–C30; Sigma, IT-Milan) in n-hexane was directly injected into the GC device. This yielded a spectrum which could be used to calculate the retention indices of the peaks from the samples.

EO components were identified following the recommendations by the International Organization of the Flavour Industry (IOFI). Computer matching with mass spectra available in the home-made library ONOSMA and other libraries including NIST08 (2008), FFNSC2 (2011), ADAMS (2007), WILEY275 (2009) was combined with calculated Van den Dool indices which in turn were compared with data from the ADAMS database. Data of ONOSMA were obtained by analyzing commercial available standard compounds and EO references.
The software used was MSD ChemStation software (Agilent, Version G1701DA D.01.00). A few peaks were assigned by comparing the \( m/z \) ratio of the molecular ion and its fragments with data previously published. (50-52) To calculate the temperature-programmed Van den Dool retention index (AI) the following formula was applied (53):

\[
AI_x = 100n + 100(t_x-t_n) / (t_{n+1}-t_n) \tag{3.3}
\]

\( n \) = the number of carbons in the \( n \)-alkane standard eluting prior to the chemical substance \( X \)

\( t_n \) = retention time of the reference \( n \)-alkane eluting prior to the chemical substance \( X \)

\( t_{n+1} \) = retention time of the \( n \)-alkane eluting immediately after the chemical substance \( X \)

\( t_x \) = retention time of compound “X”, the compound of interest

The difference between using retention indices instead of relative retention times, retention volumes, or capacity ratios, is that column dimensions, film thickness, and phase ratio have no influence on the retention indices. (54) The following analytical standards purchased from Sigma Aldrich (Milan, Italy) were used to identify EO components: \( \alpha \)-pinene, camphene, \( \beta \)-pinene, myrcene, \( \delta \)-2-carene, \( \alpha \)-phellandrene, \( \delta \)-3-carene, \( \rho \)-cymene, limonene, 1,8-cineole, \( \gamma \)-terpinene, terpinolene, linalool, camphor, isoborneol, \( \alpha \)-terpineol, isobornyl acetate, carvacrol, thymol, neryl acetate and \( \alpha \)-humulene. All compounds were of analytical standard grade.

3.2.6 HPLC

The separation of phenolic compounds was accomplished by direct injection of the plant extracts on an Synergi Polar-RP C18 analytical column (4,6 mm x 150 mm, 4 µm) purchased from Phenomenex (Chesire, UK). The mobile phase was composed of 60% 0,1% formic acid (v/v) in water and 40% methanol, working at isocratic mode at a flow rate of 0,7 mL/min. HPLC-DAD studies were performed on a Hewlett-Packard HP-1090 Series II instrument coupled with a model HP 1046 photodiode-array-detector. The HPLC system was further made by an autosampler, a binary pump, a vacuum degasser and a security cartridge preceding the reversed phase column. The samples were prepared by redissolving 10 mg of each extract in 1 mL of its corresponding extraction solvent. Deionized water (18 MΩcm resistivity) was used from a Milli-Q SP Reagent Water System (Millipore,Bedford, MA, USA).
After ultrasonification the solution was filtered through a nylon membrane filter (0.45 µm pore size, Phenex, Phenomenex, Torrance, CA, USA) before use. The sample volume injected was 5 µL. UV-spectra were recorded in the range of 210-350 nm. For quantitative analysis, the detection wavelength providing the highest intensity was chosen. The detection wavelength of 210 nm was used for quantification of shikimic acid, gallic acid, (+)-catechin hydrate and (-)-epicatechin. At 310 nm quantification of p-coumaric acid and trans-resveratrol was performed and the wavelength of 325 nm was used for quantification of caffeic acid, trans-ferulic acid, 3-O-caffeoylquinic acid (3-CQA), 5-O-caffeoylquinic acid (5-CQA), 3,5-di-O-caffeoylquinic acid (3,5-di-CQA). Identification was done by comparison of both retention times and UV spectra with those of the analytical standards.

Analytical standards used were gallic acid (CAS Number 149-91-7), p-coumaric acid (CAS Number 501-98-4), trans-ferulic acid (CAS Number 537-98-4) caffeic acid (CAS Number 331-39-5), trans-resveratrol (3,4,5’-trihydroxy-trans-stilbene, CAS Number 501-36-0), (+)-catechin hydrate (CAS Number 225937-10-0) and (-)-epicatechin (CAS Number 490-46-0), 3-O-caffeoylquinic acid (CAS Number 327-97-9), 3,5-di-O-caffeoylquinic acid (CAS Number 2450-53-5), 5-O-caffeoylquinic acid (CAS Number 906-33-2) and shikimic acid (CAS Number 138-59-0). All analytical standards were purchased from Sigma Aldrich (Milano, Italy).

### 3.2.7 Total phenolic content: Folin Ciocalteu method

The commercial available Folin Ciocalteu reagent contains a mixture of tungsten and molybdenum oxides. The colourimetric test is based on the reduction of these metal oxides by phenolic and other potential oxidizable groups, giving blue-coloured products with a maximum absorption at 765 nm. The light intensity increases with the concentration phenols present in the sample. Since the reaction only occurs in alkaline conditions, NaCO₃ has to be added. (17)

First, 100 µL of each extract (5 mg/mL) was put into the first row of a 96-well microtiter plate in triplicate, using gallic acid as a positive control (1 mg/3 mL H₂O). Second, all the other rows were filled with 50 µL of the correct solvent, according to the extraction method of the sample. 50 µL of each well in the first row was transferred to the second row and mixed. From this dilution another 50 µL was transferred to the row underneath and so on. The last row only contained solvent. Subsequently 150 µL of Folin Ciocalteu reagent (1 mL Folin-
Denis’ reagent in 4 mL H₂O) was added in the wells of the first two columns of each extract, the third column serving as a ‘blank’ for subtracting the absorbance of the original dilutions. 150 µL of solvent was added in the latter column. The plate was incubated for 10 min at 37 °C. Next, 50 µL of a saturated NaCO₃ solution in H₂O was added to each well and the plate was incubated for another 10 min. The absorbance was measured at 765 nm with the FluoSTAR Omega plate reader. Gallic acid and Folin Ciocalteu reagent were stored at 4 °C before use. Results were expressed in mg gallic acid equivalents (GAE) per gram extract.

### 3.2.8 Antioxidant experiments

All samples were prepared by redissolving 5 mg of the extract in 1 mL of extraction solvent. To get a homogeneous solution the mixture was vortexed and if necessary ultrasonificated.

#### 3.2.8.1 ABTS decolourisation assay

Reduction of the radical cation ABTS⁺⁺ by antioxidant compounds present in the plant extract, leads to decolourisation stoichiometric with the number of electrons scavenged. (8) To prepare the blue-greenish ABTS⁺⁺ stock solution, the procedure by Iqbal et al. was followed. (55) 4,9 mg ABTS and 0,3 g of MnO₂ were dissolved in 1,8 mL of distilled water. After 30 min incubation the MnO₂ was removed by filtration. For the working solution 300 µL of the filtered solution was added to 20 mL distilled water. The same amount of the filtered solution was added to 20 mL of ethanol. Trolox was prepared by dissolving 1 mg of Trolox in 1 mL of ethanol, followed by a 1:10 dilution to obtain a concentration of 0,1 mg/mL. The solution was stored at 4 °C until use. First, 100 µL of each sample and the positive control Trolox was put in triplicate into the first row of a 96-well microtiter plate. Dilutions were made like the test described above. Thereafter 150 µL of the ABTS solution in H₂O or ethanol was added into the first two columns of each extract, while 150 µL of solvent was put into the third column. The plate was incubated for at least 20 min at 37°C before it was introduced into the plate reader. The absorbance was determined at 734 nm.

#### 3.2.8.2 DPPH assay

The reduction of the purple DPPH cation by a free radical scavenging antioxidant leads to the yellow form proportional to the amount of electrons captured. (8). The DPPH solution was prepared according to the previously described procedure by Srinivasan et al. (56) 1,2 mg DPPH was solved into 30 mL of ethanol. The solution was covered from light before use.
The same procedure as the ABTS experiment was applied, except for using the DPPH solution instead of the ABTS working solution. The absorbance was read at 517 nm.

3.2.8.3 FRAP assay
The ferric reducing antioxidant power (FRAP) assay is based on the reaction of Fe$^{2+}$ with 2,4,6-tripyridyl-s-triazine (TPTZ). Fe$^{3+}$ can be reduced to Fe$^{2+}$ by antioxidant compounds present in the sample. The intense blue-coloured product formed is a ferrous tripyridyltriazine complex, which shows an absorption maximum at 593 nm. (8)

The assay was performed according to Firuzi et al. with some modifications. (57) The FRAP solution was prepared by mixing 5 mL of FeCl$_3$ solution (16.2 mg in 5 mL H$_2$O) and 5 mL of TPTZ solution (15.6 mg TPTZ in 5 mL 40 mM HCl) with 50 mL acetate buffer. The buffer was made by dissolving 2.46 g sodium acetate in 80 mL distilled water. The pH was adjusted to 3.6 by adding acetic acid and distilled water was added until a total volume of 100 mL was reached. The mixture was incubated at 37 °C. The 96 well plate was filled as follows: 50 µL of sample and the positive control Trolox in triplicate into the first row, 25 µL of the solvent (H$_2$O or ethanol) in all the other rows. The dilutions were made as described above. Finally 175 µL of the FRAP solution was added to each well, except for the third column of each extract, which was filled with the correct solvent for colour correction. The plate was incubated for 30 min at 37 °C, protected from light. The results were measured with the plate reader at 593 nm.

3.2.8.4 Chemicals
ABTS, DPPH, TPTZ, Trolox and Folin-Denis’ reagent were purchased from Sigma Aldrich (Milan, Italy), anhydrous FeCl$_3$ from J.T. Baker D.V. (Deventer, Holland) and NaCO$_3$ from Carlo Erba reagents (Milan, Italy). Ethanol, gallic acid and activated MnO$_2$ were purchased from Fluka (Buchs, Switzerland). Flat-bottomed 96-well microplates (FALCON 96, BD Biosciences) were used to do the colourimetric measurements with a FluoSTAR omega spectrophotometer, BMG Labtech (Offenburg, Germany). The temperature of the microtiter plate reader was set on 25°C. Calculations were made with GraphPad Prism 5 (GraphPad Software, S. Diego, CA, USA).
3.2.9 DPPIV activity assay

To test if the extracts had DPPIV inhibitory activity, the velocity of para-nitroanilide (pNA, Sigma Ltd. USA) release from the chromogenic substrate glycyl-prolyl-para-nitroanilide (Gly-Pro-pNA) was recorded spectrophotometrically at 410 nm. Phosphate-buffered saline (PBS, pH 7.4, 0.05 mM) was incubated at 37°C. For the control 460 μL of PBS was put into a 1-cm length cuvette together with 20 μL bovine kidney enzyme and 10 μL distilled water or ethanol. After 5 min incubation 10 μL of 2.5 mM Gly-Pro-pNA was added. The kinetics were measured for 2 min and the slope was expressed in abs/min.

For the samples the same procedure was followed, except for using 10 μL of extract (5 mg/mL) instead of 10 μL distilled water or ethanol. The measurements were performed in duplicate. Kinetic measurements were recorded with the Varian Cary 1E UV-Visible spectrophotometer and calculations were made using the computer software Cary100WinUV Kinetics.

3.2.10 Antimicrobial activity: disk diffusion test

Antimicrobial activity of aqueous and ethanol extracts of S. sericea and L. caerulea was tested using the agar disk diffusion method. Bacterial and fungal strains included S. aureus ATCC 25923, E. coli ATCC 25922, P. aeruginosa ATCC 27853, E. faecalis ATCC 29212 and C. albicans ATCC 24433. Clinical and Laboratory Standards Institute (CLSI) guidelines were followed to perform the antimicrobial disk susceptibility test. A few colonies of each bacterial strain were transferred from an overnight culture on a blood agar plate into 5 mL saline. The optical density (OD) of 1 mL of the suspension was measured with a UV-1700 Pharmaspec UV-Vis spectrophotometer (Shimadzu, Kyoto, Japan). To obtain an OD of 0.1 (1 x 10^8 cells per mL saline), further dilutions were made.

A liquid culture of C. albicans was grown overnight in Sabouraud Dextrose Agar. 1 mL of the culture was added to 5 mL of saline, followed by measuring the optical density and further diluting to obtain an OD of 0.1. 1 mL of saline was used as a blank. The prepared bacteria suspensions were spread onto Mueller Hinton agar plates using a sterile cotton swab. For C. albicans a Petri dish with solid Roswell Park Memorial Institute (RPMI) medium (thickness 4 mm) was used. Sterile paper disks (Oxoid, Basingstoke Hampshire, England) were placed onto the media with forceps. 10 μL extract (20 mg/mL) was spotted onto the blank disks
(6 mm in diameter). Ciprofloxacin (5 μg disk) and nystatin (100 Units disk) were used as reference antimicrobials against bacteria and fungi, respectively. The plates with the bacteria were incubated at 37 °C ± 1 °C for 24 h, while the plates of *C. albicans* were incubated for 48 h. Clear and transparent halos indicated antimicrobial activity. The inhibition zone diameter (IZD) was measured with a ruler and expressed in mm. The paper disk diameter was included in reported IZDs, therefore only a reading of more than 6 mm meant activity.

3.2.11 Pyocyanin assay

Quantitative analysis of pyocyanin production of *P. aeruginosa* was performed by using a liquid assay according to a previously described protocol with some modifications. (59) Assays were carried out using *P. aeruginosa* ATCC 27853 cultured in Luria Bertani (LB) broth (1% peptone, 0.5% yeast extract, 0.5% NaCl, per 100 mL distilled water). Briefly, 10 mL LB broth was inoculated and incubated for 24 h at 37°C. The OD_{600} was determined and dilutions were made to obtain an OD of 0.1 in 5 mL.

Preparation of the tubes was performed in the following way: 125 μL of *S. sericea* ethanol extract from Palm Mar (20 mg/1 mL dimethylsulfoxide (DMSO) was mixed with 4,875 mL of prepared diluted culture. 125 μL of DMSO and 125 μL of (+)-catechin (160 mM in DMSO) was added to 4,875 mL culture for the negative and positive control respectively. For the blank, 125 μL of plant extract was mixed with 4,875 fresh LB medium. After 24 h of incubation, the OD_{600} was determined. Pyocyanin was extracted by the following procedure: first, 4 mL was centrifuged for 12 min at 4000 rpm. Second, 2,4 mL chloroform was added to the supernatant. After vortexing, the aqueous layer was discarded and 1 mL of HCl 0,2 M was added. The tubes were shaken vigorously and the HCl layer was transferred into a cuvette to scan the spectrum from 800 nm to 351 nm. Values at 520 nm were chosen to make the graph. Spectra were recorded with the Varian Cary 1E UV-Visible spectrophotometer and calculations were made using the computer software Cary100WinUV Scan.

3.2.12 Cytotoxic activity: MTT assay

Thiazolyl Blue Tetrazolium Bromide (MTT) can be used in measurement of cell proliferation. MTT produces a yellowish solution that is converted to dark blue, water-insoluble MTT formazan by mitochondrial dehydrogenases of living cells.
The A375 human malignant melanoma cell line and the MDA-MB 231 human breast adenocarcinoma cell line were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM) with 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin, and supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS). HCT-116 human colon carcinoma cells were cultured in RPMI 1640 medium, with the same supplements. All cells were grown in a humidified atmosphere at 37 °C in the presence of 5% CO₂. Constituents and supplements of the growth media were purchased from Sigma (St. Louis, MO, USA).

The assay was carried out following the protocol of Quassinti et al. (60): firstly medium from the overnight culture was removed and 1,5 mL of trypsin-EDTA (0,5% and 0,2% in PBS) was added. After 5 min incubation at 37°C, cells were detached from the monolayer and 750 µL was transferred in a test tube together with 3 mL medium. The solution was centrifuged for 5 min at 1000 rpm, the medium was discarded and cells were resuspended in 3 mL of fresh medium. Next, 30 µL was mixed with 30 µL Trypan blue and the number of cells was determined with a counting chamber (Fuchs-Rosenthal) under the microscope. The culture was diluted to obtain a density of 2 x 10⁴ cells/well. 100 µL of this cell suspension was put into each well of a 96-well plate. After 24 h, samples were exposed to different concentrations of extracts (0,39–200 µg/mL). The water extracts were prepared by dissolving 10 mg of plant extract in 1 mL of water, while the ethanol extracts were dissolved in 1 mL H₂O/ethanol (1:1 v/v) The vehicle used was applied as a negative control and the chemotherapy drug cisplatin served as a positive control. Cells were incubated for 72 h in the same conditions as they were grown.

At the end of incubation, 10 µl of MTT (5 mg/mL in PBS) was added to each well and the plates were incubated for 4 h at 37 °C. Next, the medium was discarded and the purple formazan product was dissolved in 100 µL DMSO. The absorbance was recorded at 540 nm with a Titertek Multiscan microElisa spectrophotometer (Labsystems, Helsinki, Finland). Experiments were conducted in duplicate. Cytotoxicity was expressed as the concentration of extract showing a lethal effect on 50% of the cells (IC₅₀). To calculate the IC₅₀ values the GraphPad Prism 4 computer program was used (GraphPad Software, 4. Diego, CA, USA). PBS was purchased from Lonza (Verviers, Belgium), cisplatin and MTT from Sigma Aldrich (Milan, Italy).
4 RESULTS

4.1 GC-MS

Table 4.1 reports the essential oil composition of *S. sericea* harvested at three different localities. The yield of essential oil was 1.2%, 0.4% and 0.7% for *S. sericea* collected in Palm Mar, Fasnia and La Barranquera, respectively. Fifty volatile components were identified in the EO of *S. sericea* from Fasnia, accounting for 87% of the total composition. In the EO of *S. sericea* from La Barranquera, 45 compounds were detected (91% of the total composition).

For *S. sericea* harvested in Palm Mar, 43 volatile components were identified in the essential oil, representing 93% of the total composition.

Of the identified components the major part belonged to the monoterpene hydrocarbons (59-74%), followed by oxygenated monoterpenes (8,1-16%). The most abundant compound of the aromatic monoterpenes was *p*-cymene, which was also the main component of all three oils (43-57%). Thymol derivatives constituted 5,7-6,3% of the total composition, with thymol isobutyrate as the main component, followed by 8,9-dehydrothymol isobutyrate. Other thymol derivatives identified were 10-acetoxy-8,9-dehydro-6-methoxy-thymol isobutyrate, 10-acetoxy-8,9-epoxy-2-methoxy-thymol, 10-acetoxy-8,10-dehydro-9-isobutyryloxy-6-methoxy-thymol and 6-methoxythymol isobutyrate. The last one was found only in the EO of *S. sericea* from Fasnia.

Other principal compounds in all three EOs belonging to the monoterpenes hydrocarbons were limonene (4,7-5,6%), camphene (1,8-4,8%), β-pinene (0,7-5,3%) and α-phellandrene (2,2-4,8%). In the EO of La Barranquera β-pinene was present at a lower level. Among the oxygenated monoterpenes, isobornyl acetate was the most abundant component (4,5-11%).

In the sesquiterpene fraction the hydrocarbons were represented mainly by germacrene D (0,7-1,7%) and γ-himachalene (0,4-1,7%), while the oxygenated compounds were mainly represented by spathulenol (0,6-1,3%), α-cadinol (0,7-2,0%) and its isomer *epi*-α-cadinol (0,4-1,9%). 38 Common constituents were identified in the three EOs. About one third (20 out of 57 constituents) of the EO components was identified by means of co-injection with authentic standards.
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<th>Al lit. (^c)</th>
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<th>Fasnia (%)</th>
<th>La Barranquera (%)</th>
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Oil yield (%)  
Total identified (%)  
Grouped compounds (%)  
Aromatic monoterpenes  
Monoterpene hydrocarbons  
Oxygenated monoterpenes  
Sesquiterpene hydrocarbons  
Oxygenated sesquiterpenes  
Others

| 1,2 | 0,4 | 0,7 |
| 93  | 87  | 91  |

| 0,6 | 1,6 | 0,4 |
| 74  | 59  | 70  |

| 16  | 8,1 | 8,2 |
| 2,0 | 5,5 | 2,8 |

| 1,9 | 5,7 | 3,2 |
| 6,0 | 6,3 | 5,7 |

**Notes:**
- Oil yield (%)
- Total identified (%)
- Grouped compounds (%)
- Aromatic monoterpenes
- Monoterpene hydrocarbons
- Oxygenated monoterpenes
- Sesquiterpene hydrocarbons
- Oxygenated sesquiterpenes
- Others
- Compounds are listed in order of their elution from a HP-5MS column.
- Van den Dool Index on HP-5MS column, experimentally determined using homologous series of C8-C30 alkanes.
- Van den Dool Index taken from Adams (61) and FFNSC2 (62) for apolar capillary column.
- Percentage relative abundance.
- Identification methods: MS, by comparison of the mass spectrum with those of the computer mass libraries Wiley, Adams, FFNSC2 and NIST 08; RI, by comparison of Al with those reported in literature (61-63); Std, by comparison of the retention time and mass spectrum of available authentic standards.
- Tr, traces (value<0,1%).
- Retention index value taken from Weyerstahl et al. (52).
- MS fragmentations according to Gonzalez et al. (51, 64).
- MS data for unidentified components: RI 1790 m/z (%) = 276(4, M+), 148(100), 146(87), 145(84), 43(73), 135(46), 71(30), 133(30), 206(24), 147(17), 149(14).
4.2 HPLC-DAD

The polyphenolic profiles of *L. caerulea* and *S. sericea* harvested at three different locations on Tenerife are reported in Table 4.2. The flavan-3-ol (-)‐epicatechin was only found in the ethanol extract of *S. sericea* from La Barranquera (18,00 µg/g plant material dry weight). The chromatograms recorded at 210 nm showed low levels of gallic acid in all *Schizogyne* extracts (7,961-31,58 µg/g). Compared to the other two regions, the extracts from Fasnia were especially rich in caffeic acid (112,8-483,0 µg/g).

The highest amount of 3-CQA was present in the aqueous extract from Palm Mar (2075 µg/g), while in its ethanol extract 3,5-di-CQA was the main phenolic compound (714,4 µg/g). Noteworthy, this latter compound was present in a 10-fold amount with respect to the extract from La Barranquera. 5-CQA was detected in all aqueous extracts (48,88-519,4 µg/g), whereas it was not found in the ethanolic extracts from Fasnia and La Barranquera. Of each compound, except for gallic and caffeic acid, the highest amount was present in Palm Mar extracts.

As a result, the total phenolic content of its ethanol extract was 2 and even 5 times higher than those of Fasnia and La Barranquera respectively. For the aqueous extracts the difference was not so big between the three localities. With values of 1302 µg/g (ethanol extract) and 4181 µg/g (aqueous extract), Palm Mar extract was rich in phenolics. Except for the ethanol extract of Fasnia, 3,5-di-CQA and 3-CQA were the predominant compounds in all *Schizogyne* extracts (19-55% and 36-51%). In this first mentioned extract next to 3-CQA, caffeic acid was most present (21%).

In both chromatograms obtained after injection of the *L. caerulea* extracts a peak characteristic for shikimic acid was seen (51,16-204,4 µg/g). In the same chromatograms, two different hydroxycinnamate derivates were detected at 325 nm, namely 5-CQA (29,01-67,89 µg/g) and 3-CQA (233,1-741,4 µg/g). The concentration of phenolic compounds identified in the ethanol extract (1014 µg/g) was at least 3 times higher than that found in the aqueous extract (313,2 µg/g). In none of the samples (+)-catechin hydrate, *p*-coumaric acid or *trans*-resveratrol have been found.
Table 4.2: Quantification of phenolic compounds by HPLC-DAD analysis. Results expressed in µg/g plant material.

Corrections were made for the water content (90% in *L. caerulea* and 6% in *S. sericea*).

<table>
<thead>
<tr>
<th>Phenolic compounds</th>
<th><em>S. sericea</em> Palm Mar</th>
<th><em>S. sericea</em> Fasnia</th>
<th><em>S. sericea</em> La Barranquera</th>
<th><em>Lonicera caerulea</em></th>
<th><em>S. sericea</em> Palm Mar</th>
<th><em>S. sericea</em> Fasnia</th>
<th><em>S. sericea</em> La Barranquera</th>
<th><em>Lonicera caerulea</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Shikimic acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>204.4</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>51.16</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>7.961</td>
<td>16.73</td>
<td>18.45</td>
<td>N.D.</td>
<td>19.48</td>
<td>31.58</td>
<td>12.30</td>
<td>N.D.</td>
</tr>
<tr>
<td>(+)-Catechin hydrate</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>(−)-Epicatechin</td>
<td>N.D.</td>
<td>N.D.</td>
<td>17.99</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>p-Coumaric acid</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td><em>trans</em>-Resveratrol</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>5-CQA</td>
<td>48.88</td>
<td>N.D.</td>
<td>67.89</td>
<td>519.4</td>
<td>461.5</td>
<td>244.1</td>
<td>29.01</td>
<td></td>
</tr>
<tr>
<td>3-CQA</td>
<td>471.6</td>
<td>318.1</td>
<td>125.4</td>
<td>741.4</td>
<td>2075</td>
<td>1583</td>
<td>597.2</td>
<td>233.1</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>6.349</td>
<td>112.8</td>
<td>11.83</td>
<td>N.D.</td>
<td>83.28</td>
<td>483.0</td>
<td>152.4</td>
<td>N.D.</td>
</tr>
<tr>
<td><em>trans</em>-Ferulic acid</td>
<td>52.91</td>
<td>21.52</td>
<td>14.06</td>
<td>N.D.</td>
<td>151.3</td>
<td>133.9</td>
<td>66.61</td>
<td>N.D.</td>
</tr>
<tr>
<td>3,5-di-CQA</td>
<td>714.4</td>
<td>75.98</td>
<td>60.13</td>
<td>N.D.</td>
<td>1333</td>
<td>620.8</td>
<td>259.1</td>
<td>N.D.</td>
</tr>
<tr>
<td><strong>Total content</strong></td>
<td>1302</td>
<td>545.2</td>
<td>247.9</td>
<td>1014</td>
<td>4181</td>
<td>3314</td>
<td>1332</td>
<td>313.2</td>
</tr>
</tbody>
</table>

N.D.: Not detected
4.3 ANTIOXIDANT EXPERIMENTS

In Table 4.3 all data are expressed as means ± standard deviations of duplicate measurements. The total phenolic content of the *Schizogyne* ethanol extracts, expressed as GAE, was 69,9 mg/g, 21,2 mg/g and 23,5 mg/g for extracts from Palm Mar, Fasnia and La Barranquera, respectively. In comparison, the total phenolic content of the aqueous extracts was higher, being 61,0, 54,7 and 40,4 mg/g for the same locations, respectively. The ethanol extract as well as the water extract of Palm Mar contained the highest amount of phenolic compounds. The extracts of *L. caerulea* displayed a lower phenolic content, with values of 18,6 and 32,7 mg/g for the ethanol and aqueous extract, respectively.

The IC₅₀ value of the standard control Trolox amounted to 4,89 μg/mL for the DPPH assay with the *Schizogyne* samples. In this assay, the sample from Fasnia exhibited the highest antioxidant activity, with a TEAC value of 224 μmol TE/g ethanol extract (IC₅₀ value of 87,2 μg/mL) and 283 μmol TE/g aqueous extract (69,2 μg/mL). The fruits of *L. caerulea* showed lower TEAC values in the range of 72,5-103 μmol TE/g. (212-332 μg/mL).

Regarding the ABTS assay, IC₅₀ values obtained for the standard control Trolox were 1,43 μg/mL and 3,50 μg/mL for the ethanol and water extracts of *Schizogyne*, respectively. Ethanol extract of *S. sericea* from Fasnia showed the highest activity (10,7 μg/mL) while among the water extracts, that from Palm Mar was the most active, with a TEAC value up to 960 μmol TE/g (14,6 μg/mL). The same was observed in the FRAP assay.

Contradictory to the DPPH test, in the ABTS assay *L. caerulea* gave higher TEAC values. Similar values were obtained for both kind of extracts in the FRAP assay. With a TEAC value of 376 μmol TE/g for the ethanol extract, *L. caerulea* possessed even a higher antioxidant capacity than *Schizogyne*.
Table 4.3: Total phenolic content and antioxidant activity of ethanol and aqueous extracts of *Lonicera caerulea* and *Schizogyne sericea* from different locations.

<table>
<thead>
<tr>
<th></th>
<th>TOTAL PHENOLS</th>
<th>DPPH</th>
<th>ABTS</th>
<th>FRAP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mgGAE/g (^a)</td>
<td>μmol TE/g</td>
<td>IC(_{50})</td>
<td>μmol TE/g</td>
</tr>
<tr>
<td>Ethanol extracts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. sericea</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palm Mar</td>
<td>69,9(±7,3)</td>
<td>129(± 2,0)</td>
<td>151(±2,4)</td>
<td>196(±30)</td>
</tr>
<tr>
<td><em>S. sericea</em></td>
<td>21,2(±1,6)</td>
<td>224(±1,1)</td>
<td>87,2(±0,41)</td>
<td>536(±42)</td>
</tr>
<tr>
<td>Fasnia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. sericea</em></td>
<td>23,5(±1,4)</td>
<td>121(±7,1)</td>
<td>161,5(±9,4)</td>
<td>347(±47)</td>
</tr>
<tr>
<td>La Barranquera</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>Trolox</td>
<td>-----</td>
<td>4,89(±0,070)</td>
<td>-----</td>
</tr>
<tr>
<td><em>L. caerulea</em></td>
<td>18,6(± 6,1)</td>
<td>103(± 4,5)</td>
<td>212(±9,2)</td>
<td>421(±37)</td>
</tr>
<tr>
<td>Positive control</td>
<td>Trolox</td>
<td>-----</td>
<td>5,47(±0,26)</td>
<td>-----</td>
</tr>
<tr>
<td>Aqueous extracts</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. sericea</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Palm Mar</td>
<td>61,0(±4,8)</td>
<td>208(±19)</td>
<td>94,2(±8,4)</td>
<td>960(±17)</td>
</tr>
<tr>
<td><em>S. sericea</em></td>
<td>54,7(±5,8)</td>
<td>283(±13)</td>
<td>69,2(±3,2)</td>
<td>543(±32)</td>
</tr>
<tr>
<td>Fasnia</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. sericea</em></td>
<td>40,4(±3,8)</td>
<td>210(±15)</td>
<td>93,1(±6,5)</td>
<td>450(±51)</td>
</tr>
<tr>
<td>La Barranquera</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive control</td>
<td>Trolox</td>
<td>-----</td>
<td>4,89(±0,070)</td>
<td>-----</td>
</tr>
<tr>
<td><em>L. caerulea</em></td>
<td>32,7(±4,8)</td>
<td>72,5(±7,7)</td>
<td>332(±35)</td>
<td>596(±1,3)</td>
</tr>
<tr>
<td>Positive control</td>
<td>Trolox</td>
<td>-----</td>
<td>6,00(±0,51)</td>
<td>-----</td>
</tr>
</tbody>
</table>

\(^a\) GAE = Gallic acid equivalent

\(^b\) TEAC = Trolox equivalent (TE) antioxidant capacity

\(^c\) IC\(_{50}\): The concentration giving a reduction of 50%
4.4 DPPIV ACTIVITY ASSAY

Results are summarised in Table 4.4. Except for the aqueous extract of *S. sericea* collected at La Barranquera, the calculated slopes of the kinetic curves were all higher than the control. Too little difference with the control was measured of the former. This means that none of the samples showed inhibitory activity against the DPPIV enzyme.

Table 4.4: DPPIV activity of aqueous and ethanol extracts of *S. sericea* and *L. caerulea*

<table>
<thead>
<tr>
<th></th>
<th>Aqueous extracts</th>
<th>Ethanol extracts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slope (abs/min)</td>
<td>Inhibition (%)</td>
</tr>
<tr>
<td><em>S. sericea</em> Palm Mar</td>
<td>0.0130</td>
<td>--</td>
</tr>
<tr>
<td><em>S. sericea</em> Fasnia</td>
<td>0.0131</td>
<td>--</td>
</tr>
<tr>
<td><em>S. sericea</em> La Barranquera</td>
<td>0.0123</td>
<td>3.15</td>
</tr>
<tr>
<td><em>Lonicera caerulea</em></td>
<td>0.0168</td>
<td>--</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>0.0127</td>
<td>--</td>
</tr>
</tbody>
</table>

4.5 DISK DIFFUSION TEST

Table 4.5 reports the IZDs observed. Activity of the extracts was in the range of 6.0 (no activity) to 10 mm (low activity) inhibition zone diameter. For both kind of extracts of *S. sericea* and *L. caerulea* no activity was observed against *P. aeruginosa*. The aqueous extract of *L. caerulea* showed low activity against *S. aureus* and *E. coli*, while the ethanol extract inhibited only the growth of *C. albicans*.

The ethanol extracts of *S. sericea* from Fasnia and La Barranquera showed little activity against the bacterial species *S. aureus* and *E. faecalis*. The former extract was also responsible for the most growth inhibition of the fungal strain, while the latter showed no activity. Neither of the bacterial species where inhibited by ethanol extract of *S. sericea* from Palm Mar; however, low antifungal activity was detected. Aqueous extracts of both plants did not inhibit growth of *E. faecalis*, *P. aeruginosa* and *C. albicans*. An extremely low activity of the aqueous *Schizogyne* extracts against *S. aureus* and *E. coli* was observed.
Table 4.5: Antimicrobial activity of ethanol and aqueous extracts from *S. sericea* and *L. caerulea* determined by the disk diffusion test. Values indicate the diameter of the growth inhibition zone (mm).

<table>
<thead>
<tr>
<th></th>
<th><em>S. aureus</em></th>
<th><em>E. faecalis</em></th>
<th><em>E. coli</em></th>
<th><em>P. aeruginosa</em></th>
<th><em>C. albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ethanol extracts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. sericea</em> Palm Mar</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>7-9</td>
</tr>
<tr>
<td><em>S. sericea</em> Fasnia</td>
<td>7</td>
<td>8</td>
<td>6</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td><em>S. sericea</em> La Barranquera</td>
<td>8</td>
<td>8</td>
<td>6</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><em>L. caerulea</em></td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td><strong>Aqueous extracts</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. sericea</em> Palm Mar</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><em>S. sericea</em> Fasnia</td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><em>S. sericea</em> La Barranquera</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><em>L. caerulea</em></td>
<td>7</td>
<td>6</td>
<td>7</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td><strong>Reference antibiotics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>28</td>
<td>23</td>
<td>30</td>
<td>31</td>
<td>n.r.</td>
</tr>
<tr>
<td>Nystatin</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>n.r.</td>
<td>27-28</td>
</tr>
</tbody>
</table>

n.r.: Not recommended for this species.

Figure 4.6: (A) Disks on the *P. aeruginosa* plate spotted with ethanol extracts of *S. sericea* from three locations and with *L. caerulea* (lower left); (B) aqueous extracts

After 24 h incubation, a circle of spots was visible around the paper disks of the ethanol extracts of *S. sericea* on the *P. aeruginosa* plate. After 48 h the plate was coloured green. On the basis of these observations, further studies were carried out to investigate potential anti-quorum sensing activity of these extracts.
4.6 PYOCYANIN ASSAY

Results are displayed in Figure 4.7. To determine the degree of inhibition, pyocyanin was extracted and quantified spectrophotometrically. The absorbances at 520 nm showed that *Schizogyne* ethanol extract from Palm Mar reduced the QS-regulated pyocyanin production by ca. 50%, while the authentic standard (+)-catechin reduced the pyocyanin production in the range of 70%. The \( \text{OD}_{600} \) varied between 1,018 and 1,118.

![Figure 4.7: Histogram reporting inhibition of *P. aeruginosa* ATCC 27853 pyocyanin production. (A) DMSO; (B) (+)-catechin; (C) *S. sericea* ethanol extract from Palm Mar.](image)

4.7 MTT ASSAY

Table 4.8 reports the cytotoxic activity of *S. sericea* from Palm Mar and *L. caerulea*. The aqueous extract of Palm Mar had little cytotoxic potential, with an IC\(_{50}\) over 200 \( \mu \text{g/mL} \). Since both *L. caerulea* extracts and the aqueous extract of *S. sericea* showed no or little cytotoxic activity on the A375 and MDA-MB 231 tumour cell lines, they were not further evaluated on the HCT116 cell line.

Results showed a potential tumour cell growth inhibitory effect of *S. sericea* ethanol extract from Palm Mar on all tumour lines tested. IC\(_{50}\) values were 0,74 \( \mu \text{g/mL} \), 0,32 \( \mu \text{g/mL} \) and 0,52 \( \mu \text{g/mL} \) on A375, MDA-MB 231 and HCT116 tumour cell lines, respectively. The IC\(_{50}\) values of cisplatin after 72 h were 0,43 \( \mu \text{g/mL} \), 2,9 \( \mu \text{g/mL} \), and 2,4 \( \mu \text{g/mL} \), respectively.
Table 4.8: *In vitro* cytotoxic activity of aqueous and ethanol extracts of *S. sericea* and *L. caerulea*.

<table>
<thead>
<tr>
<th></th>
<th>A375&lt;sup&gt;b&lt;/sup&gt;</th>
<th>MDA-MB 231&lt;sup&gt;c&lt;/sup&gt;</th>
<th>HCT116&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aqueous extracts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. sericea</em> Palm Mar</td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>---</td>
</tr>
<tr>
<td><em>Lonicera caerulea</em></td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>---</td>
</tr>
<tr>
<td><strong>Ethanol extracts</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>S. sericea</em> Palm Mar</td>
<td>0,74</td>
<td>0,32</td>
<td>0,52</td>
</tr>
<tr>
<td>95% C.I.&lt;sup&gt;e&lt;/sup&gt;</td>
<td>0,66-0,83</td>
<td>0,28-0,37</td>
<td>0,49-0,52</td>
</tr>
<tr>
<td><em>Lonicera caerulea</em></td>
<td>&gt;200</td>
<td>&gt;200</td>
<td>---</td>
</tr>
<tr>
<td><strong>Positive control</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cisplatin</td>
<td>0,43</td>
<td>2,9</td>
<td>2,4</td>
</tr>
<tr>
<td>95% C.I.</td>
<td>0,30-0,48</td>
<td>2,2-3,1</td>
<td>2,0-2,9</td>
</tr>
</tbody>
</table>

<sup>a</sup> IC<sub>50</sub> = The concentration of extract that gives 50% reduction in cell growth (after 72 h of incubation).  
<sup>b</sup> Human malignant melanoma cell line.  
<sup>c</sup> Human breast adenocarcinoma cell line.  
<sup>d</sup> Human colon carcinoma cell line.  
<sup>e</sup> Confidence interval.
5 DISCUSSION

5.1 GC-MS

Regarding the oil yield, the low amount of EO in plant material from Fasnia could be attributed to differences in soil composition, plant age and drought with respect to the other 2 locations. (1) The detected composition was similar to that reported for S. sericea collected at Montaña de Guaza reported in a previous work, apart from some minor components. (65) p-Cymene, the main volatile component, is also the dominant compound in S. glaberrima. Furthermore, the thymol derivatives 8,9-dehydrothymol isobutyrate and thymol isobutyrate are of chemotaxonomic interest since they also occur in the EO of S. glaberrima and in other Asteraceae (Arnica amplexicaulis Nutt. and Carpesium divaricatum Siebold & Zucc.). (66, 67) p-Cymene, the most abundant compound found in the EO (43-57%), can be formed via isomerization, cyclization or oxidation reactions during the extraction process. It is the precursor of thymol and carvacrol, which explains the presence of thymol derivatives. This compound is also the degradation product of y-terpinene, only present in trace amounts in the EO. (1, 68)

p-Cymene displays analgesic and anti-inflammatory properties in mice. From an industrial point of view, it serves as a heat transfer medium and as an intermediate in the production of pesticides, fungicides, perfumes and flavouring agents. (69) It can be isolated from the oil for these applications.

5.2 HPLC-DAD AND TOTAL PHENOLIC CONTENT

The hydroxycinnamates 3-CQA and 5-CQA are abundant in L. caerulea berries. The amount found (80,93 mg/100 g) is in agreement with levels reported in literature (30,4-156,2 mg/100 g), but since only 2 hydroxycinnamic acids were quantified, comparison is difficult. (70) To caffeoylquinic acid derivatives, such as 3-CQA, 5-CQA and 3,5-di-CQA, a lot of pharmacological properties can be ascribed, among which antioxidant, antibacterial, antihistaminic, hepatoprotectant and neuroprotective activities. In particular, 3,5-di-CQA exhibits protection against neuronal cell death, which could be useful in the treatment of Alzheimer’s and Parkinson’s disease. (71)

(-)-Epicatechin, ferulic, caffeic and coumaric acid that have been mentioned in literature were not detected. (72) Probably their level was below the limit of detection (LOD in the
range of 0.01-0.15 mg/l) or they were not present in these particular berries. High content of anthocyanins has also been described, but we did not determine their concentration. Paliková et al. determined the total phenolic content of the polyphenolic fraction with the Folin-Ciocalteu method. With a value of 140.5 mg/100 g fresh weight, the amount of phenolics reported is comparable with that determined in our assay (130.8-186.0 mg/100 g berries). (73) Variation between our results and those reported in other studies can be described to different extraction conditions, determination methods, reference standards used etc. The antioxidant content might vary for a specific berry species due to different cultivars, growth and storage conditions. In addition, the stage of maturation might differ with the time of harvest. (74, 75) All these factors make comparison of results among studies quite difficult.

A disadvantage of the Folin Ciocalteu reagent is that it does not differentiate between phenols and other reducing substances. These include aromatic amines, iron, sulphur dioxide, ascorbic acid and endiols. Endiols are formed in alkali medium out of sugars. In the Folin-Ciocalteu assay we worked in alkali conditions, so these substances will definitely have contributed to the results. According to Waterhouse, different sugars lead to different interferences. Protein oxidation also occurs. All these interferences are allowed in this kind of general colour formation reaction via the reduction of the reagent. Therefore the total reducing capacity is measured besides determination of the phenolic content. (76)

The phenolic compounds of S. sericea were similar in all three localities. The phenolic acids 3,5-dicaffeoylquinic acid, caffeic acid and 3-cafeoylquinic acid are also present in the leaves of Cynara syriaca Boiss. (77) The latter is highly distributed in the genus Crepis and in Baccharis retusa DC. (78, 79) In combination with 3,5-dicaffeoylquinic acid it was identified in Arnica montana L., Artemisia vulgaris L., Calendula officinalis L. and Chamomilla recutita (L.) Rauschert. (75) HPLC-DAD analysis of specific phenolic compounds of the Schizogyne plant material showed a total phenolic content ranging from 247.9 to 4181 µg/g, whereas L. caerulea berries showed a lower level of these particular compounds (313.2-1014 µg/g). Considering Schizogyne, the content of all phenolic compounds tested in aerial parts from Palm Mar was higher than that determined in samples from Fasnia and La Barranquera, except for caffeic acid which was more abundant in the sample from Fasnia.
In overall, *S. sericea* showed a high level of hydroxycinnamic acids. The aqueous extract of Palm Mar displayed the highest concentration 3-CQA (2075 µg/g) of all analyzed samples.

The quantitative variation in the phenolic content of *S. sericea* can be explained by the different geographical origin, which is associated with other ecological, environmental and physiological factors. (7) Results of the antioxidant tests are in agreement with the total phenolic content estimated by HPLC-DAD analysis. A positive relationship between phenolic content and antioxidant potential is reported in literature. (72) For *S. sericea*, the extraction of phenolic compounds was more efficient when the solvent was changed from ethanol to water. For *L. caerulea*, the contrary was observed, probably owing to the different plant matrix and extraction method.

5.3 BIOLOGICAL ACTIVITY

It is difficult to assign the biological activity of a plant extract to one of its many individual components. Some components of the EO act synergistically, as revealed when their principal compounds used as references displayed less activity than the EO per se. Ethanol is a more general extraction solvent than water because it extracts compounds with a broader spectrum of polarities. If the activity of aqueous extracts of the same plant is higher than that of the ethanol extracts, this suggests that the active compounds are the more polar ones. Likewise, if the alcohol extract shows stronger activity than the aqueous extract, this suggests that less polar compounds are responsible for the activity. Finally, it has to be kept in mind that the employed extraction process is not specific, which leads to crude plant extracts consisting of active as well as non-active constituents. (8, 80, 81)

5.3.1 Antioxidant experiments

The three antioxidant assays did not show noteworthy differences between *Schizogyne* samples from different localities. Generally, we observed that the water extracts of *Schizogyne* showed higher antioxidant capacity compared to the respective ethanol extracts. This is in agreement with the higher total phenolic content of the aqueous extracts determined by HPLC-DAD analysis and the Folin-Ciocalteu method. Both ethanol and water extracts from *Palm Mar* have got the highest phenolic contents in the Folin Ciocalteu test as well as in HPLC-analysis.
In a preliminary work, the same antioxidant assays were performed on the essential oil of *S. sericea* collected in Montaña de Guaza (South-Tenerife), and lower activity was recorded. The lower TEAC values in comparison with those of Naviglio extracts can be attributed to the poorness of phenolic compounds in the EO.

For *L. caerulea*, both the ABTS and FRAP assays gave high TEAC values (376-596 μmol TE/g). Our results were consistent with those reported in literature. (72) The good antioxidant activity of *L. caerulea*, next to its low content of lipids and relatively good nutritional value, may support research for its use in dietary supplements. The relatively strong antioxidant capacity of *S. sericea* may justify its traditional use as wound-healing agent: it is well-known that oxidative stress impairs the wound healing process. (82)

5.3.2 DPPIV activity assay

Despite the fact that caffeic acid is a potent inhibitor of DPPIV, no inhibitory activity is seen among the extracts tested. Gallic acid is little active, while catechin, epicatechin and 3-CQA show no activity according to literature. (83) The low content of these metabolites determined in all the samples tested may explain the poor inhibition on the enzyme. Therefore, the obtained data were not promising enough to do further experiments to determine the IC$_{50}$.

5.3.3 Antimicrobial activity: disk diffusion test

The aqueous extracts of both plants showed little activity against *S. aureus* and *E. coli*. Hence, no association of inhibitory activity with the subdivision in Gram-positive and Gram-negative strains of bacteria could be made. Worthy of mention is that *E. faecalis* and *P. aeruginosa* are natural resistant to diverse antibiotics. The comparison of the three *S. sericea* extracts from samples collected in different localities showed no big differences.

The activity of the ethanolic extract in case of the yeast may be ascribed to its content of thymol derivatives present. (84) They are little soluble in ethanol and not in water, which explains why no activity was observed for the water extracts at the assayed concentrations. The phenolic acids 3,5-dicaffeoylquinic acid and 3-caffeoylquinic acid are also known to exhibit antimicrobial activity. (85) Since the antimicrobial activity determined by the disk diffusion method was poor, the extracts were not further investigated by other susceptibility testing methods.
5.3.4 Pyocyanin assay

According to Vandeputte et al., catechin is one of the flavonoids responsible for the anti-quorum sensing effect. (19) However, (+)-catechin and (−)-epicatechin were not detected in the extract with HPLC-analysis, so the observed inhibition is due to other flavonoids present in the plant. The OD$_{600}$ proved that the decreased pyocyanin production was not related to a bacteriostatic or bactericidal effect of catechin or the extract. Therefore there is no selection pressure for the development of resistant bacteria. (20)

The preliminary results suggest that it would be interesting to further investigate the effect of the plant extract for anti-quorum-sensing activities on bacteria. Since absorbance values were very low, future experiments are required to define the right conditions to obtain enough pyocyanin production. This is important in order to exclude fluctuations of the spectrophotometer lamp. A possible explanation for the low pyocyanin production may be that the _P. aeruginosa_ strain used differs from the one in previously described studies (PA01). (59) We used _P. aeruginosa_ ATCC 27853 to be consistent with the effect observed during the disk diffusion test with this particular strain.

5.3.5 MTT assay

The cytotoxic activity of the ethanol extract of _S. sericea _collected in Palm Mar is probably due to the presence of thymol derivatives. These kind of molecules are endowed with strong antiproliferative effects on different tumour cell lines. (86) Among them, one of the most active molecules is believed to be 10-acetoxy-8,9-epoxy-6-methoxythymol isobutyrate. (65) According to Aponte et al. its activity is to be ascribed to the 8,9-epoxy-10-acetoxy group. (86) Moreover, 8,9-dehydrothymol isobutyrate is an active ingredient in a patented anticancer formulation due to its excellent anti-tumour activity. (67) According to the National Cancer Institute (NCI) plant screening program of the USA, a crude extract is considered to possess active _in vitro_ cytotoxic effect if the IC$_{50}$ value on carcinoma cells, after 48–72 h incubation, is below 20 μg/mL. (87) Thus, regarding the obtained IC$_{50}$ values, the ethanol extract of _S. sericea_ from Palm Mar is considered promising.

On the above it will be interesting to conduct further studies in order to investigate cytotoxic effects on non-tumour cell lines, anti-cancer mechanisms, and _in vivo_ anti-cancer activity of these compounds.
6 CONCLUSION

The objective of this thesis was to perform a phytochemical and biological investigation of *S. sericea* from different areas of Tenerife (Canary Islands) and of *L. caerulea* berries from Russia. Analysis of the hydrodistilled essential oils of *S. sericea* revealed a similar chemical profile for all collection sites. The main component of all three essential oils was *p*-cymene, an aromatic monoterpeno used in the production of pesticides, perfumes and flavouring agents. Furthermore, the oxygenated monoterpeno isobornyl acetate and the monoterpeno hydrocarbons limonene, camphene, *β*-pinene, and *α*-phellandrene occurred in noteworthy levels.

HPLC-DAD analysis of a specific set of compounds showed that *S. sericea* is a rich source of dicaffeoylquinic acids such as 3-CQA, 5-CQA and 3,5-di-CQA. The dry plant material from Palm Mar contained the highest level of each compound analyzed. *L. caerulea* contained 3-CQA, shikimic acid and 5-CQA, in descending order of concentration.

Results from antioxidant assays revealed a profound antioxidant ability of both investigated plant species. TEAC values of DPPH, ABTS and FRAP assays were in the range 72,5-596 and 121-960 µmol TE/g for *L. caerulea* and *S. sericea*, respectively. Therefore, the traditional use of *S. sericea* as a wound-healing balm seems reasonable. Furthermore it makes blue honeysuckle fruits an interesting target for food supplement and cosmetic research. *S. sericea* and *L. caerulea* did not inhibit DPPIV activity. The antimicrobial activity of both *S. sericea* and *L. caerulea* extracts towards all species tested was absent or low. Regarding the pyocyanin assay, further investigation in the experimental set up is necessary in order to confirm the anti-quorum sensing potential of the ethanol extract of *S. sericea* collected in Palm Mar. Analysis of cytotoxic activity showed that aqueous and ethanol extracts of *L. caerulea* were ineffective in inhibiting A375, MDA-MB 231 and HCT-116 human tumour cell lines. On the other hand, the ethanol extract of *S. sericea* collected in Palm Mar was able to exert strong cytotoxic effects on all three cell lines tested. With IC$_{50}$ values of 0,74 µg/mL, 0,32 µg/mL and 0,52 µg/mL, the cytotoxicity was even higher than that of cisplatin. Hence, according to NCI guidelines, *S. sericea* extract may be a lead in the search of new chemotherapeutic agents. However, cytotoxic activity towards normal human cells needs to be determined in future experiments.
7 SOURCES

15. Daayf F, Lattanzio V. Recent advances in polyphenol research: John Wiley & Sons; 2009.


76. Rover MR. Analysis of sugars and phenolic compounds in bio-oil. 2013.


FIGURES


