

“G. d’Annunzio” University of Chieti-Pescara

“G. d’Annunzio” School of Advanced Studies

Doctorate School in Biomolecular and Pharmaceutical Sciences

XXXVII Cycle

“New trends in natural products valorisation: development of selective extraction methods, isolation, and structural elucidation from complex matrices by hyphenated techniques”

Department of Medical, Oral and Biotechnological Science

Disciplinary Area CHIM/08

PhD Student

Dr.ssa Chiara Collevocchio

Coordinator

Prof.ssa Assunta Pandolfi

Tutor

Prof. Salvatore Genovese

ACADEMIC YEARS 2021/2024

Introduction **3**

Chapter 1 - Development of innovative extraction techniques **6**

1.1	Concentration of pomegranate juice for an enhanced antioxidant activity	20
1.1.1	Plant material	22
1.1.2	Extraction procedure and results	22
1.1.3	Conclusions	29
1.1.4	Experimental procedures	30
1.2	Reducing environmental pollution through solid phase adsorption	34
1.2.1	Sample preparation procedures and results	37
1.2.2	Isotherm adsorption experiments	43
1.2.3	Conclusions	45
1.2.4	Experimental procedures	45
1.3	Removal of synthetic dyes from foods and beverages	49
1.3.1	Sample preparation procedures and results for the aqueous mixtures	52
1.3.2	Sample preparation procedures and results for the food and drink matrices	56
1.3.3	Isotherm adsorption experiments	65
1.3.4	Conclusions	68
1.3.5	Experimental procedures	68
1.4	Valorisation of agro-waste: use of olive leaves for the pre-concentration of oleuropein and elenolic acid	72
1.4.1	Plant material	77
1.4.2	Olive leaves extraction	77
1.4.3	Sample preparation procedures and results of solid phase adsorption of oleuropein	78
1.4.4	Sample preparation procedures and selective isolation of elenolic acid by hydrolysis of oleuropein	81
1.4.5	Conclusions	86
1.4.6	Experimental procedures	87
1.5	Leaves of <i>Gentiana lutea</i> L. as an alternative source of bitter principles	93
1.5.1	Plant material	97
1.5.2	<i>G. lutea</i> L. leaves extraction	97
1.5.3	Sample preparation procedures and results for the original set of solid supports	99
1.5.4	Sample preparation procedures and results for the second set of solid supports	108
1.5.5	Conclusions	110
1.5.6	Experimental procedures	111

Chapter 2 - Isolation and structural characterization of plant secondary metabolites **114**

2.1	High resolution PTP1B inhibition profiling combined with HPLC-PDA/NMR for identification of bioactive constituents in the leaves of <i>Eremophila tietkensii</i>.	115
2.1.1	High-resolution inhibition profiling of the crude leaves extract	118
2.1.2	Isolation and structure elucidation	120
2.1.3	PTP1B inhibitory activity of the isolated compounds	164

2.1.4	Conclusions	165
2.1.5	Experimental procedures	165

Chapter 3 – *Set-up of novel chemical assays for preliminary biological investigation* **173**

3.1 Novel approach for assessing the GPx-like activity of natural and semisynthetic compounds: A revised version of the Iwaoka's assay. **174**

3.1.1	Sample preparation procedures	180
3.1.2	HPLC-DAD and GC-MS methods validation	181
3.1.3	GPx-like activity quantification	186
3.1.4	Application of the revised version of Iwaoka's assay: Evaluation of phenylpropanoids and oxyprenylated derivatives GPx-like effects	190
3.1.5	Synthetic scheme of the oxyprenylated phenylpropanoids	200
3.1.6	Application of the revised version of Iwaoka's assay: Evaluation of cannabinoids and semisynthetic derivatives GPx-like effects	202
3.1.7	Synthetic scheme of cannabigerol derivatives	211
3.1.8	Conclusions	213
3.1.9	Experimental procedures	214

Conclusions **225**

References **227**

List of publications **252**

Acknowledgements **256**

Introduction

With the necessity to discover new and novel therapeutic leads, the plant kingdom, with its numerous bioactive compounds, can be considered a valuable source. Indeed, these secondary metabolites, which are produced by the plants to exert a wide range of effects on itself and other living organisms, exhibit noteworthy therapeutic properties, making them promising leads, both directly or as models for the design and semisynthesis of new drugs [1].

Discovering and creating therapeutics from natural substances found in plants is an intricate and diverse journey that encompasses several essential stages, each of which has a vital part in transforming the potential of natural metabolites into successful medications.

Due to the increase interest towards this field, innovative techniques for the identification, isolation and characterization of these molecules must be developed, featuring high-throughput biological screening assays for the preliminary evaluation of their activity.

It's also important to design new and sustainable methodologies to acquire plant materials, keeping in mind threatened and vulnerable species, and be able to scale up the supply of bioactive molecules without compromising biodiversity [2].

Coming from this, the Ph.D. research project was developed by involving multiple topics correlated with the natural world, including pharmaceutical chemistry, phytochemistry, pharmacognosy and other disciplines.

In the first phase the focus was on the development of innovative extraction techniques from complex matrices, mainly plants and foods. This has been done by using and comparing both traditional and innovative methodologies and by adhering to the concepts of “Green Extraction”, a branch of the “Green chemistry” principles focused on designing sustainable extraction methods of natural products [3]. For example, the valorisation of olive leaves, the main by-product generated during the production of olive oil [4], as an important source of bioactive compounds such as oleuropein and elenolic acid, or the use of *Gentiana lutea* leaves for the extraction of bitter active principles instead of the root, safekeeping this endangered species.

Indeed, the main goal was to obtain and pre-concentrate active compounds with pharmaceutical, nutraceutical and cosmeceutical approaches. This was achieved through solid phase adsorption (SPA) by using layered double hydroxides of variable composition, biocompatible lamellar clays with great potential for the selective extraction of active components, also representing a powerful tool to enhance their chemical stability, bioactivity and bioavailability, thus facilitating their use in multiple formulations.

Analytical techniques such HPLC-DAD-FLD, GC-MS, LC-MS and mono- and bi-dimensional NMR were used for the quantification but also for the characterization and structural elucidation of newly recovered and isolated secondary metabolites. These last parts were done at the University of Copenhagen, where, after building a high-resolution inhibition profile, 21 secondary metabolites were isolated from an Australian plant, *Eremophila tietkensii*, and identified through these techniques.

In addition, some of these compounds were discovered to be promising leads for the management of type 2 diabetes since they were able to successfully inhibit PTP1B, a negative intracellular regulator of insulin signalling pathway [5].

Moreover, in case of low concentration of a product in the plant matrix, the chemical synthesis of the natural compounds and of their semisynthetic derivatives was performed, by using non-toxic, non-polluting and affordable solvents and reagents.

The final part of the projects consisted in the set-up of novel and alternative chemical assays by using hyphenated techniques for the preliminary biological investigation of the GPx-like mimicking properties of the so recovered natural and semisynthetic products.

Chapter 1 - *Development of innovative extraction techniques*

Extraction is a fundamental process in natural products research, used to obtain and to separate bioactive compounds from a complex matrix, playing an important role in sample preparation procedures [6]. The extraction solvent, the particle size of the raw materials, the solvent-to-solid ration, the temperature and the duration are all crucial choices to evaluate when developing an extraction technique. These parameters can be modified specifically based on the raw starting materials, but also depending on the type of extraction process selected [7].

Nowadays these different methodologies can be separated in conventional and innovative extraction techniques, the latter developed to try and overcome the disadvantages of the more traditional processes, such as the need of larger volumes of solvents and longer and more tedious procedures [8]. First in the more conventional and traditional techniques we have maceration and digestion, two methods where the raw matrix (course or powdered) is left soaking in the extraction solvent at room temperature, in the first case, or by slightly warming the mixture, for the second. The aim is to break the plant's cells wall to release the soluble secondary metabolites, making the choice of the extraction solvent fundamental for the determination of the compounds that can be recovered. After a variable interval of time, the mixture can then be filtered.

Decoction uses the same principles but is more suitable for the extraction of heat-stable molecules, since the raw material is boiled in water for a precise

interval of time and then, after filtration, brought to the required volume of solution by adding cold water [9].

On the other hand, in the percolation process the plant material, shredded and packed in to a percolator, is continuously being passed through by the solvent, which is being pushed down by the addition of fresh volumes of it added to the instrument. In this way the solvent can penetrate the plant cells, gradually getting enriched in phytochemicals [10].

A continuous extraction is also employed in the Soxhlet apparatus, where the grounded sample is placed inside a porous bag. In a bottom flask the solvent is heated, generating vapours that go through the powdered material, reaching a condenser and then dripping back into the bottom flask to repeat the process [11].

However, these techniques are accompanied by a series of disadvantages, such as the need of high volumes of solvents, long processes, reduced selectivity and lower yields of extraction. This is linked with the development of the so called innovative or modern extraction technologies, having the aim of reducing those limitations.

Ultrasound-assisted extraction (UAE), for example, exploits the energy derived from ultrasounds to induce a phenomenon called cavitation, where the propagation of the sound waves in the solvent leads to a series of compression and rarefaction cycles, creating cavitation bubbles. As temperature and pressure rise in the system, these bubbles explode, forming hot spots that cause fragmentation, pore formation and swelling in the cellular matrix of the plant cells, leading to the solubilisation of the bioactive compound in the solvent [12-13].

In microwave-assisted extraction (MAE) the energy generated by the microwave radiations is used for warming the solvent and then the heat is transferred by conduction. The interaction causes the rotation of the dipoles of polar molecules, disrupting hydrogen bonds, ultimately leading to an enhancement of the migration of ions and of the penetration of the solvent in the plant cells wall. In case of non-polar solvents, the energy is transferred only through dielectric absorption [14-15].

Supercritical fluid extraction (SFE) is a technology that exploits the properties of supercritical fluids (SF), also known as dense-gasses, that have the physical characteristics of both gas and liquids at their critical point, which is determined both by pressure and temperature. The SF dissolves the molecules in the raw material while passing through a packed compartment in the apparatus. As the solvent leaves the extractor, the restoration of the temperature and pressure values causes the extract to become solvent free [16-17] The most famous SF is CO₂, when at temperature and pressure above 31.1 °C and 7380 kPa, since it's cheap, non-reactive, non-toxic and non-inflammable [18-19].

Another well-known technique is pressurized liquid extraction (PLE), where, by working with high temperature and pressure, there's an increase in the solubility of the active principles in the solvent. This is achieved by avoiding the boiling point, enhancing even more the liquid penetration in the sample matrix [20]. In multiple application, water was used as the solvent, in what can be called pressurized hot water extraction (PHWE) [21].

For plants where the separation of bioactive compounds is made complicated by the presence of a polysaccharide lignin systems, to which

they remain bound, a simple extraction process is not enough. In this case an enzyme-assisted extraction (EAE) is required. Specific enzymes, such as cellulase, pectinase, and amylase, are added to break down cellular walls and facilitate the procedure [22].

Although these innovative techniques surely overcome some of the more limiting steps of simpler processes such as maceration, decoction and so on, they do also have some disadvantages, such as the need for specialized and costly instruments and high energy consumption.

In addition, considering the great effort given in the last years for the development and design of green and sustainable extraction methods, it's important to follow directions given by the so called "Six Principles of Green Extraction of Natural Products" [23-24]. These principles, not to be followed as rules but as suggestions, are the following:

- "Principle 1: Innovation by selection of varieties and use of renewable plant resources."
- "Principle 2: Use of alternative solvents and principally water or agro-solvents."
- "Principle 3: Reduce energy consumption by energy recovery and using innovative technologies."
- "Principle 4: Production of co-products instead of waste to include the bio-and agro-refining industry."
- "Principle 5: Reduce unit operations and favour safe, robust and controlled processes."
- "Principle 6: Aim for a non-denatured and biodegradable extract without contaminants."

Specifically focusing on principle 3, for more innovative techniques such as MAE and PLE, although we can for sure notice a reduction in the process duration and the use of organic solvents, the energy consumption is still quite high, as shown in table 1.

Table 1. Different extraction techniques characteristics.

	Use of organic solvents	Energy consumption	Extraction time	Reusability	Automation
Soxhlet extraction	High	High	High	No	No
Ultrasound-assisted extraction	High	High	Low	No	No
Microwave-assisted extraction	Medium	High	Low	No	Yes
Pressurized liquid extraction	Low	High	Low	No	Yes
Supercritical fluid extraction	None	High	Medium	Yes	No

On the other hand, solid phase extraction (SPE) and solid phase adsorption (SPA) are now widely used and validated methods for the pre-concentration, extraction and purification of active compounds from complex matrices. The needed sorbents are mostly inexpensive, easily synthesized, easy to handle, recyclable and reusable. In addition, these processes can be combined with different detection methods, their energy consumption is extremely low, especially compared to other methods, and they guarantee rather high yields of extraction [25]. Table 2 summarises the main characteristics of SPE.

Table 2. Solid phase extraction (SPE) characteristics.

	Use of organic solvents	Energy consumption	Extraction time	Reusability	Automation
Solid phase extraction	Medium	Low	High	Yes	Yes

SPE and SPA are versatile techniques based on the repartition of the analytes of interest in a heterogeneous biphasic mixture, often a liquid solution of the plant matrix, and a solid phase, a sorbent. Retention of the compounds on the solids can be achieved mainly through Van Der Waals interactions, hydrogen bonds, dipole-dipole interactions and ionic interactions [26]. In addition, the chemical and physical properties of the solid sorbents can easily be modified or adapted to increase their affinity with the compounds of interest. Chemical synthesis and physical treatments can be performed to modify their particle size, internal spaces polarity, superficial area or, by introducing ionic moieties, changing their charge and polarity.

Indeed, the choice of the ideal solid sorbent is strictly dependant on the type of analyte to be extracted, but also for the purpose of the application. Nowadays the most used sorbents in SPE are the classic silica gel, or the C18 functionalized silica gel, copolymers of styrene- and divinylbenzene, activated carbon, graphene and carbon nanotubes [27-28-29-30-31]. These have a porous three-dimensional structure and a large superficial area. High selectivity towards different analyte can be achieved using sorbents such as molecular imprinted polymers, immunosorbents, nano-materials and clays [32].

However, the extraction capacity and selectivity of these supports can be limited, making the research of newer, efficient and versatile materials an important topic. Indeed, there's a great interest towards the multiple applications of SPE and SPA, especially in the pharmaceutical, cosmeceutical, nutraceutical and agricultural fields.

In this project the attention was focus on lamellar solids, microfine flowing powders modified by intercalation technologies that can act as vehiculation and controlled release systems of active ingredients and bioactive compounds. In the lamellar spaces of their structure, they can intercalate more than 50% of their weight in active compounds. These characteristics can be employed for the selective extraction of molecules from natural products, making them a valuable alternative to the typical sorbent used in SPE and SPA processes.

Due to their special layered structure and high biocompatibility, they can be used not only as excipients but also as active ingredients able to improve the formulation efficacy. They are employed as antacid and antipepsin, as absorbers to eliminate the excess of water, or as skin protectors for dermatological treatment [33]. All the solid sorbents tested in this project for their selective absorption capacity in regard of several active compounds from different raw matrices are reported in table 3.

Layered double hydroxides (LDH), also known as hydrotalcites or ionic clays, are a group of heterogeneous solids. They are bidimensional nanostructured materials having positively charged layers of metal hydroxides with charge balancing anions [34]. They can also host some water molecules in their interlamellar spaces (figure 1).

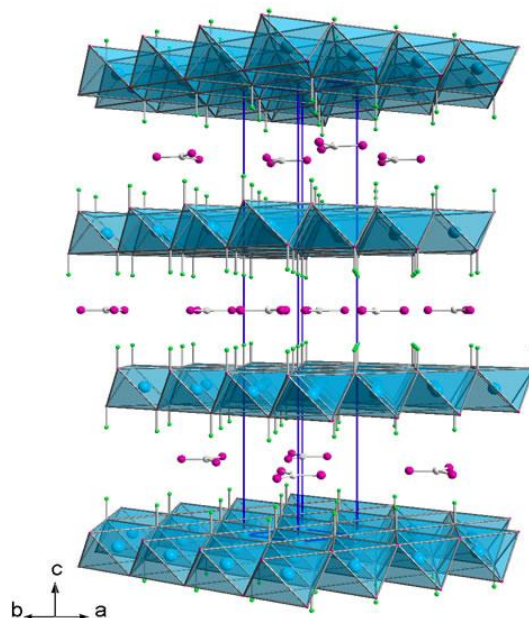
Table 3. List of inorganic and inorganic/organic solid sorbents studied for the selective absorption of multiple active principles from different raw matrices.

SOLID SORBENT	ENTRY
Layered double hydroxides	
Zinc aluminum oleate	A
Zinc aluminum nitrate	B
Zinc aluminum chloride	C
Magnesium aluminum nitrate	D
Magnesium aluminum azelate	E
Magnesium aluminum hydroxide chloride	F
Magnesium aluminum hydroxide acetate	G
Magnesium aluminum hydroxide carbonate	H
Magnesium aluminum acetate	I
Zinc hydroxide chloride	L
Lamellar solids	
Zirconium phosphate (type B)	M
Zirconium phosphate (type B) + octadecylamine	N
Oxides / Hydroxides	
Magnesium oxide	O
Magnesium hydroxide	P
Phyllosilicates	
Bentonite	Q
Talc	R
Mica L	S
Mica F	T
Mica SFG 20	U
Magnesium aluminum D. BenzenSulfonate SDS 01 H8L	V
Zinc aluminum D. BenzenSulfonate SDS 02 H8L	Z
Florisil ®	X
Alumina	Y

The insertion of specific metals into hydrotalcites structures produces highly selectivity and heterogeneity. They can intercalate a great variety of

molecules into their interlayer region, making them highly suitable to work as solid sorbent for SPE and SPA.

Figure 1. Layered double hydroxide (LDH) structure.



Hydrotalcite can easily be prepared on a laboratory scale, and their general formula is:

$$[M(II)_{1-x}M(III)_x(OH)_2]^{x+}[A_x^{n-}]^{x-} \times nH_2O$$

where M(II) is a bivalent metal cation, usually Mg or Zn, and M(III) is a trivalent metal cation, like Al. A^{n-} is an organic or inorganic anion, used as a counterion to the positive charge of the lamellar layers, while n indicates the water molecules moles [35].

Together with hydrotalcites, other solid supports were studied, some of them already well known, such as magnesium hydroxide and magnesium oxide, but also other lamellar structures like zirconium phosphate and phyllosilicates.

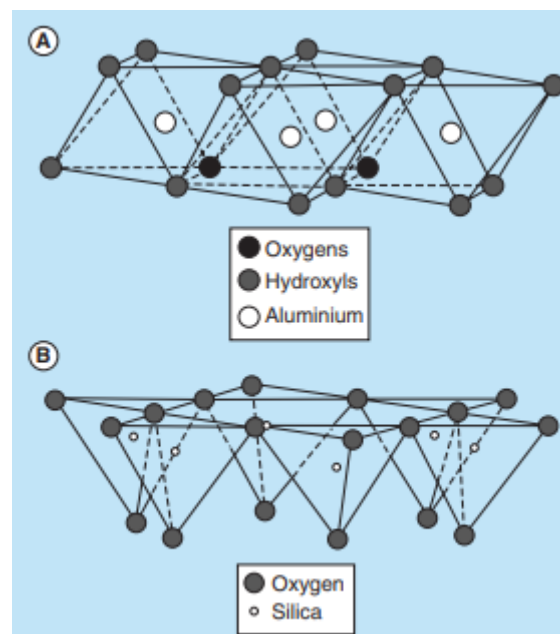
Zirconium phosphates and phosphonates are ionic exchangers and intercalating agents that possess very high stability against thermic, mechanic and irradiative stress and they're highly insoluble even in very acid not-complexant solutions [35].

Phyllosilicate are stacked polymeric substances of tetrahedral and octahedral sheet structures, with each layer having a different number of them, usually two, three or four. Every sheet structure can be formed either by tetrahedrons of silica (SiO_4^{4-}), shortened as "T", or by octahedrons of bivalent and trivalent cations, abbreviated as "O".

Usually, inside each tetrahedral and octahedral sheet structure, there's a smaller metal cation, and each apex is occupied by a hydroxide.

All the structural elements are rearranged to form a symmetrical hexagonal composition (Figure 2).

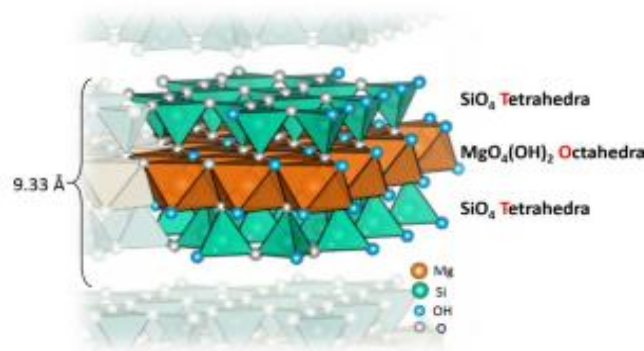
Figure 2. Octahedral (A) and tetrahedral (B) sheets structures.



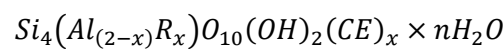
Based on the coordination between the “T” and “O” sheets, and the type of cation inside each layer, there are numerous combinations that are used to classify phyllosilicates, for example mineral clays like talk and bentonite or micas [36].

Mineral clays are usually organised in structures with T-O (1:1), T-O-T (2:1) or T-O-T-O (2:1:1) layers. Between them, talc, $\text{Mg}_3\text{Si}_4\text{O}_{10}(\text{OH})_2$, has the simpler T-O-T layered structure [37] (Figure 3).

Figure 3. Talc structure.

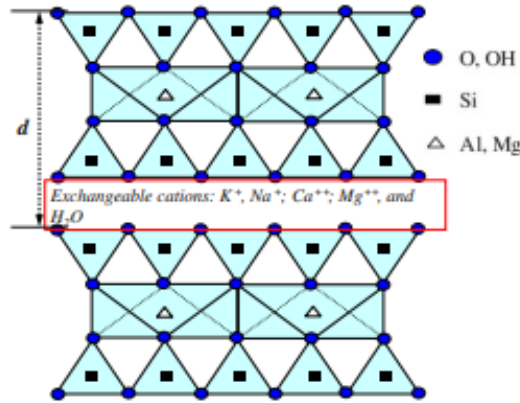


Another mineral clay with T-O-T layers is bentonite, but in this case some Si^{4+} ions in the tetrahedral sheets are replaced by Al^{3+} , while bivalent cations like Fe^{2+} and Mg^{2+} substitute Al^{3+} cations in the octahedral sheets. These substitutions dictate a negative charge that must be counteracted by the presence of cations like Na^+ , Ca^{2+} , Mg^{2+} and water molecules in the interlayer sheets. Its general formula is:

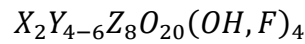


where CE indicates an interchangeable cation like Na^+ , Ca^{2+} , and Mg^{2+} and R_x represents Mg, Fe, Mn, Zn or Ni [38] (Figure 4).

Figure 4. Bentonite structure.

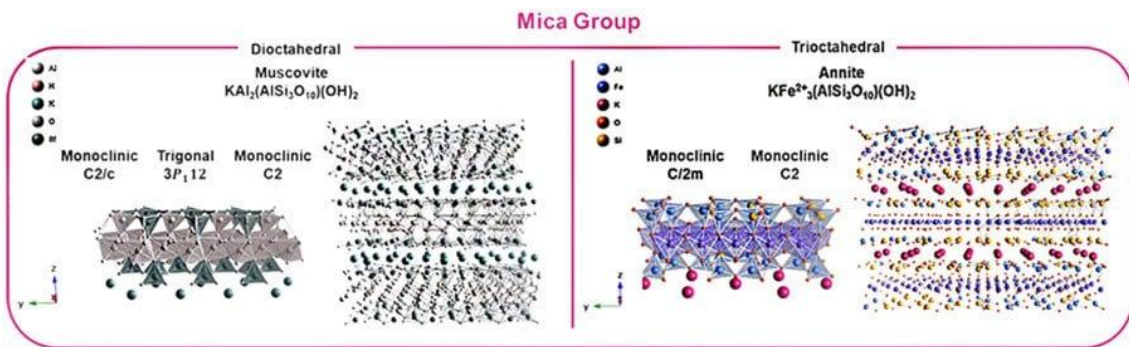


A T-O-T structure is also typical of micas, but for them the charge imbalance is usually counteracted by the presence of K^+ as the interlayer cations. Their general formula is:



Where X is K, Na or Ca, Y is Al, Mg or Fe and Z is Si or Al. Based on Y , micas can be dioctahedral ($Y = 4$), like muscovite, or trioctahedral ($Y = 6$), like lepidolite [39] (figure 5).

Figure 5. Structure of two different micas.



Considering the great potential of layered double hydroxides and of the other lamellar solids for the intercalation and adsorption of active compounds

from complex matrices, part of this project was focused on evaluating their efficiency in selectively extract multiple compounds with different fields of applications in mind.

The workflow for the treatment with the solids was different for each of the matrices considered, but the main steps included were:

Pre-treatment of the raw starting materials: Grounding or shredding of the dried plants, or treatment of the food matrices.

Pre-extraction of the raw starting materials: Directly in water if feasible, or with other solvents like MeOH or EtOH. At the beginning this step consisted in a simple maceration, but later more innovative processes such as MAE and UAE were employed.

HPLC analysis of the matrix: Directly after filtration in case of aqueous extracts, while for other solvents the extract was evaporated to dryness after filtration and re-suspend in water to a concentration for the analysis. This step was needed to quantify the concentration of bioactive molecules in the whole extract.

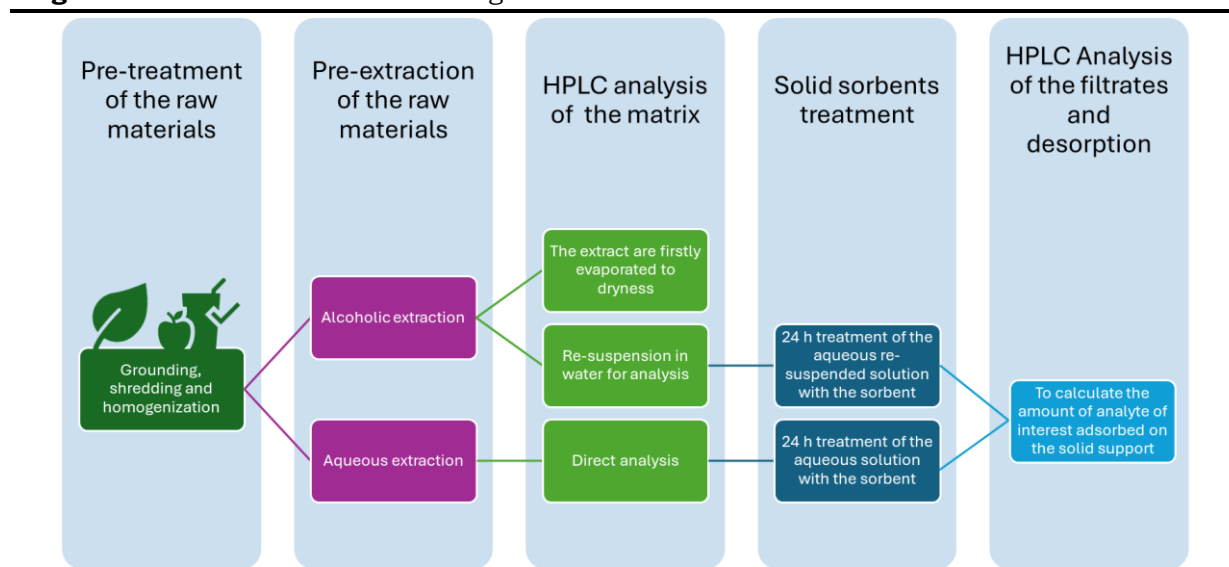
Treatment with the solid sorbents: Different quantities of solid supports were added to the aqueous solutions of the extracts, re-suspended in water when necessary, and were left under magnetic stirring at room temperature for fixed intervals of time. This was followed by filtration under vacuum and centrifugation.

HPLC analysis of the filtrates: This step allowed us to calculate the amount of analyte of interest adsorbed on the solid support as a percentage difference in the quantity of compound present in the raw extract and that present in the filtrates as determined by HPLC.

Desorption: The desorption process varied based on the nature of the analyte but was fundamental for double checking the absorption capacity of the solid and its reusability.

A schematic representation of this general workflow is reported in figure 6.

Figure 6. Solid sorbents treatment general workflow.



Five were the applications considered for the evaluation of the selective extraction abilities of the solid sorbents in study: the concentration of pomegranate juices to gain higher antioxidant activity, the removal of unwanted azo-dyes from waste waters and from foods and beverages, the valorisation of olive leaves as a source of oleuropein and the use of leaves of *Gentiana lutea*, instead of the roots, to recover bitter principles.

1.1 Concentration of pomegranate juice for an enhanced antioxidant activity

Oxidative stress can be caused by the excessive production of ROS (Reactive oxygen species), that can induce damage to DNA, proteins and lipids, leading to the development of several pathological conditions and diseases, like cancer, cardiovascular diseases, neurological disorders, and so on [40-41-42-43-44].

To help restore redox homeostasis and counteract ROS damages, antioxidants are fundamental. They were defined by Khlebnikov et al. as “any substance that directly scavenges ROS or indirectly acts to up-regulate antioxidant defences or inhibit ROS production”, followed also by their ability to form a new and more stable radical on the next oxidation after its scavenging activity [45-46]. Antioxidants act as cell’s defence mechanism, and they can be classified as endogenous or exogenous.

The endogenous one can additionally be distinguished in non-enzymatic, like glutathione, coenzyme Q, ferritin and so on, and enzymatic, such as glutathione peroxidases, superoxide dismutase, catalases, etc.. Glutathione and glutathione peroxidases will be discussed in more detail in next chapters. ROS can also be counteracted by exogenous antioxidants, like carotenoids, polyphenols, tocopherols, flavonoids, vitamins, and many others, that can be introduced through diet, pharmaceuticals, nutraceuticals and food supplements. Indeed, consuming plants and foods rich in antioxidants, has been shown to greatly improve the human’s body capacity to endure oxidative stress, significantly reducing the pathogenesis of the aforementioned diseases [47-48].

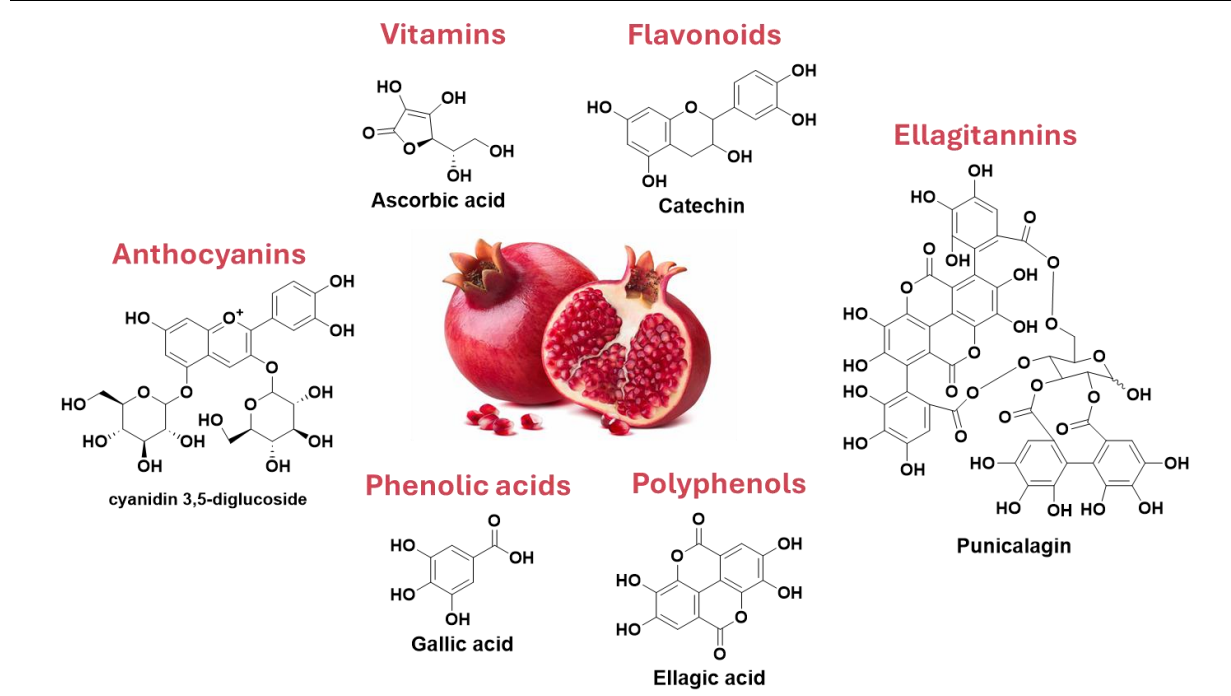
In this context, the use of both food supplements, as agents to compensate deficiencies in micro- and/or macronutrients, and nutraceuticals, born from the combinations of the words “nutrition” and “pharmaceutical” and with the aim to offer both nutritional supplementation and health-preventive effects, is highly beneficial [49].

Thus, the creation of new preparations with a high concentration of bioactive compounds, using plants and food plants with strong and improved antioxidant properties is of great importance in the nutraceutical research.

Since lamellar solids can effectively extract numerous secondary metabolites from matrices, the aim of this project was to evaluate their efficiency in pre-concentrating antioxidant active principles from pomegranate juice, with the aim of obtaining a potential novel nutraceutical formulation with enriched polyphenol content.

Pomegranate, the fruit of *Punica granatum* L., tree of the *Punicaceae* family, was chosen as the food matrix of this study thanks to wide arrays of biological properties ascribed to its extract [50]. The anticancer, cardioprotective, anti-inflammatory and neuroprotective activities, but also the protective effects on blood glucose levels and blood pressure and its prebiotic effects, can all be linked to the fruit richness in antioxidant compounds, like ascorbic acid, flavonoids, ellagitannins, anthocyanins, phenolic acids and other polyphenols (figure 7) [51].

Figure 7. *Punica granatum* L. fruit and its main antioxidant phytochemicals.



For this reason, the use of pomegranate extract as an ingredient in nutraceuticals and functional foods has been quite popular in recent years [52].

1.1.1 Plant material

Pomegranate fruits were bought from a local market in the Abruzzo region, Italy (voucher specimen POM-2021-1 stored in our laboratory). All the fruits arils were pressed, and the so obtained juice was filtered and lyophilized.

1.1.2 Extraction procedure and results

The first extraction step consisted in the treatment of 20 mL aliquots of 500 mg of freeze-dried pomegranate powder aqueous solutions with 1 g of each of the solid sorbents listed in table 4.

Being one of the first studies in the series of research activities on this topic, the number of lamellar solids we were in possession of was limited to the 13 listed in table 4.

Table 4. List of inorganic and inorganic/organic solid sorbents studied for the concentration of pomegranate juice.

SOLID SORBENT	ENTRY
Layered double hydroxides	
Zinc aluminum oleate	A
Zinc aluminum nitrate	B
Zinc aluminum chloride	C
Magnesium aluminum nitrate	D
Magnesium aluminum azelate	E
Magnesium aluminum hydroxide chloride	F
Magnesium aluminum hydroxide acetate	G
Magnesium aluminum hydroxide carbonate	H
Magnesium aluminum acetate	I
Zinc hydroxide chloride	L
Lamellar solids	
Zirconium phosphate (type B)	M
Zirconium phosphate (type B) + octadecylamine	N
Phyllosilicates	
Bentonite	Q

The resulting mixtures were kept under magnetic stirring at room temperature for 24 hours and then filtered to recover the powder. This simple process led to a complete discoloration of the filtrates, especially for entries D to H, L and O, so for magnesium based hydrotalcites, zinc hydroxide chloride and bentonite. The solids instead, appeared to be bright blue, the typical colour assumed by anthocyanins in a basic environment, like the one provided by most of the sorbents in consideration.

The blue powders were dried, collected and then desorbed with 15 mL of a 5% solution of citric acid and then filtered again. All the filtrates, now of a bright red colour because of the acidic environment, were then freeze-dried, to obtain a powder to be used for the quantifications and antioxidant activities tests.

Firstly, methods like the Folin-Ciocalteu, the AlCl_3 , and the pH-differential assays were performed to quantify the content of total phenols, flavonoids and anthocyanins, expressed as mg GAE/g dry powder, mg QE/g, and mg CGCE/g, respectively.

All the results regarding the most active lamellar solids, entries D to H, L and O, that granted a complete bleaching of the juice, are reported in table 5, while for the missing entries we recorded result that were totally comparable to the one obtained with the parent matrix.

Table 5. Concentrated pomegranate juice powder total phenols, flavonoids and anthocyanins content.

ENTRY	Total phenolics (mg GAE/g)*	Total flavonoids (mg QE/g)*	Total anthocyanins (mg CGCE/g)*
Parent matrix	176.9 ± 9.33	24.1 ± 1.21	190.6 ± 3.28
D	387.2 ± 7.11	44.6 ± 0.97	212.4 ± 4.01
E	536.8 ± 12.44	84.9 ± 2.66	366.1 ± 6.77
F	301.8 ± 4.56	39.7 ± 1.41	233.1 ± 3.99
G	223.4 ± 4.08	29.9 ± 3.02	200.3 ± 2.96
H	237.8 ± 6.51	31.7 ± 2.14	222.1 ± 3.88
L	298.4 ± 6.02	38.7 ± 0.88	264.9 ± 5.06
O	199.8 ± 2.49	26.9 ± 0.71	199.1 ± 2.37

*Values expressed as mean ± SD (n = 3).

Comparing the results obtained by performing the test on the freeze-dried pomegranate juice and on the powder obtained after the treatment with each

of the solids, significant higher concentrations of all three of the secondary metabolite classes were recorded. It's especially true for magnesium aluminum azelate (entry E), that appeared to be the most efficient, with an increase in the content of total phenolics, flavonoids and anthocyanins of 203%, 252% and 92% respectively. This is mostly likely linked to the chemical structure of these bioactive compounds, which are rich in -OH moieties that can easily be coordinated with Mg^{+2} ions, thus remaining retained on the solid [53].

To further evaluate if this concentration procedure was able to efficiently enrich and enhance the antioxidant capacity of the juice the Trolox equivalent antioxidant capacity (TEAC) coupled with ABTS decolorization assay was performed. The results, always referring to powders obtained with the most active sorbents, are reported in table 6.

Comparing to the parent matrix, Table 6 clearly show how the solid treatment was effective in improving the antioxidant activity, especially for magnesium aluminum azelate (entry E), magnesium aluminum hydroxide chloride (entry F) and zinc hydroxy chloride (entry L). Indeed, increases in the antioxidant effects, recorded for all the concentration values, were in the range of 1.9% and 48.8%.

As the last step of the study, desorption experiments were performed, to evaluate the stabilizing effect of the lamellar solids on the antioxidant compounds and thus their activity. After desorbing at 24 h, 48 h and 96 h, the same antioxidant assays were performed again, and the results are listed in table 7.

Table 6. Pomegranate juice powder TEAC and ABTS^{••} assay results.

% Inhibition*								
$\mu\text{g/mL}$	Parent matrix	D	E	F	G	H	L	O
5	3.8 $\pm$ 0.01	6.2 $\pm$ 0.02	8.4 $\pm$ 0.03	8.0 $\pm$ 0.02	4.3 $\pm$ 0.01	4.6 $\pm$ 0.05	8.1 $\pm$ 0.06	3.9 $\pm$ 0.02
10	7.2 $\pm$ 0.04	11.4 $\pm$ 0.05	12.3 $\pm$ 0.07	11.7 $\pm$ 0.04	8.3 $\pm$ 0.04	8.9 $\pm$ 0.01	11.8 $\pm$ 0.05	7.4 $\pm$ 0.02
25	14 $\pm$ 0.04	31.7 $\pm$ 0.08	35.9 $\pm$ 0.07	32.7 $\pm$ 0.04	15.9 $\pm$ 0.02	17.1 $\pm$ 0.05	33.5 $\pm$ 0.05	14.2 $\pm$ 0.01
50	32.7 $\pm$ 0.07	56.7 $\pm$ 0.02	65.8 $\pm$ 0.06	59.2 $\pm$ 0.03	35.8 $\pm$ 0.04	38.2 $\pm$ 0.01	62.1 $\pm$ 0.06	33.9 $\pm$ 0.03
75	50 $\pm$ 0.06	71.6 $\pm$ 0.03	94.7 $\pm$ 0.09	74.7 $\pm$ 0.02	54.7 $\pm$ 0.08	59.2 $\pm$ 0.03	76.2 $\pm$ 0.01	51.8 $\pm$ 0.06
100	62.6 $\pm$ 0.04	95.4 $\pm$ 0.11	100 $\pm$ 0.01	93.9 $\pm$ 0.05	66.9 $\pm$ 0.02	69.6 $\pm$ 0.03	96.2 $\pm$ 0.04	64.2 $\pm$ 0.01
150	73.4 $\pm$ 0.03	100 $\pm$ 0.01	100 $\pm$ 0.03	100 $\pm$ 0.03	78.4 $\pm$ 0.04	80.1 $\pm$ 0.01	100 $\pm$ 0.01	75.1 $\pm$ 0.04
250	79.6 $\pm$ 0.03	100 $\pm$ 0.02	100 $\pm$ 0.01	100 $\pm$ 0.01	85.9 $\pm$ 0.02	87.6 $\pm$ 0.04	100 $\pm$ 0.01	81.2 $\pm$ 0.04
300	89.1 $\pm$ 0.01	100 $\pm$ 0.04	100 $\pm$ 0.01	100 $\pm$ 0.01	93.2 $\pm$ 0.02	95.9 $\pm$ 0.01	100 $\pm$ 0.01	92.4 $\pm$ 0.02
500	98.3 $\pm$ 0.05	100 $\pm$ 0.01	100 $\pm$ 0.01	100 $\pm$ 0.01	100 $\pm$ 0.01	100 $\pm$ 0.02	100 $\pm$ 0.02	99.9 $\pm$ 0.04
TEAC	149.1 $\pm$ 0.12	185.6 $\pm$ 0.21	221.9 $\pm$ 0.16	208.2 $\pm$ 0.09	161.1 $\pm$ 0.12	164.0 $\pm$ 0.08	207.9 $\pm$ 0.11	152 $\pm$ 0.09

*Values expressed as mean $\pm$ SD (n = 3).

Table 7. Antioxidant activities of treated pomegranate powder after desorption at 0 h, 24 h, 48 h, and 96h.

% Inhibition*				
Entry D (Magnesium aluminum nitrate)				
µg/mL	0 h	24 h	48 h	96 h
5	6.2 ± 0.02	6.4 ± 0.03	6.1 ± 0.02	6.2 ± 0.02
10	11.4 ± 0.05	11.9 ± 0.04	11.2 ± 0.05	11.4 ± 0.05
25	31.7 ± 0.08	30.8 ± 0.06	30.5 ± 0.04	31.7 ± 0.08
50	56.7 ± 0.02	55.3 ± 0.06	55.2 ± 0.04	56.7 ± 0.02
75	71.6 ± 0.03	70.1 ± 0.04	69.3 ± 0.03	71.6 ± 0.03
100	95.4 ± 0.11	93.2 ± 0.09	93.1 ± 0.08	95.4 ± 0.11
150	100 ± 0.01	96.8 ± 0.02	96.4 ± 0.06	100 ± 0.01
250	100 ± 0.02	99.9 ± 0.06	99.4 ± 0.05	100 ± 0.02
300	100 ± 0.04	100 ± 0.03	100 ± 0.04	100 ± 0.04
500	100 ± 0.01	100 ± 0.02	100 ± 0.05	100 ± 0.01
Entry E (Magnesium aluminum azelate)				
	0 h	24 h	48 h	96 h
5	8.4 ± 0.03	8.7 ± 0.02	8.3 ± 0.01	7.6 ± 0.02
10	12.3 ± 0.07	12.9 ± 0.04	12.1 ± 0.06	11.0 ± 0.04
25	35.9 ± 0.07	36.5 ± 0.05	35.4 ± 0.04	34.1 ± 0.03
50	65.8 ± 0.06	66.7 ± 0.04	65.2 ± 0.03	64.5 ± 0.05
75	94.7 ± 0.09	95.0 ± 0.07	94.1 ± 0.03	92.6 ± 0.04
100	100 ± 0.01	100 ± 0.02	99.8 ± 0.03	96.3 ± 0.01
150	100 ± 0.03	100 ± 0.02	100 ± 0.01	98.2 ± 0.02
250	100 ± 0.01	100 ± 0.02	100 ± 0.01	100 ± 0.02
300	100 ± 0.01	100 ± 0.03	100 ± 0.01	100 ± 0.03
500	100 ± 0.01	100 ± 0.03	100 ± 0.01	100 ± 0.02
Entry F (Magnesium aluminum hydroxide chloride)				
	0 h	24 h	48 h	96 h
5	8.0 ± 0.02	7.8 ± 0.03	7.5 ± 0.02	7.4 ± 0.02
10	11.7 ± 0.04	11.2 ± 0.02	10.5 ± 0.03	10.2 ± 0.01
25	32.7 ± 0.04	31.1 ± 0.02	30.5 ± 0.05	30.0 ± 0.03
50	59.2 ± 0.03	57.6 ± 0.04	57.1 ± 0.02	55.4 ± 0.02
75	74.7 ± 0.02	72.9 ± 0.06	70.1 ± 0.02	68.3 ± 0.05
100	93.9 ± 0.05	91.8 ± 0.03	89.6 ± 0.04	87.2 ± 0.04
150	100 ± 0.03	98.3 ± 0.02	96.9 ± 0.04	95.2 ± 0.01
250	100 ± 0.01	99.8 ± 0.01	97.4 ± 0.05	97.1 ± 0.04
300	100 ± 0.01	100 ± 0.02	99.6 ± 0.02	98.2 ± 0.04
500	100 ± 0.01	100 ± 0.01	100 ± 0.02	99.3 ± 0.05

Table 7 (continued). Antioxidant activities of treated pomegranate powder after desorption at 0 h, 24 h, 48 h, and 96h.

Entry G (Magnesium aluminum hydroxide acetate)				
	0 h	24 h	48 h	96 h
5	4.3 ± 0.01	4.2 ± 0.02	4.2 ± 0.02	3.7 ± 0.03
10	8.3 ± 0.04	7.9 ± 0.03	7.4 ± 0.04	7.0 ± 0.02
25	15.9 ± 0.02	14.6 ± 0.03	14.0 ± 0.03	13.1 ± 0.01
50	35.8 ± 0.04	33.9 ± 0.02	34.6 ± 0.03	33.2 ± 0.02
75	54.7 ± 0.08	53.4 ± 0.06	52.1 ± 0.01	50.6 ± 0.04
100	66.9 ± 0.02	64.1 ± 0.05	61.9 ± 0.07	59.4 ± 0.03
150	78.4 ± 0.04	73.4 ± 0.02	70.8 ± 0.02	68.5 ± 0.05
250	85.9 ± 0.02	79.9 ± 0.05	76.2 ± 0.04	72.4 ± 0.03
300	93.2 ± 0.02	88.6 ± 0.05	85.1 ± 0.04	81.1 ± 0.05
500	100 ± 0.01	94.7 ± 0.05	91.1 ± 0.05	86.4 ± 0.03
Entry H (Magnesium aluminum hydroxide carbonate)				
	0 h	24 h	48 h	96 h
5	4.6 ± 0.05	4.4 ± 0.03	4.1 ± 0.02	3.8 ± 0.03
10	8.9 ± 0.01	8.6 ± 0.04	8.4 ± 0.02	7.9 ± 0.02
25	17.1 ± 0.05	16.4 ± 0.04	15.9 ± 0.04	14.9 ± 0.01
50	38.2 ± 0.01	37.0 ± 0.04	35.9 ± 0.05	34.6 ± 0.07
75	59.2 ± 0.03	57.4 ± 0.03	55.8 ± 0.03	53.4 ± 0.03
100	69.6 ± 0.03	67.1 ± 0.04	64.5 ± 0.04	61.7 ± 0.01
150	80.1 ± 0.01	77.2 ± 0.03	75.0 ± 0.02	72.4 ± 0.02
250	87.6 ± 0.04	82.4 ± 0.02	80.1 ± 0.02	76.5 ± 0.01
300	95.9 ± 0.01	90.1 ± 0.06	87.6 ± 0.03	82.2 ± 0.05
500	100 ± 0.02	95.4 ± 0.03	93.1 ± 0.03	88.7 ± 0.04
Entry L (Zinc hydroxy chloride)				
	0 h	24 h	48 h	96 h
5	8.1 ± 0.06	8.0 ± 0.04	7.8 ± 0.02	7.7 ± 0.02
10	11.8 ± 0.05	11.2 ± 0.03	10.7 ± 0.03	10.0 ± 0.02
25	33.5 ± 0.05	32.1 ± 0.04	31.3 ± 0.01	30.6 ± 0.01
50	62.1 ± 0.06	61.0 ± 0.04	59.4 ± 0.03	57.4 ± 0.02
75	76.2 ± 0.01	74.1 ± 0.02	71.6 ± 0.02	69.2 ± 0.03
100	96.2 ± 0.04	93.1 ± 0.01	89.4 ± 0.03	85.6 ± 0.02
150	100 ± 0.01	95.0 ± 0.04	91.2 ± 0.01	87.4 ± 0.04
250	100 ± 0.01	97.2 ± 0.02	92.8 ± 0.04	89.7 ± 0.03
300	100 ± 0.01	99.1 ± 0.04	94.6 ± 0.04	91.2 ± 0.02
500	100 ± 0.02	100 ± 0.01	97.1 ± 0.03	93.4 ± 0.04

Table 7 (continued). Antioxidant activities of treated pomegranate powder after desorption at 0 h, 24 h, 48 h, and 96h.

	Entry O (Bentonite)			
	0 h	24 h	48 h	96 h
5	3.9 ± 0.02	3.7 ± 0.03	3.6 ± 0.01	3.3 ± 0.01
10	7.4 ± 0.02	7.1 ± 0.03	6.5 ± 0.04	6.2 ± 0.01
25	14.2 ± 0.01	13.6 ± 0.05	13.0 ± 0.03	12.4 ± 0.02
50	33.9 ± 0.03	32.4 ± 0.01	30.6 ± 0.02	28.4 ± 0.05
75	51.8 ± 0.06	49.4 ± 0.04	47.2 ± 0.01	44.7 ± 0.04
100	64.2 ± 0.01	61.6 ± 0.04	59.3 ± 0.05	56.3 ± 0.04
150	75.1 ± 0.04	71.0 ± 0.06	68.4 ± 0.03	64.3 ± 0.05
250	81.2 ± 0.04	76.9 ± 0.03	71.8 ± 0.02	69.6 ± 0.01
300	92.4 ± 0.02	87.0 ± 0.02	83.3 ± 0.01	77.9 ± 0.05
500	99.9 ± 0.04	92.6 ± 0.03	88.4 ± 0.05	84.1 ± 0.02

*Values expressed as mean ± SD (n = 3).

These results clearly indicate how there's a delay in the chemical degradation of the polyphenols fraction of the fruits, granting a stabilizing effect on those compounds. Of course, especially at the activity evaluated after the desorption at 96 h, there's a small loss of antioxidant capacities, but that still did not exceed 10 % of the values obtained after the immediate analysis.

1.1.3 Conclusions

Considering the richness of pomegranate juice in thermolabile phytochemicals like polyphenols, this methodology allowed to perform an efficient concentration, without their degradation, overcoming some of the limitation associated with more conventional plant extraction methods. At the same time, the enhanced antioxidant and radical scavenging capacities of the successfully concentrated powder, together with its improved stability

over a long period of time, will provide a potentially novel nutraceutical formulation.

1.1.4 Experimental procedures

Chemicals

Citric acid (purity grade $\geq 99.5\%$), 2,2'-azino-bis(3-ethyl- benzothiazoline)-6-sulfonic acid (ABTS) (purity grade $\geq 98.0\%$), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) (purity grade $\geq 97.0\%$), potassium persulfate (purity grade $\geq 99.0\%$), ethanol (HPLC grade), methanol (HPLC grade), Folin Ciocalteu reagent, gallic acid (purity grade $\geq 97.5\%$), quercetin (purity grade $\geq 95.0\%$), aluminum trichloride hexahydrate (purity grade $\geq 99.0\%$), sodium carbonate decahydrate (purity grade $\geq 99.0\%$), cyanidin-3-glucoside chloride (USP reference standard) were purchased from Merck Sigma- Aldrich (Milan, Italy). Water ($>18.2 \text{ M}\Omega \text{ cm}$ resistivity) was obtained by passage through an Elix 3 and Milli-Q purification system (Millipore, Bedford, MA, USA). All solid sorbents were donated by Prolabin & Tefarm Srl (Perugia, Italy).

Total phenols, flavonoids and anthocyanins content

A Varian Cary 50 Uv-Vis spectrophotometer (Agilent Technologies, Santa Clara, CA, USA) was used for all the readings.

The total phenolic content was calculated as mg of gallic acid equivalents (GAE)/g dry powder. The calibration curve of the standard was drawn on 8 points in the concentration range of 5 – 700 $\mu\text{g/L}$ ($y = 0.0031x - 0.0063$, $r^2 = 0.9992$). The sample solution was prepared by dissolving 20 mg of the

freeze-dried powders in 5 mL of MeOH. A 0.2 mL solution of the test sample was diluted with distilled H₂O to a final volume of 0.6 mL and then 0.2 mL solution of a 1:1 Folin-Ciocalteaus reagent mix was added.

The resulting mixture was allowed to react for 5 min., then a saturated solution of Na₂CO₃ (1.0 mL) was added, finally diluting with H₂O to a total volume of 3.0 mL. This solution was kept in the dark for 30 min., centrifuged, and subsequently the absorbance was determined at 765 nm.

For the total flavonoids content, the calibration curve of quercetin, used as the standard, was obtained by plotting 8 concentration points in the range of 5 – 200 µg/mL ($y = 0.0152x + 0.0051$, $r^2 = 0.9988$) dissolving 5.0 mg of the pure standard in 1 mL of H₂O and diluting it.

The quantitative assay was carried out by adding the diluted quercetin solutions or the freeze-dried pomegranate powder one (0.6 mL) with the same volume of a 2 % w/v solution AlCl₃. The resulting mixture was allowed to react for 60 min. at room temperature, and finally the absorbance was read against the blank at 420 nm. The total flavonoid content was expressed as mg of quercetin equivalent (QE)/g dry powder.

Both methodologies were assessed by following Chandra and Coworkers procedures [54].

The total anthocyanin content was determined using the pH-differential method [55]. The calibration curve of cyanidin-3-glucoside chloride, used as the reference standard, was obtained by plotting 8 concentration points in the range of 5–500 µg/mL ($y = 0.097x + 0.0044$, $r^2 = 0.9989$).

For each sample solutions aliquot of 0.2 mL, one dilution with equal volumes of a KCl buffer pH 1.0 and another with AcONa buffer pH 4.5 were

prepared. The resulting mixtures were diluted to a final volume of 1.0 mL and kept for 15 min. at room temperature and then the absorbance was determined at 510 nm to assess the anthocyanin content and at 700 nm for haze correction. The desired parameter was expressed as mg of cyanidin-3-glucoside chloride (CGCE) equivalents/g dry powder using the equations reported in the literature.

Trolox equivalent antioxidant capacity (TEAC)/ABTS assay

ABTS was dissolved in distilled H₂O to a 7 mM concentration. The radical cation ABTS⁺• was produced by the reaction between the stock aqueous solution (5 mL) of ABTS and an aqueous solution of K₂S₂O₈ (88 µL, 140 mM). The resulting mixture was kept in the dark at room temperature for 12–16 h. The sample containing the ABTS⁺• solution was diluted with EtOH to have an absorbance of 0.700 (±0.02) at 734 nm.

To accomplish the assay, to 2.97 mL of this solution 30 µL of the solution of pomegranate freeze-dried powder deriving from the treatment with individual clays at selected concentrations (5, 10, 25, 50, 75, 100, 150, 250, 300, 500 µL each diluted to 1 mL final volume with distilled H₂O) were added. The absorbance at the same wavelength was read rigorously 2 min. after initial mixing. The ABTS⁺• scavenging activity was calculated by the following equation.

$$\% inhibition = \left[\frac{(A_B - A_A)}{A_B} \right] \times 100$$

where A_A and A_B represent the absorbance values of the ABTS⁺• solution with and without the test samples, respectively. Trolox was used as the

standard and the antioxidant capacities of pomegranate juice fractions expressed as μM values of Trolox equivalent per samples volume. This procedure was accomplished by following Re et al. method [56].

1.2 Reducing environmental pollution through solid phase adsorption

Synthetic dyes represent a huge concern in regards of environmental pollution [57]. Various industrial sites such as textile, paper manufacturing, dyeing fabrics, printing, but also pharmaceutical, food and cosmetics, use them in favour of natural dyes because of their cost-effectiveness, easy availability, bright colours and stability [58]. However, this increasing dependence on synthetic dyes raises significant environmental and health concerns, especially when huge quantities of waste fluids are directly discharged in the environment. Indeed, despite the undeniable importance of the textile industries, they represent one of the biggest pollutants worldwide, with both the use of drinking water in various of their operations and the discharge of untreated effluents into water bodies [59-60]. Consequently, their uncontrolled diffusion denotes a great risk, not just for human health, but even for the soil and the aquatic ecosystems [61].

They do not only cause an aesthetic damage to water through coloration, but they also limit photosynthetic reaction and oxygen levels, affecting all aquatic life forms, moreover, acting as toxic, mutagenic and carcinogenic agents [62-62-64-65]. Addressing the ecological implications of dye effluents is crucial for safeguarding biodiversity and ecosystem health. The development of eco-friendly and cost-effective solutions to manage these effluents is a global priority, which is made difficult by their complex and large chemical structure.

Currently, the removal of dyes from water is often achieved using both physical and biological methods. For example, chemical oxidation, in which

a $\text{Cu}^{2+}\text{-HCO}_3^-$ system was left to react with H_2O_2 to produce a higher oxidation state of copper, Cu(III) , as the reactive species responsible for the elimination of organic dyes in aqueous solution [66]. Membrane based wastewater treatment processes, which include microfiltration, ultrafiltration, nanofiltration, reverse osmosis or hybridization of two or more, can be a valid alternative [67]. Another possibility are electrochemical processes, like the one by Candia-Onfray and Coworkers where they studied the effect of four electrocatalytic materials (Boron doped diamond (BDD), PbO_2 , $\text{Ti/Ru}_{0.3}\text{Ti}_{0.7}\text{O}_2$ and Ti/Pt) for the electro-oxidation of the industrial textile dye DRBG [68].

These methodologies, while capable of decolorizing azo dyes, are limited by high costs, energy demands, specific instrumentations, the production of byproducts and are, in most cases, hard to perform. Their effectiveness in detoxifying dye effluents remains questionable and limited [69].

On the other hand, as already discussed, adsorption-based methodologies can represent a valid and effective alternative, since they can be safe, precise, easy to handle, rapid and accurate. There's also a great number of solid sorbent available, that can also be modified based on the target dye. Indeed, a great number of solid materials have been used in recent years for azo dyes removal like graphene, silica, alumina, chitosan, polystyrene and polysulfone/montmorillonite composites and so on [70-71-72-73-74-75].

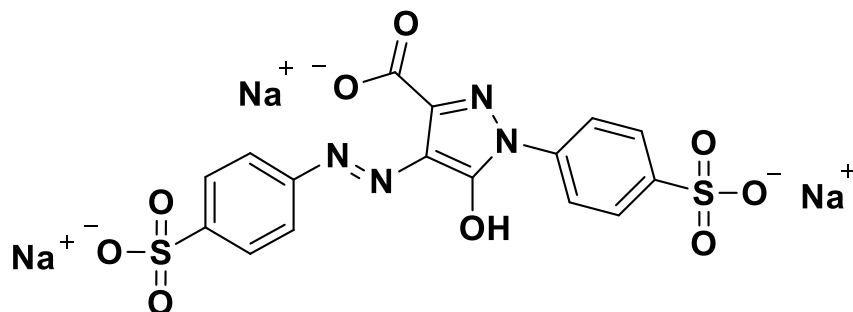
And although these solids are quite versatile and can be used in numerous adsorption procedures, there are some disadvantages that are linked to an incomplete elimination of the dye, followed by the production of byproducts due to the occurrence of secondary reactions [76]. In addition, it's important

to develop methods that are easy to handle and that do not require further modifications of the experimental conditions, like temperature and pH, and that can also be upgraded to be reproduced on a larger scale.

So in search of what can be considered the “ideal sorbent”, the one that can achieve a complete removal of azo dyes with zero environmental impact, this project was developed to try and evaluate the adsorption capacities of a panel of 25 solid sorbents, the ones listed in table 3 plus silica (entry K) and montmorillonite (entry J), for the efficient elimination of azo dyes from aqueous solutions.

Tartrazine (TZ) [(trisodium 1-(4-sulfonatophenyl)-4-(4-sulfonatophenylazo)-5-pyrazolone-3-carboxylate)], also known as E102, Yellow 5, Acid Yellow 23, and Food Yellow 4, was selected as the azo dye object of the study (figure 8).

Figure 8. Tartrazine (**TZ**) chemical formula.



This bright yellow water soluble monoazo-anionic dye is widely used in the textile industry to color wool, cotton, polyamide and silk but also as a food colorant [77]. Once E102 enters the environment through any water body, it is rarely removed, and its discharge it's not only responsible for the destruction of the soil and aquatic ecosystems, but there are also direct adverse effects for human health.

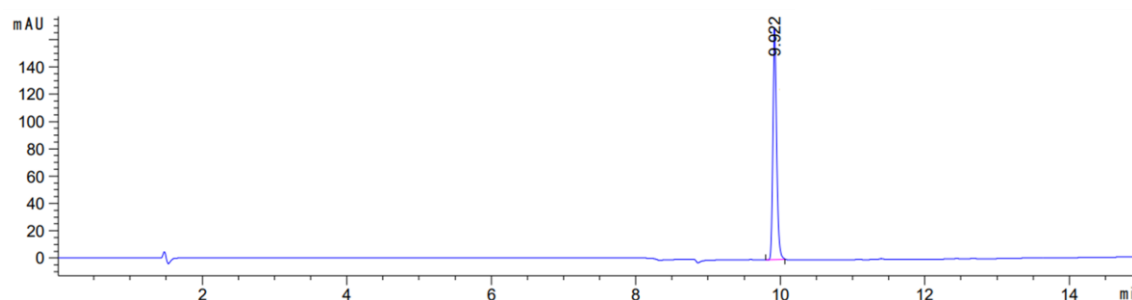
These include depression, anxiety, hyperactivity (especially in children), migraine, sleep disorders, cancer and kidney diseases, but also allergic reactions with symptoms like asthma, rhinitis, urticaria, atopic eczema and anaphylactic shock [78-79].

Considering the potential risks associated with the presence of tartrazine in water bodies, it's fundamental to develop methods for the efficient and rapid removal of this dye from aqueous solutions.

1.2.1 Sample preparation procedures and results

A concentration of 10 µg/mL was obtained by dissolving the solid and pure standard of tartrazine in HPLC-grade H₂O and then diluting to a final volume of 3 mL. This solution was analysed in HPLC-DAD, with the chromatogram shown in figure 9, and was considered as the blank reference.

Figure 9. HPLC-DAD chromatogram of 10 µg/mL of tartrazine.



Solutions prepared with the same procedure were treated by adding 100 mg of each of the 23 solid supports reported in in table 3 plus silica (entry K) and montmorillonite (entry J) to evaluate their capacity to adsorb tartrazine.

All the suspensions were left at room temperature and under magnetic stirring for 24 hours, although for some of them the decolorization of the solution was immediate. Each mixture was then centrifugated (5000 rpm for 5 min) in an Eppendorf tube and the supernatant was analysed in HPLC-DAD. The amount of E102 adsorbed on each solid, so the amount that was removed from the aqueous solutions, was quantified by subtracting the concentration of each analysed supernatant from the blank value. These results are reported in table 8.

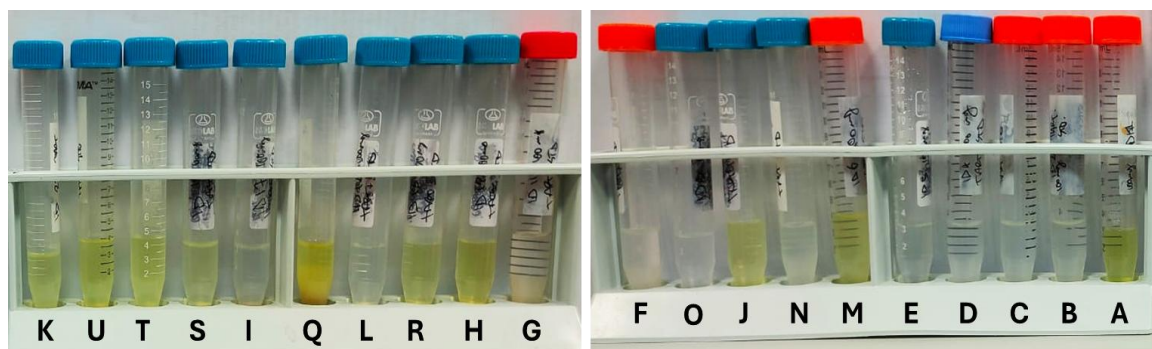
Indeed, the treatment of the yellow solutions of tartrazine with most of the solid sorbents caused a complete bleaching, providing a colourless and clear supernatant and a bright yellow solid deposit (figure 10).

Table 8. Quantification of tartrazine absorbed on solid sorbents (entries A-Y) in $\mu\text{g/mL}$ and percent (%), after treatment and HPLC-DAD analysis.

Sample entry	Tartrazine*	
	$\mu\text{g/mL}$	%
A	2.65 ± 0.13	26.2 ± 0.12
B	9.99 ± 0.16	99.9 ± 0.23
C	9.99 ± 0.02	99.9 ± 0.06
D	9.98 ± 0.07	99.8 ± 0.28
E	9.99 ± 0.26	99.9 ± 0.21
F	9.99 ± 0.09	99.9 ± 0.05
G	2.91 ± 0.26	29.1 ± 0.13
H	5.27 ± 0.18	52.7 ± 0.18
I	1.35 ± 0.10	13.5 ± 0.20
J	3.45 ± 0.12	34.5 ± 0.22
K	4.82 ± 0.06	$48.2 \pm .14$
L	9.99 ± 0.01	99.9 ± 0.02
M	3.47 ± 0.04	34.7 ± 0.05
N	9.99 ± 0.03	99.9 ± 0.09
O	9.98 ± 0.07	99.8 ± 0.05
P	9.97 ± 0.10	99.7 ± 0.11
Q	2.20 ± 0.26	22.0 ± 0.20
R	3.92 ± 0.13	39.2 ± 0.15
S	4.30 ± 0.16	43.0 ± 0.17
T	6.56 ± 0.23	65.6 ± 0.18
U	5.20 ± 0.25	52.0 ± 0.22
V	4.17 ± 0.21	41.7 ± 0.19
Z	3.44 ± 0.17	34.4 ± 0.20
X	0.78 ± 0.13	7.8 ± 0.14
Y	2.31 ± 0.5	23.1 ± 0.07

*Values expressed as mean $\pm$ SD (n = 3).

Figure 10. Supernatants of tartrazine after the treatment with some of the solid sorbents (Entries A-O, Q-U).

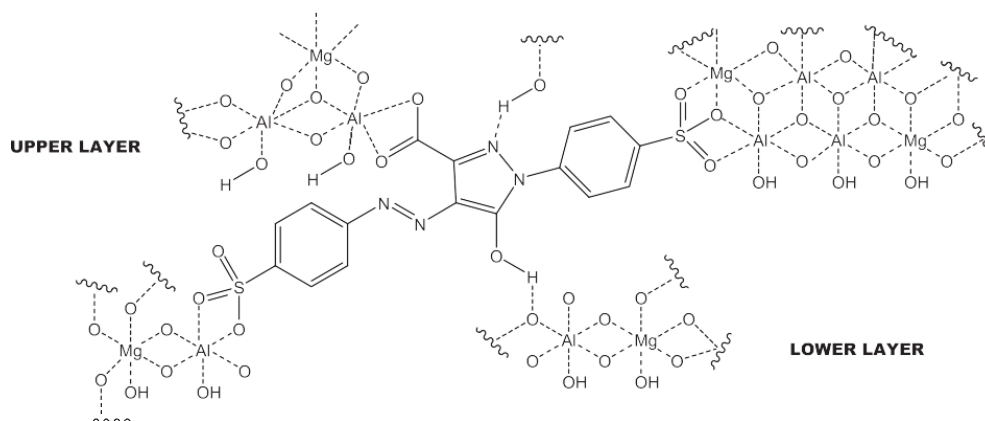


Among the 25 solid sorbents tested, 6 layered double hydroxides, namely zinc aluminum nitrate (entry B), zinc aluminum chloride (entry C), magnesium aluminum nitrate (entry D), magnesium aluminum azelate (entry E), magnesium aluminum hydroxy chloride (entry F) and zinc hydroxy chloride (entry L) provided nearly quantitative yields.

Indeed, the retention of E102 on aluminum and magnesium containing hydrotalcites could be due to a phenomenon called “chemisorption”, the formation of ionic bond between the Mg^{+2} and Al^{+3} cations that are part of the surface of the lamellar clays and the negatively charged sulfonate moieties of tartrazine, so a Lewis acid-base interaction. In addition, the solid layers comprise hydrogen containing groups that could interact through hydrogen bonds formation with the oxygens and nitrogen present in the E102 molecules.

A representative scheme of the possible interactions is depicted in figure 11, where tartrazine is shown intercalated between two layers, interacting with them through ionic and hydrogen bonds.

Figure 11. Representative scheme of the possible interaction between E102 and Mg and Al containing hydrotalcites.



The chemisorption mechanism was also proposed by Ouassif and Coworkers, who specifically studied the interaction between tartrazine and zinc aluminum chloride [80].

Additionally, this type of interaction has been frequently observed in different studies involving layered double hydroxides as solid sorbents for the adsorption and intercalation of multiple biologically active compounds [81].

The good performance of a lamellar sorbent like zirconium phosphate (type B) intercalated with octadecylamine (Entry N), can be explained by its high ion-exchange properties and intercalation chemistry, indeed this class of solid has already been widely used for the removal of pollutants, specifically heavy metals and harmful gasses from water and air [82-83].

For what concerns magnesium oxide (Entry O) magnesium hydroxide (Entry P), the results in this study further confirmed their role as good sorbent materials in regards of the removal of dyes and heavy metals from aqueous solutions. This is because of their excellent physical properties such as large surface area, high pore volume and large numbers of reactive edges [84].

On the other hand, all phyllosilicates, as well as other commonly used solids like silica (Entry K) and alumina (Entry Y), that were used as a comparison, were less effective, with percentages of adsorption between 23.1% and 65.6%.

Additional experimental routes were assessed to try and increase the adsorption capacities of the less effective solids, like adding up to 500 mg of sorbents for each treatment, leaving the reaction under magnetic stirring for 96 h and by application of temperatures in the range of 60 °C and 90 °C. All these steps did not lead to an improvement in yields of removal, and sometimes caused even a loss of efficacy, especially for the most effective clays. The solutions did not require any pH modifications, thus keeping the procedure easier to reproduce, even eventually, on a larger scale.

Finally, desorption experiments were performed by washing each solid with a solution of NaOH 0.01 M until a clear white solid was recovered and then dried in the oven at 80 °C overnight. This allowed to evaluate the reusability and recyclability of the clays, and that occurred for all of them. Further adsorption experiments were performed using magnesium aluminum azelate (entry E) as reference to be reused more than once (5 times) with the same protocol. The percentages of adsorption of tartrazine were all between 99.1% and 99.6%, meaning no significant loss of adsorption capacity was recorded, confirming the possible recyclability of these solid sorbents.

1.2.2 Isotherm adsorption experiments

These experiments were performed by following a modified protocol of the one reported by Sahnoun and Coworkers and using magnesium aluminum azelate (entry E) as the reference solid [85]. Solution of tartrazine at different concentrations, from 20 to 550 µg/mL were prepared and treated with a fixed amount of entry E. The quantity of dye adsorbed on the solid at equilibrium q_e , and at any time q_t , expressed as mg/g, were calculated as follows:

$$q_{e,t} = (C_0 - C_{e,t})V/m$$

where $q_{e,t}$ is the quantity of adsorbed E102 at equilibrium (q_e) or at any time (q_t), V is the volume in L of the aqueous solution, C_0 is the initial E102 concentration, $C_{e,t}$ is the E102 concentrations at equilibrium (C_e) or at any time (C_t) and m is the amount of solid in mg. All the determinations were accomplished in HPLC.

The highest amount of E102 adsorbed onto the 9 most effective sorbents (entries B-F, L, N-P), calculated from each respective adsorption isotherms, are reported in table 9.

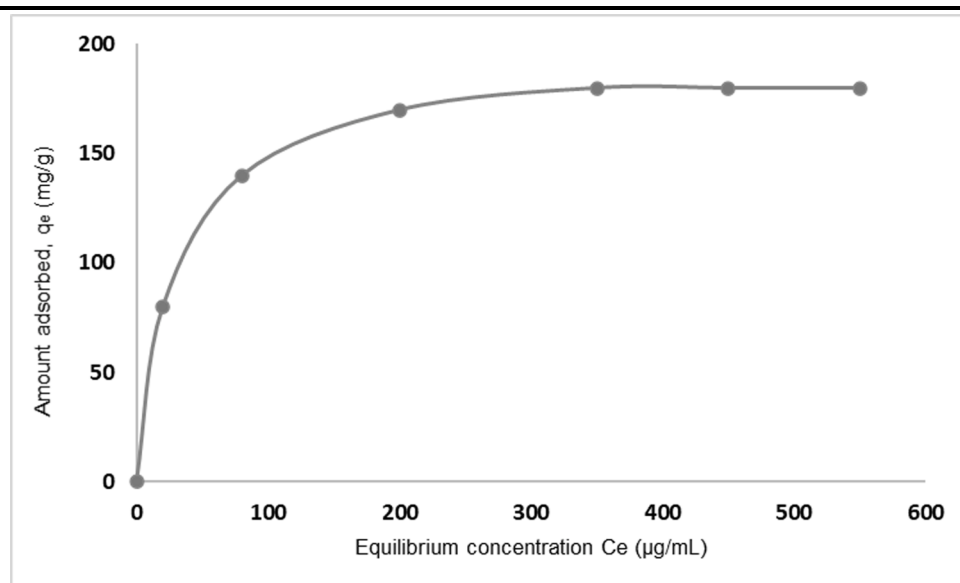
Table 9. Highest amount of E102 adsorbed onto the 9 most effective sorbents (entries B-F, L, N-P).

Sample entry	q_e (mg/g)	C_e ($\mu\text{g/mL}$)
B	185.7 ± 0.88	366.9 ± 0.12
C	181.0 ± 0.27	376.3 ± 0.41
D	169.9 ± 0.52	374.8 ± 0.59
E	173.2 ± 0.38	371.3 ± 0.44
F	171.8 ± 0.42	382.3 ± 0.33
L	179.3 ± 0.40	380.1 ± 0.22
N	168.9 ± 0.17	377.4 ± 0.33
O	174.6 ± 0.32	375.1 ± 0.65
P	174.5 ± 0.44	369.9 ± 0.59

*Values expressed as mean $\pm$ SD (n = 3).

In figure 12, an equilibrium isotherm curve for magnesium aluminum azelate is shown as reference, but the profiles of the curves are basically the same for all the most efficient solids.

Figure 12. Equilibrium isotherm for adsorption of E102 onto magnesium aluminum azelate (entry E).



The outline of the curve shows how at higher concentrations of the tartrazine solution there's an increase in the adsorption rate until a stall is

reached, meaning that the process appears to be favourably chemical in nature. Probably, at higher concentrations of dye, all the binding sites available on the surface and on the lamellar spaces of entry E get saturated, causing a plateau in the curve.

1.2.3 Conclusions

The removal of synthetic azo dyes from water bodies remains a challenge that still needs to be addressed. Solid phase adsorption offers a valid and interesting alternative. Indeed, this methodology is associated with numerous advantages, like an easy-to-handle procedure, which can be coupled with many analytical hyphenated techniques, such as HPLC-DAD, and that can be highly versatile based on the type of dye that has to be removed. In this context, 9 out of the 25 solid sorbents herein tested, appeared to be particularly efficient in the removal of tartrazine, all falling within the definition of ideal sorbent. They were confirmed to be recyclable and reusable and, considering their characteristics of being non-toxic and completely biocompatible, they could efficiently be employed for the removal of E102 from wastewaters, even on a larger scale.

1.2.4 Experimental procedures

Chemicals

CH₃CN and H₂O, all HPLC grade, were purchased by Honeywell Research Chemicals (Charlotte, North Carolina, USA) and Merck Sigma-Aldrich (Darmstadt, Germany). Ammonium acetate and HPLC standard tartrazine

(purity grade 99%) were purchased from Merck Sigma-Aldrich (Darmstadt, Germany). All solid sorbents were synthesized “in house” and kindly provided by Prolabin & Tefarm Srl (Perugia, Italy).

HPLC-DAD analyses

HPLC analyses were performed using an Agilent 1100 (Santa Clara, CA, USA) series instrument equipped with an autosampler, a binary solvent pump, and a diode-array detector (DAD). The qualitative and quantitative analysis were achieved employing a Kromasil RP C18 (4.6 mm $\varnothing$ x 150 mm, 5 μ m particle size). The column temperature was 25 °C and the injection volume was 20 μ L, while the wavelength set for the acquisition was 427 nm. The mobile phase consisted of an aqueous ammonium acetate solution 1 % (w/v) (solvent A) and an 8:2 MeOH/CH₃CN mixture (solvent B) following a gradient elution program as depicted in table 10 at a flow rate of 1.0 mL/min.

Table 10. Gradient elution program for HPLC analysis of tartrazine.

Time (min)	A %	B %
0	100	0
5	100	0
10	52.5	47.5
15	0	100
18	100	0

The presence of ammonium acetate was essential, as it provided good chromatographic profile, with an optimal peak shape. Under these conditions, the retention time of tartrazine was 9.92 min ($\pm$ 0.02).

The HPLC-DAD method was validated according to ICH recommendations, and all the relevant analytical parameters are reported in table 11 [86].

Table 11. HPLC method validation parameters

Slope	14,080
Intercept	-907
r²	0.9991
SD_s (slope)	1024
SD_i (intercept)	76
SD_r (residuals)	0.259
LOD (µg/mL)	0.24
[3.3 X SD_s/S]	
LOQ (µg/mL)	0.73
[10 X SD_s/S]	
Precision	
Intra-day	% RSD (n=3)
QC _{low}	100.1/0.990
QC _{medium}	99.9/0.992
QC _{high}	100.2/1.008
Inter-day	% RSD (n=3)
QC _{low}	99.8/1.011
QC _{medium}	99.9/0.999
QC _{high}	100.1/1.005
Accuracy	
Intra-day	% Recovery (n=3)
QC _{low}	101.2/1.001
QC _{medium}	101.7/0.998
QC _{high}	100.6/0.996
Inter-day	% Recovery (n=3)
QC _{low}	100.2/1.001
QC _{medium}	101.1/0.992
QC _{high}	104.4/0.984
Capacity factor	1.2 ± 0.01
Resolution	1.3 ± 0.02
Peak symmetry	1.02 ± 0.01
Tailing	0.99 ± 0.02
Theoretical plate numbers	7715 ± 108

Calibration curves were drawn by injecting standard mixture solutions at 9 concentrations values, namely 0.20 µg/mL, 0.35 µg/mL, 0.55 µg/mL, 1.0 µg/mL, 5.0 µg/mL, 12.5 µg/mL, 15 µg/mL, 20 µg/mL, and 25 µg/mL.

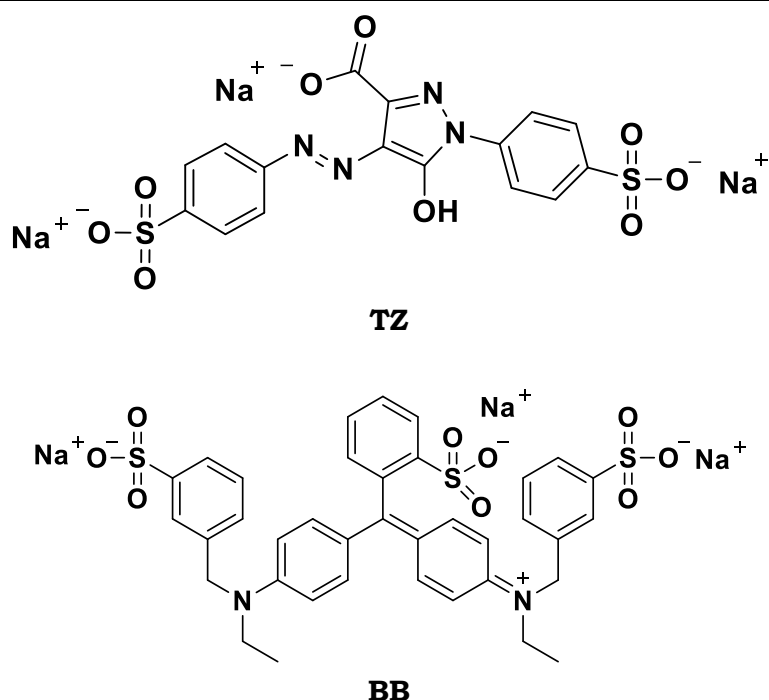
LODs and LOQs were obtained by injecting serial dilutions of the corresponding standard solutions, taking the signal-to-noise (S/N) ratio of 3.3 and 10 as criteria, respectively. The analyte tartrazine under investigation demonstrated a very good linearity providing an r^2 value of 0.9991. The repeatability of the HPLC method was assessed by performing six consecutive injections in the same day on QC samples spiked at three different standard E102 concentration levels, namely $QC_{low} = 0.20 \mu\text{g/mL}$, $QC_{medium} = 1.0 \mu\text{g/mL}$, and $QC_{high} = 20.0 \mu\text{g/mL}$. QC samples were also analysed in separate days to determine the intermediate precision of the process. Trueness of the method was established at the same analyte concentration levels by comparison of QC samples concentrations with their nominal values. RSD values of the intra- and inter-day precisions were not higher than 2.2 %, while the BIAS % ones referred to the intra- and inter-day accuracies were in the range - 1.3 % to 2.1 %.

In conclusion, the overall recorded data indicate that the method set up for the recovery of tartrazine from its aqueous solutions is featured by appropriate accuracy, precision, robustness, and reproducibility, with reference to ICH guidelines.

1.3 Removal of synthetic dyes from foods and beverages

Synthetic dyes are widespread and employed, not only in the textile industry, but also in the food and beverage sectors, where they are used to adjust the color loss linked to light, air or temperature, and to obtain and enhance a more brilliant “natural” color [87]. They’re often added to sweets, candies, drinks, spices, seasonings, cheeses and other products since they’re preferred over natural dyes because of their stability, coloring capacity, absence of any flavour and their low cost [88]. Among the most used synthetic pigments, tartrazine (**TZ**) (trisodium 1-(4-sulfonatophenyl)-4-(4-sulfonatophenylazo)-5-pyrazolone-3-carboxylate), or E102, an azo dye, and Brilliant Blue FCF (**BB**) (disodium 2-[[4-[ethyl-[(3-sulfonatophenyl)methyl]amino]phenyl]-[4-[ethyl-[(3-sulfonatophenyl)methyl]azaniumylidene]cyclohexa-2,5-dien-1-ylidene]methyl]benzenesulfonate), or E133, a triphenylmethane dye, are well known for their bright lemon yellow and blue colors (figure 13).

Figure 13. Tartrazine (**TZ**) and brilliant blue FCF (**BB**) chemical structures.



They are added to foods separately, but often also in combination to provide different shades of green to foods and drinks. For example, both tartrazine and blue brilliant FCF are added to peas for granting them a more interesting green colour [89]. Although their use as additives is quite common nowadays, their prolonged intake can lead to serious health problems. For example, brilliant blue FCF, when consumed in high doses, can deposit in the kidneys and in the lymphatic system, but it has also been showed to cause hyperactivity and skin rashes, and in even higher doses, DNA damages and cancer in animals [90-91].

Tartrazine is responsible of most of the allergic reaction caused by synthetic dyes, especially for asthmatics and individuals with aspirin intolerance, inducing urticaria, skin rashes and angioedema because of the massive release of histamine [92-93]. In children between the age of 3 and 9, E102 has been extensively linked with hyperactivity [94-95]. In addition, similarly

to E133, it has been linked to kidney and liver diseases, DNA damages and carcinogenesis [94-95-96-97].

For these reasons, both EFSA (European Food Safety Authority, and later WHO (World Health Organization) set a limit for the average daily intake of tartrazine, in 2009, as 7.5 mg/kg body weight, and for blue brilliant FCF, in 2010, as 12.5 mg/kg body weight [98-99].

Therefore, it's important to find and develop innovative methods that can identify and quantify, especially in different food matrices, the content of these two dyes. In literature, few papers are dedicated to the simultaneous removal of both dyes in a mixture, and most of the techniques proposed are based on electrochemical analysis.

For example, the use of a carbon paste electrode chemically modified with multi walled carbon, or of an ionic liquid-modified expanded graphite paste electrode for the determination of blue brilliant FCF and tartrazine using voltametric methods [100-101]. Although these electrochemical techniques are associated with good precision, reproducibility, stability and with a reusable electrode sensor, they also come with some disadvantages, such as a possible interference between the two analytes, limited applications, and the costly instruments.

On the other hand, adsorption-based techniques could be highly advantageous methods for the removal of these two dyes, since they are precise, easy to perform, granting high extraction yields and pre-concentration capacities, mostly inexpensive and with the possibility to recycle the employed sorbents [102]. For instance, the studies conducted by Fernández-Andrade and Coworkers, are based on the use of rice husk as

adsorbent material for the removal of both E102 and E133 from aqueous solutions. But in both sets of experiments the materials were showing a higher adsorption capacity in regards of one of the two dye, revealing a competitive behaviour between the two [103-104].

Thus, in the study herein proposed, a method with the aim of evaluating the panel of 23 solid supports reported in in table 3 plus silica (entry K) and montmorillonite (entry J) was developed, for both the selective and non-selective adsorption and removal of tartrazine and blue brilliant FCF.

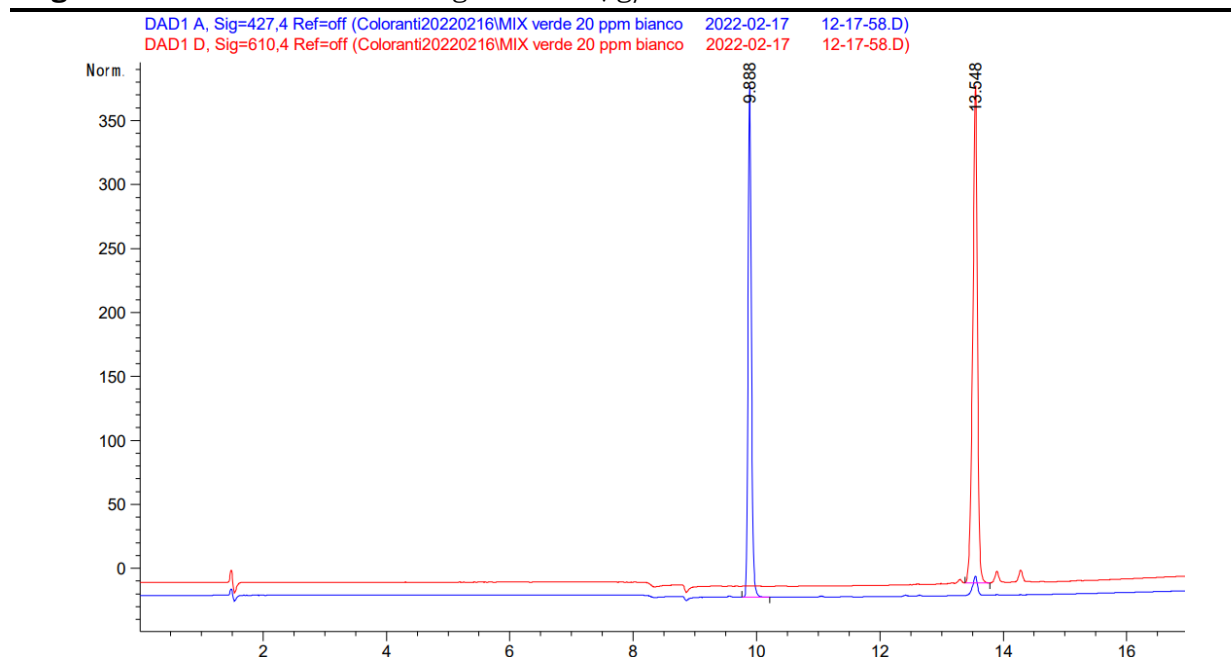
A preliminary step consisted in the application of this technique to aqueous mixtures of the two dyes, this was then followed by the direct treatment to food matrices and beverages, characterized by the presence in different percentages of E102 and E133.

1.3.1 Sample preparation procedures and results for the aqueous mixtures

A concentration of 20 µg/mL was obtained by dissolving the pure standards of tartrazine and brilliant blue FCF in HPLC-grade H₂O. This solution was analysed in HPLC-DAD and was considered as the blank reference.

Figure 14 shows the chromatogram of the two compounds: **TZ** (blue signal at 427 nm, retention time 9.888) and **BB** (red signal at 610 nm, retention time 13.548)

Figure 14. HPLC-DAD chromatogram of 20 µg/mL of **TZ** and **BB**.



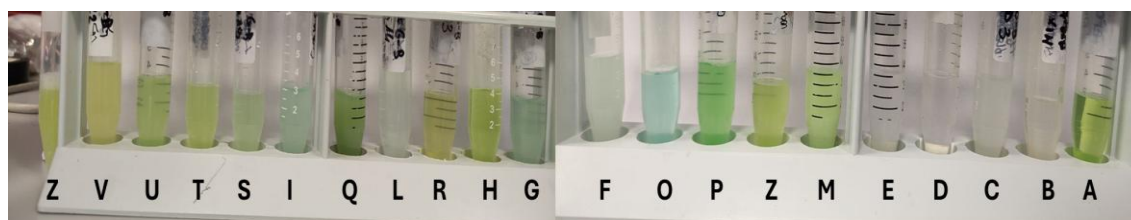
Solutions of 5 mL prepared with the same procedure were treated by adding 100 mg of each of the 23 solid supports reported in in table 3 plus silica (entry K) and montmorillonite (entry J) to evaluate their capacity to adsorb tartrazine and brilliant blue FCF.

All the suspensions were left at room temperature and under magnetic stirring for 5 min.

Each mixture was then centrifugated (5000*g* for 5 min) in an Eppendorf tube and the supernatant was analysed in HPLC-DAD.

The adsorption results and the selectivity of some sorbents for one of the two dyes was visible even before the quantification (figure 15). Indeed, the supernatants appeared completely bleached when the sorbents were particularly efficient in adsorbing both dyes, yellow or blue if they had selectivity in regards of BB or TZ, respectively. In the case of a low removal capacity, the supernatant remained green.

Figure 15. Supernatants of **TZ** and **BB** after the treatment with some of the solid sorbents (Entries A-I, L, M, O-V, Z).



The amount of E102 and E133 adsorbed on each solid, so the amount that was removed from the aqueous solutions, was quantified by subtracting the concentration of each analysed supernatant from the blank value. These results are reported in table 12.

7 out of 25 solid sorbents tested, 6 layered double hydroxides and a lamellar solid, namely zinc aluminum nitrate (entry B), zinc aluminum chloride (entry C), magnesium aluminum nitrate (entry D), magnesium aluminum azelate (entry E), magnesium aluminum hydroxy chloride (entry F), zinc hydroxy chloride (entry L) and zirconium phosphate (type B) with octadecylamine, provided nearly quantitative yields, together with a complete decoloration of the treated solution. The putative mechanism responsible for the efficient interaction between tartrazine and hydrotalcites has already been explained in the previous chapter and can also be applied to blue brilliant FCF, which is also rich in negatively charged moieties, and oxygen and nitrogen for the formation of hydrogen bonds. Thus, the intercalation between the layer of the lamellar clays is facilitated by chemisorption through ionic bonds, hydrogen bonds, acid-base interactions and Wand der Waals interactions.

Table 12. Quantification of TZ and BB absorbed on solid sorbents (entries A-DZ) in $\mu\text{g/mL}$ and percent (%), after treatment and HPLC-DAD analysis

Sample entry	TZ*		BB*	
	$\mu\text{g/mL}$	%	$\mu\text{g/mL}$	%
A	7.0 ± 0.04	36.4 ± 0.02	19.7 ± 0.02	92.9 ± 0.01
B	20.0 ± 0.01	100 ± 0.01	20.0 ± 0.02	100 ± 0.01
C	20.0 ± 0.02	100 ± 0.01	20.0 ± 0.01	100 ± 0.01
D	20.0 ± 0.02	100 ± 0.01	20.0 ± 0.01	100 ± 0.01
E	20.0 ± 0.01	100 ± 0.01	20.0 ± 0.01	100 ± 0.01
F	20.0 ± 0.01	100 ± 0.01	20.0 ± 0.01	100 ± 0.01
G	14.5 ± 0.08	75.2 ± 0.04	6.4 ± 0.06	30.2 ± 0.04
H	6.4 ± 0.03	32.9 ± 0.01	19.7 ± 0.03	94.7 ± 0.02
I	12.6 ± 0.07	65.2 ± 0.04	8.3 ± 0.06	39.1 ± 0.03
J	6.4 ± 0.04	33.0 ± 0.02	8.4 ± 0.05	39.7 ± 0.03
K	6.4 ± 0.05	33.0 ± 0.03	17.5 ± 0.09	82.5 ± 0.05
L	19.99 ± 0.01	99.9 ± 0.02	19.7 ± 0.05	94.7 ± 0.03
M	12.6 ± 0.07	65.2 ± 0.04	8.3 ± 0.06	39.1 ± 0.03
N	20.0 ± 0.01	100 ± 0.01	20.0 ± 0.01	100 ± 0.01
O	20.0 ± 0.02	100 ± 0.01	2.4 ± 0.05	11.7 ± 0.02
P	12.8 ± 0.06	66.1 ± 0.03	8.4 ± 0.05	39.7 ± 0.03
Q	2.8 ± 0.01	22.8 ± 0.01	10.9 ± 0.06	51.5 ± 0.04
R	8.5 ± 0.04	43.9 ± 0.02	19.7 ± 0.05	94.7 ± 0.03
S	12.2 ± 0.08	62.8 ± 0.05	17.6 ± 0.07	83.2 ± 0.04
T	8.5 ± 0.07	43.8 ± 0.04	18.2 ± 0.07	86.0 ± 0.04
U	8.7 ± 0.07	45.0 ± 0.03	17.1 ± 0.05	80.6 ± 0.02
V	2.1 ± 0.03	10.5 ± 0.03	20.0 ± 0.01	100 ± 0.01
Z	6.6 ± 0.02	34.0 ± 0.04	14.8 ± 0.08	69.9 ± 0.05
X	1.6 ± 0.02	8.0 ± 0.01	20.0 ± 0.02	100 ± 0.01
Y	9.9 ± 0.09	51.4 ± 0.05	10.9 ± 0.06	51.4 ± 0.04

*Values expressed as mean $\pm$ SD (n = 3).

An interesting response was recorded for magnesium oxide (entry O), that showed a complete selectivity towards tartrazine adsorption, and magnesium aluminum D. benzenesulfonate SDS 01 H8L (entry V) and

Florisil (entry X), that concentrated only blue brilliant FCF. This can easily be explained by describing the structures of both the dyes and of the solids. Entries V and X are multilayered sorbents rich in Al^{+3} and Mg^{+2} cations, that are known to interact with sulfonate moieties. This interaction can be dependant by the higher presence of such negatively charged groups in blue brilliant FCF, and together with its larger planar structure, the better selectivity can be explained [104].

In contrast, magnesium oxide has a brucite-like structure, with lamellar spaces that are much closer to each other. Tartrazine, having also a few basic sites, can easily interact with both Mg^{+2} and O^{-2} ions, forming ionic and hydrogen bonds. Additional experimental routes were assessed to try and increase the adsorption capacities of the less effective solids, like adding up to 500 mg of sorbents for each treatment, leaving the reaction under magnetic stirring for 30 min. and by apply temperatures up to 75 °C. All these steps did not lead to an improvement in yields of removal, and sometimes caused even a loss of efficacy, especially for the most effective clays. The solutions did not require any pH modifications, at least for the aqueous artificial green mixtures, thus keeping the procedure easier to reproduce.

1.3.2 Sample preparation procedures and results for the food and drink matrices

After having screened the efficiency of all the solids in adsorbing TZ and BB from artificial aqueous solutions, the attention was focused on evaluating

the trend of extraction on food and beverages with the presence of E102 and E133, but not the quantity, declared on the label.

The selected products were a high alcoholic grade liquor, apple flavoured candies, dried pomelo peels, green icing and low alcoholic grade soft liquor.

The first step consisted in the specific preparation of the matrices, making them suitable for HPLC-DAD analysis, thus for the quantification of each dye. 2 mL of the liquor were diluted with 3 mL of HPLC-grade H₂O. 2.5 mg of green icing and 4 g of dried pomelo peels were weighted and left macerating in 5 mL of HPLC-grade H₂O and after 24 hours under magnetic stirring, the resulting suspensions were filtered. 1.4 g of apple candies were triturated and dissolved in 5 mL of HPLC-grade H₂O. 3 mL of the soft drink were subjected to a treatment with 290 µL of NaOH 1 N to adjust the pH at 7, since citric acid was one of the main ingredients and the acidic solution could alter the protonation of the sulfonate moieties necessary for the chemisorption process.

All the so obtained solutions were used firstly as blank references and then prepared again, with the same procedures, for the treatment with the most effective solid sorbents in term of removal yields and selectivity. Specifically, magnesium oxide (entry O), magnesium aluminum D. benzenesulfonate SDS 01 H8L (entry V) and Florisil (entry X), were added in different quantities going from 25 mg to 400 mg. The amount of E102 and E133 adsorbed on each solid, so the amount that was removed from the different matrices, was quantified by subtracting the concentration of each analysed supernatant from the blank reference of each food and beverage. The results are reported in table 13 for tartrazine and in table 14 for blue brilliant FCF.

Table 13. Adsorption capacity of TZ in food and beverage matrices for magnesium oxide (entry O), magnesium aluminum D. benzenesulfonate SDS 01 H8L (entry V) and Florisil (entry X) in µg/mL (and percent in parenthesis), after treatment and HPLC-DAD analysis

Entry	TZ*				
	1	2	3	4	5
PARENT MATRIX	18.1 ± 0.05	14.4 ± 0.04	13.3 ± 0.04	36.5 ± 0.07	1.2 ± 0.02
O					
50 mg	9.7 ± 0.04 (53.6 ± 0.05)	7.0 ± 0.03 (48.8 ± 0.04)	7.7 ± 0.02 (57.9 ± 0.06)	17.5 ± 0.06 (48.1 ± 0.07)	0.9 ± 0.02 (79.4 ± 0.04)
100 mg	15.6 ± 0.06 (87.0 ± 0.06)	9.3 ± 0.02 (64.7 ± 0.05)	9.2 ± 0.03 (69.2 ± 0.04)	31.3 ± 0.08 (85.8 ± 0.05)	1.0 ± 0.02 (90.1 ± 0.02)
200 mg	17.6 ± 0.04 (97.2 ± 0.03)	15.6 ± 0.06 (88.1 ± 0.02)	11.2 ± 0.04 (84.3 ± 0.06)	34.5 ± 0.07 (94.5 ± 0.02)	1.2 ± 0.01 (100 ± 0.01)
300 mg	18.1 ± 0.03 (100 ± 0.02)	14.4 ± 0.04 (100 ± 0.02)	13.3 ± 0.03 (100 ± 0.03)	36.5 ± 0.01 (100 ± 0.01)	1.2 ± 0.01 (100 ± 0.02)
V					
25 mg	0.4 ± 0.02 (2.4 ± 0.01)	ND	ND	ND	ND
50 mg	0.5 ± 0.03 (2.6 ± 0.02)	ND	ND	ND	ND
100 mg	0.5 ± 0.01 (2.7 ± 0.01)	0.2 ± 0.01 (1.3 ± 0.02)	ND	1.0 ± 0.02 (2.8 ± 0.03)	ND
200 mg	0.5 ± 0.01 (2.8 ± 0.02)	0.3 ± 0.02 (1.9 ± 0.01)	ND	1.6 ± 0.04 (4.5 ± 0.04)	ND
X					
200 mg	0.4 ± 0.01 (2.1 ± 0.02)	ND	ND	0.4 ± 0.02 (1.1 ± 0.02)	ND
300 mg	0.4 ± 0.03 (2.2 ± 0.01)	ND	ND	0.4 ± 0.03 (1.3 ± 0.03)	ND
400 mg	0.4 ± 0.02 (2.4 ± 0.04)	0.3 ± 0.01 (2.3 ± 0.04)	ND	0.5 ± 0.02 (2.1 ± 0.02)	ND

ND = not detected or below LOQ; **1** = liqueur, **2** = apple candies, **3** = dried pomelo peels, **4** = green icing, **5** = soft drink.

*Values expressed as mean ± SD (n = 3).

Table 14. Adsorption capacity of **BB** in food and beverage matrices for magnesium oxide (entry O), magnesium aluminum D. benzenesulfonate SDS 01 H8L (entry V) and Florisil (entry X) in $\mu\text{g/mL}$ (and percent in parenthesis), after treatment and HPLC-DAD analysis

Entry	BB*				
	1	2	3	4	5
PARENT MATRIX	19.3 $\pm$ 0.04	20.1 $\pm$ 0.03	1.1 $\pm$ 0.01	17.9 $\pm$ 0.02	1.0 $\pm$ 0.01
O					
50 mg	ND	ND	ND	ND	ND
100 mg	ND	ND	ND	ND	ND
200 mg	0.3 $\pm$ 0.01 (1.9 $\pm$ 0.01)	0.3 $\pm$ 0.02 (1.7 $\pm$ 0.02)	ND	0.4 $\pm$ 0.01 (2.1 $\pm$ 0.03)	ND
300 mg	0.7 $\pm$ 0.03 (3.6 $\pm$ 0.02)	0.8 $\pm$ 0.02 (4.2 $\pm$ 0.04)	ND	0.9 $\pm$ 0.03 (5.1 $\pm$ 0.02)	ND
V					
25 mg	19.3 $\pm$ 0.02 (100 $\pm$ 0.02)	14.0 $\pm$ 0.03 (69.8 $\pm$ 0.02)	0.9 $\pm$ 0.01 (79.5 $\pm$ 0.02)	17.2 $\pm$ 0.02 (96.3 $\pm$ 0.04)	0.9 $\pm$ 0.04 (94.2 $\pm$ 0.04)
50 mg	19.3 $\pm$ 0.03 (100 $\pm$ 0.03)	15.8 $\pm$ 0.06 (78.9 $\pm$ 0.03)	0.9 $\pm$ 0.02 (83.0 $\pm$ 0.03)	17.9 $\pm$ 0.02 (100 $\pm$ 0.02)	1.0 $\pm$ 0.02 (100 $\pm$ 0.01)
100 mg	19.3 $\pm$ 0.05 (100 $\pm$ 0.01)	19.2 $\pm$ 0.07 (95.5 $\pm$ 0.04)	1.1 $\pm$ 0.01 (100 $\pm$ 0.01)	17.9 $\pm$ 0.02 (100 $\pm$ 0.02)	1.0 $\pm$ 0.01 (100 $\pm$ 0.03)
200 mg	19.3 $\pm$ 0.01 (100 $\pm$ 0.01)	20.1 $\pm$ 0.03 (100 $\pm$ 0.02)	1.1 $\pm$ 0.04 (100 $\pm$ 0.03)	17.9 $\pm$ 0.01 (100 $\pm$ 0.01)	1.0 $\pm$ 0.01 (100 $\pm$ 0.02)
X					
200 mg	18.9 $\pm$ 0.07 (97.9 $\pm$ 0.05)	19.1 $\pm$ 0.02 (95.2 $\pm$ 0.04)	1.0 $\pm$ 0.01 (98.7 $\pm$ 0.03)	16.7 $\pm$ 0.06 (93.6 $\pm$ 0.04)	1.0 $\pm$ 0.02 (100 $\pm$ 0.01)
300 mg	19.3 $\pm$ 0.01 (100 $\pm$ 0.01)	20.1 $\pm$ 0.02 (100 $\pm$ 0.01)	1.1 $\pm$ 0.01 (100 $\pm$ 0.03)	17.9 $\pm$ 0.04 (100 $\pm$ 0.03)	1.0 $\pm$ 0.02 (100 $\pm$ 0.02)
400 mg	19.3 $\pm$ 0.03 (100 $\pm$ 0.02)	20.1 $\pm$ 0.02 (100 $\pm$ 0.02)	1.1 $\pm$ 0.01 (100 $\pm$ 0.01)	17.9 $\pm$ 0.04 (100 $\pm$ 0.02)	1.0 $\pm$ 0.01 (100 $\pm$ 0.01)

ND = not detected or below LOQ; **1** = liqueur, **2** = apple candies, **3** = dried pomelo peels, **4** = green icing, **5** = soft drink.

*Values expressed as mean $\pm$ SD (n = 3).

The data obtained from these experiments fully confirmed what was previously recorded with the artificial aqueous mixtures, highlighting a great efficacy and selectivity of the sorbents for the two dyes. A dose dependant

removal capacity was recorded, but in some instances, based on the complexity of the matrix, the quantitative extractive yields were recorded even when adding the smallest amount of sorbent (for example 25 mg of entry X and entry V were able to completely adsorb blue brilliant from the soft drink and the liqueur, respectively).

Chromatograms regarding the analysis of some of the parent matrices and of the supernatant after treatment with the most efficient amount of the 3 solids (entries O, V and X) are reported in figure 16, 17 and 18.

Figure 16. HPLC chromatograms recorded at 427.4 nm and 610.4 nm of liqueur before **(a)** and after treatment with 200 mg of Magnesium oxide (O) **(b)**, 50 mg of Magnesium aluminium D. Benzen Solphonate SDS 01 H8L (V) **(c)** and 400 mg of Florisil (X) **(d)**

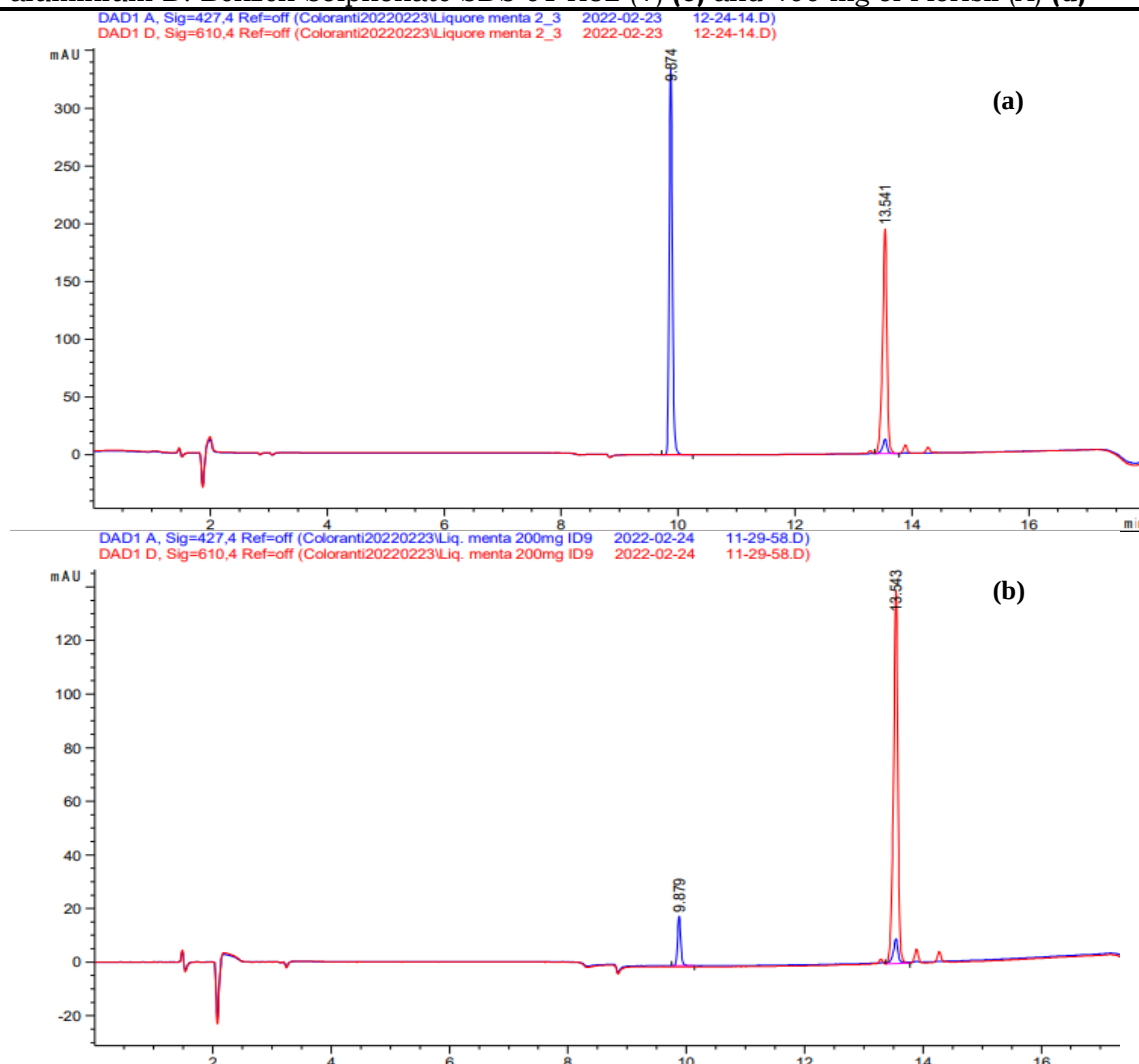


Figure 16 (continued). HPLC chromatograms recorded at 427.4 nm and 610.4 nm of liqueur before **(a)** and after treatment with 200 mg of Magnesium oxide (O) **(b)**, 50 mg of Magnesium aluminium D. Benzen Solphonate SDS 01 H8L (V) **(c)** and 400 mg of Florisil (X) **(d)**

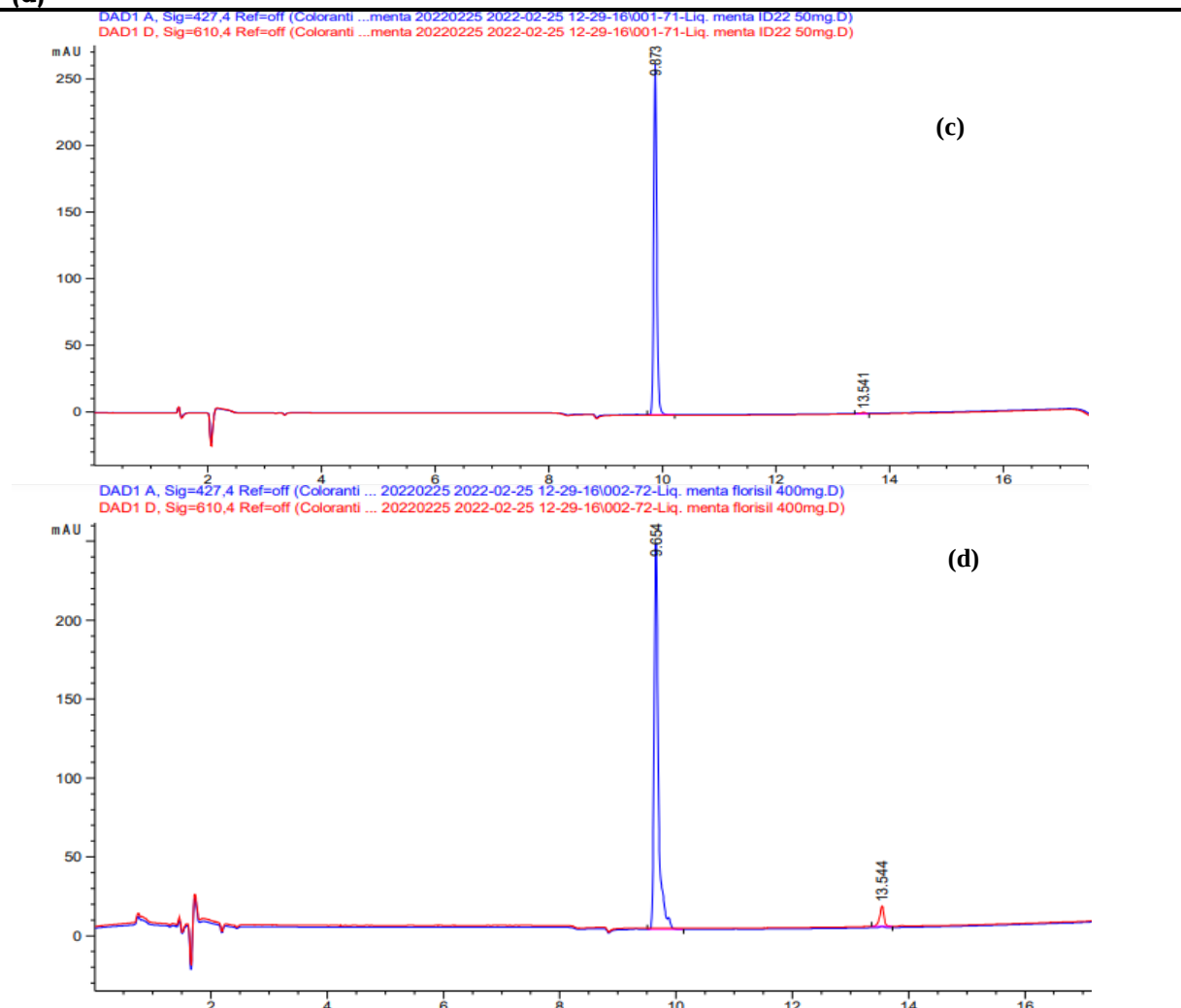


Figure 17. HPLC chromatograms recorded at 427.4 nm and 610.4 nm of green icing before **(a)** and after treatment with 200 mg of Magnesium oxide (O) **(b)**, 50 mg of Magnesium aluminium D. Benzen Solphonate SDS 01 H8L (V) **(c)** and 400 mg of Florisil (X) **(d)**

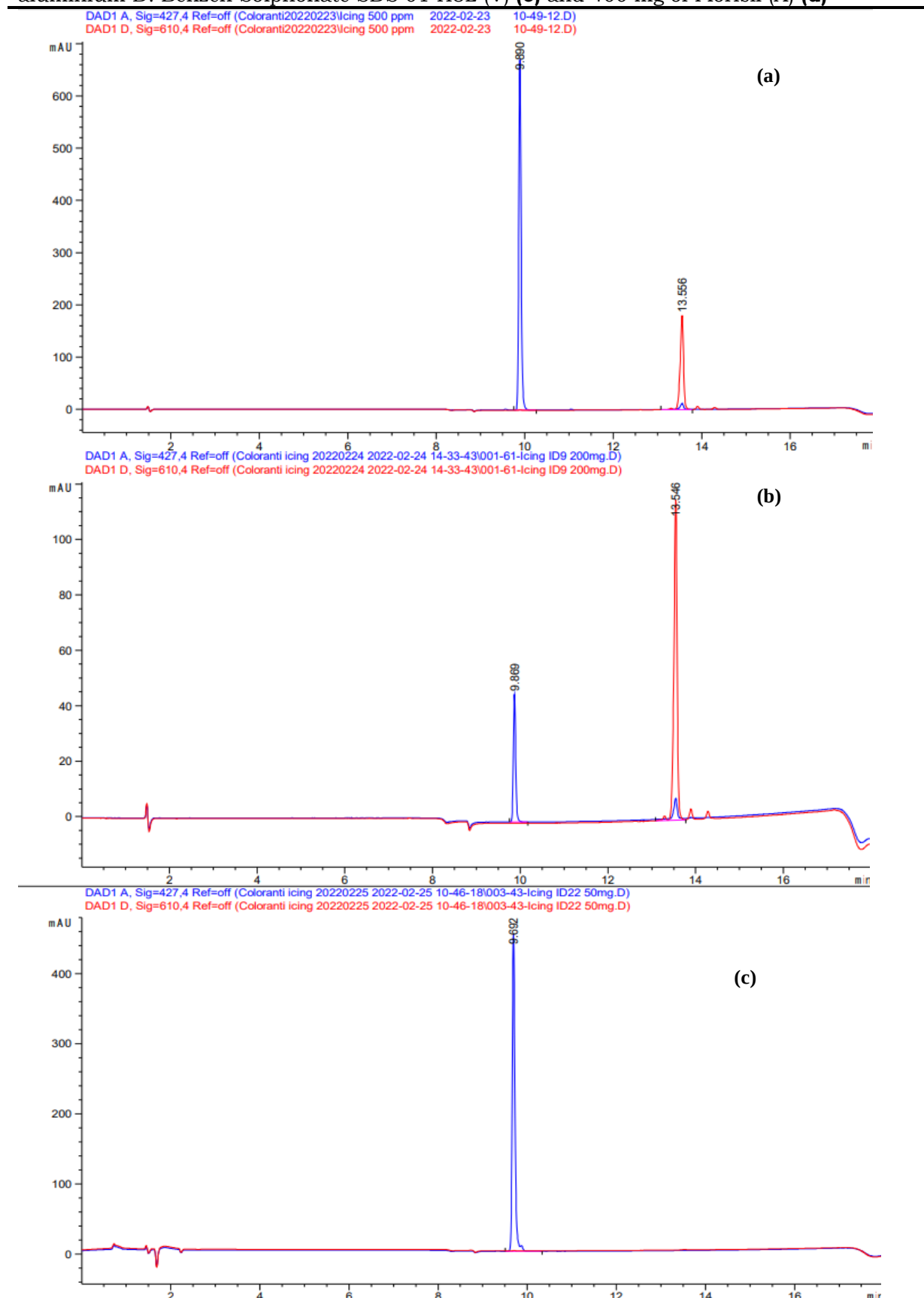


Figure 17 (continued). HPLC chromatograms recorded at 427.4 nm and 610.4 nm of green icing before **(a)** and after treatment with 200 mg of Magnesium oxide (O) **(b)**, 50 mg of Magnesium aluminium D. Benzen Solphonate SDS 01 H8L (V) **(c)** and 400 mg of Florisil (X) **(d)**

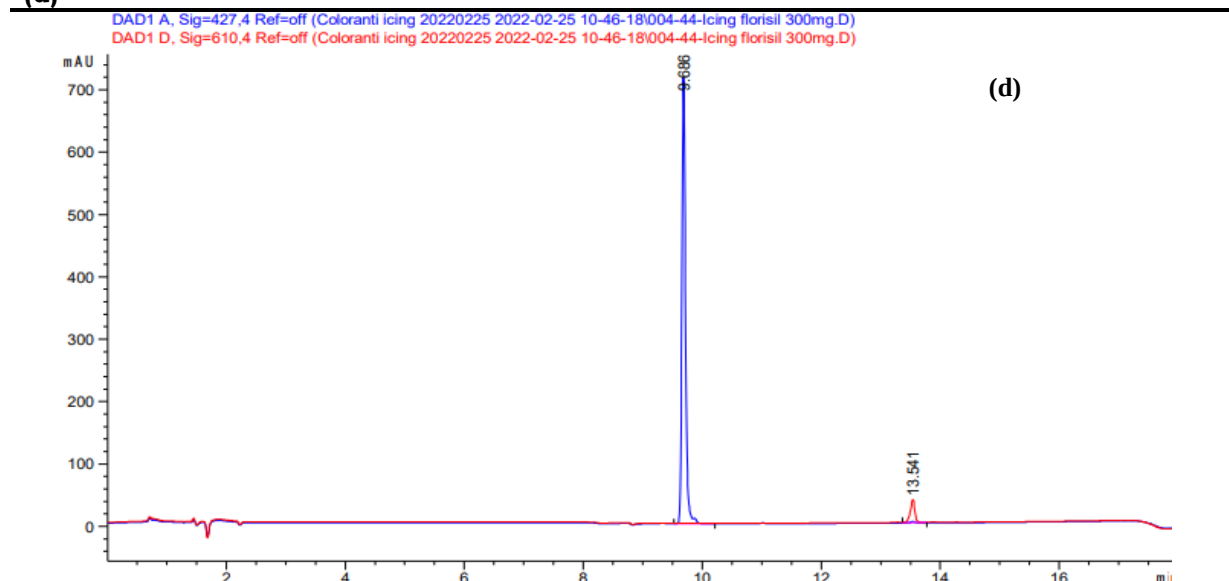


Figure 18. HPLC chromatograms recorded at 427.4 nm and 610.4 nm of dried pomelo peels before **(a)** and after treatment with 200 mg of Magnesium oxide (O) **(b)**, 50 mg of Magnesium aluminium D. Benzen Solphonate SDS 01 H8L (V) **(c)** and 400 mg of Florisil (X) **(d)**

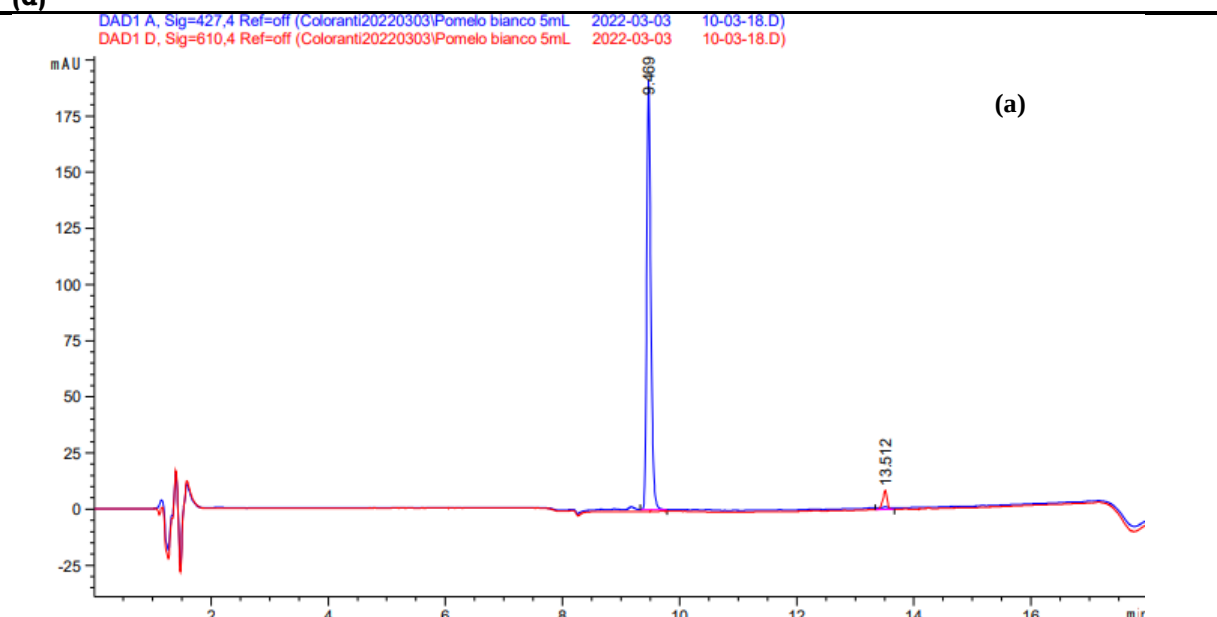
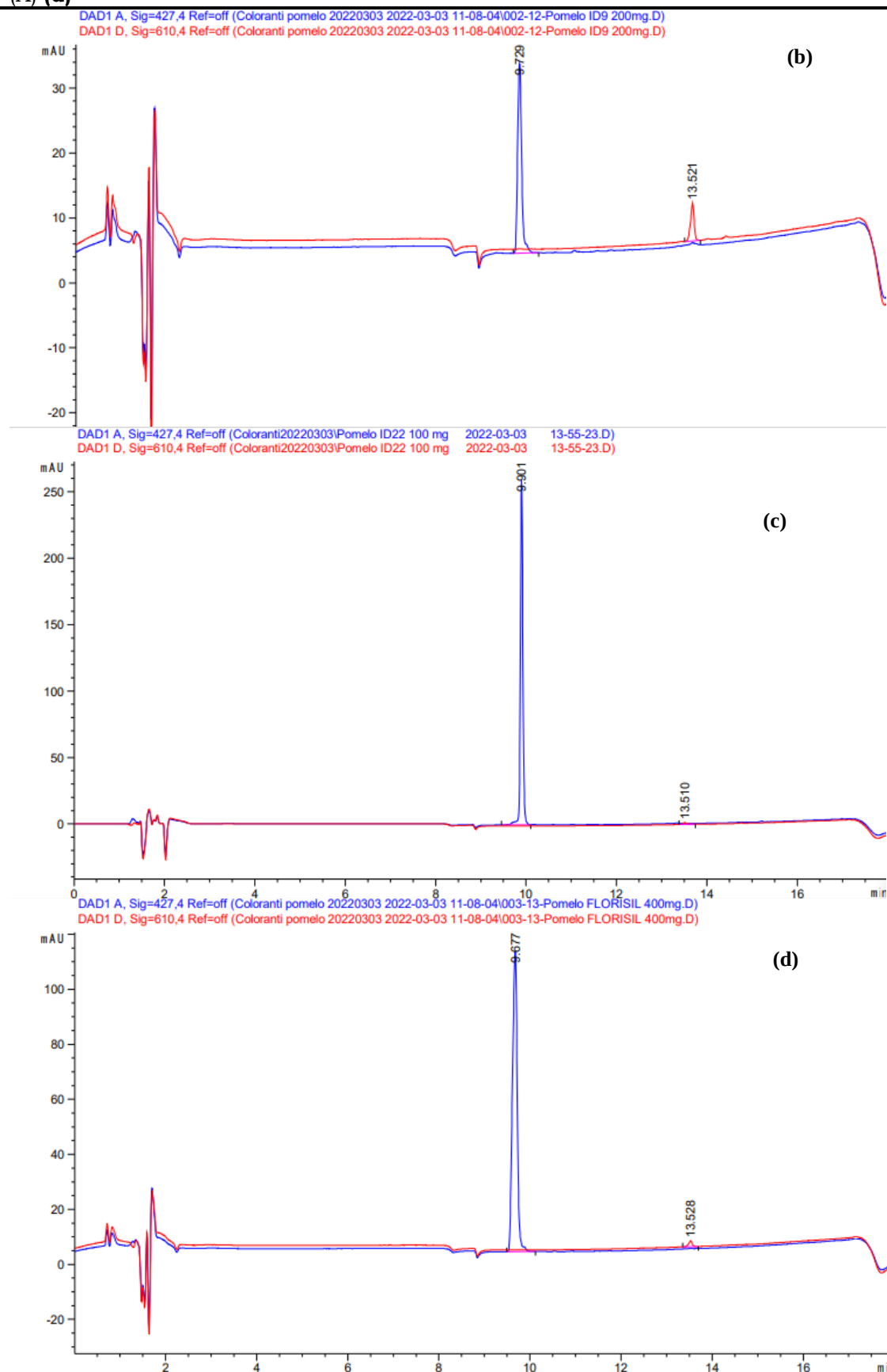


Figure 18 (continued). HPLC chromatograms recorded at 427.4 nm and 610.4 nm of dried pomelo peels before (a) and after treatment with 200 mg of Magnesium oxide (O) (b), 50 mg of Magnesium aluminium D. Benzen Solphonate SDS 01 H8L (V) (c) and 400 mg of Florisil (X) (d)



1.3.3 Isotherm adsorption experiments

These experiments were performed by following a modified protocol of the one reported by Zhang and Coworkers [105]. The Langmuir and Freundlich isotherm equations were selected as models and described with the following equations:

Langmuir equation

$$\frac{C_e}{q_e} = \frac{1}{(K_L \times q_m)} + \frac{C_e}{q_m}$$

Freundlich equation:

$$\log q_e = \log K_F + \frac{1}{n} \log C_e$$

Where C_e is the equilibrium concentration of TT and BB ($\mu\text{g/mL}$), q_e is the amount of dyes adsorbed on the solid ($\mu\text{g/mg}$), q_m is the maximum amount of dyes per unit weight of sorbent ($\mu\text{g/mg}$), K_L is the Langmuir adsorption equilibrium constant ($\text{mL}/\mu\text{g}$), K_F is the Freundlich constant ($\mu\text{g/mg} (\text{mL}/\mu\text{g})^{1/n}$), and $1/n$ is a dimensionless parameter representing the measurement of adsorption intensity.

For the experiments the liqueur and the green icing were selected as the matrices, and then they were both treated with 10, 25, 50, 100 and 200 mg of magnesium oxide (entry O), and 25, 50, 100, 200, 300 and 400 mg of florisil (entry X).

The isotherms constants and parameters are reported in table 15, while the related curves are shown in figure 19 and 20.

Table 15. Isotherm constants for **TZ** onto magnesium oxide (entry O), and for **BB** onto florisil (entry X).

Matrix*	TZ					
	Langmuir**			Freundlich**		
	q_m	K_L	r^2	K_F	$1/n$	r^2
1	0.219	0.56	0.9979	0.172	0.3153	0.9666
2	1.483	0.205	0.9967	0.110	0.2576	0.9564

	BB					
	Langmuir**			Freundlich**		
	q_m	K_L	r^2	K_F	$1/n$	r^2
1	0.588	0.085	0.983	0.038	0.2899	0.9882
2	1.57	0.077	0.9832	0.0487	0.1671	0.9792

***1** = green icing, **2** = liqueur.

** Values are expressed as the following: q_m (μg/mg), K_L (mL/μg), K_F (μg/mg (mL/μg)^{1/n})

Figure 19. Equilibrium isotherms **(a)** Langmuir isotherm **(b)**, and Freundlich isotherm **(c)** for adsorption of **TZ** in the green icing **(1)** and in the liqueur **(2)**

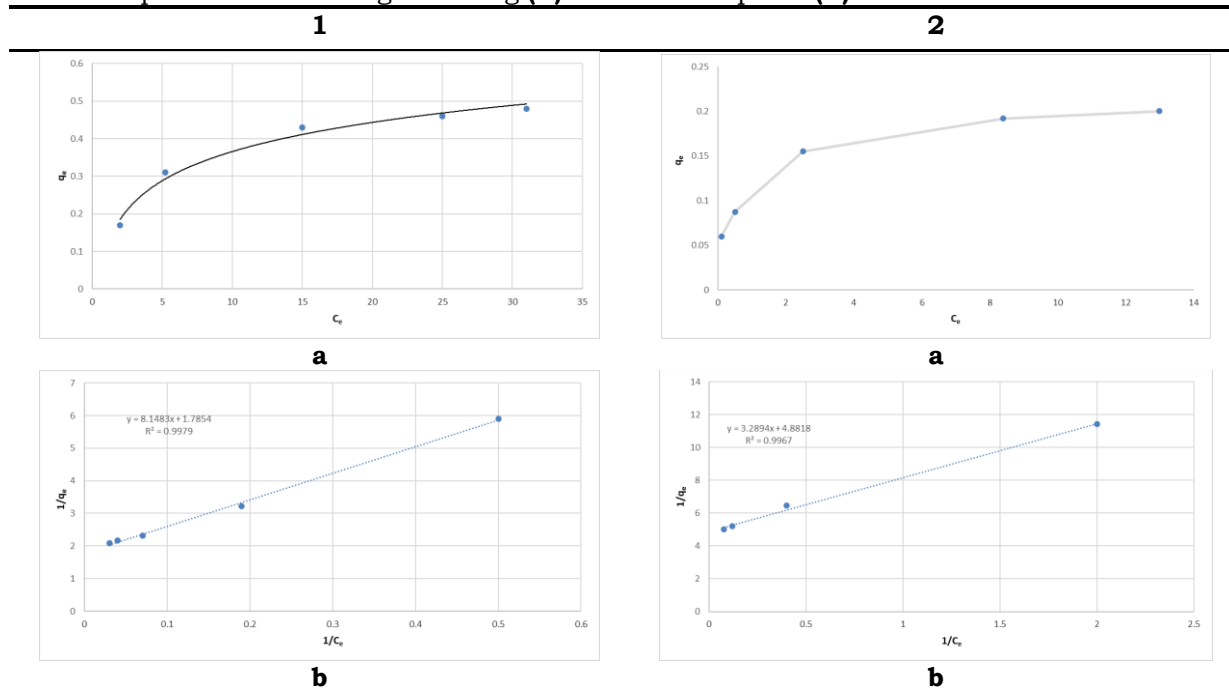


Figure 19 (continued). Equilibrium isotherms (a) Langmuir isotherm (b), and Freundlich isotherm (c) for adsorption of **TZ** in the green icing (1) and in the liqueur (2)

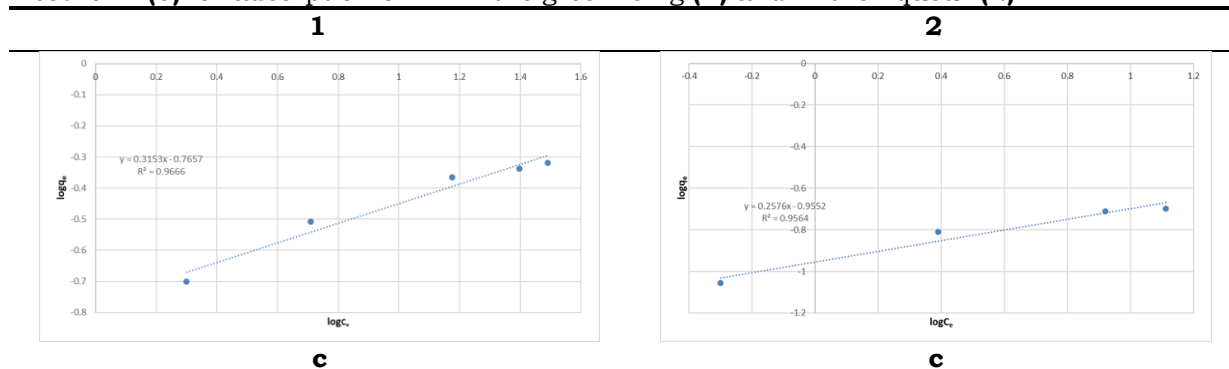
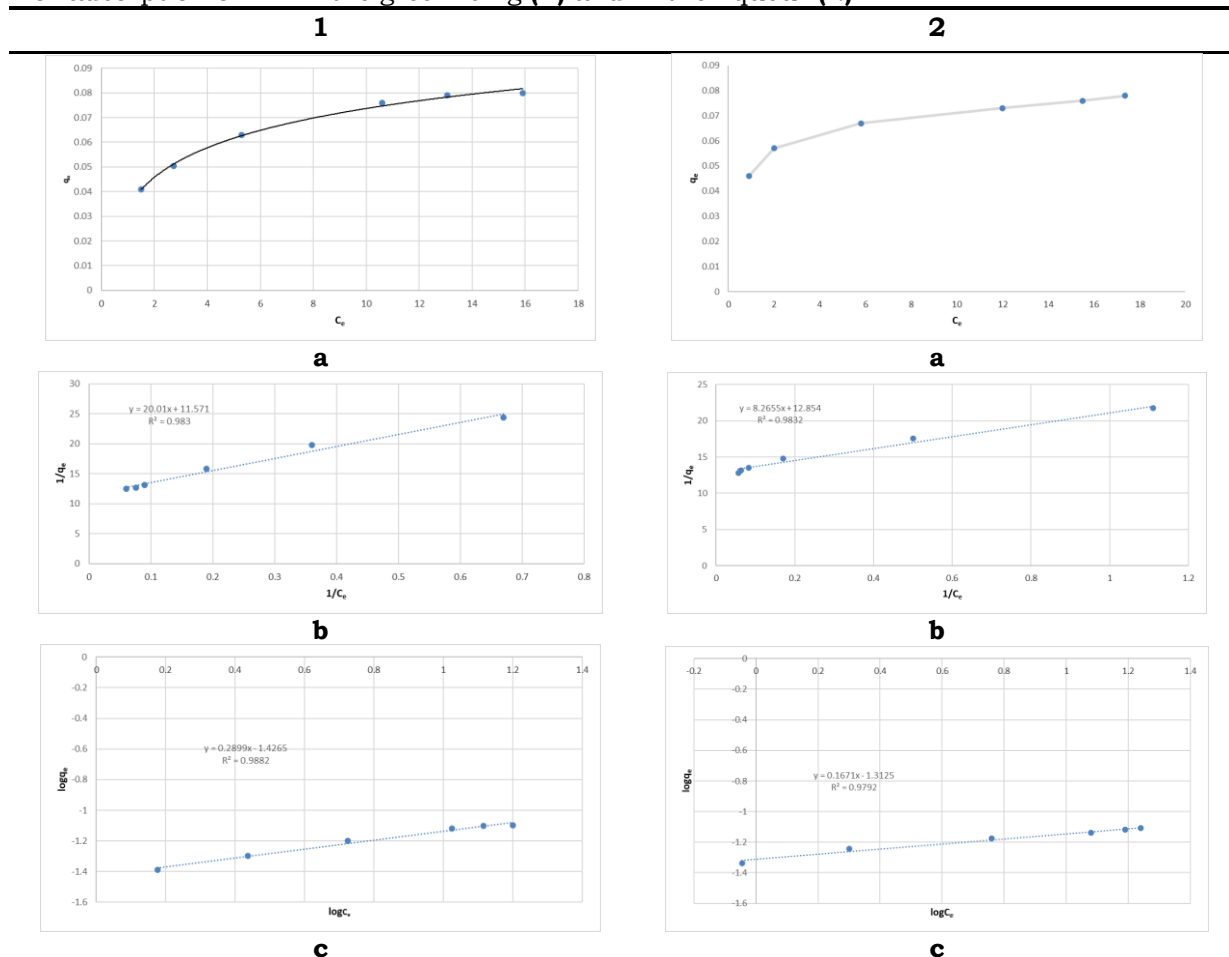


Figure 20. Equilibrium isotherms (a) Langmuir isotherm (b), and Freundlich isotherm (c) for adsorption of **BB** in the green icing (1) and in the liqueur (2)



Considering the r^2 values, the Langmuir model seems to better describe the adsorption for both solids, entries O and X, suggesting that that this process occurs in the monomolecular layers of the sorbents. Additionally, the

Freundlich model provided $1/n$ values that were all below 1, meaning that the extraction of the two dyes was thermodynamically favoured.

1.3.4 Conclusions

The application of SPA technology herein proposed for the efficient removal of tartrazine and blue brilliant FCF from aqueous artificial mixtures and from foods and beverages was successful. The validated method consisted in a simple procedure, with HPLC-DAD analysis and with no need to adjust the pH of the solutions. This provided additional information on the mechanism of action of the panel of the inexpensive and easy to handle lamellar solids in study, focusing specifically on their selectivity, allowing for the first time in literature, to observe the single removal of a dye from a mixture of two.

1.3.5 Experimental procedures

Chemicals

CH₃CN and H₂O HPLC grade, ammonium acetate, tartrazine (purity grade ≥ 97.0 %), and brilliant Blue FCF (purity grade ≥ 97.0 %) were acquired from Honeywell Research Chemicals (Charlotte, North Carolina, USA) and Merck Millipore (Darmstadt, Germany). The solid sorbents used are products commercialized and kindly supplied by Prolabin & Tefarm Srl (Perugia, Italy). All the colored food and beverages were purchased from a local market and stored at room temperature before use.

HPLC-DAD analysis

HPLC analyses were performed using an Agilent 1100 (Santa Clara, CA, USA) series instrument equipped with an autosampler, a binary solvent pump, and a diode-array detector (DAD). The qualitative and quantitative analysis were achieved employing a Kromasil RP C18 (4.6 mm $\varnothing$ x 150 mm, 5 μ m particle size). The column temperature was 25 °C and the injection volume was 20 μ L, while the wavelength set for the acquisition was at 427 nm, for tartrazine, and at 610 nm, for blue brilliant FCF. The mobile phase consisted of an aqueous ammonium acetate solution 1 % (w/v) (solvent A) and an 8:2 MeOH/CH₃CN mixture (solvent B) following a gradient elution program as depicted in table 10 at a flow rate of 1.0 mL/min, for a total run time of 18 min. The HPLC-DAD method was validated according to ICH recommendations, and all the relevant analytical parameters are reported in table 16 [86]. Calibration curves were constructed by injecting standard mixture solutions at the following ten concentration values: 0.1 μ g/mL, 0.5 μ g/mL, 1.0 μ g/mL, 2.5 μ g/mL, 5.0 μ g/mL, 10.0 μ g/mL, 15.0 μ g/mL, 20.0 μ g/mL, 30.0 μ g/mL, and 40.0 μ g/mL. Precision was determined at the following concentration values for quality control (QC): QC_{low} = 0.5 μ g/mL, QC_{medium} = 10.0 μ g/mL, and QC_{High} = 40.0 μ g/mL. Accuracy was assessed by spiking the filtrate obtained after application of MgO (entry O) to the TZ aqueous solution and after application of Florisil (entry X) to the one containing BB with the just mentioned concentrations (low, medium, and high) of the respective pure chemical standard solutions. LODs and LOQs were obtained by injecting serial dilutions of the corresponding standard

solutions, taking the signal-to-noise (S/N) ratio of 3.3 and 10 as criteria, respectively.

Table 16. HPLC method validation parameters for **TZ** and **BB**.

	TZ	BB
Retention time	9.90 ± 0.02	13.52 ± 0.02
Slope	14,059	12.733
Intercept	-804	-274
r²	0.9991	0.9992
SD_s (slope)	997	821
SD_i (intercept)	57	66
SD_r (residuals)	0.241	0.198
LOD (µg/mL) [3.3 X SDs/S]	0.23	0.21
LOQ (µg/mL) [10 X SDs/S]	0.71	0.64
Precision		
Intra-day	% RSD (n=3)	
QC _{low}	100.2/0.995	100.3/0.997
QC _{medium}	100.1/0.994	100.2/1.003
QC _{high}	100.3/1.004	100.2/0.998
Inter-day	% RSD (n=3)	
QC _{low}	100.1/1.008	99.7/1.001
QC _{medium}	99.7/0.992	99.8/1.002
QC _{high}	100.2/1.003	99.7/0.996
Accuracy		
Intra-day	% Recovery (n=3)	
QC _{low}	101.1/1.005	100.7/1.003
QC _{medium}	101.4/0.999	101.1/1.002
QC _{high}	100.8/0.999	100.6/0.997
Inter-day	% Recovery (n=3)	
QC _{low}	100.1/0.997	100.1/0.997
QC _{medium}	101.4/0.999	100.8/0.999
QC _{high}	104.1/0.991	102.0/1.006
Capacity factor	1.1 ± 0.03	1.2 ± 0.04
Resolution	1.2 ± 0.03	1.1 ± 0.05
Peak symmetry	1.03 ± 0.01	1.02 ± 0.02
Tailing	0.99 ± 0.01	0.98 ± 0.02

In conclusion, the overall recorded data indicate that the method set up for the recovery of tartrazine and blue brilliant FCF from aqueous solutions and food and beverages matrices is featured by appropriate accuracy, precision, robustness, and reproducibility, with reference to ICH guidelines.

1.4 Valorisation of agro-waste: use of olive leaves for the pre-concentration of oleuropein and elenolic acid

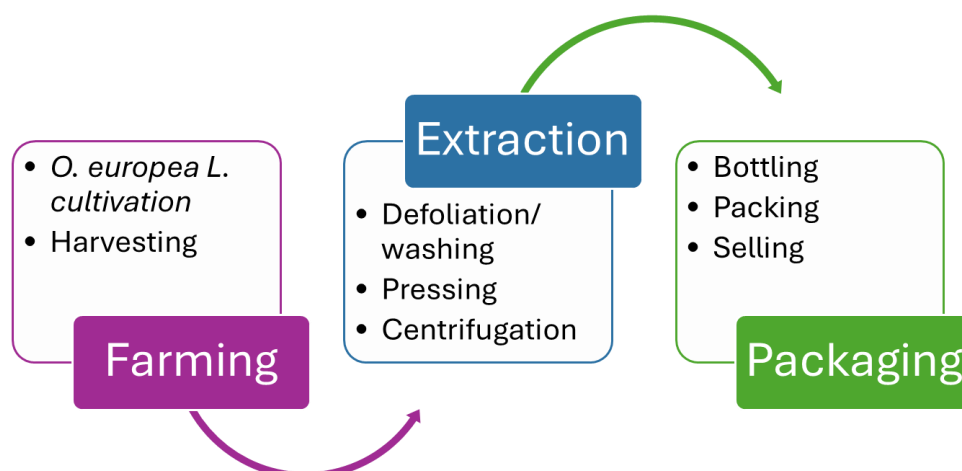
Each year, considerable amounts of agricultural waste are produced and then improperly disposed, causing major environmental issues. Methods such as burning, dumping or landfilling of the untreated agro-industrial waste are quite common, even though they are known to have a big negative impact on the ecosystem, but also on public health, since they can affect soil, air and water quality [106-107-108]. In fact, the great amount of waste is effectively contributing to greenhouse gas emissions, worsening climate change in more ways than one [109].

These issues could be overcome by developing renewable and green technologies with the aim of creating valuable products from agro-waste [110]. Indeed, these by-products are rich in multiple bioactive compounds that could be used for the development of nutraceuticals, food supplements and pharmaceuticals having health-promoting effects [111-112].

Currently, olive oil production represents a significant source of economic income for the Mediterranean countries, accounting for about 98% of its global production, with Spain, Italy and Greece as the forerunner manufacturers [113].

All the stages involved in the production of olive oil are reported in figure 21 [114].

Figure 21. Flow chart of the olive oil production process.



The first fundamental step consists in the cultivation of olive trees, *Olea europaea* L., belonging to the Oleaceae family. But before the plant can effectively produce olives it has to grow from four to eight years [115]. The fruits start to grow around the end of spring, and they are ready to be harvested in September-November. Harvesting can be accomplished manually by farmers, if tools are used for helping in the process it's a mechanized harvesting, or it can be considered mechanical if machines are employed.

The quality of olive oil can deeply depend on the harvesting steps, especially if it's premature or delayed, or if it's followed by an incorrect storage of the olives [116]. Subsequently, the fruits are washed and defoliated to be prepared for pressing, traditionally in a muller, or by using a hammer or a disc crusher, with or without the stone. The resulting paste is subjected to the oil extraction, in which the liquid phase is separated from the solid phase, the pomace. This step can be achieved by pressure, with a hydraulic press, or by using a three- or two-phase centrifuge. At the end of all these phases, the oil can be bottled [117].

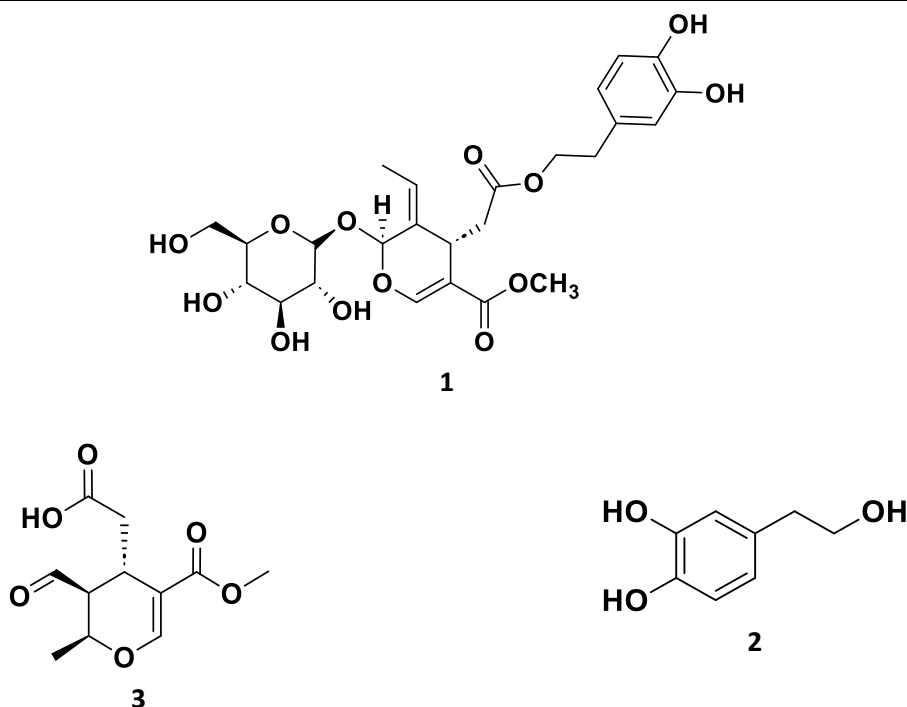
Depending on the different techniques used during each of these steps, the overall process is linked with the production of massive amounts of by-products, including tree pruning, leaves, stones, olive pomace and mill wastewaters [118].

Among them olive leaves are the most abundant waste, representing more than 10 % in weight of the total harvest and about 30 kg per tree during pruning [119]. Although they are currently mostly discarded or burned by olive oil producers, they could be considered as a cheap source of important bioactive compounds with antimicrobial, antioxidant, hypoglycemic, anti-hypertensive, hypocholesterolemia, and anti-inflammatory properties [120-121]. Indeed, nowadays several pharmaceutical and nutraceutical products having olive leaves extract as one of the main ingredients are available in the market.

These beneficial effects can all be attributed to their richness in several polyphenols and phenylpropanoids like verbascoside, luteolin, rutin, apigenin, kaempferol, tyrosol, quercetin, oleuroside, ferulic acid, *p*-coumaric acid, cinnamic acid, caffeic acid and many more [122].

Among the most abundant active principles found in olive leaves is oleuropein (**1**), a bitter tasting secoiridoid glycoside. During fruit maturation and leaves growth, it can undergo hydrolysis, returning different products, among which two of its main aglycons are elenolic acid (**2**) and hydroxytyrosol (**3**) (figure 22).

Figure 22. Chemical structure of oleuropein (**1**), elenolic acid (**2**) and hydroxytyrosol (**3**).



The beneficial effects of oleuropein on human health are multiple. Its antioxidant potential is linked to its ability to form intramolecular hydrogen bonds with radicals, effectively counteracting ROS damages. These effects are at the base of the numerous proven activities of this bioactive compound, like the anti-inflammatory, anti-tumor, neuroprotective and hepatoprotective properties. In addition, antimicrobial and antiviral effects have been recorded [123].

Although oleuropein it's considered to be one of the most abundant secondary metabolites, on average more then 9 % of the dry weight of leaves, its content strongly depends on many endogenous and exogenous factor, like different botanical variety and cultivars, the time of the harvest, climate, soil quality, the extraction process conditions (temperature, pH, time, number of steps, extraction solvent), and so on [124-125]. All these factors

can deeply influence the quality and efficacy of eventual olive leaves-based phyto-preparation.

Thus, one of the aims of this project involved the search for methodologies to optimize and standardize the content of oleuropein from olive leaves extracts and other matrices, which is of great importance, both for quality control during olive oil production and for the development of oleuropein rich phyto-preparations and nutraceuticals [126]. Based on this, the application of SPA technology, by assessing the adsorption capacity of the solids reported in table 3, to selectively pre-concentrate and extract oleuropein from an olive leaves aqueous extract was developed and validated.

In addition, a method for the isolation of elenolic acid (**2**) in pure form from the same extract has been set up. Indeed, due to its chemical instability and the different isoforms occurring from its keto-enolic tautomeric equilibrium, few studies in literature have been devoted to developing methods for its isolation, which instead have been more focused on obtaining hydroxytyrosol [127-128-129]. Most of these procedures required the use of isolated enzymes, which in some cases induced an extreme degradation of oleuropein, preventing elenolic acid obtainment or its recovery in pure form [130]. Even Isenolic®, the branded olive leaf extract product containing elenolic acid, has a standardized content of this compound in the concentration range between 4 % and 8 %, so in low yields. Nonetheless its anti-influenza virus activity is really promising, even in comparison to oseltamivir, a neuraminidase enzyme inhibitor [131]. Moreover, it has also been shown that this bioactive compound is able to exert anti-diabetic properties and hepatoprotective effects [132-133].

Considering the great potential associated with elenolic acid, part of the focus of this project was also dedicated on its isolation through hydrolysis of oleuropein.

1.4.1 Plant material

Leaves of *O. europaea* L., cultivar “Gentile”, were obtained during harvest season (September) in the Abruzzo region, Italy (voucher specimen OEL-1-2023 stored in our laboratory). The leaves were air-dried and then grounded with an electric spice mill for 2 minutes.

The resulting powder was stored in the dark at 4 °C before use.

1.4.2 Olive leaves extraction

The first step consisted in the extraction of the olive leaves powder to obtain high yields of oleuropein. For this aim, several procedures, mostly employing H₂O as the solvent, were evaluated. A simple maceration at room temperature for 24 hours, a 2h Soxhlet extraction and an ultrasound-assisted extraction for 10 min at a temperature of 60 °C were all performed but, in the end, the optimized condition consisted in a microwave-assisted extraction of 20 g of triturated leaves with 300 mL of H₂O for 1 minute at 200 W (equivalent to 80 °C). This was confirmed, after filtration under vacuum, through HPLC-DAD analysis of the filtrates to quantify the content in Oleuropein (table 17).

Table 17. Comparison of extractive yields of oleuropein from powdered olive leaves employing different methodologies.

Method of extraction	Extractive yield (mg/g)*
Maceration	44.3 ± 0.16
Soxhlet	27.9 ± 0.08
Ultrasound-assisted	29.9 ± 0.24
Microwave-assisted	81.3 ± 0.34

*Values expressed as mg/g and as mean ± SD (n = 3).

As shown in table 17, the extract obtained through MAE was the one with the higher content in oleuropein, about 2.3 times higher than the others. Although oleuropein is a thermolabile compound, 1 min of microwave irradiations did not lead to its degradation. Higher time of extractions were evaluated but resulted in a lower yield of the compound of interest. In addition, other extraction solvents were evaluated, like a mixture of H₂O: MeOH in a ratio of 8:2 and EtOH, but they were not as efficient as H₂O.

1.4.3 Sample preparation procedures and results of solid phase adsorption of oleuropein

All the layered biocompatible clays were evaluated for their capacity to selectively adsorb oleuropein from the olive leaves extract. To achieve this, 100 mg of each solid sorbent listed in table 3 was added to 5 mL of the aqueous extract obtained as previously described. The resulting suspension were left under magnetic stirring for 24 hours at room temperature and then centrifugated (5000g for 5 min) and filtrated under vacuum. The amount of oleuropein adsorbed on each solid, so the amount that was removed from the aqueous solutions, was quantified by subtracting the concentration of each analysed supernatant from the blank value by HPLC-DAD analysis.

This was also double checked by washing the solid 5 times with 3 mL of MeOH and then analysing these filtrates with HPLC-DAD. Data regarding the adsorption of oleuropein on the solid supports is reported in table 18.

Table 18. Quantification of oleuropein absorbed on solid sorbents (entries A-Y) from olive leaves aqueous extract in $\mu\text{g/mL}$ and percent (%), after treatment and HPLC-DAD analysis

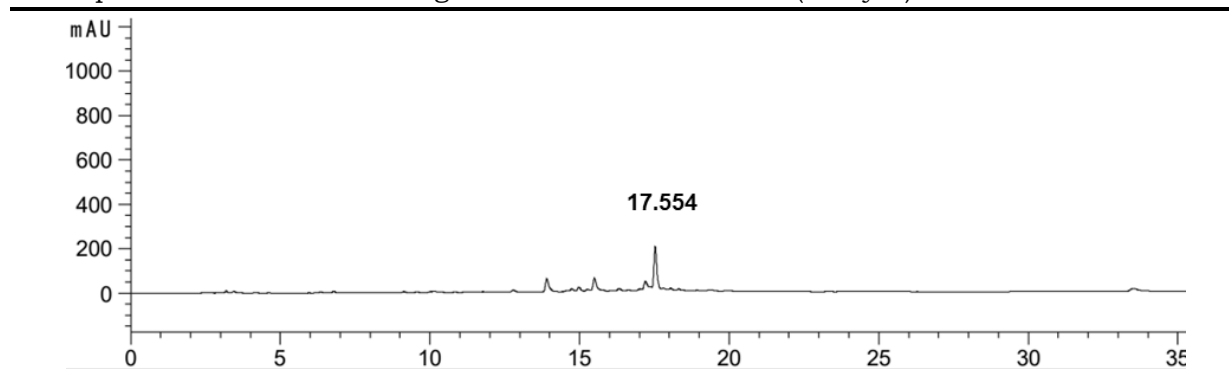
Sample entry	Oleuropein*	
	$\mu\text{g/mL}$	%
Raw MW extract	81.3 ± 0.34	-
A	79.4 ± 0.21	97.6 ± 0.02
B	65.0 ± 0.31	79.9 ± 0.01
C	64.8 ± 0.24	79.7 ± 0.02
D	66.2 ± 0.22	81.4 ± 0.02
E	81.2 ± 0.12	99.9 ± 0.01
F	67.4 ± 0.32	82.9 ± 0.03
G	76.2 ± 0.18	93.7 ± 0.02
H	62.4 ± 0.23	76.7 ± 0.01
I	72.6 ± 0.17	89.2 ± 0.02
L	68.8 ± 0.26	84.6 ± 0.01
M	49.1 ± 0.19	60.4 ± 0.02
N	52.2 ± 0.25	64.2 ± 0.02
O	41.6 ± 0.19	51.1 ± 0.01
P	40.1 ± 0.20	49.3 ± 0.03
Q	55.2 ± 0.21	67.9 ± 0.02
R	57.3 ± 0.20	70.4 ± 0.01
S	61.8 ± 0.18	76.0 ± 0.02
T	64.4 ± 0.17	79.2 ± 0.01
U	62.8 ± 0.17	77.2 ± 0.01
V	80.4 ± 0.13	99.1 ± 0.03
Z	80.4 ± 0.12	99.1 ± 0.02
X	65.9 ± 0.22	81.0 ± 0.03
Y	52.8 ± 0.24	64.9 ± 0.01

*Values expressed as mean $\pm$ SD (n = 3).

As reported in table 18, all solid materials were able to adsorb oleuropein from the aqueous leaves' extract from good to excellent yields. This could be associated with the complexation of the metal cations that are scattered on the surface and on the inner layers of most of the sorbents with the many hydroxy moieties present in oleuropein. In addition the most efficient lamellar solids, namely zincum aluminum oleate (entry A), magnesium aluminum azelate (entry E), magnesium aluminum hydroxide acetate (entry G), magnesium aluminum D.benzenesulfonate SDS 01 H8L (entry V) and zinc aluminum D.benzenesulfonate SDS 02 H8L (entry Z), were all characterized by the presence of an organic anionic counterpart, meaning that supplementary Van Der Waals interaction were fundamental to further facilitate the adsorption of a molecule such as oleuropein. Additional experimental routes were assessed to try and increase the adsorption capacities of the less effective solids, like adding up to 500 mg of sorbents for each treatment, leaving the reaction under magnetic stirring for 72 hours and by apply temperatures above 35 °C. All these steps did not lead to an improvement in yields of removal, and mostly lead to the degradation of oleuropein.

Furthermore, the intercalation of this active principle in the lamellar layers of the clays could be the main reason why the treatment was able to enhance the chemical stability of oleuropein over time. Indeed, when desorbing the compound from the solid every 48 hours over a period of 60 days, it remained unaltered, with a loss in content of less than 1 %. A chromatogram of the desorbed oleuropein from magnesium aluminum azelate (entry E) is reported in figure 23.

Figure 23. HPLC-DAD chromatogram recorded at 280 nm of oleuropein obtained by desorption with MeOH from magnesium aluminum azelate (Entry E)



1.4.4 Sample preparation procedures and selective isolation of elenolic acid by hydrolysis of oleuropein

Finally, the desorption from the solid supports with MeOH, provided high amounts of almost completely pure oleuropein that could easily be further processed to obtain elenolic acid. In fact, a hydrolysis of the glycosidic linkage of the sugar moiety, and of the ester of the hydroxytyrosol portion, was proposed to lead to the release of this terpenoid unit.

Considering how easily oleuropein can undergo chemical degradation, any type of homogenous phase hydrolysis was unsuccessful, both by using inorganic and organic acids and with different reaction conditions.

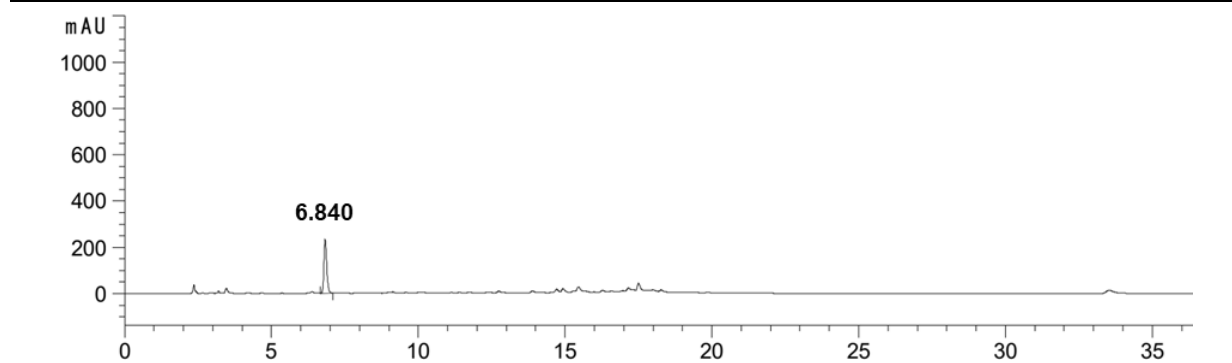
The best solution appeared to be working with a heterogenous phase hydrolysis, and specifically by selecting $\text{-SO}_3\text{H}$ functionalized silica as a catalyst, which is characterized by easy recovery, thermal stability and recyclability, but especially by the ability to work under mild conditions, potentially preserving more labile compounds such as oleuropein [134].

$\text{SiO}_2\text{-OSO}_3\text{H}$ was prepared by dispersing 15 g of SiO_2 gel in 70 mL of Et_2O . After 10 min. under magnetic stirring, 3 mL of H_2SO_4 conc. dissolved in 5

mL of Et₂O were added drop by drop. 1 hour later the solvent was evaporated in a hot air oven at 60 °C followed by heating for 12 hours at 120 °C, finally obtaining a light brown powder.

Next the desorbed secoiridoid was made to react with the solid support in a heterogeneous phase by suspending 1 g of SiO₂-OSO₃H in 10 mL of double distilled H₂O and 200 mg of desorbed oleuropein and leaving under magnetic stirring for 2 hours at room temperature. After filtration under vacuum of this mixture, the filtrates were analysed in HPLC-DAD (figure 24).

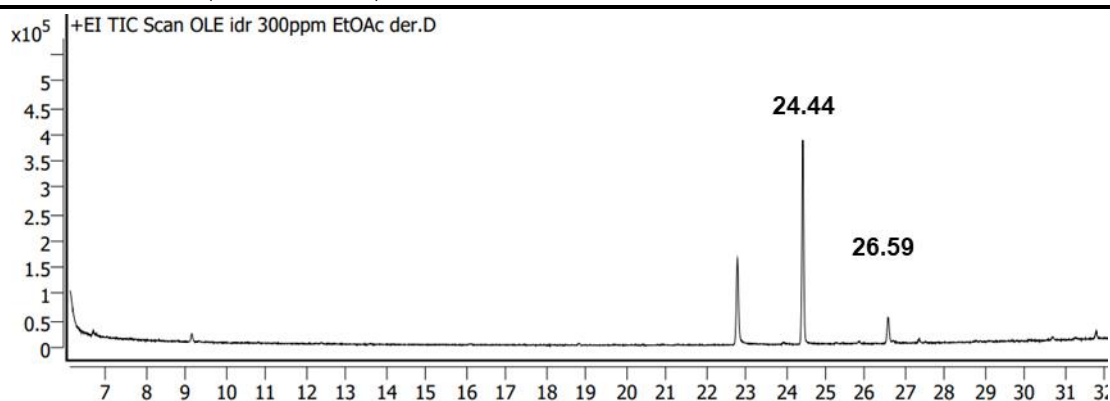
Figure 24. HPLC-DAD chromatogram recorded at 280 nm obtained after treatment of the desorbed oleuropein with silica sulfuric acid (SiO₂-OSO₃H).



The so obtained chromatogram in figure 24 showed the loss of the peak related to oleuropein, at a R_t of 17.55 min., in favour of the appearance of a new unresolved peak at R_t 6.84 min., attributed to the overlapping peaks of hydroxytyrosol and elenolic acid, having a similar polarity.

To further confirm this, after a derivatization procedure to obtain TMS ethers, the same sample was also analysed in GC-MS (figure 25).

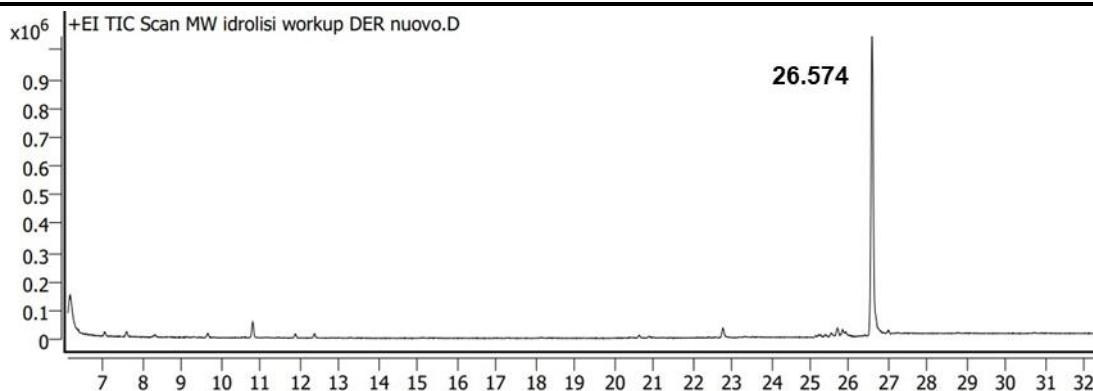
Figure 25. GC-MS chromatogram obtained after treatment of the desorbed oleuropein with silica sulfuric acid ($\text{SiO}_2\text{-OSO}_3\text{H}$).



As shown in figure 25, two different peak can be distinguished from the GC-MS chromatogram, one at R_t 24.44 min. and one at R_t 26.59 min., attributed to be the TMS ethers of hydroxytyrosol and elenolica acid, respectively.

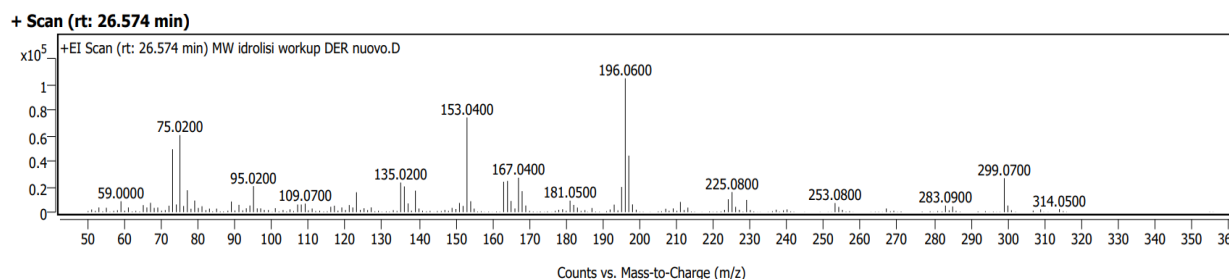
To validate this hypothesis, the two products were separated by an acid-base work up. The filtrate obtained after the hydrolysis was further treated with a 9 % solution of NaHCO_3 to reach a pH of 8.5 and then extracted with EtOAc (3 x 5 mL). Consequently, the aqueous phase was recovered and acidified with a 10 % solution of HCl, to reach a pH of 3.0, and then extracted again with EtOAc (3 x 5 mL). The organic phase was collected and dried over anhydrous NaSO_4 and then evaporated to dryness with a rotary evaporator, providing 45 mg of a raw solid. This was dissolved in EtOAc and derivatized, to be then analysed in GC-MS to further confirm the presence of elenolic acid (figure 26).

Figure 26. GC-MS chromatogram obtained after acid-base work up of the desorbed oleuropein treated with silica sulfuric acid ($\text{SiO}_2\text{-OSO}_3\text{H}$).



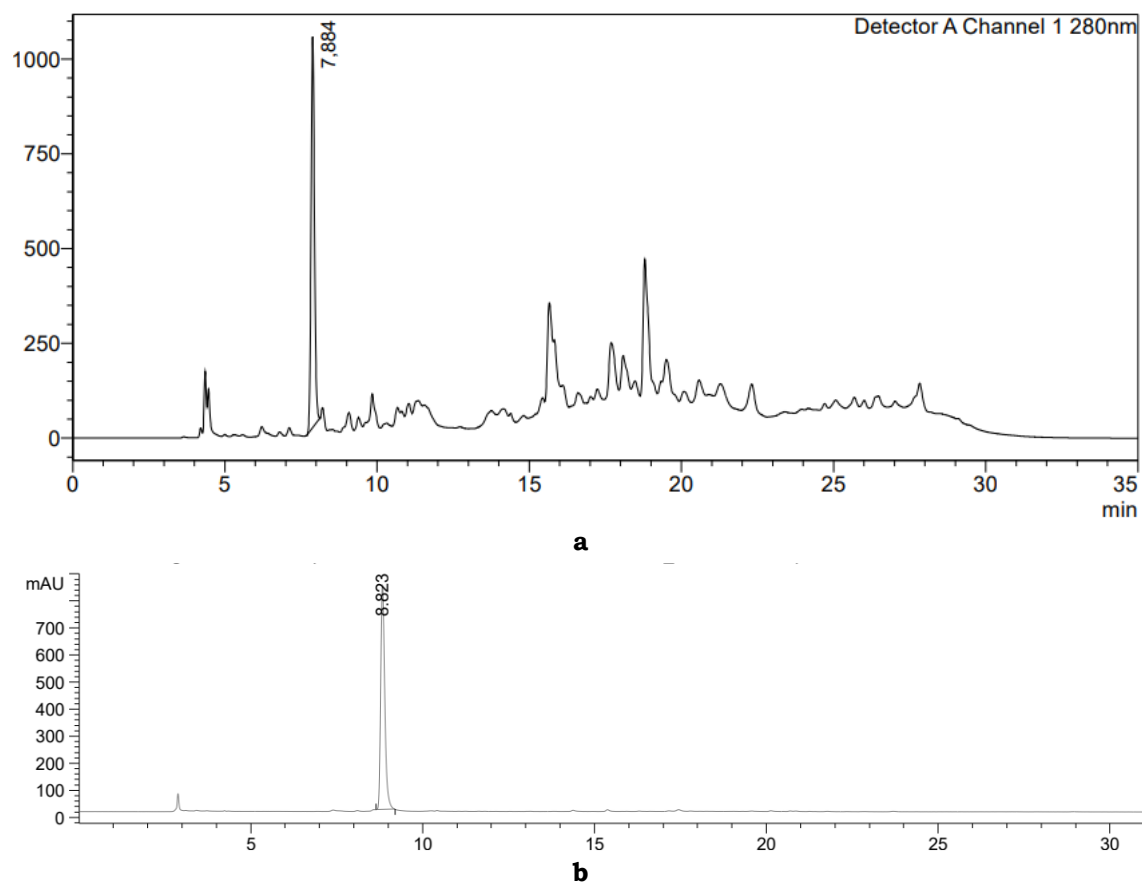
Indeed, the GC-MS chromatogram, reported in figure 26, showed a single peak at a R_t of 26.57 min, that was confirmed to be elenolic acid by comparison of the MS fragmentation pattern with that reported in the NIST library database (figure 27).

Figure 27. MS fragmentation spectrum of elenolic acid.



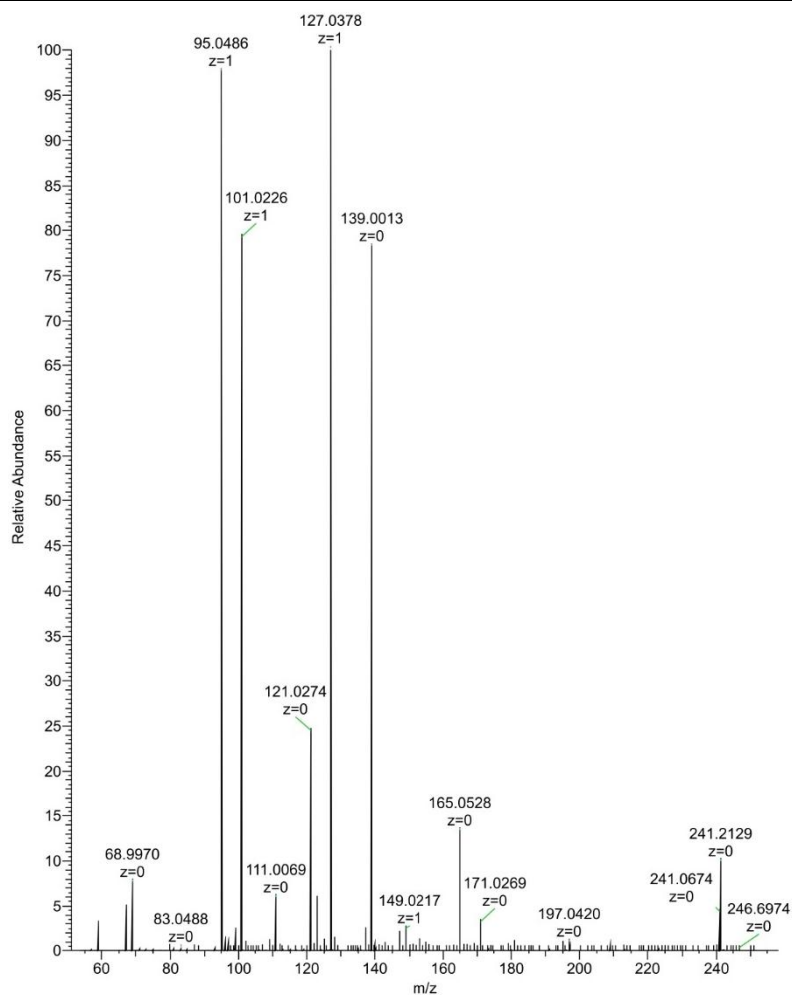
The material was further purified through semi-preparative HPLC (figure 28), providing 7 mg of a white waxy solid, with an overall yield of 7.8 % starting from the desorbed oleuropein.

Figure 28. Semipreparative HPLC chromatogram recorded at 280 nm of elenolic acid before purification (**a**) and HPLC-DAD chromatogram recorded at 280 nm of elenolic acid after purification (**b**)



The structure of pure elenolic acid in its monoaldehydic dihydropyran form was also additionally characterized by HRMS analysis (figure 29).

Figure 29. HRMS spectrum of pure elenolic acid.



1.4.5 Conclusions

The results herein reported represented an example of a successful method for the valorisation of by-products deriving from olive processing. The selective adsorption of oleuropein by lamellar clays, that also led to the enhancement of its chemical stability, could be considered useful for the enrichment with this bioactive secoiridoid of potential olive-leaf based phytopreparations and nutraceutical.

In addition, through the application of simple, easy to handle experimental procedures and by using low-cost materials and equipment, a new procedure to selectively obtain elenolic acid by hydrolysis of oleuropein was developed.

1.4.6 Experimental procedures

Chemicals

H₂O HPLC-grade (>18.2 MΩ/cm resistivity), MeOH Chromasolv® (purity grade ≥ 99.9 %), EtOAc (purity grade ≥ 99.5 %), HCl 37 %, HCOOH 98 %, 3-hydroxytyrosol (analytical standard), and oleuropein (analytical standard) were purchased from Merck Sigma Aldrich (Darmstadt, Germany). CH₃CN (purity grade ≥ 99.9 %) was obtained from Fisher Scientific Italia (Rodano MI, Italy). NaHCO₃ was purchased from Carlo Erba reagents (Cornaredo, Milan, Italy). For GC analyses, standard solutions as well as all samples have been derivatized using BSTFA reagent (N,O-bis(trimethylsilyl)trifluoroacetamide containing 1 % of TMCS, trimethylchlorosilane, LiChropur™) obtained from Supelco® (a Sigma-Aldrich Corporation subsidiary). The procedure was performed by adding an aliquot of 50 μL of the BSTFA derivatizing agent to the solution to be injected in the GC-MS instrument, the vial was capped, mixed, and finally left for 30 min at 70 °C.

Silica sulfuric acid (SiO₂-OSO₃H) was prepared in house by the reaction between silica gel (high-purity grade), purchased from Honeywell Research Chemicals (Charlotte, NC, USA) and H₂SO₄ 96 %, obtained from Carlo Erba reagents (Cornaredo, Milan, Italy).

All solid sorbents tested for oleuropein adsorption from aqueous olive leaf extracts were all commercial products and were kindly donated by Prolabin & Tefarm Srl (Perugia, Italy).

HPLC-DAD analysis

HPLC analyses were performed using an Agilent 1100 (Santa Clara, CA, USA) series instrument equipped with an autosampler, a binary solvent pump, a diode-array detector (DAD), and a fluorescence detector (FLD). The qualitative and quantitative analysis were achieved employing a Phenomenex® RP C18 (4.6 mm Ø x 250 mm, 5 µm particle size) column. The column temperature was 25 °C and the injection volume was 20 µL, while the wavelength set for the acquisition was at 280 nm. The mobile phase consisted of a solution of H₂O/HCOOH 99.6/0.4 as solvent A and of CH₃CN/HCOOH as solvent B, following a gradient elution program as depicted in table 19 at a flow rate of 1.0 mL/min, for a total run time of 35 min.

Table 19. Gradient elution program for HPLC analysis.

Time (min)	A %	B %
0	95	5
10	80	20
15	70	30
18	70	30
25	50	50
30	95	5
35	95	5

The fluorescence detector signal was monitored at 520 nm and 460 nm, both with excitation at 240 nm.

The HPLC-DAD method was validated according to ICH recommendations, and all the relevant analytical parameters are reported in table 20 [86].

Table 20. HPLC method validation parameters for oleuropein

Slope	6402
Intercept	-766
r²	0.9993
SD_s (slope)	971
SD_i (intercept)	47
LOD (µg/mL) [3.3 X SDs/S]	0.50
LOQ (µg/mL) [10 X SDs/S]	1.51
Precision	
Intra-day	% RSD (n=3)
QC _{low}	100.1/0.997
QC _{medium}	100.2/0.996
QC _{high}	100.1/0.999
Inter-day	% RSD (n=3)
QC _{low}	100.2/0.998
QC _{medium}	100.2/0.999
QC _{high}	100.3/0.999
Accuracy	
Intra-day	% Recovery (n=3)
QC _{low}	100.8/0.995
QC _{medium}	101.0/0.996
QC _{high}	100.9/0.998
Inter-day	% Recovery (n=3)
QC _{low}	100.1/0.998
QC _{medium}	100.6/0.996
QC _{high}	101.2/0.996
Capacity factor	1.05 ± 0.02
Resolution	1.10 ± 0.02
Peak symmetry	1.01 ± 0.01
Tailing	0.98 ± 0.02

Calibration curves of oleuropein were drawn by injecting pure standard solutions at twelve concentrations values, namely 0.50 µg/mL, 1 µg/mL, 2.5 µg/mL, 5.0 µg/mL, 10.0 µg/mL, 20.0 µg/mL, 25.0 µg/mL, 50.0 µg/mL, 75.0 µg/mL, 150.0 µg/mL, 300.0 µg/mL and 600.0 µg/mL. The

retention time recorded for oleuropein was 17.5 min. with an r^2 value of 0.9993. Precision was measured at three concentration levels for quality control (QC) samples, namely $QC_{Low} = 1.0 \mu\text{g/mL}$, $QC_{Medium} = 75.0 \mu\text{g/mL}$, and $QC_{High} = 600.0 \mu\text{g/mL}$. Accuracy was calculated by spiking samples deriving from extraction of olive leaves with three concentrations of the oleuropein pure chemical standard (low, medium, and high spikes). LOD and LOQ values were determined by the injection of serial dilutions of the corresponding standard solutions, featured by a signal-to-noise (S/N) ratio of 3.3 and 10.0 as the reference, respectively. All calculations were done in triplicate.

GC-MS qualitative analysis

Gas chromatography-mass spectrometry (GC-MS) analyses have been accomplished using an 8860 GC with 5977B GC/MSD Agilent apparatus. Separation has been obtained by a J&W DB-5 ms Ultra Inert GC Agilent Technologies Column (30 m length, $\emptyset$ 0.25 mm capillary column coated with a 1 μm film thickness stationary phase). The sample volume of 0.2 μL was injected using an AOC-20i + s autoinjector. The temperature of the injection port has been set at 240 °C in split mode (25:1 ratio).

The GC oven temperature ramp was set as follows: 5 min. at 140 °C, increased by 4 °C / min. to the final value of 310°C (with 2.5 min hold at 300 °C). Solvent delay was set to 7 min. The ion source temperature in the MS has been set at 230 °C and the interface at 280 °C. Total Ion Chromatogram (TIC) has been created for m/z range 50-600. GC peaks have been identified by comparing their mass spectra to the database of the

National Institute of Standards and Technology (NIST 11, Mass Spectral Library 2011/EPA/NIH, version updated on June 18th, 2021).

Semipreparative-scale HPLC purifications

Semipreparative-scale HPLC purification of elenolic acid was carried out on a Shimadzu system equipped with a SIL-20A autosampler, a LC20AD pump, a CTO-10AS column oven, an SPD-20A UV-VIS detector, and a FRC-10A fraction collector. The separations were performed on a Phenomenex C18(2) Luna column (10.0 mm $\varnothing$ x 250 mm, 5 μ m particle size, 100 Å pore size), with the oven temperature set at 25 °C. The mobile phase consisted of a solution of H₂O/HCOOH 99.6/0.4 as solvent A and of CH₃CN/HCOOH as solvent B mixture working in a gradient mode at a flow rate of 4.0 mL/min with the same gradient elution program reported in table 16. The peak corresponding to elenolic acid, having a retention time of 7.88 min, was collected from injections of 90 μ L of the raw solid extract, obtained after the acid-base work up, at a concentration of 45 mg/mL.

High Resolution Mass Spectrometry Analysis (HRMS)

50 μ g/mL of the investigated compound was dissolved in CH₃CN/ H₂O 60/40 and injected into the mass spectrometer through a syringe pump at a flow rate of 30 μ L/min. The mass spectrometer was a Thermo Fischer Orbitrap Fusion™ Tribrid™ operating in negative mode MS scan in the m/z range of 50 to 1000 m/z by using the Orbitrap as detector type at 120,000 of mass resolution (FWHM). Precursors were isolated by the quadrupole for subsequent HCD fragmentation using a collision energy of 30% for

241.0669. Fragment ions were scanned in the ion trap (AGC target value of 100, MIT of 100 ms).

1.5 Leaves of *Gentiana lutea* L. as an alternative source of bitter principles

Gentiana lutea L. (common name “yellow gentian”) is a perennial herb of the Gentianaceae family that grows in the mountains of Northern and Central Italy, in the Alpine regions of France, Switzerland, Germany, Austria and Slovenia, but also in Western Asia, and Turkey [135]. It's characterized by erect stems and yellow flowers and its root appear as branched, single subcylindrical pieces of different lengths, usually 10–40 mm thick. The rhizomes are more prominent in diameter and often bear one or more apical buds and surrounding leaf scars (figure 30) [136].

Figure 30. *Gentiana lutea* L. plant and roots.

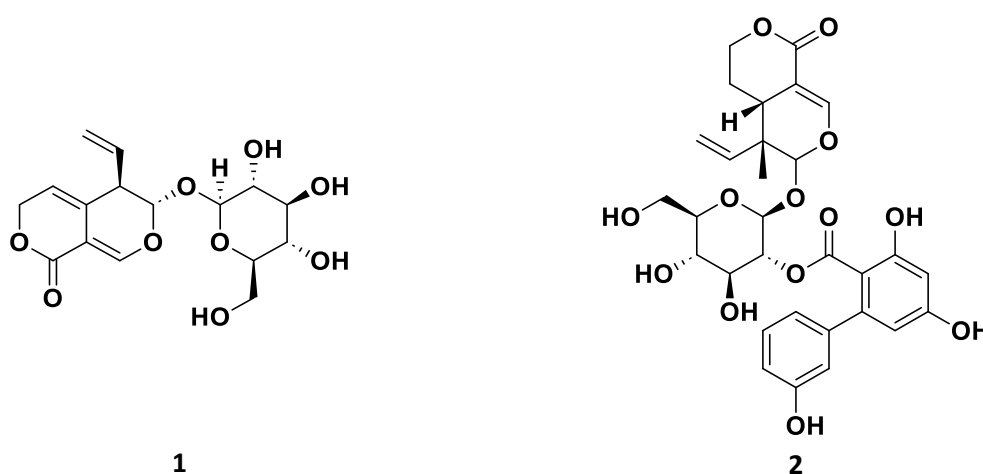


Traditionally, extracts of roots and rhizomes of yellow gentian have been used in the treatment of gastrointestinal disorders, as a stomachic agent and as an external anti-rheumatic and anti-neuralgic remedies [137-138]. Thus, representing a medicinal preparation that has been included in

several pharmacopeia [139]. Indeed, in recent years, *G. lutea* extracts, of roots and of other parts of the plants, or compounds isolated from it have been demonstrated to exert several biological effects, among which antioxidant, antimicrobial, anti-inflammatory, anti-atherosclerotic, antihypertensive, anti-obesogenic, hepatoprotective, radioprotective, and antidepressant properties [140-141-142-143-144].

The roots infusion of yellow gentian is also the main ingredient of a digestive alcoholic beverage widely produced and consumed in the Central regions of Italy, mainly Abruzzo. This liqueur, called “genziana”, is known for its bitter taste, which can be attributed to the presence of two secoiridoid, gentiopicroside (**1**) and amarogentin (**2**) (figure 31) [145].

Figure 31. Chemical structures of gentiopicroside (**1**) and amarogentin (**2**)



These compounds have been evaluated as the most naturally occurring bitter tasting molecules, with bitterness index of 58×10^6 for amarogentin and of 12×10^3 for gentiopicroside [146]. Because of this characteristic, the infusions are also used as ingredients in other alcoholic beverages. Considering the presence of these bitter principles in the roots and rhizomes

of the plants, the production of this liqueur causes the collection and the destruction of large numbers of yellow gentian plants.

The harvest of *G. lutea* takes place in autumn, between September and November, and, in controlled crops, only plants older than 5 years of age can be collected. Once extracted and divided lengthwise, the gentian root is cut into pieces and exposed to air. The drying causes the transformation of its outer color from yellow to reddish-brown.

The slow growth of the plant, together with the risk of overharvesting, in many instances led to a major depletion of *G. lutea* in different territories. Mount Genzana, in Scanno (Abruzzo, Italy), is an interesting example of this, since the name of the mountain itself derived from the past big population of this species on its slopes. In this case, the excessive collection of the roots almost caused the extinction of yellow gentian in that area [147]. To avoid this, yellow gentian is not only a protected species in several countries, but the collection of its roots is now regulated by national and regional laws.

Today the collection of yellow gentian is regulated at the EU level under the Council Directive 92/43/EEC of May 21, 1992, on the conservation of natural habitats and of wild fauna and flora, and EU Commission Regulation no. 1320/2014 of December 1, 2014, amending Council Regulation (EC) no. 338/97 on the protection of species of wild fauna and flora.

For this purpose, it has been demonstrated that in some plants of the Gentianaceae family, especially several *Swertia* spp., that the bitter secoiridoid, gentiopicroside and amarogentin are firstly biosynthesized in the

leaves and then translocated and stored in the root parenchyma [148-149]. This could mean that both of these compounds could be extracted not only from the root, which would imply the complete destruction of the plant, but also from the leaves, concretely reducing the risk of overharvesting.

Therefore, a methodology that could allow the selective extraction of the bitter tasting compounds without harming the plant in the process, could be useful to produce the genziana liqueur, limiting the number of roots needed in the infusion process.

At the same time, the isolation of this two secoiridoid could be the key for the formulations of blends to be used as food additives, for beverages and foods, but also as ingredients with pharmaceutical, nutraceutical and cosmetic purposes.

Thus, the aim of the project was to firstly investigate the content in both gentipicroside and amarogentin in the leaves of *G. lutea* as an alternative source to the roots, and later test the performance of the panel of solids reported in table 3 to selectively isolate and pre-concentrate both compounds. In a second set of experiments, additional solid sorbent reported in table 21, specifically layered double hydroxides with organic anions counterparts of different compositions, were tested to better understand the adsorption mechanism.

Table 21. List of inorganic/organic solid sorbents, with mono and dicarboxylic acid of different length as organic anions, studied for the selective absorption of gentiopicroside and amarogentin from different raw matrices.

SOLID SORBENT	ENTRY
Layered double hydroxides	
Magnesium aluminum oxalate (C2)	AA
Magnesium aluminum malonate (C3)	AB
Magnesium aluminum succinate (C4)	AC
Magnesium aluminum glutarate (C5)	AD
Magnesium aluminum pentanoate (C5)	AE
Magnesium aluminum adipate (C6)	AF
Magnesium aluminum pimelate (C7)	AG
Magnesium aluminum suberate (C8)	AH
Magnesium aluminum nonanoate (C9)	AI
Magnesium aluminum dodecandioate (C12)	AL
Magnesium aluminum carbonate 3:1	AM

1.5.1 Plant material

Leaves of *G. lutea* were collected on Majella Mountain (altitude > 1500 m, Abruzzo region, Italy) between May and August 2020 with the permission obtained from the local government authorities (voucher specimen GL-L-2020-1 stored in our laboratory). The leaves were air-dried and then grounded with an electric spice mill for 2 minutes.

1.5.2 *G. lutea* L. leaves extraction

Leaves extracts for the first experiments were obtained through a simple overnight maceration in absolute EtOH. 10 g of the finely triturated leaves were added to 120 mL of EtOH and after an overnight maceration the mixture was filtrated and evaporated to dryness with a rotary evaporator.

The raw solid extract was resuspended to a concentration in H₂O and analysed in HPLC-DAD to record the content in gentiopicroside and amarogentin in the leaves of yellow gentian (table 22).

Table 22. Comparison of extractive yields of gentiopicroside and amarogentin from powdered *G. lutea* roots and leaves employing different extraction methodologies.

Raw matrix and method of extraction	Extractive yield (mg/g)*	
	Gentiopicroside	Amarogentin
Roots maceration (EtOH)	108.2 ± 0.12	16.0 ± 0.08
Leaves maceration (EtOH)	70.5 ± 0.08	20.6 ± 0.05
Microwave-assisted (H ₂ O)	168.8 ± 0.14	72.1 ± 0.12

*Values expressed as mg/g and as mean ± SD (n = 3).

As shown in table 22, the values of the contents of the two secoiridoid, expressed in mg/g of dry extract, are completely comparable to those obtained in a previous study from the extraction of the roots of the same plant for gentiopicroside, and even higher in the case of amarogentin [150].

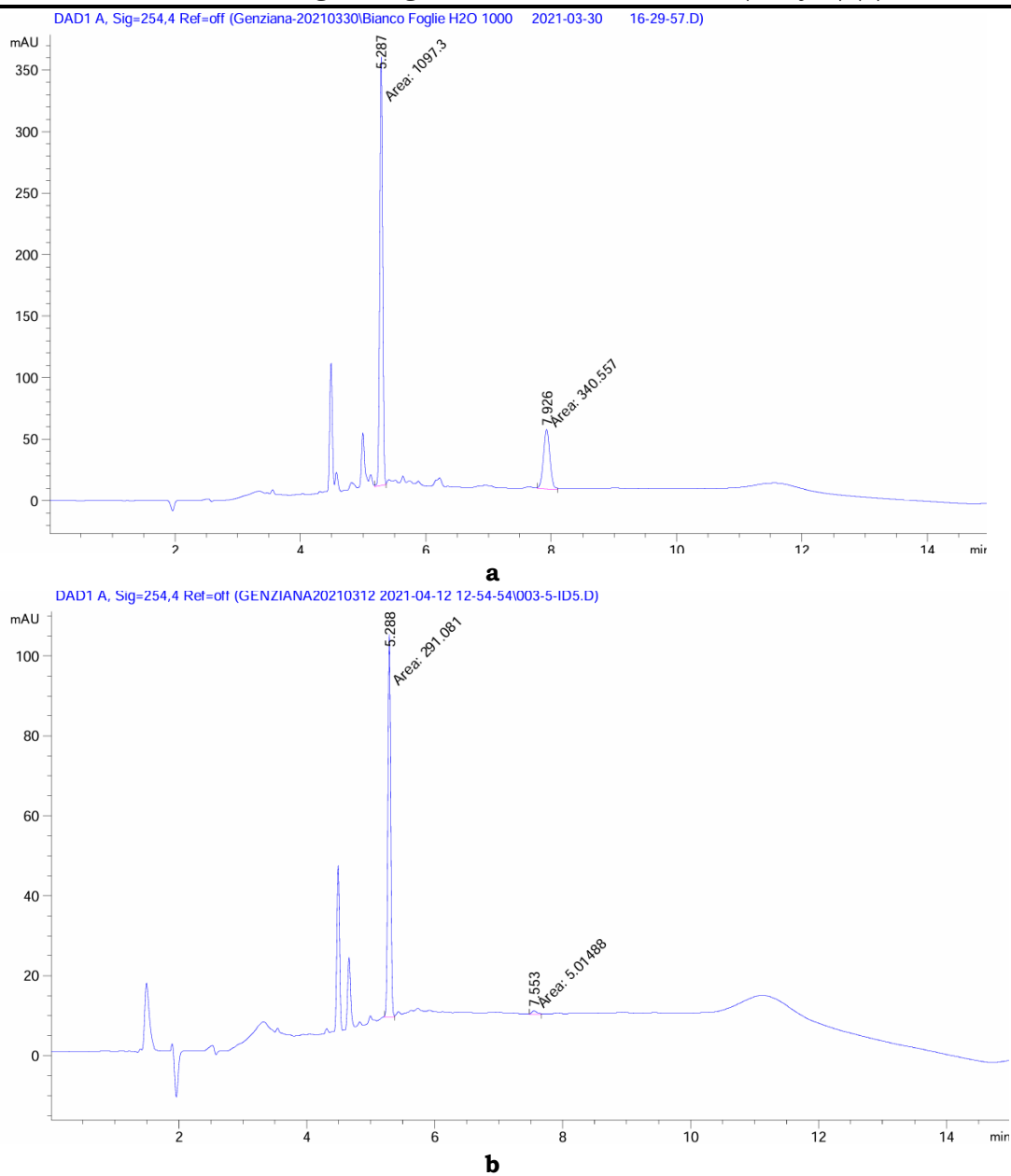
In a second set of experiments, to enhance even more the sustainability of the method, a new extraction procedure was performed. 800 mg of finely triturated leaves were suspended in 80 mL of HPLC-grade H₂O and then subjected to microwave-assisted extraction for 40 sec at 600-675 W. After filtration the same mixture was analysed in HPLC-DAD to evaluate the content of the two molecules of interest. The results reported in table 19 show how this optimized procedure led to higher concentration values of both gentiopicroside and amarogentin in a shorter period.

1.5.3 Sample preparation procedures and results for the original set of solid supports

The raw solid extract obtained through overnight maceration was resuspended in EtOH to reach a final concentration of 1000 µg/mL. The alcoholic solution extract was then divided into 23 aliquots of 1 mL each followed by evaporation to dryness of the solvent. 200 mg of each of the solid sorbents were suspended in 5 mL of double-distilled water and the resulting mixtures were added to the vials containing the leaves raw extracts. Finally, after overnight magnetic stirring at room temperature all the suspension were filtered and centrifugated (5000*g* for 5 min).

The amount of gentiopicroside and amarogentin adsorbed on each solid, so the amount that was removed from the aqueous solutions, was quantified by subtracting the concentration of each analysed supernatant from the blank value by HPLC-DAD analysis. An example of the leaves extract before and after treatment with magnesium aluminum azelate (entry E) is shown in figure 32.

Figure 32. HPLC chromatograms recorded at 254.4 nm *G. lutea* leaves extract before (a) and after treatment with 200 mg of magnesium aluminum azelate (entry E) (b).



The quantification data regarding the adsorption results are reported in table 23.

Table 23. Quantification of gentiopicroside and amarogentin absorbed on solid sorbents (entries A-Z) in $\mu\text{g/mL}$ and percent (%), after treatment and HPLC-DAD analysis

Sample entry	Gentiopicroside*		Amarogentin*	
	$\mu\text{g/mL}$	%	$\mu\text{g/mL}$	%
A	11.1 ± 0.07	33.7 ± 0.3	2.9 ± 0.03	47.5 ± 0.1
B	12.2 ± 0.09	37.1 ± 0.1	5.2 ± 0.04	85.2 ± 0.3
C	11.4 ± 0.07	34.5 ± 0.3	5.6 ± 0.04	91.8 ± 0.1
D	11.9 ± 0.06	36.2 ± 0.1	6.1 ± 0.03	100 ± 0.2
E	11.4 ± 0.06	34.6 ± 0.4	6.1 ± 0.04	100 ± 0.1
F	13.9 ± 0.04	42.0 ± 0.1	6.1 ± 0.05	100 ± 0.2
G	16.5 ± 0.05	50.1 ± 0.2	6.1 ± 0.05	100 ± 0.2
H	11.9 ± 0.07	36.0 ± 0.2	5.8 ± 0.07	95.1 ± 0.2
I	9.2 ± 0.03	27.8 ± 0.2	6.1 ± 0.01	100 ± 0.2
L	13.7 ± 0.08	41.6 ± 0.1	4.4 ± 0.02	72.1 ± 0.1
M	11.2 ± 0.10	33.8 ± 0.2	4.8 ± 0.07	78.7 ± 0.5
N	13.1 ± 0.08	39.7 ± 0.3	4.7 ± 0.06	77.0 ± 0.4
O	12.1 ± 0.14	38.0 ± 0.3	2.8 ± 0.04	45.9 ± 0.2
P	12.5 ± 0.11	38.1 ± 0.1	3.0 ± 0.04	49.2 ± 0.1
Q	18.3 ± 0.09	55.5 ± 0.2	4.5 ± 0.05	73.7 ± 0.1
R	14.1 ± 0.09	42.8 ± 0.2	6.1 ± 0.04	100 ± 0.1
S	29.5 ± 0.15	89.5 ± 0.3	4.7 ± 0.04	77.0 ± 0.3
T	8.5 ± 0.02	25.7 ± 0.1	4.6 ± 0.03	75.4 ± 0.4
U	27.7 ± 0.08	83.9 ± 0.3	1.8 ± 0.01	29.5 ± 0.5
V	6.2 ± 0.03	18.8 ± 0.1	6.1 ± 0.03	100 ± 0.1
Z	7.0 ± 0.03	21.1 ± 0.1	6.2 ± 0.04	100 ± 0.3
X	6.7 ± 0.04	20.3 ± 0.2	2.4 ± 0.05	39.2 ± 0.2
Y	5.8 ± 0.15	17.6 ± 0.3	1.5 ± 0.02	24.5 ± 0.1

*Values expressed as mean $\pm$ SD (n = 3).

The general trend highlighted a better retention capacity towards amarogentin. Indeed, only two clays were able to adsorb gentiopicroside with satisfactory values, namely mica L (entry S) and mica SFG20 (entry U), with

percentages of adsorption of 89.5 % and 83.9 % respectively, while for the others they were lower than 50.1 %.

On the other hand, almost all the solid supports performed quite well regarding the adsorption of amarogentin. In particular, Mg and Al containing clays, namely magnesium aluminum nitrate (entry D), magnesium aluminum azelate (entry E), magnesium aluminum hydroxy chloride (entry F), magnesium aluminum hydroxy acetate (entry G), magnesium aluminum hydroxy carbonate (entry H), magnesium aluminum acetate (entry I), talc (entry R) and magnesium aluminum d. benzene sulfonate SDS 01H8L (entry V), but also zinc d. benzene sulfonate SDS 02H8L (entry Z), provided almost quantitative extractive yields. This clear selectivity towards the adsorption of amarogentin may be due to its larger chemical structure which is also richer in phenolic hydroxy groups.

To better evaluate the adsorption capacity of the nine most active sorbents towards amarogentin, the effect of the solids loading was investigated. The same treatment was repeated but this time using 10 mg, 25 mg, 50 mg and 100 mg of the selected clays (entries D-I, R, V and Z). The quantification results are reported in table 24.

Table 24. Effects of sorbent loading on amarogentin adsorption (percentage %) with entries D-I, R, V, Z after treatment and HPLC-DAD analysis.

Sample entry	Sorbent loading and percentages (%) [*]			
	100 mg	50 mg	25 mg	10 mg
D	100 ± 0.2	100 ± 0.4	100 ± 0.1	94.4 ± 0.2
E	100 ± 0.1	100 ± 0.2	100 ± 0.3	100 ± 0.3
F	100 ± 0.3	100 ± 0.3	100 ± 0.1	83.3 ± 0.4
G	100 ± 0.5	100 ± 0.3	93.0 ± 0.2	76.1 ± 0.1
H	100 ± 0.3	64.4 ± 0.2	44.1 ± 0.2	28.2 ± 0.2
I	100 ± 0.1	100 ± 0.1	98.7 ± 0.4	98.3 ± 0.1
R	100 ± 0.4	62.1 ± 0.3	48.9 ± 0.2	11.7 ± 0.1
V	100 ± 0.2	100 ± 0.3	100 ± 0.1	100 ± 0.1
Z	92.1 ± 0.5	85.4 ± 0.1	83.7 ± 0.2	55.1 ± 0.2

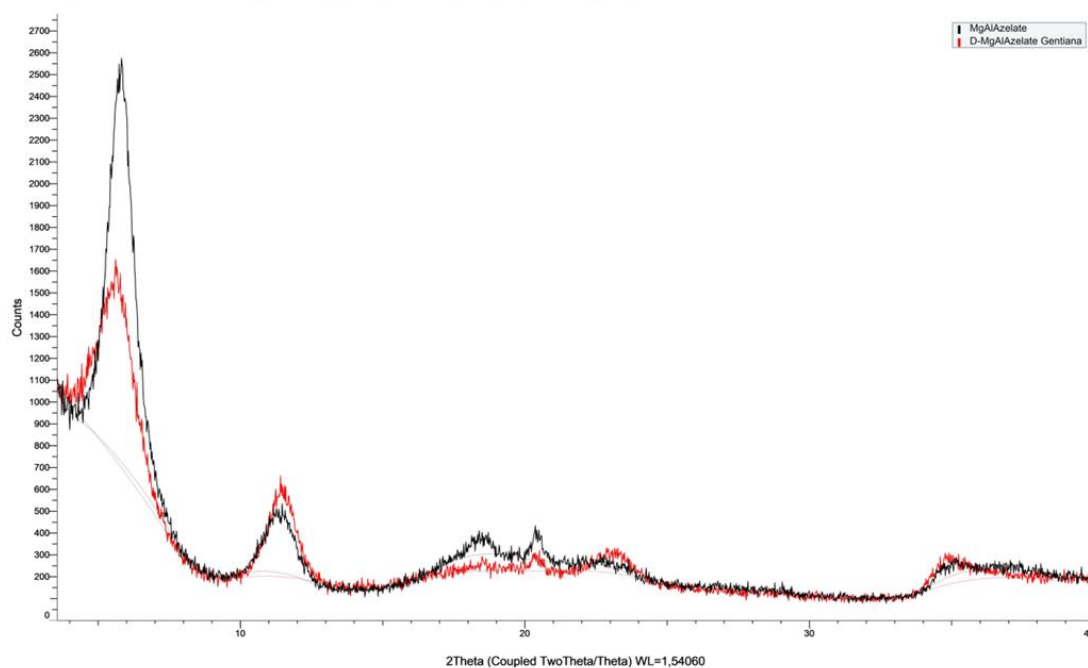
^{*}Values expressed as mean ± SD (n = 3).

Out of the nine selected for these additional tests, five sorbents remained efficient in their adsorption even with at the lowest loading amount. Specifically, magnesium aluminum azelate (entry E), magnesium aluminum d. benzene sulfonate SDS 01H8L (entry V) and magnesium aluminum acetate (entry I), gave quantitative or nearly quantitative adsorption yields for amarogentin even when tested at 10 mg. Considering how these layered double hydroxides are all characterized by the presence of an organic anion in their lamellar layers, this could link their good performance to the establishment of additional Wand der Waals interaction with the more hydrophobic portions of the bitter secoiridoid.

To further investigate and confirm this theory, additional characterizations of one of the most performing solids were performed, specifically X-ray diffraction, FT-IR/ATR spectroscopy, BET specific surface area and scanning

electron microscope (SEM) analysis on magnesium aluminum azelate (entry E), before and after the adsorption with the two compounds of interest.

Figure 33. XRD spectra of magnesium aluminum azelate (entry E) in black, and of magnesium aluminum azelate (entry E) after gentiopicroside and amarogentin adsorption in red.

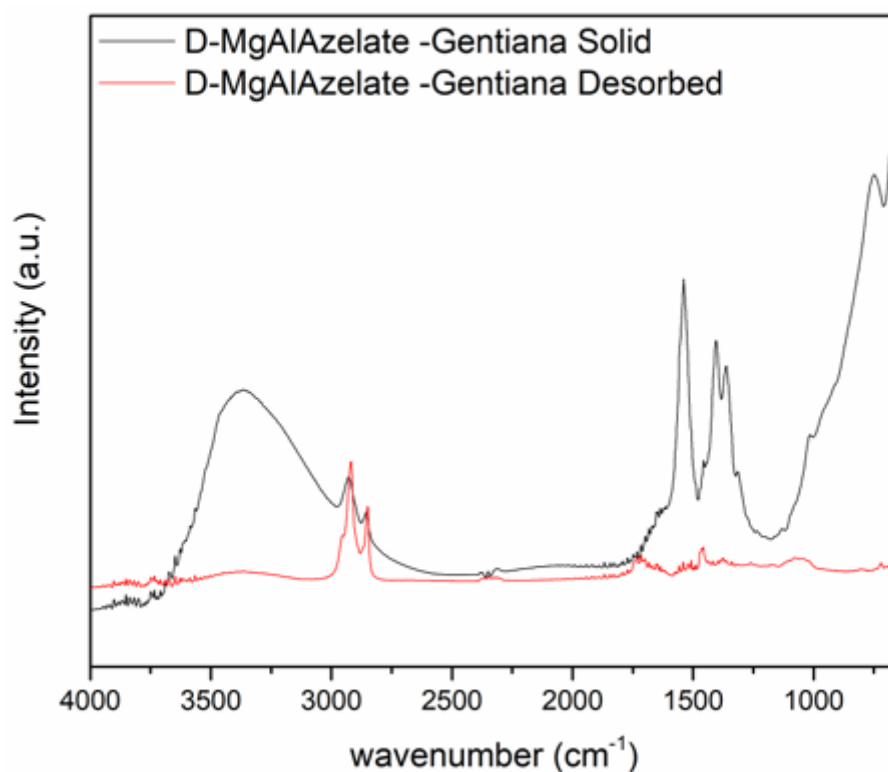


The XRD spectra were recorded using an X-Ray Powder Diffractometer Bruker D2 PHASER 2nd generation, and, as shown in figure 33, no variations of the interlayer spacing was detected in the solid before and after the adsorption process. The unaltered distances between the lamellar layers, could indicate that big molecules, such as gentiopicroside and amarogentin, were not able to get intercalated in the intermediate spaces.

At the same time, it cannot be excluded that the active principles could intercalate in the lamellar layers without altering their distance spacing. So, following Pàlinkò and Coworkers procedures, FT-IR/ATR analysis on the solid before and after desorption with EtOH, in the latter case directly on the

resulting desorbed extract, were performed on a JASCO FT/IR-4600 spectrometer [151].

Figure 34. FT-IR/ATR spectra of the yellow gentian extract adsorbed on magnesium aluminum azelate (entry E) in black, and of the same extract after desorption from the solid with EtOH in red.



The FT-IR/ATR spectra are reported in figure 34. The correct desorption of the solid can be observed by the absence of an intense peak at around 1700 cm⁻¹, confirming that the EtOH did not cause the detachment of the azelaic acid, linked to the sorbent with ionic interactions, while it led to the release of gentipicroside and amarogentin.

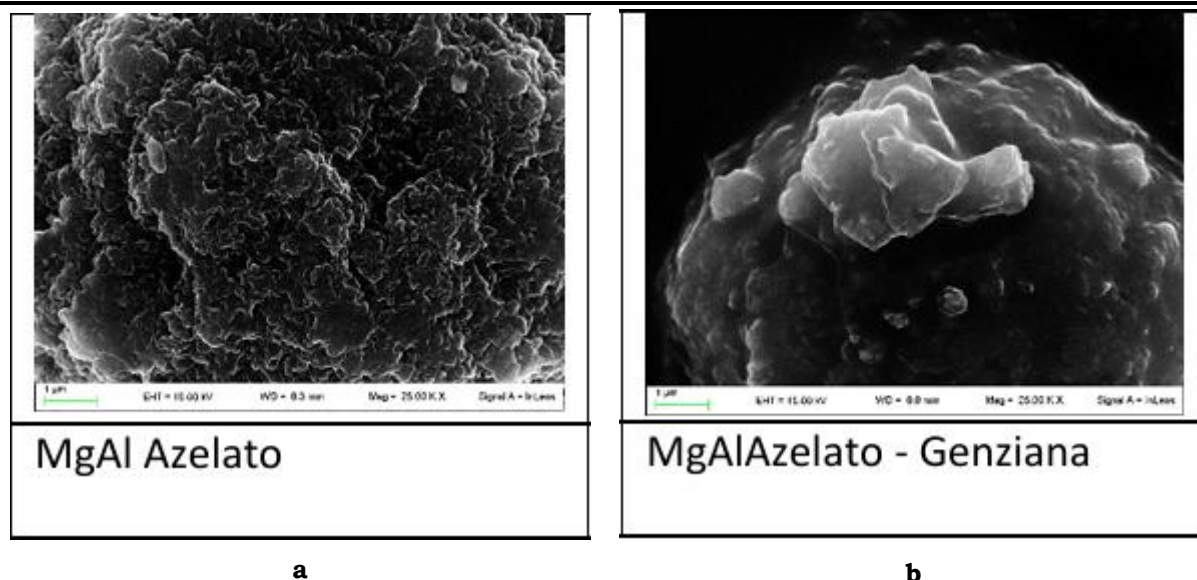
This can prove that a weak interaction is taking place between the clay and the compounds of interest. Additionally, since no significant shift between the signals of the two samples can be detected, it's still difficult to confirm

whether the molecules are only adsorbed on the surfaces of the solid or also in the interlayer spaces.

To evaluate the differences on the surface of the sorbent before and after the adsorption process BET specific surface area was calculated on a BET Micromeritics Gemini VII. The value obtained from magnesium aluminum azelate (entry E) before the treatment, 1,3103 m²/g, increased of about 190.8% to reach a value of 3.8108 m²/g after the adsorption of the active secoiridoids. This can be interpreted as a confirmation of the interaction of the compounds of interests with the surface of the clay.

Finally, scanning electron microscopy (SEM) was performed to further assess the changes in the surface of the solid. In figure 35, a clear difference in the superficial morphology of magnesium aluminum azelate before and after the adsorption process can be detected.

Figure 35. Scanning electron micrograph of magnesium aluminuma azelate (entry E) before (a) and after the adsorption process with yellow gentiana (b).



In conclusion, all the data obtained suggests that the secoiridoids interact with the sorbent mainly through weak interactions on a surface level. Therefore, there is no exchange between the target molecules and those intercalated at the start, but mainly an adsorption based on hydrophobic interaction, such as Van der Waals interactions, between their non-polar portions.

Further changes of the experimental conditions, like the treatment time and an increase in temperature, did not lead to better results. And after having selected magnesium aluminum azelate (entry E), magnesium aluminum acetate (entry I) and magnesium aluminum d. benzene sulfonate SDS 01H8L (entry V) as the most performing solid sorbents, even at the lowest loading levels (table 21), they were desorbed with EtOH to release the active principles and then recycled by drying in an oven at 70 °C for 2 h.

Subsequently, five more treatments were performed using 10 mg of each of the solids and with the same experimental procedures. The percentage of adsorption of amarogentin are reported in table 25.

Table 25. Percentages of adsorption (%) of amarogentin by 10 mg of magnesium aluminum azelate (entry E), magnesium aluminum acetate (entry I) and magnesium aluminum d. benzene sulfonate SDS 01H8L (entry V) obtained for each treatment after recycling of the solids.

Treatment cycle	Sample entry and percentages (%)*		
	E	I	V
1	100 ± 0.2	99.2 ± 0.2	100 ± 0.3
2	100 ± 0.4	98.7 ± 0.4	99.9 ± 0.2
3	99.8 ± 0.1	98.8 ± 0.3	99.9 ± 0.3
4	100 ± 0.2	99.1 ± 0.1	100 ± 0.25
5	99.6 ± 0.15	98.4 ± 0.2	99.8 ± 0.1

*Values expressed as mean ± SD (n = 3).

Such results clearly indicate how the solid sorbents can be recycled and reutilized without any loss of adsorption capacity.

1.5.4 Sample preparation procedures and results for the second set of solid supports

The *G. lutea* L. aqueous extract obtained with the new procedure, so with the microwave-assisted extraction, was filtrated and then divided into 14 aliquots of 5 mL each and kept under magnetic stirring with 100 mg of each of the solids reported in table 21 together with magnesium aluminum azelate (entry E), magnesium aluminum hydroxide carbonate (entry H), and magnesium aluminum carbonate (entry I), as references.

After 24 hours each mixture was filtrated under vacuum and centrifugated (5000*g* for 5 min), analysing the resulting solutions in HPLC-DAD. The amount of gentiopicroside and amarogentin adsorbed on each solid, so the amount that was removed from the aqueous solutions, was quantified by subtracting the concentration of each analysed supernatant from the blank value by HPLC-DAD analysis. The quantification data regarding the adsorption results are reported in table 26.

Table 26. Quantification of gentiopicroside and amarogentin absorbed on solid sorbents (entries E-H-I, AA-AM) in $\mu\text{g/mL}$ and percent (%), after treatment and HPLC-DAD analysis

Sample entry	Gentiopicroside*		Amarogentin*	
	$\mu\text{g/mL}$	%	$\mu\text{g/mL}$	%
E	107.3 ± 0.06	36.3 ± 0.1	0.1 ± 0.03	99.9 ± 0.3
H	108.1 ± 0.07	36.0 ± 0.2	9.8 ± 0.04	84.6 ± 0.1
I	118.7 ± 0.3	29.7 ± 0.4	6.3 ± 0.02	91.2 ± 0.2
AA	102.9 ± 0.1	39.0 ± 0.05	32.5 ± 0.1	54.9 ± 0.1
AB	101.1 ± 0.15	40.1 ± 0.3	5.4 ± 0.02	92.5 ± 0.1
AC	96.4 ± 0.04	42.9 ± 0.2	4.6 ± 0.05	93.6 ± 0.2
AD	96.0 ± 0.03	43.1 ± 0.1	3.8 ± 0.04	94.7 ± 0.3
AE	99.8 ± 0.03	40.9 ± 0.1	14.2 ± 0.03	80.3 ± 0.1
AF	115.9 ± 0.08	31.3 ± 0.1	20.3 ± 0.06	91.9 ± 0.1
AG	89.3 ± 0.03	47.1 ± 0.1	1.5 ± 0.03	98.5 ± 0.1
AH	87.6 ± 0.08	48.1 ± 0.3	0.9 ± 0.01	98.7 ± 0.5
AI	95.2 ± 0.02	43.6 ± 0.1	20.1 ± 0.03	72.1 ± 0.4
AL	71.8 ± 0.02	57.4 ± 0.5	0.4 ± 0.02	99.4 ± 0.4
AM	35.4 ± 0.15	79.0 ± 0.3	6.8 ± 0.04	90.6 ± 0.3

*Values expressed as mean $\pm$ SD (n = 3).

For all the new tested sorbents, quite good results in term of extractive yields and selectivity were recorded. Additionally, the gap in the percentage of adsorption between amarogentin and gentiopicroside can still be observed.

Indeed, the presence in the solids of an organic anion of medium to high lipophilicity intercalated in the lamellar layers, greatly facilitates the interaction with the two compounds.

For example, when focusing on the percentages of adsorption of amarogentin obtained with magnesium aluminum oxalate (entry AA) and magnesium aluminum dodecandioate (entry AL), we can see how the increase in the lipophilicity of the organic anions greatly enhances the

adsorption capacity from 54.9 % to 99.4 %. Amarogentin, having a bigger structure, is better adsorbed thanks to additional Van der Waals and lipophilic interactions.

For what concerns magnesium aluminum carbonate 3:1 (entry AM), this solid is characterised by a bigger interlayer distance in comparison to the corresponding magnesium aluminum hydroxide carbonate (entry H). This led to an enhancement in the adsorption capacity of gentiopicroside, followed by a loss of selectivity towards amarogentin.

1.5.5 Conclusions

Following the “Green extraction principles”, two secoiridoids compounds of high commercial value, like gentiopicroside and amarogentin, were quantified and extracted from renewable plant sources and using green extraction methodologies. Solid materials, characterized by low cost, recyclability and reusability, were used in an easy-to-handle procedure to obtain excellent preconcentration yields of the two compounds of interest, with a concrete possibility to transfer this technology to pilot plant and industrial reactor applications.

Indeed, this project allowed the formulation of nature-derived blends to potentially become ingredients for the preparation of yellow gentian-based extracts, in the liqueur industry, but also for pharmaceuticals, nutraceutical and cosmeceutical purposes.

1.5.6 Experimental procedures

Chemicals

Solvents (CH₃CN, H₂O both HPLC grade, EtOH, MeOH, and HCOOH) were purchased from Honeywell Research Chemicals (Charlotte, North Carolina, USA) and Merck Sigma-Aldrich (Milan, Italy). HPLC standards, gentiopicroside and amarogentin (purity grade $\geq$ 99%) were purchased from PhytoLab (Vestenbergsgreuth, Germany). All solid materials were supplied by Prolabin & Tefarm Srl (Perugia, Italy).

HPLC-DAD analysis

An Agilent 1100 (Santa Clara, CA, USA) series instrument equipped with an autosampler, a binary solvent pump, a diode array detector (DAD) and a fluorescence detector (FLD), was used to accomplish HPLC analyses. The qualitative and quantitative analysis were achieved employing a Kromasil RP C18 (4.6 mm $\varnothing$ x 150 mm, 5 μ m particle size) column. The column temperature was 25 °C and the injection volume was 20 μ L, while the wavelength set for the acquisition was at 254 nm. The mobile phase consisted of a solution of H₂O/HCOOH 99.6/0.4 as solvent A and of CH₃CN/HCOOH as solvent B, following a gradient elution program as depicted in table 27 at a flow rate of 1.0 mL/min, for a total run time of 15 min.

Table 27. Gradient elution program for HPLC analysis.

Time (min)	A %	B %
0	97	3
3	70	30
9	70	30
12	97	2
15	97	2

The HPLC-DAD method was validated according to ICH recommendations, and all the relevant analytical parameters are reported in table 28 [86].

Table 28. HPLC method validation parameters for oleuropein

	Gentiopicroside	Amarogentin
Slope	114112	112397
Intercept	1650	1501
r²	0.9997	0.9999
LOD (µg/mL) [3.3 X SDs/S]	0.15	0.05
LOQ (µg/mL) [10 X SDs/S]	0.30	0.10
Precision		
Intra-day (n = 6)	2.1 – 3.9	2.0 – 3.8
Inter-day (n = 6)	2.3 – 4.0	2.4 – 4.1
Accuracy		
Intra-day (n = 6)	2.1 – 3.9	2.0 – 3.7
Inter-day (n = 6)	1.2 – 2.1	1.0 – 1.9

The intra-day precision was determined by the injection of the standard mixture solution five times a day. For the inter-day precision, measurements were recorded once a day on three consecutive days. All these were expressed as relative standard deviations (RSDs). Precision was calculated at three concentration levels for quality control (QC) samples, namely QC_{Low} = 1.0 µg/mL, QC_{Medium}=25.0 µg/mL, and QC_{High} = 100.0 µg/mL.

Accuracy was determined by spiking samples deriving from yellow gentian leaves extracts treated with the hydrotalcite magnesium aluminium azelate (entry E) with three concentrations of the two standard compounds (low, medium, and high spikes). Calibration curves were drawn by injecting gentiopicroside and amarogentin pure standard stock solutions at the following 9 concentrations values, namely 0.50 µg/mL, 1 µg/mL, 5.0 µg/mL, 10.0 µg/mL, 25.0 µg/mL, 50.0 µg/ mL, 75.0 µg/mL, 100.0 µg/mL, 200.0 µg/mL. LODs and LOQs were obtained by injecting serial dilutions of the corresponding standard solutions, having a signal to-noise (S/N) ratio of 3.3 and 10 as the reference, respectively. RSD values for intraand interday precisions were not higher than 3.4%, while BIAS % ones of accuracies related to the same phytochemicals ranged from – 1.7% to 2.9%. The carry over effect (<0.12%), was not observed. Recoveries of the two compounds were above 99.6% with a good precision (RSD < 1.1%).

The retention time recorded for gentiopicroside and amarogentin was 5.3 ± 0.02 min. and 7.9 ± 0.05 min., respectively. All calculations were done in triplicate.

Chapter 2 - *Isolation and structural characterization of plant secondary metabolites*

Plants are a remarkable source of bioactive compounds that are commonly biosynthesised as secondary metabolites, playing an important role in the defence, communication, and adaptation to the environment [152-153-154-155]. Nonetheless, these plant-derived natural products have important medicinal values, and their still directly used as remedies for multiple health related problems all over the world [156-157-158]. New or less studied alkaloids, terpenoids and phenolic compounds recovered from different plant matrices, are often discovered to be potent pharmacologically active molecules [159-160-161]. In addition, because of their scaffold diversity and structural complexity, natural products play a significant role in the drug discovery processes, working as innovative leads in multiple fields of research [162]. Thus, the isolation, characterization and structural elucidation of plant secondary metabolites is fundamental.

2.1 High resolution PTP1B inhibition profiling combined with HPLC-PDA/NMR for identification of bioactive constituents in the leaves of *Eremophila tietkensis*.

The Myoporeae tribe, of the Scrophulariaceae family, comprehends 7 genera. Among them, the *Eremophila* genus is the largest with around 230 species and 58 subspecies [163]. Plants of *Eremophila* genus appear as perennial shrubs and small trees and they are commonly widespread in the Eremean biome, an arid inland region that comprises about 70 % of the Australian continent [164]. Indeed, the name *Eremophila* indicates how these plants grow in the desert harsh conditions, since it's derived from the Greek words “*Eremos*”, desert, and “*Phileo*”, love [165]. These species are characterized by alternate, petiolate and lanceolate leaves, acute apex and both entire or dentate margins, and they are often grey and hairy. The axillary single flowers have a yellowish, green or red corolla [166]. Considering their vast diffusion, the Aboriginal Peoples of Australia, who commonly refers to them as “Fuchsia bush” or “Emu bush”, have always recognized various *Eremophila* species as a valuable source of traditional medicine, frequently using the leaves in their preparations for treatment of various ailments or for ceremonial uses [167].

The research for the identification of secondary metabolites from *Eremophila* species began in the 70s and led to the discovery of a wide array of structurally unique terpenoids, such as serruletanes, viscidanes, cembrane and eramane, all exhibiting promising biological activity [168-169-170-171]. They have been tested, both as isolated compounds and in the extracts, for their promising activity as antiviral, antibacterial, anticancer agents and

they also showed antimalarial, anti-inflammatory and antidiabetic effects [172-173-174-175-176-177-178]. Despite the discovery of many bioactive compounds, only a fraction of species within the *Myoporeae* tribe have undergone in-depth metabolite analysis. Given the wealth of bioactive molecules identified so far, further research in this field is needed, for the discovery of novel compounds with potential pharmaceutical applications, but also to better understand the chemistry of these species.

Eremophila tietkensii, firstly described by von Mueller and Tate, is a species widely distributed from the Cape Range and Carnarvon biogeographic region of western Australia to the North Territory border region (figure 33) [179-180-181].

Figure 36. *Eremophila tietkensii*. Photograph: R. Fowler.



In this context, no previous works have characterized secondary metabolites from *E. tietkensii*.

Nowadays, approximately 537 million adults globally are affected by diabetes, mainly diabetes mellitus type 2 [182]. The physiopathology of this

disease involves the onset of insulin resistance, with the failure of the involved tissues to respond to its stimuli, coupled together with a progressive decline in its production by the pancreatic β cells. This progresses in the inability to maintain glucose homeostasis and thus, hyperglycaemia [183]. Subsequently, vascular supply and integrity are compromised, causing organ dysfunction and death [184].

Treatment and management of type 2 diabetes involves a combination of intensive lifestyle changes, like weight loss and exercise, and use of antihyperglycemic drugs [185-186]. Among the already well-known drugs, PTP1B inhibitors could be effective not only for the treatment of type 2 diabetes, but also for the management of obesity [187].

PTP1B (Protein tyrosine phosphatase 1B) is a ubiquitous, negative intracellular regulator of insulin and leptin signalling pathway [188-189]. This tyrosine phosphatase can dephosphorylate the insulin receptor and its substrate proteins, inactivating its signalling [190]. Because of its role, PTP1B inhibition could enhance insulin action in the targeted tissues, representing an important therapeutic alternative. But to date, most of PTP1B inhibitors previously approved for clinical use have been discontinued due to lack of efficiency, specificity and unwanted side effects [191].

Eremophila species have been shown to be rich in PTP1B inhibitors. [192-193]. Indeed, the crude acetonitrile leaf resin extract of *E. tietkensisii*, redissolved to a concentration of 50 $\mu\text{g/mL}$, provided 75.2 ± 1.3 % for inhibition against PTP1B. Additionally, the assessment of the dose-

dependent inhibitory activity against the same target led to IC₅₀ values of 17.84 ± 1.1 µg/mL.

Thus, the aim of this study was to investigate a crude *E. tietkensii* leaves extract using high-resolution PTP1B inhibition profiling, HPLC-DAD and NMR. This is a technique used to identify active secondary metabolites towards a specific target, in this case the inhibition of PTP1B.

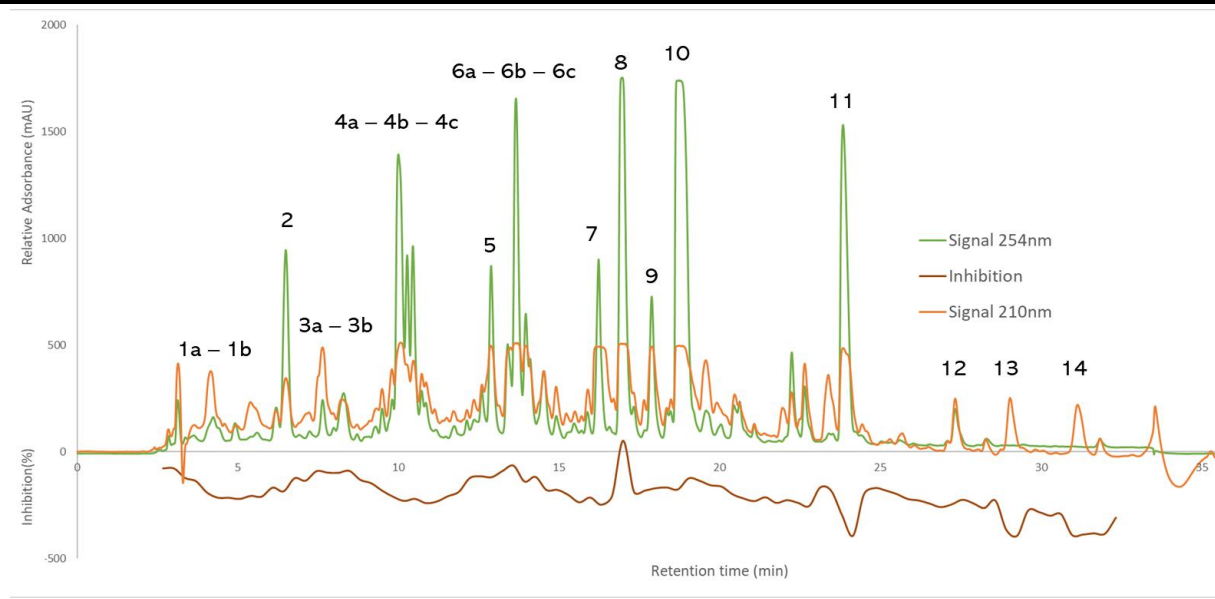
The crude extract is fractionated through analytical HPLC in a 96 well microplates, then the fractions are dried in the wells and directly subjected to the assay. The resulting percentage of inhibition are used to build the high-resolution inhibition profiling by plotting them with the average retention time of the fraction in each well. This method allows to easily visualize the constituents associated with bioactivity, speeding up their isolation and structure elucidation process, with techniques such as HPLC-DAD, both analytical and semi-preparative scale, and NMR [194-195]. For completion of the study, even the constituents associated with no activity were isolated to be structurally elucidated.

2.1.1 High-resolution inhibition profiling of the crude leaves extract

To identify all the constituents responsible for the inhibitory activity, a reversed-phase analytical-scale HPLC method was developed, following a 37 min gradient elution profile. The eluate obtained from 2.5 to 33 min after the injection of 25 µL of the crude leaves extract was microfractionated into a 96-well microplate. After evaporation of the eluate, the material in each well

was assayed and the result, expressed as percentage of PTP1B inhibition, were plotted at the respective retention times for each fraction, providing the high-resolution PTP1B inhibition profile, also called biochromatogram, shown in figure 37.

Figure 37. HPLC chromatogram of crude leaves extract of *E. tietkensii* monitored at 210 nm (orange) and 254 nm (green) with corresponding high-resolution PTP1B (red) inhibition profiles.



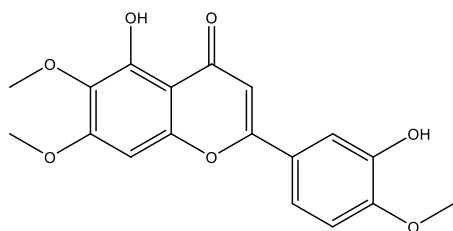
Constituents eluted within the retention time range 9-12 min were correlated with weak PTP1B inhibitory activity, while those eluted with peaks 11, 13 and 14 showed much stronger inhibition. At the same the fraction eluted with peak 8 showed an inverse activity response. This could be explained by two hypotheses: this constituent could be an activator of the enzyme, or it experienced a precipitation in the wells when the assay was performed due to limited solubility in DMSO.

2.1.2 Isolation and structure elucidation

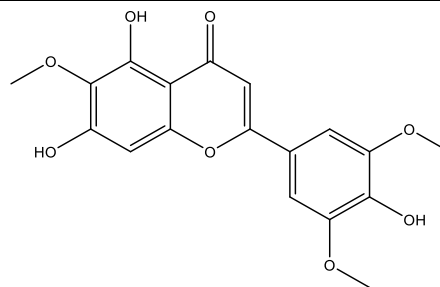
Each of the fractions were collected using semi-preparative scale HPLC and further purified with analytical-scale HPLC, leading to the isolation of compounds from **1-14**.

By comparison of the NMR data obtained with those reported in literature, compound **5** was identified as eupatorin, **6c** as 6-methoxytricin, **9** as murrayanol, **13** as lantanolic acid and peak **14** as an inseparable mixture of oleanonic acid and ursonic acid in a 2:1 ratio (figure 38) [196-197-198-199-200-201]. The ¹H NMR and ¹³C NMR spectroscopic data of compounds **5**, **6c**, **9**, **13** and of fraction **14** are reported in table 29 and 30.

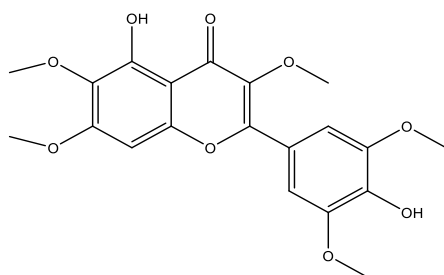
Figure 38. Structures of previously reported compounds isolated from *E. tietkensii*, namely eupatorine (**5**), 6-methoxytricin (**6c**), murrayanol (**9**), lantanolic acid (**13**) and oleanolic acid and ursonic acid (**14**)



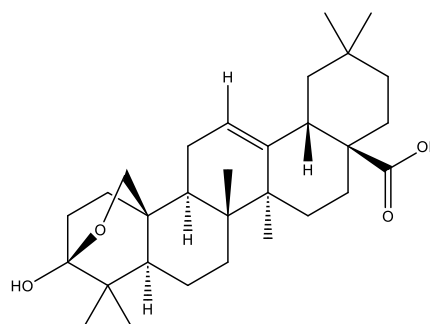
5



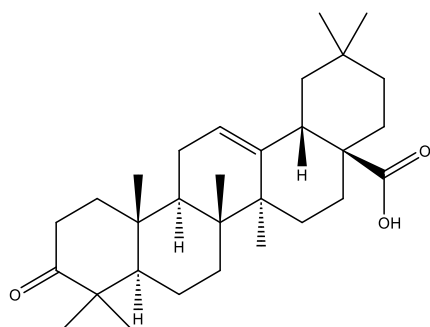
6c



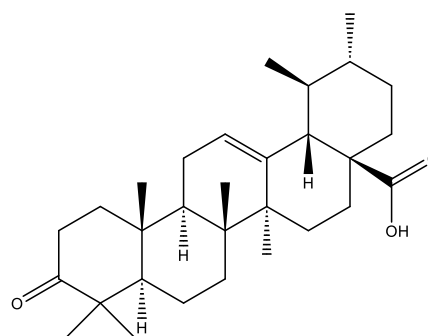
9



13



14a



14b

Table 29. ¹H and ¹³C NMR data of **13**, **6c** and **9**.

Pos.	5^a		6c^b		9^a	
	δ_{H} , nH, m, <i>J</i> (Hz) ^c	δ_{C} , type ^d	δ_{H} , nH, m, <i>J</i> (Hz) ^c	δ_{C} , type ^d	δ_{H} , nH, m, <i>J</i> (Hz) ^c	δ_{C} , type ^d
1		O		O		O
2		163.9, C		165.3, C1		155.1, C
3	6.78 (1H, s)	103.2, CH	6.73 (1H, s)	105.1, CH		138.7, C
4		182.1, C		184.0, C		178.5, C
5		164.3, C		105.3, C		152.9, C
6		131.8, C		132.8, C		131.9, C
7		158.6, C		158.9, C		158.9, C
8	6.90 (1H, bs)	91.5, CH	6.62 (1H, s)	95.2, CH	6.97 (1H, s)	91.4, CH
9		152.7, C		154.5, C		151.9, C
10		105.1, C		105.6, C		105.8, C
11	3.72 (3H, s)	60.0, CH ₃	3.89 (3H, s)	60.6, CH ₃	3.74 (3H, s)	59.8, CH ₃
12	3.92 (3H, s)	56.4, CH ₃	-	-	3.94 (3H, s)	56.3, CH ₃
13	3.85 (3H, s)	55.7, CH ₃	-	-	3.85 (3H, s)	59.7, CH ₃
1'		122.8, C		127.8, C		125.1, C
2'	7.46 (1H, d, <i>J</i> _{H2', H6'} = 2.3)	112.9, CH	7.26 (1H, s)	104.9, CH	7.40 (1H, s)	105.8, CH
3'		146.8, C		142.5, C		140.2, C
4'		151.3, C		105.1, C		106.1, C
5'	7.09 (1H, d, <i>J</i> _{H5', H6'} = 8.7)	112.1, CH		154.8, C		152.8, C
6'	7.56 (1H, dd, <i>J</i> _{H6', H5'} = 8.7, <i>J</i> _{H6', H2'} = 2.3)	118.8, CH	7.26 (1H, s)	104.9, CH	7.40 (1H, s)	105.8, CH
7'	-	-	3.85 (3H, s)	60.9, CH ₃	3.78 (3H, s)	60.0, CH ₃
8'	-	-	3.95 (3H, s)	56.7, CH ₃	3.88 (3H, s)	55.9, CH ₃

^a ¹H NMR (600.13) data obtained with samples in DMSO-*d*₆. ^b ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^c Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. n.d. = not detected. ^d Assignments based on HSQC and HMBC experiments.

Table 30. ¹H and ¹³C NMR data of **13**, **14a** and **14b**.

Pos.	13^a		14a^b		14b^b	
	δ_{H} , nH, m, J (Hz) ^c	δ_{C} , type ^d	δ_{H} , nH, m, J (Hz) ^c	δ_{C} , type ^d	δ_{H} , nH, m, J (Hz) ^c	δ_{C} , type ^d
1	α . 1.11 (1H, m, overlap)	34.5, CH ₂	A. 1.42 (1H, m, overlap)	39.3, CH ₂	A. 1.42 (1H, m, overlap)	39.3, CH ₂
	β . 1.99 (1H, ddd, $J_{\text{H1}\alpha, \text{H1}\beta}$ = overlap, $J_{\text{H1}\alpha, \text{H2}\beta}$ = 12.4, $J_{\text{H1}\alpha, \text{H2}\alpha}$ = 5.6)		B. 1.89 (1H, m, overlap)		B. 1.89 (1H, m, overlap)	
2	α . 1.94 (1H, m, overlap)	29.3, CH ₂	α . 2.37 (1H, m, overlap)	34.3, CH ₂	α . 2.37 (1H, m, overlap)	34.3, CH ₂
	β . 1.59 (1H, m, overlap)		β . 2.53 (1H, ddd, $J_{\text{H2}\beta, \text{H2}\alpha}$ = 15.9, $J_{\text{H2}\beta, \text{H1A}}$ = 9.7, $J_{\text{H2}\beta, \text{H1B}}$ = 7.3)		β . 2.53 (1H, ddd, $J_{\text{H2}\beta, \text{H2}\alpha}$ = 15.9, $J_{\text{H2}\beta, \text{H1A}}$ = 9.7, $J_{\text{H2}\beta, \text{H1B}}$ = 7.3)	
3		96.9, C		217.8, C		217.8, C
4		39.4, C		47.6, C		47.6, C
5	1.09 (1H, m, overlap)	49.5, CH	1.31 (1H, m, overlap)	55.4, CH	1.31 (1H, m, overlap)	55.4, CH
6	1.43 (2H, m, overlap)	19.0, CH ₂	1.47 (2H, m, overlap)	19.7, CH ₂	1.47 (2H, m, overlap)	19.7, CH ₂
7	1.31 (2H, m, overlap)	30.7, CH ₂	A. 1.35 (1H, m, overlap)	32.3, CH ₂	A. 1.38 (1H, m, overlap)	32.5, CH ₂
			B. 1.49 (1H, m, overlap)		B. 1.51 (1H, m, overlap)	
8		37.7, C		39.4, C		39.6, C
9	1.57 (1H, m, overlap)	41.3, CH	1.63 (1H, m, overlap)	47.0, CH	1.60 (1H, m, overlap)	49.9, CH
10		34.0, C		36.9, C		36.9, C
11	α . 1.90 (1H, m, overlap)	23.0, CH ₂	A. 1.90 (1H, m, overlap)	23.6, CH ₂	A. 1.93 (1H, m, overlap)	23.5, CH ₂
	β . 1.63 (1H, m, overlap)		B. 1.97 (1H, m, overlap)		B. 1.98 (1H, m, overlap)	
12	5.18 (1H, t, $J_{\text{H12}, \text{H11}\beta} \approx J_{\text{H12}, \text{H11}\alpha} = 3.3$)	121.5, CH	5.30 (1H, t, $J_{\text{H12}, \text{H11A}} \approx J_{\text{H12}, \text{H11B}} = 3.5$)	122.5, CH	5.26 (1H, t, $J_{\text{H12}, \text{H11A}} \approx J_{\text{H12}, \text{H11B}} = 3.5$)	125.7, CH
13		143.4, C		143.8, C		138.2, C
14		41.4, C		41.9, C		42.2, C
15	α . 1.07 (1H, m, overlap)	27.2, CH ₂	A. 1.12 (1H, m, overlap)	27.8, CH ₂	A. 1.10 (1H, m, overlap)	28.1, CH ₂
	β . 1.60 (1H, m, overlap)		B. 1.72 (1H, m, overlap)		B. 1.88 (1H, m, overlap)	
16	α . 1.88 (1H, m, overlap)	22.6, CH ₂	A. 1.63 (1H, m, overlap)	22.9, CH ₂	A. 1.67 (1H, m, overlap)	24.2, CH ₂
	β . 1.48 (1H, m, overlap)		B. 1.99 (1H, m, overlap)		B. 2.01 (1H, m, overlap)	
17		45.5, C		46.7, C		48.1, C
18	2.74 (1H, dd, $J_{\text{H18}, \text{H19}\alpha} = 14.1$, $J_{\text{H18}, \text{H19}\beta} = 4.1$)	41.2, CH	2.83 (1H, dd, $J_{\text{H18}, \text{H19A}} = 14.0$, $J_{\text{H18}, \text{H19B}} = 4.4$)	41.2, CH	2.20 (1H, d, $J_{\text{H18}, \text{H19}} = 11.4$)	52.7, CH

Table 30 (continued). ¹H and ¹³C NMR data of **13**, **14a** and **14b**.

Pos.	13 ^a		14a ^b		14b ^b	
	δ_{H} , nH, m, <i>J</i> (Hz) ^c	δ_{C} , type ^d	δ_{H} , nH, m, <i>J</i> (Hz) ^c	δ_{C} , type ^d	δ_{H} , nH, m, <i>J</i> (Hz) ^c	δ_{C} , type ^d
19	α . 1.04 (1H, m, overlap)	45.3, CH ₂	A. 1.16 (1H, m, overlap)	45.9, CH ₂	1.33 (1H, m, overlap)	39.2, CH
	β . 1.62 (1H, m, overlap)		B. 1.62 (1H, m, overlap)			
20		30.1, C		30.8, C	1.01 (1H, m, overlap)	38.9, CH
21	α . 1.13 (1H, m, overlap)	33.3, CH ₂	A. 1.22 (1H, m, overlap)	34.0, CH ₂	A. 1.31 (1H, m, overlap)	30.7, CH ₂
	β . 1.30 (1H, m, overlap)		B. 1.35 (1H, m, overlap)		B. 1.52 (1H, m, overlap)	
22	α . 1.60 (1H, m, overlap)	31.8, CH ₂	A. 1.58 (1H, m, overlap)	32.5, CH ₂	A. 1.67 (1H, m, overlap)	36.8, CH ₂
	β . 1.42 (1H, m, overlap)		B. 1.77 (1H, m, overlap)		B. 1.73 (1H, m, overlap)	
23	0.86 (3H, s)	18.4, CH ₃	1.08 (3H, s)	26.7, CH ₃	1.08 (3H, s)	26.7, CH ₃
24	0.91 (3H, s)	27.0, CH ₃	1.03 (3H, s)	21.5, CH ₃	1.03 (3H, s)	21.5, CH ₃
25	A. 3.71 (1H, dd, $J_{\text{H25A}, \text{H25B}} = 8.8$, $J_{\text{H25A}, \text{H5}} = 1.3$)	66.1, CH ₂	1.05 (3H, s)	15.2, CH ₃	1.05 (3H, s)	15.2, CH ₃
	B. 4.07 (1H, dd, $J_{\text{H25b}, \text{H25a}} = 8.8$, $J_{\text{H25b}, \text{H1a}} = 2.6$)					
26	0.67 (3H, s)	17.0, CH ₃	0.81 (3H, s)	17.1, CH ₃	0.83 (3H, s)	17.2, CH ₃
27	1.08 (3H, s)	24.8, CH ₃	1.14 (3H, s)	25.9, CH ₃	1.09 (3H, s)	23.7, CH ₃
28		178.5, C		184.0, C		183.9, C
29	0.87 (3H, s)	32.7, CH ₃	0.93 (3H, s)	23.7, CH ₃	0.86 (3H, s)	17.1, CH ₃
30	0.86 (3H, s)	23.1, CH ₃	0.90 (3H, s)	33.2, CH ₃	0.95 (3H, s)	21.3, CH ₃

^a ¹H NMR (600.13) data obtained with samples in DMSO-*d*₆. ^b ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^c Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. n.d. = not detected. ^d Assignments based on HSQC and HMBC experiments.

In figure 39 and 40 all the structure and the key COSY, HMBC and ROESY correlection of all the previously unreported compounds isolated from *E. tietkensii* leaves crude extract are shown.

Figure 39. Structures of previously unreported compounds isolated from *E. tietskensis* leaves crude extract.

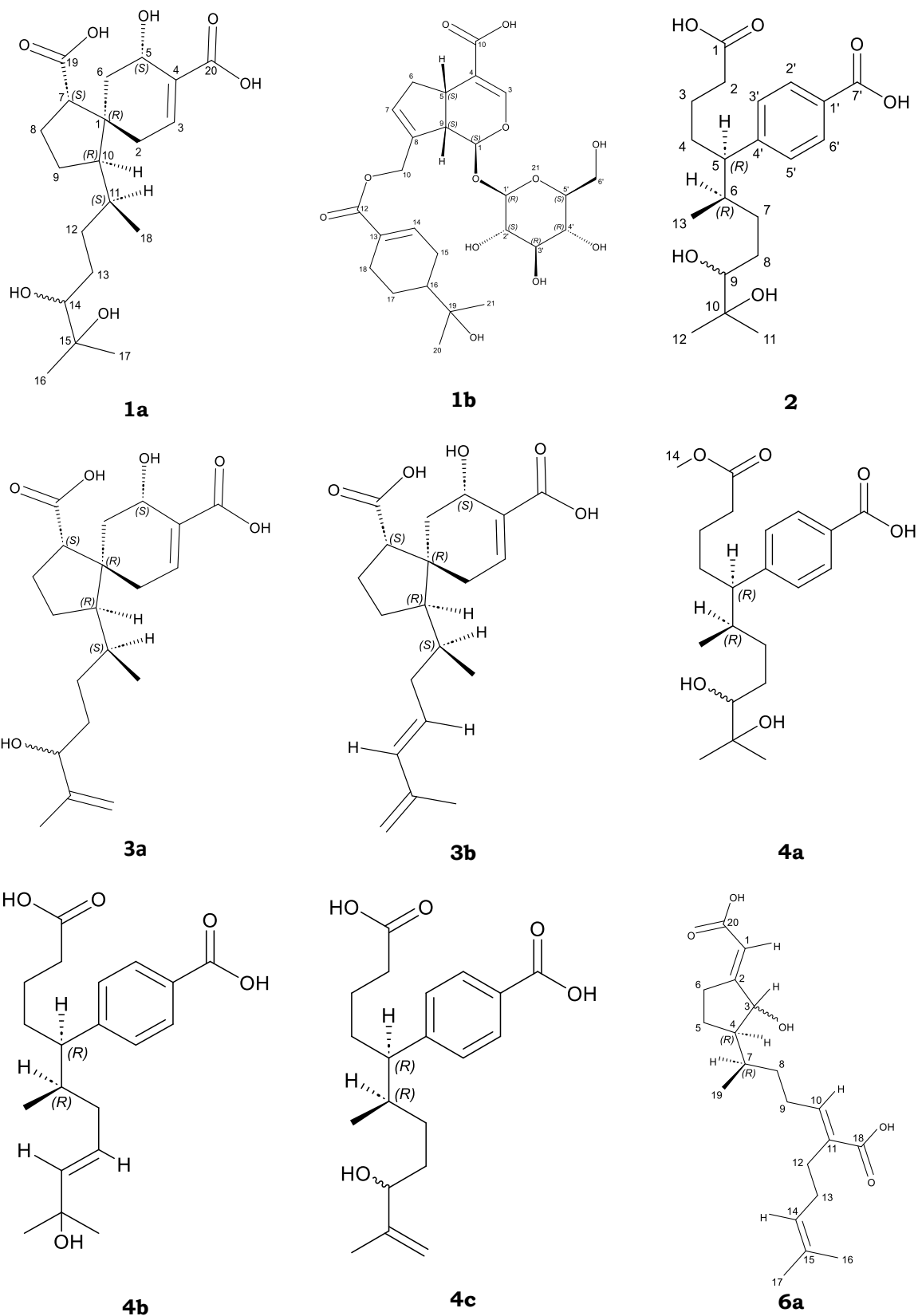


Figure 39 (continued). Structures of previously unreported compounds isolated from *E. tietkensii* leaves crude extract.

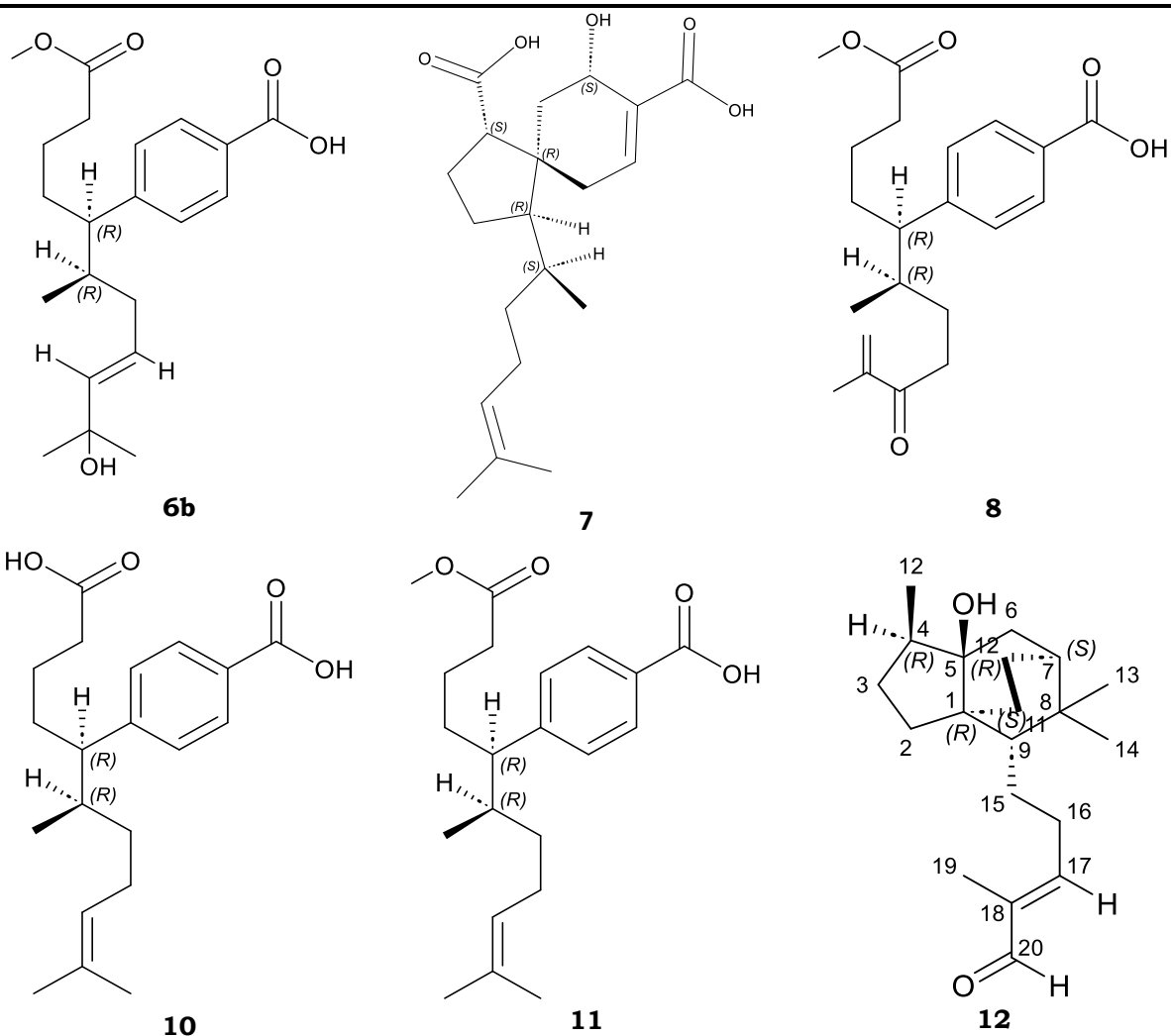


Figure 40. Selected COSY (bold), HMBC (blue arrows from H to C), and ROESY (red double arrows) correlations used for structure elucidation of previously unreported compounds isolated from *E. tietkensii* leaves crude extract.

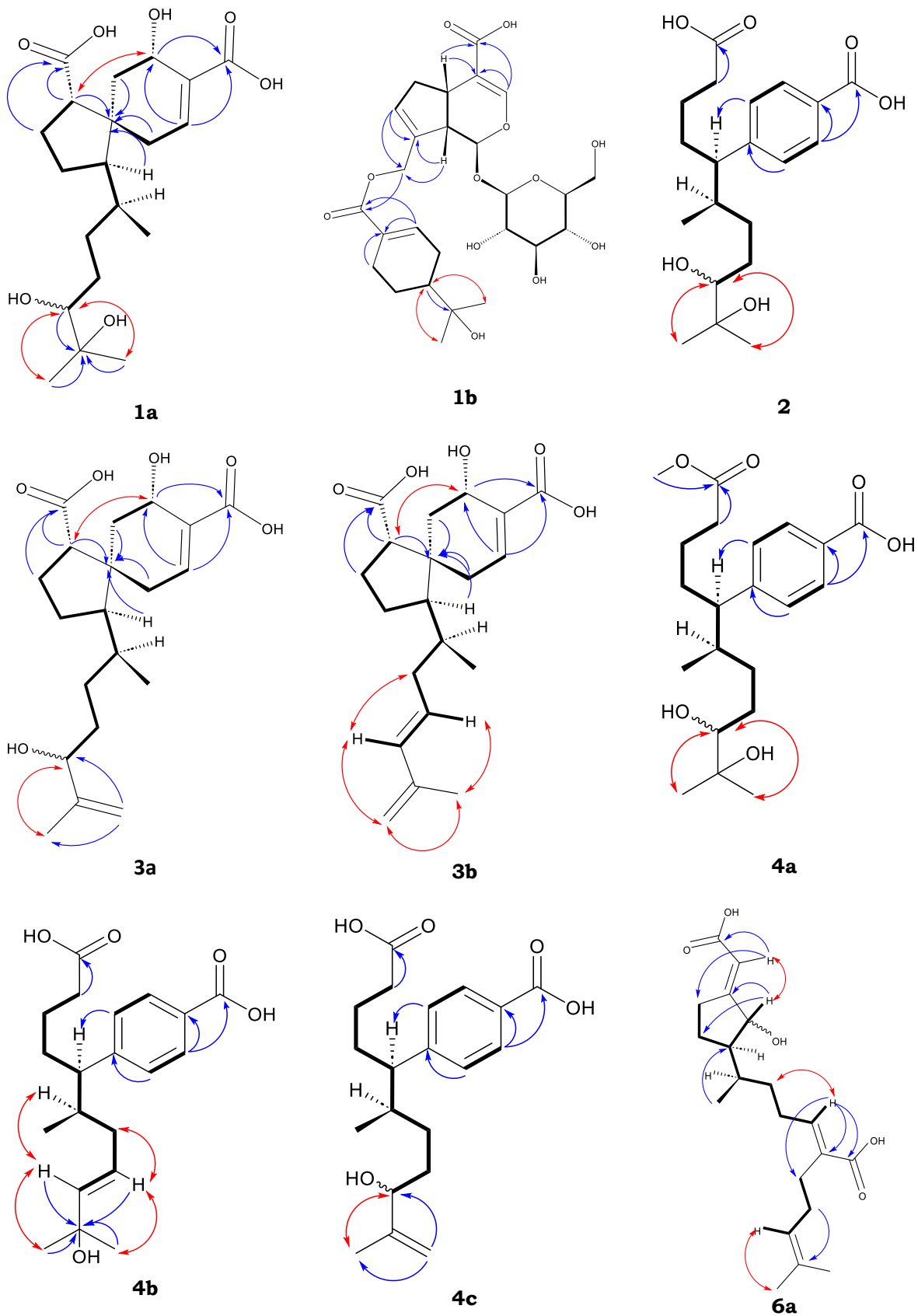
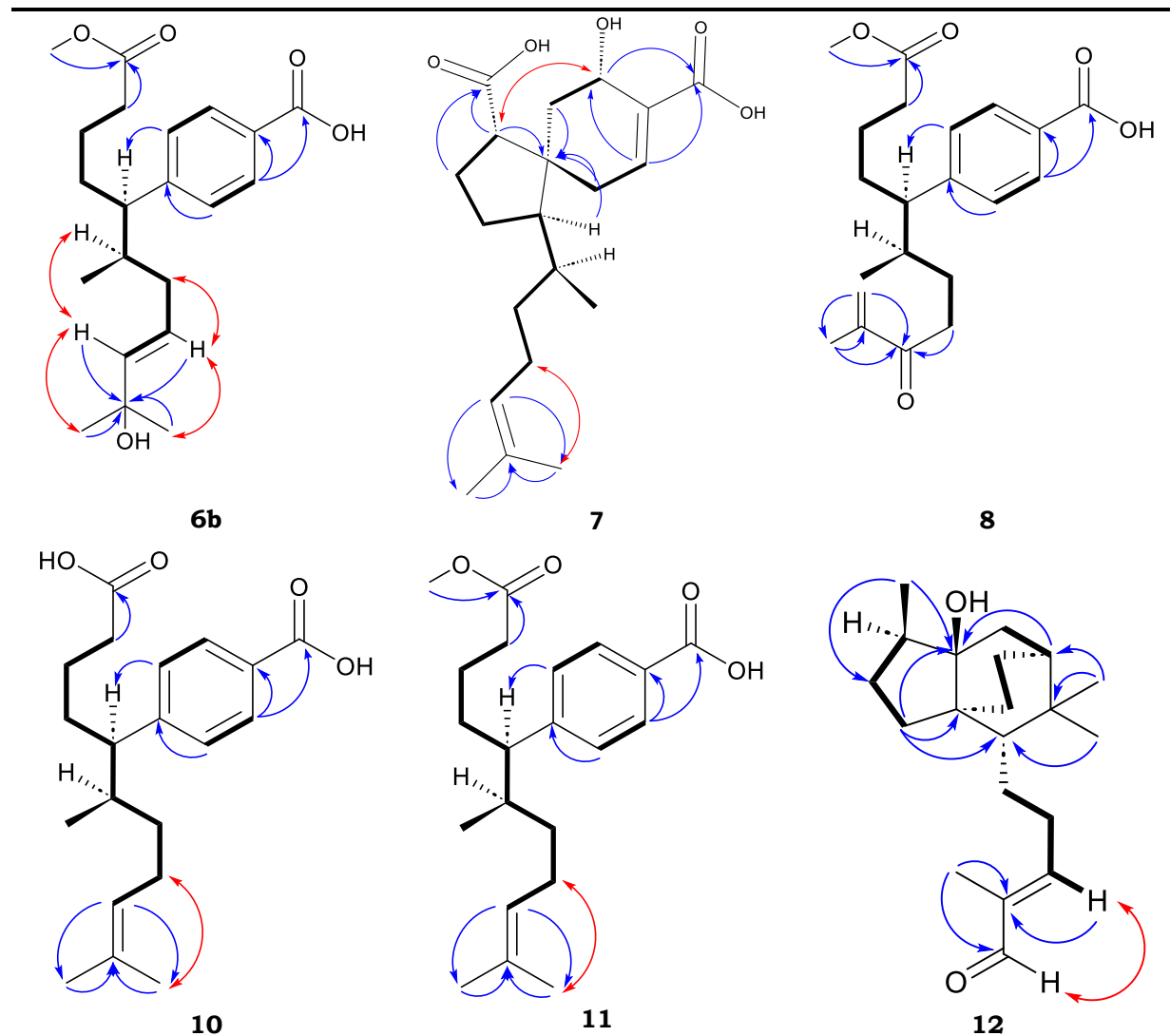


Figure 40 (continued). Selected COSY (bold), HMBC (blue arrows from H to C), and ROESY (red double arrows) correlations used for structure elucidation of previously unreported compounds isolated from *E. tietskensis* leaves crude extract.



The ^1H NMR and 2D homo- and heteronuclear NMR data of the material eluted with peak **1a** revealed it to be a previously undescribed viscidane type diterpenoid, whose framework is featured by a spiro [4,5] decane ring system of ring A (five-membered ring) and ring B (six-membered ring) (table 31). The structure of ring A in conjunction with the linear side chain was established by the $\text{H-7} \leftrightarrow \text{H}_2\text{-8} \leftrightarrow \text{H}_2\text{-9} \leftrightarrow \text{H-10} \leftrightarrow \text{H-11}(\text{H}_3\text{-18}) \leftrightarrow \text{H}_2\text{-12} \leftrightarrow \text{H}_2\text{-13} \leftrightarrow \text{H-14} \leftrightarrow \text{H}_3\text{-16/H}_3\text{-17}$ spin system, observed in the COSY spectrum, and the latter being long-range couplings, observed in the ROESY spectrum.

HMBC correlations from H-7 and H₂-8 to the quaternary carbon at δ_c 179.2 of C-19 confirmed the carboxylic acid at C-7 on ring A. The structure of ring B was established based on the H₂-2 $\leftrightarrow$ H-3 and the H-5 $\leftrightarrow$ H₂-6 spin systems, as well as HMBC correlations from H-3 to C-5. In addition, HMBC correlations from H-5 and H-3 to the quaternary carbon at δ_c 170.2 of C-20 confirmed the carboxylic acid at C-4 on ring B. Finally, the position of the side chain and the spiro junction between ring A and ring B at δ_c 49.5 were established based on HMBC correlations from H-7 to C-1 and C-2, from H₂-2 to C-1, from H₂-6 to C-1 and C-10, from H-3 to C-1 and from H-10 to C-1. Aliphatic oxymethine signals were observed at δ_H 3.25 (H-14, dd, $J_{H14, H13A} = 10.3$, $J_{H14, H13B} = 1.5$, δ_c 79.3), showing the presence of a hydroxyl group at C-14. The oxygenated quaternary C-15 at δ_c 73.6 was identified based on HMBC correlations from H-14, H₃-16, H₃-17 and H₃-18. The relative stereochemistry of **1a** was determined by analysis of the ROESY spectrum. H-11 is tentatively positioned in α , with the assignment of the 11*S* configuration based on biosynthetic considerations. Additionally, no key ROE correlations were detected between H-7 and H-2 α , H-10 and H-11 suggesting a β orientation for H-7, together with an α orientation for H-10. At the same time a roe correlation between H-7 and H-5 allowed to indicate the latter as β oriented.

Thus 1*R*,7*S*,10*R*,11*S* configuration was tentatively assigned. For what concerns the stereochemistry of C-14, the free rotation of the chain at C-11 makes it difficult to determine the stereochemistry. But considering that another compound was isolated from the same fraction, and it shared the same ¹H NMR and 2D homo- and heteronuclear NMR data of the previously

described viscidane, but with a few chemical shift variations from position 11 to 14, it can be assumed it's an epimer at C-14 of compound **1a** (table 32).

Table 31. ^1H and ^{13}C NMR data and 2D COSY, ROESY, and HMBC correlations for **1a**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
1		49.5, C spiro			
2	α . 2.37 (1H, dt, $J_{\text{H}2\alpha, \text{H}2\beta} = 19$, $J_{\text{H}2\alpha, \text{H}3} = 2.8$)	31.8, CH ₂	H-2 β , H-3, H-5(w)	C-1, C-3, C-4, C-7, C-10	H-3, H-6 α , H-11, H-18 (w), H-2 β
	β . 2.08 (1H, m, overlap)		H-2 α , H-3,	C-1, C-3, C-4, C-7, C-10,	H-2 α , H-7, H-12B
3	6.96 (1H, dd, $J_{\text{H}3, \text{H}2\beta} = 5.9$, $J_{\text{H}3, \text{H}2\alpha} = 2.8$)	140.8, CH	H-2 α , H-2 β , H-5 (w)	C-1, C-2, C-4, C-5, C-20	H-2 α , H-2 β , H-6 β , H-7
4		134.2, C			
5	4.79 (1H, t, $J_{\text{H}5, \text{H}6\alpha} \approx J_{\text{H}5, \text{H}6\beta} = 9.1$)	65.3, CH	H-6 α , H-6 β	C-3, C-4, C-6	H-2 β , H-6 α , H-6 β , H-7
6	α . 1.93 (1H, m, overlap)	40.0, CH ₂	H-5, H-6 β	C-1, C-2, C-4, C-5, C-7	H-2 α , H-5, H-11, H-18
	β . 1.98 (1H, m)		H-5, H-6 α	C-1, C-2, C-4, C-5, C-7, C-10	H-2 α , H-5, H-11, H-18
7	2.61 (1H, dd, $J_{\text{H}7, \text{H}8\beta} = 8.5$, $J_{\text{H}7, \text{H}8\alpha} = 3.0$)	53.0, CH	H-8A, H-8B	C-1, C-2, C-8, C-9, C-10, C-19	H-5, H-8A, H-8B, H-9A, H-9B
8	A. 1.82 (1H, dtd, $J_{\text{H}8\text{A}, \text{H}8\text{B}} = 14.1$, $J_{\text{H}8\text{A}, \text{H}9\alpha} \approx J_{\text{H}8\text{A}, \text{H}9\beta} = 9.1$, $J_{\text{H}8\text{A}, \text{H}7} = 3.1$)	26.5, CH ₂	H-7, H-8B, H-9A, H-9B	C-7, C-10, C-19	H-7, H-9A, H-10
	B. 1.91 (1H, m, overlap)		H-7, H-8A, H-9A, H-9B	C-1, C-7, C-9, C-10, C-19	H-7, H-9A, H-9B
9	A. 1.58 (1H, m, overlap)	27.6, CH ₂	H-8A, H-8B, H-9B, H-10	C-1, C-7, C-8, C-10, C-11,	H-7, H-8A, H-8B, H-9B, H-10
	B. 2.06 (1H, m, overlap)		H-8A, H-8B, H-9A	C-1, C-7, C-8, C-10, C-11,	H-7, H-8A, H-8B, H-9A
10	2.15 (1H, m)	52.6, CH	H-9A, H-9B, H-11	C-1, C-2, C-6, C-9, C-11, C-18	H-8A, H-9A, H-9B, H-11, H-18
11	1.58 (1H, m, overlap)	34.0, CH	H-10, H-12 α , H-18	C-1 (w), C-7, C-9, C-10, C-12, C-13, C-14, C-18	H-2 α , H-10, H-12B, H-12 α , H-18
12	A. 1.39 (1H, m, overlap)	33.4, CH ₂	H-11, H-12B, H-13B, H-13A	C-11, C-13, C-14, C-18	H-11, H-13B, H-17, H-18
	B. 1.54 (1H, m, overlap)		H-11, H-12 α , H-13B, H-13A	C-11, C-13, C-14, C-18	H-2 α

Table 31 (continued). ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **1a**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
13	A. 1.40 (1H, m, overlap)	29.0, CH ₂	H-12B, H-12a, H-13B, H-14	C-11, C-12, C-14, C-18	
	B. 1.55 (1H, m, overlap)		H-12B, H-12a, H-13A, H-14	C-11, C-12, C-14, C-18	H-2a
14	3.25 (1H, dd, $J_{\text{H14, H13A}}$ = 10.3, $J_{\text{H14, H13B}}$ = 1.5)	79.3, CH	H-13B, H-13A	C-12, C-13, C-15 C-16, C-17	H-13B, H-13A, H-16, H-17
15		73.6, C			
16	1.17 (3H, s)	25.4, CH ₃	H-14 (w)	C-14, C-15, C-17	H-14
17	1.14 (3H, s)	17.2, CH ₃	H-14 (w)	C-14, C-15, C-16	H-14
18	1.00 (3H, d, $J_{\text{H18, H11}}$ = 6.5)	20.4, CH ₃	H-11	C-10, C-11, C-12	H-2a, H-6a, H-10, H-11, H-12a
19		179.2, C			
20		170.2, C			

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

Table 32. ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **1a C-14 epimer** from position 11 to 14.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
11	1.53 (1H, m, overlap)	34.9, CH	H-10, H-12a, H-18	C-1 (w), C-7, C-9, C-10, C-12, C-13, C-14, C-18	H-2a, H-10, H-12B, H-12a, H-18
12	A. 1.06 (1H, m, overlap)	34.0, CH ₂	H-11, H-12B, H-13B, H-13A	C-11, C-13, C-14, C-18	H-11, H-13B, H-17, H-18
	B. 1.85 (1H, m, overlap)		H-11, H-12a, H-13B, H-13A	C-11, C-13, C-14, C-18	H-2a
13	A. 1.14 (1H, m, overlap)	29.7, CH ₂	H-12B, H-12a, H-13B, H-14	C-11, C-12, C-14, C-18	
	B. 1.75 (1H, m, overlap)		H-12B, H-12a, H-13A, H-14	C-11, C-12, C-14, C-18	H-2a
14	3.19 (1H, dd, $J_{\text{H14, H13A}}$ = 10.3, $J_{\text{H14, H13B}}$ = 1.8)	80.2, CH	H-13B, H-13A	C-12, C-13, C-15 C-16, C-17	H-13B, H-13A, H-16, H-17

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

The NMR data for the constituents eluted with peaks **3a**, **3b**, and **7** were similar to the abovementioned viscidane **1a**, with the same structure for the ring A and B presence, the main difference being the side chain in position 10. Indeed, for **3a** the same spin system $H-7 \leftrightarrow H_2-8 \leftrightarrow H_2-9 \leftrightarrow H-10 \leftrightarrow H-11 (H_3-18) \leftrightarrow H_2-12 \leftrightarrow H_2-13 \leftrightarrow H-14$ as **1a** was detected, also further establishing the linear side chain conjunction with ring A. In this case, the aliphatic oxymethine signals were observed at δ_H 3.96 (H-14, t, $J_{H14, H13A} \approx J_{H14, H13B} = 6.6$, δ_C 77.0), showing the presence of a hydroxyl group at C-14 (table 33). A geminal pair of vinylic protons appeared at δ_H 4.82 and δ_H 4.91 (H-16A, H16-B, m, δ_C 111.5), which both showed HMBC correlations to C-14 and C-17, suggesting a double bond between C-15 and C-16. This was further confirmed by the ROESY correlations between vinylic H₂-16 and H₃-17 and H-14. The same 1*R*,7*S*,10*R*,11*S* configuration is tentatively assigned to compound **3a** for the same reason mentioned above. Another similarity is represented by the existence of the epimer at C-14 of compound **3a** that could be isolated from the same fraction. They shared the same NMR data, but with a few chemical shift variations from position 11 to 17 (table 34).

Table 33. ^1H and ^{13}C NMR data and 2D COSY, ROESY, and HMBC correlations for 3a .					
Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
1		49.5, C spiro			
2	α . 2.34 (1H, dt, $J_{\text{H}2\alpha, \text{H}2\beta} = 19$, $J_{\text{H}2\alpha, \text{H}3} = 2.8$)	31.8, CH ₂	H-2 β , H-3, H-5(w)	C-1, C-3, C-4, C-7, C-10, C-20 (w)	H-3, H-6 α , H-11, H-18 (w), H-2 β
	β . 2.06 (1H, m, overlap)		H-2 α , H-3, H-5(w)	C-1, C-3, C-4, C-6, C-7, C-10 (w)	H-2 α , H-5, H-7, H-18 (w)
3	6.94 (1H, dd, $J_{\text{H}3, \text{H}2\beta} = 5.4$, $J_{\text{H}3, \text{H}2\alpha} = 2.8$)	140.4, CH	H-2 α , H-2 β	C-1, C-2, C-4, C-5, C-20	H-2 α , H-2 β , H-5, H-6 β , H-7
4		134.5, C			
5	4.78 (1H, m)	65.5, CH	H-2 α (w), H- 2 β (w), H- 6 α , H-6 β	C-3, C-4, C-6	H-2 β , H-6 α , H-6 β , H-7
6	α . 1.92 (1H, m, overlap)	40.0, CH ₂	H-5	C-1, C-2, C-4, C-5, C-7, C-10 (w)	H-2 α , H-5, H-11, H-18
	β . 1.98 (1H, m, overlap)		H-5	C-1, C-2, C-4, C-5, C-7 (w), C- 10, C-20	H-2 α , H-5, H-11, H-18
7	2.60 (1H, dd, $J_{\text{H}7, \text{H}8\beta} = 8.7$, $J_{\text{H}7, \text{H}8\alpha} = 3.1$)	53.1, CH	H-8 α , H-8 β	C-1, C-2, C-8, C-9, C-10, C-19	H-5, H-8 α , H-8 β , H-9A, H-9B
8	α . 1.80 (1H, dtd, $J_{\text{H}8\alpha, \text{H}8\beta} = 14.1$, $J_{\text{H}8\alpha, \text{H}9\alpha} \approx J_{\text{H}8\alpha, \text{H}9\beta} = 9.1$, $J_{\text{H}8\alpha, \text{H}7} = 3.1$)	26.5, CH ₂	H-7, H-8 β , H-9A, H-9B	C-1, C-2 (w), C-6 (w), C-7, C-9	H-7, H-9A, H-10
	β . 1.90 (1H, m, overlap)		H-7, H-8 α , H-9A, H-9B	C-1, C-2 (w), C-6 (w), C-7, C-9	H-7, H-9A, H-9B, H-18
9	A. 1.54 (1H, m, overlap)	27.8, CH ₂	H-8 α , H-8 β , H-9B, H-10	C-7, C-8, C-10	H-7, H-8 α , H-8 β , H-9B, H-10
	B. 2.03 (1H, m, overlap)		H-8 α , H-8 β , H-9A	C-7, C-8, C-10	H-7, H-8 α , H-8 β , H-9A
10	2.13 (1H, td, $J_{\text{H}10, \text{H}11} = 9.9$, $J_{\text{H}10, \text{H}9\alpha} \approx J_{\text{H}10, \text{H}9\beta} = 7.2$)	52.5, CH	H-9A, H-9B, H-11	C-1, C-2, C-6, C-9, C-11, C-12, C-18	H-8 α , H-9A, H-9B, H-11, H-18
11	1.54 (1H, m, overlap)	34.5, CH	H-10, H- 12B, H-12 α , H-18	C-1, C-9, C-10, C-12, C-18	H-2 α , H-10, H-12B, H- 12 α , H-18
12	A. 1.04 (1H, dtd, $J_{\text{H}12\alpha, \text{H}12\beta} = 14.3$, $J_{\text{H}12\alpha, \text{H}13\text{A}} \approx J_{\text{H}12\alpha, \text{H}13\text{B}} = 9.2$, $J_{\text{H}12\alpha, \text{H}11} = 4.9$)	32.6, CH ₂	H-11, H- 12B, H- 13B, H-13A	C-10 (w), C-11, C-13, C-14, C- 18	
	B. 1.57 (1H, m, overlap)		H-12 α , H- 13B, H-13A	C-10, C-11, C- 13, C-14, C-18	
13	A. 1.44 (1H, dddd, $J_{\text{H}13\text{A}, \text{H}13\text{B}} = 13$, $J_{\text{H}13\text{A}, \text{H}12\alpha} = 11.2$, $J_{\text{H}13\text{A}, \text{H}14} = 6.6$, $J_{\text{H}13\text{A}, \text{H}12\beta} = 4.6$)	32.9, CH ₂	H-12B, H- 12 α , H-13B, H-14	C-11, C-12, C- 14, C-15	
	B. 1.66 (1H, m, overlap)		H-12B, H- 12 α , H-13A, H-14	C-11, C-12, C- 14, C-15	

Table 33 (continued). ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **3a**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
14	3.96 (1H, t, $J_{\text{H14}, \text{H13A}} \approx J_{\text{H14}, \text{H13B}} = 6.6$)	77.0, CH	H-13B, H-13A	C-12, C-13, C-15, C-16, C-17	H-12 α , H12-B, H-13A, H-13B, H-16B
15		148.6, C			
16	A. 4.82 (1H, m)	111.5, CH ₂	H-17 (w)	C-14, C-15, C-17	H-17
	B. 4.91 (1H m)		H-17 (w)	C-14, C-15, C-17	H-14
17	1.71 (3H, s)	17.2, CH ₃	H-14 (w), H-16A (w), H-16B (w)	C-14, C-15, C-16	H-16A
18	1.00 (3H, d, $J_{\text{H18}, \text{H11}} = 6.7$)	20.4, CH ₃	H-11	C-10, C-11, C-12	H-2 α , H-9B, H-6 α , H-6 β , H-8A, H-10, H-13B
19		179.2, C			
20		170.5, C			

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

Table 34. ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **3a C-14 epimer** from position 11 to 17.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
11	1.55 (1H, m, overlap)	34.2, CH	H-10, H-12B, H-12A, H-18	C-1, C-5 C-9, C-10, C-12, C-13, C-18	H-2 α , H-10, H-12B, H-12 α , H-18
12	A. 1.18 (1H, dtd)	32.2, CH ₂	H-11, H-12B, H-13B, H-13A	C-11, C-13, C-14, C-18	
	B. 1.46 (1H, m, overlap)		H-12 α , H-13B, H-13A	C-10, C-11, C-13, C-14, C-18	
13	A. 1.46 (1H, m, overlap)	32.8, CH ₂	H-12B, H-12A, H-13B, H-14	C-11, C-12, C-14, C-15	
	B. 1.64 (1H, m)		H-12B, H-12A, H-13A, H-14	C-11, C-12, C-14, C-15	
14	3.97 (1H, t, $J_{\text{H14}, \text{H13A}} \approx J_{\text{H14}, \text{H13B}} = 6.6$)	76.5, CH	H-13B, H-13A	C-12, C-13, C-15, C-16, C-17	H-12 α , H12-B, H-13A, H-13B, H-16B
15		148.8, C			
16	A. 4.81 (1H, m)	111.0, CH ₂		C-14, C-15, C-17	H-17
	B. 4.91 (1H m)		H-17 (w)	C-14, C-15, C-17	H-14
17	1.71 (3H, s)	17.4, CH ₃	H-14 (w), H-16A (w), H-16B (w)	C-14, C-15, C-16	H-13A, H-16A

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

The constituent eluted with peak **3b** maintained the same viscidane structure and substitutions on ring A and B as compound **3a** (table 35). In this instance, a geminal pair of vinylic protons was present at δ_{H} 4.85 (H₂-16, m, δ_{C} 114.4), also showing HMBC correlations to C-14 and C-17, suggesting a double bond between C-15 and C-16. In addition, two alkenyl protons appeared at δ_{H} 5.67 (H-13, ddd, δ_{C} 129.8) and at δ_{H} 6.15 (H-14, d, δ_{C} 135.4), with proton coupling constants typical of a trans alkene ($^3J_{\text{H}13, \text{H}14} = ^3J_{\text{H}14, \text{H}13} = 15.6$). The trans configuration of this double bond was further confirmed through ROESY correlations: H-14 showed ROE correlations to H₂-12 and the geminal vinylic protons H₂-16, while H-13 with H₃-17. The same 1*R*,7*S*,10*R*,11*S* configuration is tentatively assigned to compound **3b** for the same reason mentioned above.

Table 35. ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **3b**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
1		49.5, C spiro			
2	α . 2.35 (1H, m, overlap)	31.6, CH ₂	H-2 β , H-5	C-1, C-3, C-4, C-7	H-3, H-6 α , H-11, H-18
	β . 2.04 (1H, m, overlap)		H-2 α , H-3,	C-1, C-3, C-4, C-6	H-5, H-7, H-9 β , H-18
3	6.88 (1H, m)	139.0, CH	H-2 α , H-2 β	C-1, C-2, C-4, C-5, C-20	H-2 α , H-2 β , H-6 β , H-7
4		135.3, C			
5	4.78 (1H, m, overlap)	65.8, CH	H-2 α , H-6 α , H-6 β	C-3, C-4, C-6	H-2 β , H-6 α , H-6 β , H-7
6	α . 2.00 (1H, m, overlap)	39.9, CH ₂	H-2 α , H-5	C-1, C-2, C-4, C-5	H-2 α , H-5, H-18 (w)
	β . 1.93 (1H, m, overlap)		H-5, H-6 α	C-1, C-2, C-4, C-5, C-7	H-5, H-7, H-18
7	2.62 (1H, dd, $J_{\text{H}7, \text{H}8\beta}$ = 8.6, $J_{\text{H}7, \text{H}8\alpha}$ = 2.7)	53.1, CH	H-8 α , H-8 β	C-1, C-2, C-8, C-9, C-10, C-19	H-2 β , H-5, H-8 α , H-8 β , H-9 β
8	α . 1.80 (1H, m)	26.5, CH ₂	H-8 β , H-9 β , H-9 α	C-1, C-7, C-9, C-19	H-7, H-9 β , H-9 α
	β . 1.93 (1H, m, overlap)		H-7, H-8 α , H-9 β	C-1, C-7, C-9, C-19	H-5, H-7, H-12 β , H-18
9	α . 2.12 (1H, m)	28.4, CH ₂	H-8 α , H-8 β , H-9 β	C-1, C-10	H-8 α , H-8 β , H-9 β
	β . 1.57 (1H, m, overlap)		H-8 α , H-8 β , H-9 α , H-10	C-1, C-8, C-10, C-11	H-8 α , H-8 β , H-9 α , H-10, H-12 β
10	2.14 (1H, m, overlap)	52.3, CH	H-9 β , H-9 α , H-11	C-1, C-2, C-9, C-11, C-12, C-18	H-11
11	1.63 (1H, m)	35.3, CH	H-10, H-12 β , H-12 α , H-18	C-1, C-9, C-10, C-12, C-18	H-2 α , H-10, H-12 α
12	α . 1.91 (1H, m, overlap)	40.2, CH ₂	H-11, H-12 β , H-13	C-10, C-11, C-13, C-14, C-18	H-11, H-12 β , H-18
	β . 2.32 (1H, m, overlap)		H-12 α , H-13	C-10, C-11, C-13, C-14, C-18	H-9 β , H-13, H-18
13	5.67, (1H, ddd, $J_{\text{H}13, \text{H}14}$ = 15.6, $J_{\text{H}13, \text{H}12\alpha}$ = 8.3, $J_{\text{H}13, \text{H}12\beta}$ = 6.4)	129.8, CH	H-12 α , H-12 β , H-14	C-11, C-12, C-15	H-12 α , H-12 β , H-17
14	6.15 (1H, d, $J_{\text{H}14, \text{H}13}$ = 15.6)	135.4, CH	H-13	C-12, C-15, C-16, C-17	H-12 α , H-12 β , H-16
15		143.1, C			
16	4.85 (2H, m, overlap)	114.4, CH ₂	H-17 (w)	C-14, C-15, C-17	H-14, H-17
17	1.83 (3H, t, $J_{\text{H}17, \text{H}16}$ = 1.0)	18.6, CH ₃	H-16(w)	C-14, C-15, C-16	H-13, H-16
18	1.00 (3H, d, $J_{\text{H}18, \text{H}11}$ = 6.7)	20.2, CH ₃	H-11	C-10, C-11, C-12	H-2 α , H-2 β , H-6 β , H-8 β , H-10
19		179.3, C			
20		171.5, C			

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

Once again, the material eluted with peak **7** maintained the same viscidane structure and substitutions on ring A and B as compounds **1a**, **3a** and **3b** (table 36). The conjunction of the linear side chain with ring A was established by the H-7 $\leftrightarrow$ H₂-8 $\leftrightarrow$ H₂-9 $\leftrightarrow$ H-10 $\leftrightarrow$ H-11(H₃-18) $\leftrightarrow$ H₂-12 $\leftrightarrow$ H₂-13 $\leftrightarrow$ H-14 $\leftrightarrow$ H₃-16/H₃-17 spin system, observed in the COSY spectrum, with the latter being long-range couplings. The double bond between C-14 (δ_c 125.5) and C-15 (δ_c 132.0) was further identified thanks to the HMBC correlations of H-14 (m, δ_H 5.12) to C-15, C-16 and C-17 and of H₃-16 and H₃-17 to C-15.

Table 36. ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **7**.

Pos.	δ_H , nH, m, J (Hz) ^a	δ_C , type ^b	COSY	HMBC	ROESY
1		49.5, C spiro			
2	α . 2.32 (1H, dt, $J_{H2\alpha, H2\beta} = 19$, $J_{H2\alpha, H3} = 2.8$)	31.7, CH ₂	H-2 β , H-3, H-5(w)	C-1, C-3, C-4, C-7, C-10, C-20 (w)	H-6 α , H-11, H-18 (weaker)
	β . 2.06 (1H, m, overlap)		H-2 α , H-3	C-1, C-3, C-4, C-7, C-10 (w), C-20 (w)	H-7, H-18
3	6.94 (1H, dd, $J_{H3, H2\beta} = 5.7$, $J_{H3, H2\alpha} = 2.8$)	140.4, CH	H-2 α , H-2 β , H-5 (w)	C-1, C-2, C-4, C-5, C-20	H-6 β , H-7
4		134.5, C			
5	4.78 (1H, m)	65.40, CH	H-2 α (w), H-6 α , H-6 β	C-3, C-4, C-6, C-20 (w)	H-2 β , H-6 α , H-6 β , H-7
6	α . 1.91 (1H, m, overlap)	39.8, CH ₂	H-5, H-6 β	C-1, C-2, C-4, C-5, C-7, C-10 (w)	H-2 α , H-18
	β . 1.96 (1H, m, overlap)		H-5, H-6 α	C-1, C-2, C-4, C-5, C-7 (w), C-10, C-20 (w)	
7	2.59 (1H, dd, $J_{H7, H8\beta} = 8.4$, $J_{H7, H8\alpha} = 3.4$)	53.1, CH	H-8 α , H-8 β	C-1, C-2, C-6 (w), C-8, C-9, C-10, C-19	H-2 β , H-5, H-9A
8	α . 1.81 (1H, dtd, $J_{H8\alpha, H8\beta} = 17.4$, $J_{H8\alpha, H9\alpha} \approx J_{H8\alpha, H9\beta} = 6.1$, $J_{H8\alpha, H7} = 3.4$)	26.5, CH ₂	H-7, H-9A, H-9B	C-1, C-6, C-7, C-9, C-19	H-10
	β . 1.90 (1H, m, overlap)		H-7, H-9A	C-6, C-7, C-9, C-19	H-18
9	A. 1.53 (1H, m, overlap)	27.6, CH ₂	H-8 α , H-8 β , H-9B, H-10	C-8, C-10	
	B. 2.03 (1H, m, overlap)		H-8 α , H-8 β , H-9A, H-10	C-8, C-10	

Table 36 (continued). ^1H and ^{13}C NMR data and 2D COSY, ROESY, and HMBC correlations for **7**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
10	2.14 (1H, td, $J_{\text{H}10, \text{H}11}$ = 9.6, $J_{\text{H}10, \text{H}9\text{A}} \approx J_{\text{H}10, \text{H}9\text{B}} = 6.9$)	52.4, CH	H-9A, H-9B, H-11	C-1, C-2, C-6, C-9, C-11, C-12, C-18	H-8 α , H-11, H-18 (weaker)
11	1.54 (1H, m, overlap)	33.8, CH	H-10, H-12 β , H-18	C-1, C-10, C-12, C-13, C-18	H-2 α , H-10, H-18
12	α . 1.48 (1H, m, overlap) β . 1.13 (1H, dtd, $J_{\text{H}12\beta, \text{H}12\alpha} = 14.3$, $J_{\text{H}12\beta, \text{H}13\text{A}} \approx J_{\text{H}12\beta, \text{H}13\text{B}} = 9.2$, $J_{\text{H}12\beta, \text{H}11} = 4.9$)	36.5, CH ₂	H-12 β , H-13A, H-13B H-11, H-12 α , H-13A, H-13B	C-10, C-11, C-13, C-14, C-18 C-11, C-13, C-14, C-18	H-18 (stronger)
13	A. 1.94 (1H, m, overlap) B. 2.07 (1H, m, overlap)	26.0, CH ₂	H-12 α , H-12 β , H-13B, H-14 H-12 α , H-12 β , H-13A, H-14	C-10, C-11, C-12, C-14, C-15 C-10, C-11, C-12, C-14, C-15	
14	5.12 (1H, m)	125.5, CH	H-13A, H-13B, H-16 (w), H-17 (w)	C-12, C-13, C-16, C-17	H-13A, H-13B, H-17
15		132.0, C			
16	1.62 (3H, s)	17.5, CH ₃	H-14 (w)	C-12, C-13, C-14, C-15, C-17	
17	1.68 (3H, s)	25.6, CH ₃	H-14 (w)	C-12, C-13, C-14, C-15, C-16	H-14
18	1.00 (3H, d, $J_{\text{H}18, \text{H}11}$ = 6.8)	20.4, CH ₃	H-11	C-10, C-11, C-12	H-2 α , H-2 β , H-10, H-13A
19		179.1, C			

^a ^1H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

The ^1H NMR and 2D homo- and heteronuclear NMR data of the material eluted with peak **1b** revealed it to be a previously undescribed iridoid (table 37). The COSY spectra displayed three spin systems H-1'↔H-2'↔H-3'↔H-4'↔H-5'↔H₂-6', H-1↔H-9↔H-5↔H₂-6↔H-7 and finally H-14↔H₂-15↔H-16↔H₂-17↔H₂-18. The first system was identified as the sugar moiety, recognised also by comparing its NMR data with the glucose δ_{H} and δ_{C} of other known iridoid [202]. The second spin system was attributed to the bicyclic *cis*-fused cyclopentane-pyran. In this case, HMBC correlations from H-3 and H-5 to the quaternary carbons at δ_{C} 170.6 of C-11 confirmed the

carboxylic acid at C-4 (δ_C 112.4) on the six terms ring. An alkenyl proton appeared at δ_H 5.85 (H-7, brs, δ_C 130.8), and together with the HMBC correlations from H-9 to the quaternary carbon at δ_C 139.5 (C-8) and δ_C 63.4 (C-10), it indicated the presence of a double bond in the cyclopentene ring between C-7 and C-8, and conjunction with the side chain in position 10. H₂-10 showed HMBC correlations with an additional quaternary carbon at δ_C 168.5 (C-12), but no COSY correlations. In addition, the H₂-10 ¹H NMR signal at δ_{HA} 4.81 and δ_{HB} 4.89 suggested the linkage with an ester moiety. The third and final COSY system highlighted the presence of a cyclohexane ring, with a double bond positioned between C-13 and C-14. This was corroborated by HMBC correlations of alkenyl proton H-14 (δ_H 7.05, m, δ_C 141.4) to the quaternary carbons at δ_C 131.0 (C-13) and to C-12. A para substitution on the same ring was observed by HMBC correlations from H-16 to a quaternary carbon at δ_C 72.6. The substitution on C-19 with two methyl groups was evident from the ROESY spectra whit correlations between H-16 to H₃-20 (δ_H 1.182, s, δ_C 26.7) and H₃-21 (δ_H 1.180, s, δ_C 26.1). The additional oxymethine group on C-19 was hypothesized because of its higher δ_C value.

Table 37. ^1H and ^{13}C NMR data and 2D COSY, ROESY, and HMBC correlations for 1b .					
Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
1	5.19 (1H, d, $J_{\text{H1}, \text{H9}}$ = 7.6)	98.0, C	H-9	C-3, C-5, C-8, C-9, C-1'	H-6A, H-9, H-1'
2		O			
3	7.52 (1H, d, $J_{\text{H3}, \text{H5}}$ = 1.1)	153.0, CH	H-5 (w)	C-1, C-4, C-5, C-11	H-1, H-1'
4		112.4, C			
5	3.20 (1H, tdd, $J_{\text{H5}, \text{H6A}} \approx J_{\text{H5}, \text{H6B}} = 17.3$, $J_{\text{H5}, \text{H9}} = 8.1$, $J_{\text{H5}, \text{H3}} = 1.1$)	36.2, CH	H-3 (w), H-6A, H-6B, H9	C-1, C-4, C-6, C-9, C-11	
6	A. 2.15 (1H, m, overlap)	39.6, CH ₂	H-5 (w), H-6B, H-7	C-4, C-5, C-7, C-8	H-1, H-7
	B. 2.86 (1H, dd, $J_{\text{H6B}, \text{H5}} = 17.3$, $J_{\text{H6B}, \text{H6A}} = 8.7$)		H-5 (w), H-6A, H-7	C-5, C-7, C-8, C-9	H-7
7	5.85 (1H, brs)	130.8, CH	H-6A (w), H-6B (w)	C-5, C-6, C-8, C-9, C-10	H-10B
8		139.5, C			
9	2.77 (1H, dd, $J_{\text{H9}, \text{H5}} = 8.1$, $J_{\text{H9}, \text{H1}} = 7.6$)	47.3, CH	H-1, H-5	C-1, C-5, C-6, C-7, C-8, C-10	H-10A
10	A. 4.81 (1H, m, overlap)	63.4, CH ₂		C-7, C-8, C-12	
	B. 4.89 (1H, m, overlap)		H-10A	C-7, C-8, C-9, C-12	
11		170.6, C			
12		168.5, C			
13		131.0, C			
14	7.05 (1H, m)	141.4, CH	H-15A(w), H-15B	C-12, C-15, C-16, C-18	H-15A
15	A. 2.04 (1H, m, overlap)	28.3, CH ₂	H-15B, H-16	C-13, C-14, C-16	
	B. 2.36 (1H, m)		H-14, H-15A, H-16	C-12, C-13, C-14, C-16, C-17	H-20, H-21
16	1.55 (1H, tdd, $J_{\text{H16}, \text{H15A}} \approx J_{\text{H16}, \text{H15B}} = 12.0$, $J_{\text{H16}, \text{H17A}} = 5.1$, $J_{\text{H16}, \text{H17B}} = 2.5$)	45.2, CH	H-15A, H-15B, H-17A, H-17B	C-15, C-17, C-18, C-19, C-20, C-21	H-20, H-21
17	A. 1.24 (1H, m, tdd, $J_{\text{H17A}, \text{H18A}} \approx J_{\text{H17A}, \text{H18B}} = 13.2$, $J_{\text{H17A}, \text{H17B}} = 12.4$, $J_{\text{H17A}, \text{H16}} = 5.1$)	24.3, CH ₂	H-16, H-17B, H-18A, H-18B	C-15, C-16, C-18	
	B. 2.02 (1H, m, overlap)		H-16, H-17A, H-18A	C-13, C-15, C-16	H-20, H-21, H-18B
18	A. 2.16 (1H, m, overlap)	26.1, CH ₂	H-17A, H-17B, H-18B	C-13	
	B. 2.52 (1H, m)		H-17A, H-17B, H-18A	C-12, C-13, C-14, C-16, C-17	H-16, H-17A
19		72.6, C			
20	1.182 (3H, s)	26.7, CH ₃		C-16, C-19, C-21	H-16, H-15A, H-15B, H-17B, H-18A

Table 37 (continued). ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **1b**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
21	1.180 (3H, s)	26.1, CH ₃		C-16, C-19, C-20	H-16, H-15A, H-15B, H-17B, H-18A
1'	4.72 (1H, d, $J_{\text{H1}', \text{H2}'} = 7.9$)	100.3, CH	H-2'	C-1, C-3', C-5'	H-5', H-3'
2'	3.24 (1H, dd, $J_{\text{H2}', \text{H3}'} = 9.3$, $J_{\text{H2}', \text{H1}'} = 7.9$)	74.6, CH	H-1', H-3'	C-1', C-3'	
3'	3.38 (1H, $J_{\text{H3}', \text{H2}'} = 9.3$, $J_{\text{H3}', \text{H4}'} = 9.0$)	77.7, CH	H-2', H-4'	C-2', C-4'	
4'	3.31 (1H, m, overlap)	71.2, CH	H-3', H-5'	C-3', C-5'	
5'	3.30 (1H, m, overlap)	78.1, CH	H-4', H-6'A	C-3', C-4', C-6'	
6'	A. 3.66 (1H, dd, $J_{\text{H6A}', \text{H6B}'} = 12.0$, $J_{\text{H6A}', \text{H5}'} = 5.5$)	62.5, CH ₂	H-5', H-6'B	C-4', C-5'	
	B. 3.86 (1H, dd, $J_{\text{H6B}', \text{H6A}'} = 12.0$, $J_{\text{H6B}', \text{H5}'} = 2.0$)		H-5', H-6'A		

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

The constituents eluted with peak **2**, **4a**, **4b**, **4c**, **6b**, **8**, **10** and **11** showed NMR data that suggested they shared the same core structure.

Considering compound **2** as an example, a AA'BB' pattern in the ¹H NMR spectra can be observed at δ_{H} 7.94 (2H, $J_{\text{Orto}} = 8.2$) and δ_{H} 7.28 (2H, $J_{\text{Orto}} = 8.2$), signifying the presence of a p-disubstituted benzene ring (table 38). HMBC correlations from H-2' and H-6' to the quaternary carbons at δ_{C} 169.9 of C-7' indicated a carboxylic acid on C-1' (δ_{C} 129.5). The conjunction with the side chain, starting from position 4' (δ_{C} 150.6), was identified through HMBC correlations from H-3' and H-5' to C-5 (δ_{C} 52.0). From here, the COSY spectra displayed a H₂-2 $\leftrightarrow$ H₂-3 $\leftrightarrow$ H₂-4 $\leftrightarrow$ H-5 $\leftrightarrow$ H-6(H₃-13) $\leftrightarrow$ H₂-7 $\leftrightarrow$ H₂-8 $\leftrightarrow$ H-9 $\leftrightarrow$ H₃-11 $\leftrightarrow$ H₃-12 spin system, the latter being long-range couplings. The presence of a carboxylic acid at one of the chain's ends was observed thanks to the HMBC correlations of H₂-2 to a quaternary carbon at δ_{C} 177.2. From position 5 onwards, the chain showed the same data pattern

as the one observed for compound **1a**. The relative stereochemistry of **2** was determined by analysis of the ROESY spectrum, even though the free rotation of the chain at C-5 and C-6 makes the determination difficult. Considering the similarity of the chain with that of already known serrulatene diterpenoids, H-6 and H-5 are tentatively positioned in β , with the assignment of the *5R,6R* configuration based on biosynthetic considerations [192-193]. For what concerns the stereochemistry of C-9, another compound was isolated from the same fraction, and it shared the same ^1H NMR and 2D homo- and heteronuclear NMR data of compound **2**, but with a few chemical shift variations from position 6 to 9. It can be assumed it's an epimer at C-9 of compound **2** (table 39).

Table 38. ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **2**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
1'		129.5, C			
2'	7.94 (1H, AA'BB', $J_{\text{Ortho}} = 8.2$)	130.3, CH	H-3', H-5'	C-1', C-3', C-4', C-5', C-6', C-7'	
3'	7.28 (1H, AA'BB', $J_{\text{Ortho}} = 8.2$)	129.7, CH	H-2', H-6'	C-1', C-2', C-6', C-4', C-5', C-7' (w), C-5	H-4 α , H-4 β , H-5, H-13
4'		150.6, C			
5'	7.28 (1H, AA'BB', $J_{\text{Ortho}} = 8.2$)	129.7, CH	H-2', H-6'	C-1', C-2', C-3', C-6', C-4', C-7' (w), C-5	H-4 α , H-4 β , H-5, H-13
6'	7.94 (1H, AA'BB', $J_{\text{Ortho}} = 8.2$)	130.3, CH	H-3', H-5'	C-1', C-2', C-3', C-4', C-5', C-7'	
7'		169.9, C			
1		177.2, C			
2	2.24 (2H, td, $J_{\text{H2, H2'}} = 15.8$ Hz, $J_{\text{H2, H3}} = 7.1$)	34.7, CH ₂	H-3	C-1, C-3, C-4	H-4 α , H-4 β
3	1.38 (2H, m, overlap)	24.2, CH ₂	H-2, H-4 α , H-4 β	C-2, C-4, C-5	H-2', H-6', H-5
4	α . 1.75 (1H, m, overlap)	33.0, CH ₂	H-3, H-4 β , H-5	C-2, C-3, C-5, C-4'	H-2, H-3, H-3', H-5'
	β . 1.85 (1H, m overlap)		H-3, H-4 α	C-3, C-5, C-6, C-4'	H-2, H-3, H-13, H-3', H-5'
5	2.58 (1H, ddd, $J_{\text{H5, H4}\alpha} = 8.8$, $J_{\text{H5, H4}\beta} = 5.5$, $J_{\text{H5, H6}} = 4.3$)	52.0, CH	H-4 α , H-4 β , H-6	C-3 (w), C-4, C-6, C-13, C-3', 5', C-4'	H-4 β , H-7 β , H-13, H-3', H-5'
6	1.77 (1H, m, overlap)	39.3, CH	H-5, H-7 β , H-13	C-5, C-7, C-13	H-9
7	α . 1.53 (1H, dddd, $J_{\text{H7}\alpha, \text{H7}\beta} = 13.3$, $J_{\text{H7}\alpha, \text{H8A}} = 11.1$, $J_{\text{H7}\alpha, \text{H8B}} = 9.8$, $J_{\text{H7}\alpha, \text{H6}} = 3.8$)	32.7, CH ₂	H-7 β , H-8B, H-8A		H-6, H-9, H-13
	β . 1.36 (1H, m, overlap)		H-6, H-7 α , H-8B	C-5, C-8, C-9, C-13	H-5
8	A. 1.35 (1H, m, overlap)	29.3, CH ₂	H-7 β , H-8B, H-9	C-7, C-9	H-9, H-13
	B. 1.61 (1H, dddd, $J_{\text{H8B, H7}\alpha} = 9.8$, $J_{\text{H8B, H8A}} = 8.2$, $J_{\text{H8B, H7}\beta} = 7.7$, $J_{\text{H8B, H9}} = 2.0$)		H-7 α , H-8A	C-6, C-7	H-13
9	3.23 (1H, dd, $J_{\text{H9, H8A}} = 10.3$, $J_{\text{H9, H8B}} = 2.0$)	79.5, CH	H-8A	C-7, C-8, C-10 C-11, C-12	H-6, H-7 α , H-11
10		73.6, C			
11	1.16 (3H, s)	25.5, CH ₃		C-9, C-10, C-12	H-8A, H-8B, H-9
12	1.13 (3H, s)	24.6, CH ₃		C-9, C-10, C-11	H-8A, H-8B, H-9
13	0.75 (3H, d, $J_{\text{H13, H6}} = 6.9$)	17.1, CH ₃	H-6	C-5, C-6, C-7	H-3, H-4 α , H-7 α , H-7 β , H-8A, H-8B

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

Table 39. ^1H and ^{13}C NMR data and 2D COSY, ROESY, and HMBC correlations for **2 C-9 epimer** from position 6 to 9.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
6	1.76 (1H, m, overlap)	39.0, CH	H-5, H-7 α H-7 β , H-13	C-5, C-7, C-13	H-9
7	α . 1.07 (1H, tt, $J_{\text{H}7\alpha, \text{H}7\beta} \approx J_{\text{H}7\alpha, \text{H}8\text{A}} = 14.8$, $J_{\text{H}7\alpha, \text{H}6} \approx J_{\text{H}7\alpha, 8\text{B}} = 4.8$)	32.9, CH ₂	H-6, H-7 β , H-8A	C-6, C-8, C-13	H-6, H-8A
	β . 1.86 (1H, m, overlap)		H-7 α , H-8A, H-8B	C-5, C-6, C-9, C-13	H-5, H-7 β , H-13
8	A. 1.22 (1H, m, overlap)	29.5, CH ₂	H-7 α , H-7 β , H-8B, H-9	C-7, C-9	H-7 α , H-8B
	B. 1.72 (1H, m, overlap)		H-7 α , H-7 β , H-8A	C-7, C-9	H-8A
9	3.20 (1H, dd, $J_{\text{H}9, \text{H}8\text{A}} = 10.3$, $J_{\text{H}9, \text{H}8\text{B}} = 1.3$)	80.0, CH	H-8A	C-7, C-8, C-10 C-11, C-12	H-6, H-7 α , H-11

^a ^1H NMR (600.13) data obtained with samples in methanol- d_4 . ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. α denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

For what concerns materials eluted with peak **4a**, a comparable NMR data pattern to compound **2** was observed, suggesting the same core skeleton (table 40). The main difference for compound **4a** was represented by the appearance of an additional singlet in the ^1H NMR at δ_{H} 3.61 (H₃-14, s, δ_{C} 51.7), that in the HMBC spectra showed correlations to the quaternary carbon C-1 (δ_{C} 175.6). Thus, the presence of a methyl ester was established.

Table 40. ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **4a**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
1'		130.0, C			
2'	7.94 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	130.3, CH	H-3', H-5'	C-1', C-3', C-4', C-5', C-6', C-7'	
3'	7.27 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	129.7, CH	H-2', H-6'	C-1', C-2', C-5', C-6', C-7' (w), C-5	H-5, H-6
4'		150.4, C			
5'	7.27 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	129.7, CH	H-2', H-6'	C-1', C-2', C-3', C-6', C-7' (w), C-5	H-5, H-6
6'	7.94 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	130.3, CH	H-3', H-5'	C-1', C-2', C-3', C-4', C-5', C-7'	
7'		170.2, C			
1		175.6, C			
2	2.28 (2H, dt, $J_{\text{H2}, \text{H2}'} = 10.0$, $J_{\text{H2}, \text{H3}} = 7.5$)	34.5, CH ₂	H-3	C-1, C-3, C-4	
3	1.39 (2H, m)	24.2, CH ₂	H-2, H-4A, H-4B	C-1, C-2, C-4, C-5	H-5
4	A. 1.73 (1H, m, overlap) B. 1.84 (1H, dddd, $J_{\text{H4B}, \text{H4A}} = 14$, $J_{\text{H4B}, \text{H3}} = 10.0$, $J_{\text{H4B}, \text{H3}} = 6.7$, $J_{\text{H4A}, \text{H5}} = 4.3$)	33.0, CH ₂	H-3, H-4B, H-5 H-3, H-4A	C-5, C-4' C-5, C-4'	H-3
5	2.56 (1H, ddd, $J_{\text{H5}, \text{H4A}} = 10.8$, $J_{\text{H5}, \text{H6}} = 6.7$, $J_{\text{H5}, \text{H4B}} = 4.3$)	52.0, CH	H-4A, H-4B, H-6	C-4, C-6, C-7, C-13, C-3', C-4', C-5'	H-7A
6	1.75 (1H, m, overlap)	38.6, CH	H-5, H-7A, H-13	C-5	
7	A. 1.37 (1H, m, overlap) B. 1.52 (1H, m)	32.7, CH ₂	H-6, H-7B, H-8B H-7A, H-8A, H-8B	C-8, C-9	
8	A. 1.35 (1H, m, overlap) B. 1.60 (1H, m)	29.3, CH ₂	H-7A, H-7B, H-8B, H-9 H-7A, H-7B, H-8A	C-7, C-9 C-7	
9	3.23 (1H, dd, $J_{\text{H9}, \text{H8A}} = 10.1$, $J_{\text{H9}, \text{H8B}} = 2.0$)	79.5, CH	H-8A, H-8B (w)	C-7, C-8, C-10, C-11, C-12	H-6, H-11, H-12
10		73.6, C			
11	1.16 (3H, s)	25.5, CH ₃		C-9, C-10, C-12	H-8A, H-8B, H-9
12	1.13 (3H, s)	24.7, CH ₃		C-9, C-10, C-11	H-8A, H-8B, H-9
13	0.74 (3H, d, $J_{\text{H13}, \text{H6}} = 6.7$)	17.1, CH ₃	H-6	C-5, C-6, C-7	H-5, H-7A, H-7B, H-8A, H-8B
14	3.61 (3H, s)	51.7, CH ₃		C-1	

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

For the constituents represented by peak **4b** and **6b**, the same p-disubstituted benzene core skeleton was observed through analysis of the ^1H NMR spectra, and an additional HMBC correlation between H₃-14 and C-1 established compound **6b** as a methyl ester of **4b** on C-1 (table 41-42). The COSY spectrum of both compounds revealed the same H₂-2 $\leftrightarrow$ H₂-3 $\leftrightarrow$ H₂-4 $\leftrightarrow$ H-5 $\leftrightarrow$ H-6(H₃-13) $\leftrightarrow$ H₂-7 $\leftrightarrow$ H-8 $\leftrightarrow$ H-9 spin system. In this instance, two alkenyl protons appeared at δ_{H} 5.61 (H-8, m, δ_{C} 125.7) and at δ_{H} 5.61 (H-9, m, δ_{C} 140.9) for **4b** and at δ_{H} 5.61 (H-8, m, δ_{C} 125.7) and at δ_{H} 5.60 (H-9, m, δ_{C} 140.9) for **6b**. The trans configuration of this double bond was tentatively assigned through ROESY correlations: H-8 showed ROE correlations to H₂-7 and H₃-11, while H-9 with H₃-13 and H₃-12. From here the oxygenated quaternary C-10 at δ_{C} 71.0 for **4b** and at δ_{C} 70.9 for **6b** was identified based on HMBC correlations from H-8, H-9, H₃-11 and H₃-12. The 5*R*,6*R* configuration has been assigned based on biosynthetic considerations for both the compounds.

Table 41. ¹ H and ¹³ C NMR data and 2D COSY, ROESY, and HMBC correlations for 4b .					
Pos.	δ _H , nH, m, <i>J</i> (Hz) ^a	δ _C , type ^b	COSY	HMBC	ROESY
1'		130.4, C			
2'	7.94 (1H, AA'BB', <i>J</i> _{Orto} = 8.3)	130.3, CH	H-3', H-5'	C-1', C-3', C-4', C-5', C-6', C-7'	
3'	7.26 (1H, AA'BB', <i>J</i> _{Orto} = 8.3)	129.7, CH	H-2', H-6'	C-1', C-2', C-5', C-6', C-7', C-5	H-4A, H-4B, H-5, H-6
4'		150.2, C			
5'	7.26 (1H, AA'BB', <i>J</i> _{Orto} = 8.3)	129.7, CH	H-2', H-6'	C-1', C-2', C-3', C-6', C-7', C-5	H-4A, H-4B, H-5, H-6
6'	7.94 (1H, AA'BB', <i>J</i> _{Orto} = 8.3)	130.3, CH	H-3', H-5'	C-1', C-2', C-3', C-4', C-5', C-7'	
7'		170.2, C			
1		177.4, C			
2	2.23 (2H, td, <i>J</i> _{H2, H2'} = 15.0, <i>J</i> _{H2, H3} = 8.2)	34.7, CH ₂	H-3	C-1, C-3, C-4	
3	1.37 (2H, dqd, <i>J</i> _{H3, H4B} = 9.7, <i>J</i> _{H3, H2} ≈ <i>J</i> _{H3, H3} = 8.2, <i>J</i> _{H3, H4} = 7.0)	24.2, CH ₂	H-2, H-4A, H-4B	C-1, C-2, C-4, C-5	H-5
4	A. 1.73 (1H, dtd, <i>J</i> _{H4A, H4B} = 14.0, <i>J</i> _{H4A, H3} ≈ <i>J</i> _{H4A, H3} = 7.0, <i>J</i> _{H4A, H5} = 5.6)	33.1, CH ₂	H-3, H-4B, H-5	C-2, C-3, C-5, C-4'	H-3', H-5'
	B. 1.85 (1H, m, overlap)		H-3, H-4A	C-3, C-5, C-4'	H-3', H-5'
5	2.58 (1H, ddd, <i>J</i> _{H5, H4B} = 10.8, <i>J</i> _{H5, H4A} = 5.6, <i>J</i> _{H5, H6} = 4.4)	51.2, CH	H-4A, H-4B, H-6	C-3, C-4, C-6, C-7, C-13, C-3', C-4', C-5'	H-3, H-7A, H-7B, H-13, H-3', H-5'
6	1.81 (1H, m, overlap)	39.3, CH	H-5, H-7A, H-13	C-5, C-7, C-8, C-13, C-4'	
7	A. 1.86 (1H, m, overlap)	38.2, CH ₂	H-7B, H-8	C-5, C-6, C-8, C-9, C-13	H-5, H-13
	B. 2.19 (1H, dt, <i>J</i> _{H7B, H6} , <i>J</i> _{H7B, H7A} ≈ 13.6, <i>J</i> _{H7B, H8} = 4.9)		H-7A, H-8	C-5, C-6, C-8, C-9, C-13	H-5, H-13
8	5.61 (1H, m, overlap)	125.7, CH	H-7A, H-7B, H-9	C-7, C-9, C-10, C-11, C-12	H-5, H-6, H-11, H-12, H-13
9	5.61 (1H, m, overlap)	140.9, CH	H-8	C-7, C-8, C-10, C-11, C-12	H-5, H-6, H-11, H-12, H-13
10		71.0, C			
11	1.28 (3H, s)	29.8, CH ₃		C-9, C-10, C-12	H-8, H-9
12	1.28 (3H, s)	29.8, CH ₃		C-9, C-10, C-11	H-8, H-9
13	0.72 (3H, d, <i>J</i> _{H13, H6} = 6.7)	17.2, CH ₃	H-6	C-5, C-6, C-7	H-5, H-7A, H-7B, H-8, H-9, H-3', H-5'

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

Table 42. ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **6b**.

Pos.	δ_H , nH, m, J (Hz) ^a	δ_C , type ^b	COSY	HMBC	ROESY
1'		130.7, C			
2'	7.94 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	130.3, CH	H-3', H-5'	C-1', C-3', C-4', C-5', C-6', C-7'	
3'	7.25 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	129.6, CH	H-2', H-6'	C-1', C-2', C-5', C-6', C-5	H-3, H-4A, H-4B, H-5, H-6, H-13
4'		149.7, C			
5'	7.25 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	129.6, CH	H-2', H-6'	C-1', C-2', C-3', C-6', C-5	H-5, H-6
6'	7.94 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	130.3, CH	H-3', H-5'	C-1', C-2', C-3', C-4', C-5', C-7'	
7'		170.7, C			
1		175.5, C			
2	2.27 (2H, td, $J_{H2, H2'} = 16.0$, $J_{H2, H3} = 7.6$)	34.5, CH ₂	H-3	C-1, C-3, C-4	H-4A, H-4B
3	1.37 (2H, ttd, $J_{H3, H4A} = 16.0$, $J_{H3, H2} \approx J_{H3, H3} = 13.0$, $J_{H3, H4B} = 6.7$)	24.1, CH ₂	H-2, H-4A, H-4B	C-1, C-2, C-4, C-5	H-5
4	A. 1.72 (1H, tdd, $J_{H4A, H3} = 13.2$, $J_{H4A, H5} = 10.8$, $J_{H4A, H4B} = 5.3$)	33.2, CH ₂	H-3, H-4B, H-5		H-2, H-3, H-5
	B. 1.83 (1H, m, overlap)		H-3, H-4A		H-2, H-3, H-5
5	2.57 (1H, ddd, $J_{H5, H4A} = 10.8$, $J_{H5, H4B} = 6.7$, $J_{H5, H6} = 4.4$)	51.2, CH	H-4A, H-6	C-6, C-13, C-3', C-4', C-5'	H-3
6	1.80 (1H, m, overlap)	39.3, CH	H-5, H-7A, H-13	C-5, C-7, C-8, C-13, C-4'	H-7A
7	A. 1.86 (1H, m, overlap)	38.3, CH ₂	H-6, H-7B, H-8	C-6, C-8, C-9, C-13	
	B. 2.18 (1H, dt, $J_{H7B, H7A} = 13.6$, $J_{H7B, H6} \approx J_{H7B, H8} = 5.0$)		H-6, H-7A, H-8	C-5, C-8, C-9, C-13	H-6
8	5.61 (1H, m, overlap)	125.7, CH	H-7A, H-7B, H-9	C-7, C-9, C-10	H-11, H-12, H-13
9	5.60 (1H, m, overlap)	140.9, CH	H-8	C-7, C-8, C-10	H-7A, H-7B, H-8A, H-8B, H-11B, H-12
10		70.9, C			
11	1.27 (3H, s)	29.8, CH ₃		C-8, C-9, C-10, C-12	H-8, H-9
12	1.27 (3H, s)	29.8, CH ₃		C-8, C-9, C-10, C-11	H-8, H-9
13	0.72 (3H, d, $J_{H13, H6} = 6.8$)	17.2, CH ₃	H-6	C-5, C-6, C-7	H-5, H-8, H-9, H-7B, H-3', H-5'
14	3.61 (3H, s)	51.7, CH ₃		C-1	

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

For the constituent eluted with fraction **4c**, the p-disubstituted benzene core skeleton was maintained, but in this case the side chain was partially similar to that of compound **3a**. In this case, the aliphatic oxymethine signals were observed at δ_{H} 3.96 (H-9, t, $J_{\text{H9, H8A}} \approx J_{\text{H9, H8B}} = 6.5$, δ_{C} 76.8), showing the presence of a hydroxyl group at C-9 (table 43). A geminal pair of vinylic protons appeared at δ_{H} 4.80 and δ_{H} 4.89 (H-11A, H11-B, t, $J_{\text{H11A, H11B}} \approx J_{\text{H11A, H11B}} = 1.0$, δ_{C} 111.4), which both showed HMBC correlations to C-10 and C-12, suggesting a double bond between C-10 and C-11. This was further confirmed by the ROESY correlations between vinylic H₂-11 and H₃-12 and H-9. The same 5*R*,6*R* configuration is tentatively assigned to compound **4c** for the same reason mentioned above.

The existence of an epimer at C-9 of compound **4c** was detected and isolated from the same fraction. They shared the same NMR data, but with a few chemical shift variations from position 4 to 13 (table 44).

Table 43. ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **4c**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
1'		130.0, C			
2'	7.94 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	130.3, CH	H-3', H-5'	C-1', C-3', C-4', C-5', C-6', C-7'	
3'	7.26 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	129.6, CH	H-2', H-6'	C-1', C-2', C-5', C-6', C-7', C-5	H-5, H-6
4'		150.4, C			
5'	7.26 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	129.6, CH	H-2', H-6'	C-1', C-2', C-3', C-6', C-7', C-5	H-5, H-6
6'	7.94 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	130.3, CH	H-3', H-5'	C-1', C-2', C-3', C-4', C-5', C-7'	
7'		170.1, C			
1		177.4, C			
2	2.23 (2H, td, $J_{\text{H2, H2}} = 16.0$, $J_{\text{H2, H3}} = 8.3$)	34.6, CH ₂	H-3	C-1, C-3, C-4	H-4A, H-4B
3	1.37 (2H, dqd, $J_{\text{H3, H4A}} = 16.0$, $J_{\text{H3, H2}} \approx J_{\text{H3, H3}} = 13$, $J_{\text{H3, H4B}} = 6.7$)	24.2, CH ₂	H-2, H-4A, H-4B	C-1, C-2, C-4, C-5	H-5
4	A. 1.72 (1H, m, overlap)	33.0, CH ₂	H-4B, H-5	C-2, C-3, C-5, C-4'	H-3, H-5
	B. 1.82 (1H, tdd, $J_{\text{H4B, H3}} = 16.0$, $J_{\text{H4B, H4A}} = 6.7$, $J_{\text{H4B, H5}} = 4.4$)		H-3, H-4A	C-3, C-5, C-4'	H-3, H-5
5	2.54 (1H, ddd, $J_{\text{H5, H4A}} = 10.6$, $J_{\text{H5, H6}} = 6.6$, $J_{\text{H5, H4B}} = 4.4$)	52.0, CH	H-4A, H-4B, H-6	C-3, C-4, C-6, C-7, C-8, C-13, C-3', C-4', C-5'	H-3, H-4A, H-4B, H-13
6	1.72 (1H, m, overlap)	39.0, CH	H-5, H-7A, H-13	C-5, C-7, C-8, C-13, C-4'	
7	A. 1.02 (1H, dtd, $J_{\text{H7A, H7B}} = 15.4$, $J_{\text{H7A, H6}} \approx J_{\text{H7A, H8B}} = 8.8$, $J_{\text{H7A, H8A}} = 4.4$)	31.3, CH ₂	H-6, H-7B, H-8A, H-8B	C-5, C-6, C-8, C-9, C-13	H-6, H-13
	B. 1.58 (1H, m, overlap)		H-6, H-7A, H-8A, H-8B	C-8, C-9, C-13	H-5, H-13
8	A. 1.50 (1H, dtd, $J_{\text{H8A, H8B}} = 16.3$, $J_{\text{H8A, H7B}} \approx J_{\text{H8A, H9}} = 6.5$, $J_{\text{H8A, H7A}} = 4.4$)	32.9, CH ₂	H-7A, H-7B, H-8B, H-9	C-6, C-7, C-9, C-10	H-5, H-13
	B. 1.60 (1H, m, overlap)		H-7A, H-8A, H-9	C-6, C-7, C-9, C-10	H-5, H-13
9	3.96 (1H, t, $J_{\text{H9, H8A}} \approx J_{\text{H9, H8B}} = 6.5$)	76.8, CH	H-8A, H-8B	C-7, C-8, C-10, C-11, C-12	H-7A, H-7B, H-11B, H-12
10		148.4, C			
11	A. 4.80 (1H, t, $J_{\text{H11A, H11B}} = 1.0$)	111.4, CH ₂		C-9, C-10, C-12	H-12
	B. 4.89 (1H, t, $J_{\text{H11B, H11A}} = 1.0$)			C-9, C-10, C-12	H-9
12	1.69 (3H, s)	17.1, CH ₃		C-9, C-10, C-11	
13	0.73 (3H, d, $J_{\text{H13, H6}} = 6.8$)	17.2, CH ₃	H-6	C-5, C-6, C-7	H-5, H-7A, H-7B, H-3', H-5'

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

Table 44. ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **4c C-9 epimer** from position 4 to 13.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
4	A. 1.73 (1H, m, overlap)	33.1, CH ₂	H-3, H-4B, H-5	C-3, C-5, C-4'	H-2, H-3, H-5
	B. 1.83 (1H, tdd, $J_{\text{H4B, H3}} = 13.2$, $J_{\text{H4B, H4A}} = 6.0$, $J_{\text{H4B, H5}} = 4.4$)		H-3, H-4A		
5	2.55 (1H, ddd, $J_{\text{H5, H4A}} = 10.7$, $J_{\text{H5, H6}} = 6.7$, $J_{\text{H5, H4B}} = 4.4$)	51.9, CH	H-4A, H-4B, H-6	C-3, C-4, C-6, C-13, C-3', C-4', C-5'	H-3, H-4A, H-4B, H-6, H-7A, H-7B, H-13
6	1.73 (1H, m, overlap)	39.0, CH	H-5, H-7A, H-13	C-5, C-8, C-13, C-4'	H-7A, H-7B, H-8A, H-8B
7	A. 1.17 (1H, dddd, $J_{\text{H7A, H7B}} = 11.0$, $J_{\text{H7A, H6}} = 8.7$, $J_{\text{H7A, H8A}} = 7.1$, $J_{\text{H7A, H8B}} = 4.3$)	31.4, CH ₂	H-6, H-7B, H-8A	C-6, C-8, C-9, C-13	H-5, H-6, H-9, H-13
	B. 1.46 (1H, m, overlap)		H-7A, H-8B		
8	A. 1.52 (1H, m, overlap)	33.0, CH ₂	H-7A, H-7B, H-8B, H-9	C-6, C-7, C-9, C-10	H-5, H-6, H-9, H-13
	B. 1.59 (1H, dddd, $J_{\text{H8B, H8A}} = 11.8$, $J_{\text{H8B, H7B}} = 10.2$, $J_{\text{H8B, H9}} = 6.4$, $J_{\text{H8B, H7A}} = 4.4$)		H-7A, H-8A, H-9		
9	3.96 (1H, t, $J_{\text{H9, H8A}} \approx J_{\text{H9, H8B}} = 6.4$)	76.6, CH	H-8A, H-8B	C-7, C-8, C-10, C-11, C-12	H-7A, H-7B, H-8A, H-8B, H-11B, H-12
10		148.7, C			
11	A. 4.80 (1H, t, $J_{\text{H11A, H11B}} = 1.0$)	111.1, CH ₂		C-9, C-10, C-12	H-12
	B. 4.90 (1H, t, $J_{\text{H11B, H11A}} = 1.0$)				
12	1.70 (3H, s)	17.4, CH ₃		C-9, C-10, C-11	H-9
13	0.74 (3H, d, $J_{\text{H13, H6}} = 6.8$)	17.2, CH ₃	H-6	C-5, C-6, C-7	H-5, H-7A, H-7B, H-8A, H-8B, H-3', H-5'

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

For the constituent eluted with peak **8**, the p-disubstituted benzene core skeleton was maintained, but the NMR data showed the presence of the methyl ester in position 1, as shown by the HMBC correlation between H₃-14 and C-1 at δ_{C} 175.5 (table 45). The COSY spectrum revealed a slightly different H₂-2 $\leftrightarrow$ H₂-3 $\leftrightarrow$ H₂-4 $\leftrightarrow$ H-5 $\leftrightarrow$ H-6(H₃-13) $\leftrightarrow$ H₂-7 $\leftrightarrow$ H₂-8 spin system. From here, the presence of a quaternary carbon at δ_{C} 204.3 was detected through HMBC correlations from H-8, suggesting the presence of a ketone moiety in position 9. A geminal pair of vinylic protons appeared at δ_{H} 5.83 and δ_{H} 6.06 (H-11A, H11-B, t, $J_{\text{H11A}, \text{H11B}} \approx J_{\text{H11A}, \text{H11B}} = 1.1$, δ_{C} 125.7), which both showed HMBC correlations to both C-9 and C-12, suggesting a double bond between C-10 and C-11. Finally, a methyl group was positioned through HMBC correlation between H₃-12 and C-9, C-10 and C-11. The same 5*R*,6*R* configuration is tentatively assigned to compound **8** for the same reason mentioned above.

Table 45. ^1H and ^{13}C NMR data and 2D COSY, ROESY, and HMBC correlations for **8**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
1'		131.7, C			
2'	7.94 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	130.3, CH	H-3', H-5'	C-3', C-4', C-5', C-6', C-7'	
3'	7.26 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	129.6, CH	H-2', H-6'	C-1', C-2', C-5', C-6', C-7' (w), C-5	H-4A, H-4B, H-5, H-6, H-13
4'		150.0, C			
5'	7.26 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	129.6, CH	H-2', H-6'	C-1', C-2', C-3', C-6', C-7' (w), C-5	H-4A, H-4B, H-5, H-6, H-13
6'	7.94 (1H, AA'BB', $J_{\text{Orto}} = 8.3$)	130.3, CH	H-3', H-5'	C-1', C-2', C-3', C-4', C-5', C-7'	
7'		170.3, C			
1		175.5, C			
2	2.27 (2H, td, $J_{\text{H2}, \text{H2}} = 15.5$ Hz, $J_{\text{H2}, \text{H3}} = 8.2$)	34.6, CH ₂	H-3	C-1, C-3, C-4	H-4A, H-4B, H-5
3	1.38 (2H, m, overlap)	24.1, CH ₂	H-2, H-4A, H-4B	C-1, C-2	H-3', H-5', H-5
4	A. 1.73 (1H, m, overlap)	32.9, CH ₂	H-3, H-4B, H-5		H-3', H-5', H-2
	B. 1.82 (1H, m, overlap)		H-3, H-4A		H-3', H-5', H-2, H-13
5	2.56 (1H, ddd, $J_{\text{H5}, \text{H4A}} = 10.6$, $J_{\text{H5}, \text{H6}} = 6.6$, $J_{\text{H5}, \text{H4B}} = 4.2$)	51.8, CH	H-4A, H-4B, H-6		H-3, H-7A, H-7B, H-13
6	1.74 (1H, m, overlap)	38.8, CH	H-5, H-7A, H-7B H-13		H-7B, H-8A
7	A. 1.32 (1H, m, overlap)	30.3, CH ₂	H-6, H-7B, H-8		H-5, H-13
	B. 1.82 (1H, m, overlap)		H-6, H-7A, H-8		H-4A, H-4B, H-5, H-13
8	2.76 (2H, ddd, $J_{\text{H8}, \text{H8}} = 15.8$, $J_{\text{H8}, \text{H7B}} = 9.2$, $J_{\text{H8}, \text{H7A}} = 5.8$)	35.7, CH ₂	H-7A, H-7B	C-6, C-7, C-9	H-7A, H-7B
9		204.3, C			
10		145.4, C			
11	A. 5.83 (1H, d, $J_{\text{H11A}, \text{H11B}} = 1.1$)	125.7, CH ₂		C-9, C-12	H-12
	B. 6.06 (1H, d, $J_{\text{H11B}, \text{H11A}} = 1.1$)			C-9, C-12	H-12
12	1.84 (3H, s)	17.4, CH ₃		C-9, C-10, C-11	H-11A, H-11B
13	0.74 (3H, d, $J_{\text{H13}, \text{H6}} = 6.7$)	17.0, CH ₃	H-6	C-5, C-6, C-7	H-5, H-7A, H-7B, H-8A, H-8B H-3', H-5'
14	3.62 (3H, s)	51.7, CH ₃		C-1	

^a ^1H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

For the constituents represented by peak **10** and **11**, the same p-disubstituted benzene core skeleton was observed through analysis of the ^1H NMR spectra, and an additional HMBC correlation between H₃-14 and C-1 established compound **11** as a methyl ester of **10** on C-1 (table 46-47). The COSY spectra of both of the compounds revealed a H₂-2 $\leftrightarrow$ H₂-3 $\leftrightarrow$ H₂-4 $\leftrightarrow$ H-5 $\leftrightarrow$ H-6(H₃-18) $\leftrightarrow$ H₂-7 $\leftrightarrow$ H₂-8 $\leftrightarrow$ H-9 $\leftrightarrow$ H₃-11/H₃-12 spin system, with the latter being long-range couplings. The double bond between C-9 (δ_{C} 125.4 for **10** and δ_{C} 125.5 for **11**) and C-10 (δ_{C} 131.8 for **10** and δ_{C} 131.9 for **11**) was further identified thanks to the HMBC correlations of H-9 (t, $J_{\text{H9, H8A}} \approx J_{\text{H9, H8B}} = 7.4$, δ_{H} 5.07 for **10** and tt, $J_{\text{H5, H8A}} \approx J_{\text{H5, H8A}} = 7.2$, $J_{\text{H5, H11}} \approx J_{\text{H5, H12}} = 1.3$, δ_{H} 5.09 for **11**) to C-10, C-11 and C-12 and of H₃-11 and H₃-12 to C-10. The same 5*R*,6*R* configuration is tentatively assigned to compound **10** and **11** for the same reason mentioned above.

Table 46. ^1H and ^{13}C NMR data and 2D COSY, ROESY, and HMBC correlations for **10**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
1'		129.4, C			
2'	7.95 (1H, AA'BB', $J_{\text{Ortho}} = 8.2$)	130.3, CH	H-3', H-5'	C-1', C-3', C-4', C-5', C-6', C-7'	
3'	7.24 (1H, AA'BB', $J_{\text{Ortho}} = 8.2$)	129.6, CH	H-2', H-6'	C-1', C-2', C-5', C-6', C-7' (w), C-5	H-4A, H-4B, H-5, H-6, H-13
4'		150.4, C			
5'	7.24 (1H, AA'BB', $J_{\text{Ortho}} = 8.2$)	129.6, CH	H-2', H-6'	C-1', C-2', C-3', C-6', C-7' (w), C-5	H-4A, H-4B, H-5, H-6, H-13
6'	7.95 (1H, AA'BB', $J_{\text{Ortho}} = 8.2$)	130.3, CH	H-3', H-5'	C-1', C-2', C-3', C-4', C-5', C-7'	
7'		169.8, C			
1		177.2, C			
2	2.22 (2H, td, $J_{\text{H2, H2}} = 15.5$ Hz, $J_{\text{H2, H3}} = 8.2$)	34.6, CH ₂	H-3	C-1, C-3, C-4	H-4A, H-4B, H-5
3	1.37 (2H, m)	24.2, CH ₂	H-2, H-4A, H-4B	C-2, C-4, C-5	H-3', H-5', H-5
4	A. 1.71 (1H, m, overlap)	33.0, CH ₂	H-3, H-4B, H-5	C-3, C-5, C-4'	H-3', H-5', H-2
	B. 1.79 (1H, dddd, $J_{\text{H4B, H4A}} = 15.3$, $J_{\text{H4B, H3}} = 11.3$, $J_{\text{H4B, H3}} = 9.8$, $J_{\text{H4A, H5}} = 4.7$)		H-3, H-4A	C-3, C-5, C-4'	H-3', H-5', H-2, H-13

Table 46 (continued). ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **10**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
5	2.54 (1H, ddd, $J_{\text{H5, H4A}} = 10.5$, $J_{\text{H5, H6}} = 6.3$, $J_{\text{H5, H4B}} = 4.7$)	52.0, CH	H-4A, H-4B, H-6	C-3, C-4, C-6, C-7, C-13, C-3', C-4', C-5'	H-3, H-7A, H-7B, H-13
6	1.70 (1H, m, overlap)	38.6, CH	H-5, H-7A, H-7B, H-13	C-5, C-7, C-13, C-4'	H-7B, H-8A
7	A. 1.07 (1H, tdd, $J_{\text{H7A, H8A}} \approx J_{\text{H7A, H8B}} = 14.4$, $J_{\text{H7A, H7B}} = 8.8$, $J_{\text{H7A, H6}} = 5.7$)	35.5, CH ₂	H-6, H-7B, H-8A, H-8B	C-5, C-6, C-8, C-9, C-13	H-5, H-13
	B. 1.47 (1H, tdd, $J_{\text{H7B, H8A}} \approx J_{\text{H7B, H8B}} = 14.4$, $J_{\text{H7B, H7A}} = 8.8$, $J_{\text{H7B, H6}} = 4.5$)		H-7A, H-8A, H-8B	C-5, C-6, C-8, C-9, C-13	H-4A, H-4B, H-5, H-13
8	A. 1.95 (1H, qd, $J_{\text{H8A, H7A}} \approx J_{\text{H8A, H7B}} \approx J_{\text{H8A, H8B}} = 14.4$, $J_{\text{H8A, H9}} = 7.4$)	26.1, CH ₂	H-7A, H-7B, H-8B, H-9	C-6, C-7, C-9, C-10	H-5, H-6, H-13
	B. 2.03 (1H, qd, $J_{\text{H8B, H7A}} \approx J_{\text{H8B, H7B}} \approx J_{\text{H8B, H8A}} = 14.4$, $J_{\text{H8B, H9}} = 7.4$)		H-7A, H-7B, H-8A, H-9	C-6, C-7, C-9, C-10	H-5, H-6, H-13
9	5.07 (1H, t, $J_{\text{H9, H8A}} \approx J_{\text{H9, H8B}} = 7.4$)	125.4, CH	H-8A, H-8B, H-11 (w), H-12 (w)	C-7, C-8, C-11, C-12	H-11, H-12 (weaker)
10		131.8, C			
11	1.65 (3H, s)	25.7, CH ₃		C-9, C-10, C-12	H-8A, H-8B, H-9
12	1.58 (3H, s)	17.5, CH ₃		C-9, C-10, C-11	H-8A, H-8B, H-9
13	0.71 (3H, d, $J_{\text{H13, H6}} = 6.9$)	17.0, CH ₃	H-6	C-5, C-6, C-7	H-3, H-4 α , H-7 α , H-7 β , H-8A, H-8B

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. α denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

Table 47. ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **11**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
1'		130.1, C			
2'	7.94 (1H, AA'BB', $J_{\text{Ortho}} = 8.3$)	130.3, CH	H-3', H-5'	C-1', C-3', C-4', C-5', C-6', C-7'	
3'	7.24 (1H, AA'BB', $J_{\text{Ortho}} = 8.3$)	129.6, CH	H-2', H-6'	C-1', C-2', C-4', C-5', C-6', C-7', C-5	H-5, H-6
4'		150.3, C			
5'	7.24 (1H, AA'BB', $J_{\text{Ortho}} = 8.3$)	129.6, CH	H-2', H-6'	C-1', C-2', C-3', C-4', C-6', C-7', C-5	H-5, H-6
6'	7.94 (1H, AA'BB', $J_{\text{Ortho}} = 8.3$)	130.3, CH	H-3', H-5'	C-1', C-2', C-3', C-4', C-5', C-7'	
7'		170.1, C			
1		175.5, C			
2	2.26 (2H, td, $J_{\text{H2,H2}} = 15.8$ Hz, $J_{\text{H2,H3}} = 8.4$)	34.4, CH ₂	H-3	C-1, C-3, C-4	H-4A, H-4B
3	1.37 (2H, dqd, $J_{\text{H3,H4B}} = 10.4$, $J_{\text{H3,H2}} \approx J_{\text{H3,H3}} = 8.0$, $J_{\text{H3,H4A}} = 7.0$)	24.1, CH ₂	H-2, H-4A, H-4B	C-1, C-2, C-4, C-5	H-5
4	A. 1.71 (1H, m)	33.2, CH ₂	H-3, H-4B, H-5	C-4', C-2, C-3, C-5, C-6, C-13	
	B. 1.78 (1H, tdd, $J_{\text{H4B,H3}} = 10.4$, $J_{\text{H4B,H4A}} = 6.2$, $J_{\text{H4B,H5}} = 4.4$)		H-3, H-4A, H-5	C-4', C-2, C-3, C-5, C-6, C-13	H-13, H-7B
5	2.54 (1H, ddd, $J_{\text{H5,H6}} = 10.6$, $J_{\text{H5,H4A}} = 6.3$, $J_{\text{H5,H4B}} = 4.4$)	51.8, CH	H-4A, H-4B, H-6	C-3', C-5', C-4', C-3, C-4, C-6, C-7, C-13	H-3, H-7A, H-7B, H-13
6	1.71 (1H, m, overlap)	38.6, CH	H-5, H-7A, H-13	C-4', C-3, C-5, C-7, C-8, C-13	
7	A. 1.08 (1H, m, overlap)	35.6, CH ₂	H-6, H-7B, H-8A, H-8B	C-5, C-6, C-8, C-9, C-13	H-13
	B. 1.48(1H, dddd, 4.3/5.8/7/13.6))		H-7A, H-8A, H-8B	C-5, C-6 (w), C-8, C-9 (w), C-13	H-6, H-13
8	A. 1.97 (1H, qd, $J_{\text{H8A,H7A}} \approx J_{\text{H8A,H7B}} \approx J_{\text{H8A,H8B}} = 14.4$, $J_{\text{H8A,H9}} = 7.1$)	26.2, CH ₂	H-7A, H-7B, H-8B, H-9	C-6, C-7, C-9, C-10	H-13
	B. 2.05 (1H, qd, $J_{\text{H8B,H7A}} \approx J_{\text{H8B,H7B}} \approx J_{\text{H8B,H8A}} = 14.4$, $J_{\text{H8B,H9}} = 7.1$)		H-7A, H-7B, H-8A, H-9	C-6, C-7, C-9, C-10	H-6, H-13
9	5.09 (1H, tt, $J_{\text{H5,H8A}} \approx J_{\text{H5,H8A}} = 7.2$, $J_{\text{H5,H11}} \approx J_{\text{H5,H12}} = 1.3$)	125.5, CH	H-8A, H-8B, H-11 (w), H-12 (w)	C-7, C-8, C-11, C-12	H-11
10		131.9, C			
11	1.67 (3H, s)	25.6, CH ₃		C-9, C-10, C-12	H-9
12	1.60 (3H, s)	17.5, CH ₃		C-9, C-10, C-11	H-8A, H-8B
13	0.73 (3H, d, $J_{\text{H13,H6}} = 6.8$)	17.0, CH ₃		C-5, C-6, C-7	H-5, H-6, H-7A, H-7B, H-8A, H-8B

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^c Assignments based on HSQC and HMBC experiments.

For the constituent eluted with peak **6a**, the COSY spectrum revealed two spin systems $\text{H-3} \leftrightarrow \text{H-4} (\text{H}_2\text{-5} \leftrightarrow \text{H}_2\text{-6}) \leftrightarrow \text{H-7} (\text{H}_3\text{-19}) \leftrightarrow \text{H}_2\text{-8} \leftrightarrow \text{H}_2\text{-9} \leftrightarrow \text{H-10}$ and $\text{H}_2\text{-12} \leftrightarrow \text{H}_2\text{-13} \leftrightarrow \text{H-14} \leftrightarrow \text{H}_3\text{-16/H}_3\text{-17}$, with the latter being long-range couplings (table 48). The presence of a five-member ring in conjunction with two side chains was observed from the HMBC correlations of an aliphatic oxymethine signal at δ_{H} 4.13 (H-3, dddd, $J_{\text{H}_3, \text{H}_4} = 9.5$, $J_{\text{H}_3, \text{H}_{6\text{A}}} = 3.5$, $J_{\text{H}_3, \text{H}_{6\text{B}}} = 2.5$, $J_{\text{H}_3, \text{H}_1} = 2.1$, δ_{C} 69.0) to a quaternary carbon at δ_{C} 133.0 (C-2) and to C-5 and C-6. An alkenyl proton at δ_{H} 6.78 (H-1, m, overlap, δ_{C} 142.6) with HMBC correlations to C-4, C-6 and a quaternary carbon at δ_{C} 171.1 (C-20) suggested the presence of a double bond between C-2 and C-1, with one of the substitutions on C-1 being a carboxylic acid. HMBC correlations of the methyl singlet H₃-19 to C-4 confirmed the side chain conjunction. Two more alkenyl protons were observed in the ^1H NMR spectrum. The first one at δ_{H} 6.79 (H-10, t, $J_{\text{H}_{10}, \text{H}_9} = 7.6$, δ_{C} 143.9), showing HMBC correlations to C-12, and to quaternary carbon, one at δ_{C} 133.1 and one at δ_{C} 171.4, being respectively an alkenyl carbon and a carboxylic acid carbon. The second alkenyl proton was observed at δ_{H} 6.79 (H-14, tsep, $J_{\text{H}_{14}, \text{H}_{13}} = 7.5$, $J_{\text{H}_{14}, \text{H}_{16}} \approx J_{\text{H}_{14}, \text{H}_{17}} = 1.4$, δ_{C} 124.6) and with HMBC correlations to C-12, C-16 and C-17. Thus, the third double bond was established between C-14 and C-15 (δ_{C} 132.8, observed through HMBC correlations from H₂-13). This unusual compound seems to maintain the terpenoid pattern. Although the free rotation of the chain at C-4 and C-7 makes the determination of the relative configuration difficult, considering the similarity of the chain with that of a serrulatene, H-7 and H-4 were tentatively positioned in α , with the assignment of the 4*R*,7*R* configuration based on biosynthetic considerations.

Table 48. ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **6a**.

Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
1	6.78 (1H, m, overlap)	142.6, CH	H-3, H-6A, H-6B	C-4, C-6, C-20	H-3
2		133.0, C			
3	4.13 (1H, dddd, $J_{\text{H3, H4}} = 9.5$, $J_{\text{H3, H6A}} = 3.5$, $J_{\text{H3, H6B}} = 2.5$, $J_{\text{H3, H1}} = 2.1$)	69.0, CH	H-1 (w), H-4, H-6A (w)	C-1, C-2, C-4	H-1, H-4, H-5A, H-5B, H-7, H-19
4	1.47 (1H, m, overlap)	46.0, CH	H-3, H-5A, H-7	C-3, C-5, C-7, C-19	H-6A, H-6B, H-19
5	A. 1.30 (1H, tdd, $J_{\text{H5A, H4}} \approx J_{\text{H5A, H5B}} = 13.2$, $J_{\text{H5A, H6A}} = 12.0$, $J_{\text{H5A, H6B}} = 5.2$)	21.4, CH ₂	H-4, H-5B, H-6A, H-6B	C-3, C-4, C-6	H-19
	B. 1.75 (1H, dddd, $J_{\text{H5B, H5A}} = 13.2$, $J_{\text{H5B, H4}} = 5.2$, $J_{\text{H5B, H6A}} = 2.8$, $J_{\text{H5B, H6B}} = 2.0$)		H-5A, H-6A	C-2, C-3, C-4, C-6	H-4, H-6B, H-19 (weaker)
6	A. 2.14 (1H, m, overlap)	25.8, CH ₂	H-5A, H-5B, H-6B		H-4
	B. 2.38 (1H, dddd, $J_{\text{H6B, H6A}} = 18.1$, $J_{\text{H6B, H5A}} = 5.2$, $J_{\text{H6B, H3}} = 2.5$, $J_{\text{H6B, H5B}} = 2.0$)		H-5A, H-6A	C-1, C-2, C-4, C-5	H-4
7	2.03 (1H, qtd, $J_{\text{H7, H19}} = 7.1$, $J_{\text{H7, H8}} = 6.8$, $J_{\text{H7, H4}} = 2.6$)	32.0, CH	H-8, H-19	C-3, C-4, C-5, C-8, C-9, C-19	H-3, H-5A, H-9
8	1.45 (2H, m, overlap)	35.3, CH ₂	H-7, H-9	C-4, C-7, C-9, C-10, C-19	H-10, H-19
9	2.24 (2H, tdd, $J_{\text{H9, H8}} = 11.4$, $J_{\text{H9, H9}} = 9.0$, $J_{\text{H9, H10}} = 7.6$)	27.7, CH ₂	H-8, H-10	C-7, C-8, C-10, C-11, C-12	H-7, H-10, H-19
10	6.79 (1H, t, $J_{\text{H10, H9}} = 7.6$)	143.9, CH	H-9	C-8, C-9, C-11, C-12, C-18	H-8
11		133.1, C			
12	2.32 (2H, td, $J_{\text{H12, H13}} = 8.2$, $J_{\text{H12, H12}} = 2.0$)	27.5, CH ₂	H-13	C-10, C-11, C-13, C-14, C-18	H-14
13	2.10 (2H, qd, $J_{\text{H13, H12}} \approx J_{\text{H13, H13}} = 8.2$, $J_{\text{H13, H14}} = 7.5$)	28.5, CH ₂	H-12, H-14	C-11, C-12, C-14, C-15, C-17 (w)	H-14, H-16
14	5.14 (1H, tsep, $J_{\text{H14, H13}} = 7.5$, $J_{\text{H14, H16}} \approx J_{\text{H14, H17}} = 1.4$)	124.6, CH	H-13, H-16 (w), H-17 (w)	C-12, C-13, C-16, C-17	H-12, H-13, H-17
15		132.8, C			
16	1.60 (3H, s)	17.5, CH ₃		C-12, C-14, C-15, C-17	H-13
17	1.67 (3H, s)	25.6, CH ₃		C-12, C-14, C-15, C-16	H-14
18		171.4, C			
19	0.87 (3H, d, $J_{\text{H19, H7}} = 7.1$)	14.0, CH ₃	H-7	C-4, C-7, C-8	H-3, H-4, H-5A, H-5B, H-8, H-9
20		171.1, C			

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Assignments based on HSQC and HMBC experiments. ^c Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^cn.d. = not detected.

For the constituent eluted with peak **12**, the spectroscopic data highlighted its eremane diterpenoid structure (table 49). Indeed, the ^1H NMR spectrum of compound **12** showed the presence of a methyl doublets at δ_{H} 0.96 (H₃-12, d, $J_{\text{H12}, \text{H4}} = 6.8$, δ_{C} 12.9) and of a broad singlet at δ_{H} 1.73 (H₃-19, bs, δ_{C} 8.9), as well as two methyl singlets at δ_{H} 1.07 (H₃-13, s, δ_{C} 25.2) and δ_{H} 1.17 (H₃-14, s, δ_{C} 32.2).

The eremane skeleton incorporates a tricyclo [5.2.2.0^{1,5}] undecane ring system and in this case the five-membered ring was identified by the H₂-2 $\leftrightarrow$ H₂-3 $\leftrightarrow$ H-4 $\leftrightarrow$ H₃-12 spin system observed in the COSY spectrum together with the HMBC correlations from H₃-12 to C-3 and C-5, and from H₂-2 to C-1, C-5 and C-9. The two fused six-membered rings were observed by the H₂-6 $\leftrightarrow$ H-7 $\leftrightarrow$ H₂-10 $\leftrightarrow$ H₂-11 spin system in the COSY spectrum and by the HMBC correlations from H-7 to C-5, from H-9 to C-1, C-5 and C-7 and finally from both H₃-13 and H₃-14 to C-7, C-8 and C-9. The linear side chain was established based on the H-9 $\leftrightarrow$ H₂-15 $\leftrightarrow$ H₂-16 $\leftrightarrow$ H-17 spin system. In this instance, the alkenyl proton at δ_{H} 6.64 (H-17, td, $J_{\text{H17}, \text{H16A}} = 7.4$, $J_{\text{H17}, \text{H16B}} = 1.3$, δ_{C} 157.2), with HMBC correlations to a quaternary carbon at δ_{C} 139.9, indicated the presence of a double bond between C-17 and C-18. The substitutions on C-18 were identified as a methyl group, confirmed by the HMBC correlations from H₃-19 to C-17, C-18 and C-20, and of an aldehydic moiety with a singlet in the ^1H NMR spectrum at δ_{H} 9.36 (1h, s, δ_{C} 197.1). The trans configuration of this double bond was tentatively assigned through ROESY correlations: H-17 showed ROE correlations to H-20. The 1*R*,4*R*,7*S*,11*S*,12*R* configuration was tentatively assigned to compound **12**.

Table 49. ¹ H and ¹³ C NMR data and 2D COSY, ROESY, and HMBC correlations for 12 .					
Pos.	δ _H , nH, m, <i>J</i> (Hz) ^a	δ _C , type ^b	COSY	HMBC	ROESY
1		49.8, C			
2	α. 1.20 (1H, m, overlap)	32.2, CH ₂	H-2β, H-3β, H-3α	C-1, C-4, C-5, C-9, C-10, C-11	
	β. 1.54 (1H, td, <i>J</i> _{H2β, H2α} ≈ <i>J</i> _{H2β, H3β} = 12.0, <i>J</i> _{H2β, H3α} = 6.4)		H-2α, H-3β, H-3α	C-1, C-3, C-4, C-9, C-10, C-11	H-4
3	α. 1.28 (1H, m, overlap)	29.8, CH ₂	H-2β, H-3α, H-4	C-1, C-2, C-4, C-9, C-12	
	β. 1.82 (1H, dtd, <i>J</i> _{H3α, H3β} = 13, <i>J</i> _{H3α, H2α} ≈ <i>J</i> _{H3α, H4} = 9.6, <i>J</i> _{H3α, H2β} = 6.4)		H-2α, H-2β, H-3β, H-4	C-1, C-2, C-4, C-5	H-12
4	2.01 (1H, ddq, <i>J</i> _{H4, H3β} = 16, <i>J</i> _{H4, H3α} = 9.6, <i>J</i> _{H4, H12} = 6.8)	42.5, CH	H-3β, H-3α, H-12	C-1, C-2, C-3, C-5, C-6, C-11, C-12	H-2β, H-6β, H-10β, H-11β
5		82.6, C			
6	α. 1.90 (1H, dt, <i>J</i> _{H6α, H6β} = 14.1, <i>J</i> _{H6α, H7} = 3.4)	37.6, CH ₂	H-6β, H-7	C-5, C-10	H-13, H-14
	β. 1.46 (1H, m, overlap)		H-6α, H-7	C-1, C-4, C-5, C-7, C-8, C-10	H-6β
7	1.18 (1H, m, overlap)	42.4, CH	H-6β, H-6α, H-10-A, H-10α	C-1, C-5, C-8, C-9, C-10	H-4, H-9, H-12
8		34.7, C			
9	2.04 (1H, m, overlap)	44.6, CH	H-15α, H-15β	C-1, C-5, C-8, C-11, C-13, C-14, C-15, C-16	H-7, H-14, H-16A
10	α. 1.22 (1H, m, overlap)	24.3, CH ₂	H-7, H-10α, H-11β	C-1, C-5, C-6, C-7, C-8, C-11	H-4
	β. 1.85 (1H, m, overlap)		H-7, H-10β, H-11α, H-11β	C-1, C-5, C-6, C-7	H-13, H-14
11	α. 1.45 (1H, m, overlap)	23.8, CH ₂	H-10β, H-10α, H-11α	C-1, C-2, C-5, C-7, C-9, C-10	H-4, H-15B
	β. 1.25 (1H, m, overlap)		H-10β, H-10α, H-11β	C-1, C-2, C-5, C-7, C-9, C-10	
12	0.96 (3H, d, <i>J</i> _{H12, H4} = 6.8)	12.9, CH ₃	H-4	C-3, C-4, C-5	H-2α, H-3α, (weaker: H-3β, H-6β)
13	1.07 (3H, s)	25.2, CH ₃		C-7, C-8, C-9, C-14	H-10α, H-11α, H-15A (w), H-15B, H-16A (w), H-16B
14	1.17 (3H, s)	32.2, CH ₃		C-7, C-8, C-9, C-13	H-9, H-12, H-16A, H-16B

Table 49 (continued). ¹H and ¹³C NMR data and 2D COSY, ROESY, and HMBC correlations for **12**.

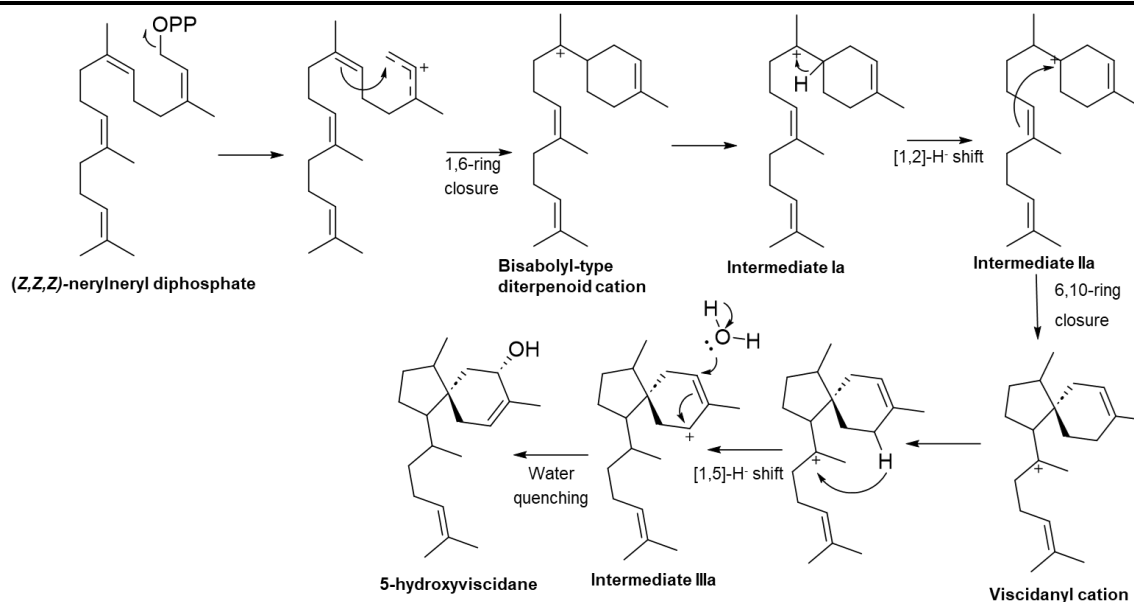
Pos.	δ_{H} , nH, m, J (Hz) ^a	δ_{C} , type ^b	COSY	HMBC	ROESY
15	A. 1.34 (1H, m, overlap)	34.0, CH ₂	H-15B, H-16A, H-16B	C-1, C-8, C-9, C-10, C-16, C-17	H-9, H-14
	B. 1.74 (1H, tdd, 10-7.2-3.0)		H-9, H-15A, H-16A, H-16-B	C-8, C-9, C-16, C-17	H-13
16	A. 2.38 (1H, dddd)	31.5, CH ₂	H-15A, H-15B, H-16B, H-17	C-9, C-15, C-17, C-18, C-20	H-9, H-13, H-14
	B. 2.48 (1H, m)		H-15A, H-15B, H-16A, H-17	C-9, C-15, C-17, C-18, C-20	H-9, H-13, H-14
17	6.64 (1H, td, $J_{\text{H17, H16A}}$ = 7.4, $J_{\text{H17, H16B}}$ = 1.3)	157.2, CH	H-16A, H-16B, H-19 (w)	C-15, C-16, C-19, C-20	H-20
18		139.9, C			
19	1.73 (3H, bs)	8.9, CH ₃	H-17 (w)	C-17, C-18, C-20	
20	9.36 (1H, s)	197.1, COH		C-17, C-18, C-19	H-17

^a ¹H NMR (600.13) data obtained with samples in methanol-*d*₄. ^b Assignments based on HSQC and HMBC experiments. ^c Multiplicities reported as apparent splittings: s = singlet, d = doublet, t = triplet, q = quartet, p = pentet, sep = septet, m = multiplet, br = broad. A denotes hydrogen pointing into the plane, β denotes hydrogen pointing out of the plane. A denotes the lowest chemical shift value and B denotes the highest chemical shift value. ^e n.d. = not detected.

For the above-described compounds, relative configurations of some of the stereocenters were tentatively assigned through ROESY correlations or by biosynthetic considerations. Recent studies have highlighted how *cis*-prenyltransferases found in *E.denticulata*, *E. lucida* and *E. drummondii* are able to synthesize a C20 all-*cisoid* precursor from, NNPP (neryl neryl diphosphate), by catalysing the reaction between isopentenyl diphosphate and dimethylallyl diphosphate. From here, class I TPS (Terpene synthases), more specifically *ETSP3*, can utilize NNPP as a substrate to form 5-hydroxyviscidanes. The reaction seems to go through the formation of a bisabolyl diterpenoid cation, obtained with a 1,6 cyclization. After a 1,2-hydride migration from C-6 to C-7, an additional 6,10 ring closure would

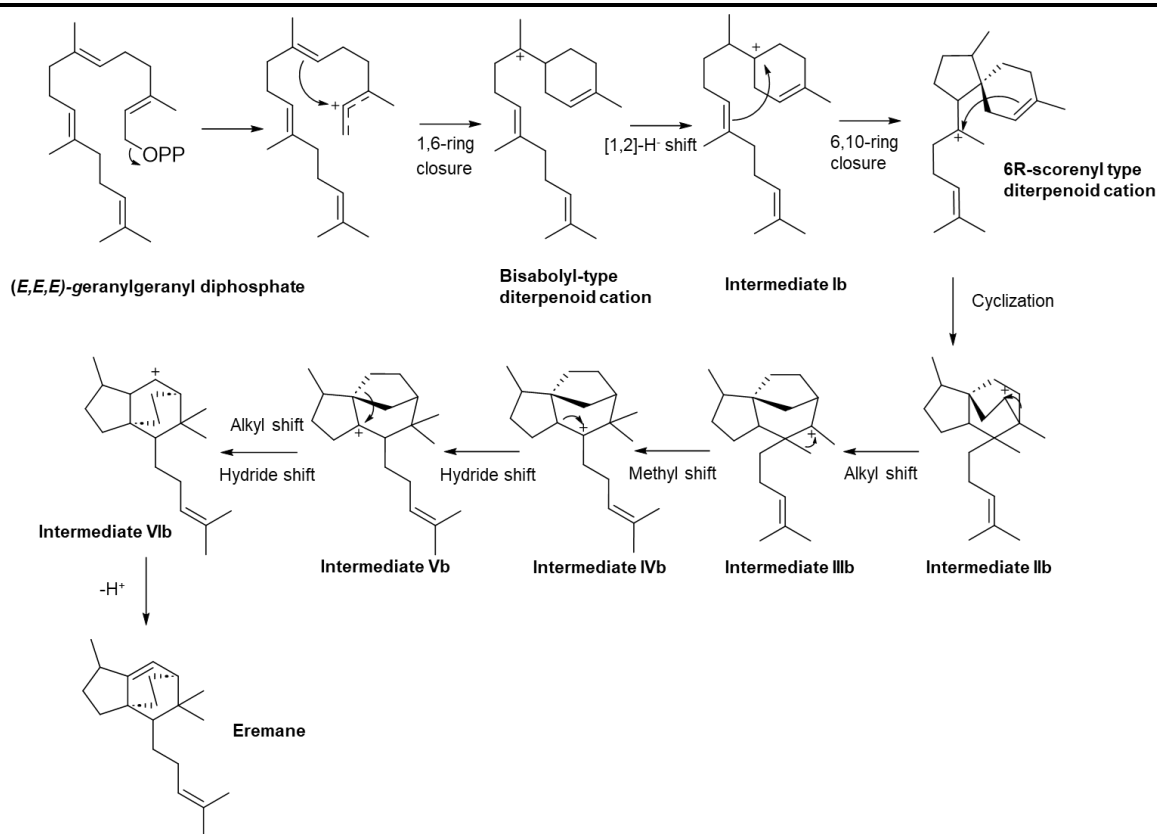
produce a viscidanyl cation. This cation would be further rearranged through a 1,5-hydride shift followed by a deprotonation in position C-5 [203-204] (figure 41).

Figure 41. Plausible biosynthetic route for formation of the 5-hydroxyviscidane core skeleton.



Even though no diterpenoid synthases have been identified yet for the biosynthesis of the ermane diterpenoids backbone, the typical C₂₀ transoid precursor GGDP ((E,E,E)-geranylgeranyl diphosphate), seems to be involved (figure 42) [205]. In this case, the biosynthesis starts with the formation of a bisabolyl-type diterpenoid cation, in its trans configuration, and from here, similar steps lead to the formation of 6*R*-acorenyl type diterpenoid cation. Subsequently, multiple steps of cyclization, alkyl and methyl shift take place, and with a final deprotonation, the core skeleton of ermane diterpenoids is generated [206]. For what concerns the origin of compounds **2**, **4a**, **4b**, **4c**, **6b**, **8**, **10**, **11**, their biosynthetic pathway is still under investigation, but they seem to derive directly from the bisabolyl-type cation.

Figure 42. Plausible biosynthetic route for formation of the eremane core skeleton.



These backbones are then subjected to additional modifications. Most of them seems to involve the cytochrome P450s activity, since esterifications and multiple oxidations with introductions of carboxylic moieties can be seen. Indeed, methyltransferases could be responsible for the methoxycarbonyl observed at C-1 in compounds **4a**, **6b**, **8** and **11**. Constituents **1a**, **3a**, **3b** and **7** may be formed through the action of P450 oxygenations of the methyl groups in position C-4 and C-7 [207-208-209]. Moreover, all these additional modifications of both the side chain and of their main core, grant the compounds with different pharmacological properties.

2.1.3 PTP1B inhibitory activity of the isolated compounds

Considering the amount of material available and the results of the biochromatogram, compounds **3a**, **3b**, **4b**, **4c**, **6a**, **6b**, **7**, **10**, **11**, **12**, **13** and fraction **14** were tested at a single concentration of 200 μ M for their PTP1B inhibitory activity (table 50).

Table 50. PTP1B inhibitory activity for the isolated compounds at 200 μ M.

Compound	Concentration (μ M)	PTP1B inhibition (%)
3a	200	24.8 $\pm$ 0.9
3b	200	13.4 $\pm$ 1.4
4b	200	10.8 $\pm$ 5.8
4c	200	30.5 $\pm$ 0.9
6a	200	3.5 $\pm$ 3.6
6b	200	32.8 $\pm$ 3.5
7	200	61.7 $\pm$ 10.4
10	200	14.6 $\pm$ 6.4
11	200	98.3 $\pm$ 0.1
12	200	8.4 $\pm$ 5.6
13	200	99.3 $\pm$ 2.4
14	200	95.5 $\pm$ 20.4

*Values expressed as mean $\pm$ SD (n = 3).

Based on these results, compounds **11**, **13** and fraction **14** were selected for further dilution tests to build a full dose-response curve. The three isolated materials provided IC₅₀ values of 60.87 $\pm$ 2.5 μ M, 24.67 $\pm$ 2.4 μ M and 7.24 $\pm$ 0.8 μ M, respectively. Considering how the IC₅₀ value of the positive control RK-682 was 19.24 $\pm$ 3.6 μ M, compounds **11**, **13** and fraction **14** are weak inhibitors of PTP1B in comparison to the reference molecule. This is in agreement with the peaks of the biochromatogram graph in figure 34. Although the triterpenoids were already well characterized in literature, no study was previously made in reference to their antidiabetic activity mediated through PTP1B inhibition.

Moreover, the inverse peak of the graph associated with compound **8**, was confirmed to be due to precipitation issues in DMSO, and not because of an activating action towards the enzyme.

2.1.4 Conclusions

To conclude, the combination of high/resolution PTP1B inhibition profiling of the acetonitrile leaves extract of *E. tietkensii* with HPLC-DAD, semi-preparative scale HPLC and NMR spectroscopy, allowed the identification of 21 compounds, 15 of them being undescribed previously. 3 known flavonoids and 3 already described triterpenoid. These findings are important to better understand *Eremophila* species, and at the same time to find new and promising pharmacologically active natural compounds.

2.1.5 Experimental procedures

Chemicals

NaCl, acetic acid, dimethyl sulfoxide (DMSO), dithiothreitol (DTT), N,N,N',N'-ethylenediaminetetraacetate (EDTA), p-nitrophenyl phosphate (*p*-NPP), tris(hydroxymethyl)-aminomethane (Tris) and bis(2-hydroxyethyl)-imino-tris(hydroxymethylmethane) (BisTris) were purchased from Sigma-Aldrich (St. Louis, MO, USA), recombinant human protein tyrosine phosphatase 1B (PTP1B) (BML-SE332-0050, EC 3.1.3.48) and RK-682 from Enzo Life Sciences (Farmingdale, NY, USA), while formic acid from Merck (Darmstadt, Germany). HPLC-grade acetonitrile was purchased from VWR International (Søborg, Denmark), and methanol-d₄ and chloroform-d from Euriso-top

(Saint-Aubin Cesex, France). Deionization and filtration of water was conducted through a 0.22- μ m membrane on a Milli-Q Plus system (Millipore, Billerica, MA, USA).

Plant material and extraction

Leaves of *Eremophila tietkensii* were collected in Western Australia and identified by Dr Bevan Buirchell. A voucher specimen has been deposited at the University of Melbourne Herbarium, Melbourne, Victoria, Australia.

The leaves extraction was done using 200 mL of acetonitrile shaken for 10 min (Ratek, Knox City, Victoria, Australia) and then filtered using a glass funnel and dried in vacuo at 40 °C using a rotary evaporator (IKA RV10). The dry extract was transferred to amber-colored vials using after redissolution and dried under nitrogen gas, then stored at – 20 °C until use.

PTP1B inhibition assay

The assay was performed using 96-well microplates following a slightly modified protocol of the one proposed by Tahtah and Coworkers [210]. To dissolve and dilute all the necessary reagents, a buffer solution was prepared by mixing 50 mM of Tris, 50 mM of bis-Tris and 100 mM of NaCl and the adjusted at pH 7 with acetic acid. 18 μ L of DMSO and 52 μ L of a 3.46 mM EDTA solution were added in to each well and shaken for 3 min. to dissolve all the constituents. A substrate solution of 1.5 mM of *p*-NPP and of 6 mM of DTT was freshly prepared and the 60 μ L of it were added to the microplate, leaving them to incubate for 10 min. at 25 °C. In the end, 50 μ L of 0.001 μ g/ μ L of PTP1B were added on each well to start the reaction. After

10 min of incubation, the absorbance of each cleavage product was recorded at 405 nm every 30 s for 10 min using a Thermo Scientific Multiscan FC microplate reader (Thermo Scientific, Waltham, MA, USA).

DMSO was considered as the blank sample and all the measurements were performed in triplicate. The enzyme activity was recorded as $\Delta AU/s$ and the inhibition rate was calculated with the following equation:

$$\text{Percentage inhibition} = \left(1 - \frac{\text{Slope}_{\text{sample}}}{\text{Slope}_{\text{blank}}} \right) \times 100\%$$

Crude extract microfractionation and high-resolution inhibition profiling

An Agilent 1200 (Santa Clara, CA, USA) series instrument equipped with a G1367C high-performance autosampler, a G1311A quaternary solvent pump, a G1322A degasser, a G1416A thermostated column compartment, a G1315C diode array detector (DAD) and a G1364C fraction collector, was used to accomplish analytical-scale HPLC analyses.

The separation was achieved employing a Phenomenex Luna RP C₁₈(2) (4.6 mm Ø x 150 mm, 3 µm particle size) column. The column temperature was 40 °C while the wavelength for the acquisition was set at 210 nm, 254 nm, 280 nm and 330 nm. For the microfractionation, the leaves crude extract of *E. tietkensii* was redissolved in a 1:1 ratio of solvent A and solvent B to a final concentration of 25 mg/mL. The mobile phase consisted of a solution of CH₃CN/H₂O/HCOOH (5/94.9/0.1) as solvent A and of CH₃CN/H₂O/HCOOH (94.9/5/0.1) as solvent B, following a gradient elution program as depicted in table 51 at a flow rate of 0.5 mL/min, for a total run time of 37 min and by injecting 25 µL of the crude extract.

Table 51. Gradient elution program for HPLC analysis.

Time (min)	A %	B %
0	65	35
23	0	100
28	0	100
29	65	35
37	65	35

The eluate from 2.5 to 33 min was collected into 88 wells while the remaining 8 wells were left out for the blank samples with DMSO.

The microplate was then evaporated to dryness with a Savant SPD121P speed vacuum concentrator equipped with an OFP400 oil-free pump and a RVT400 refrigerated vapor trap. The dried microfractionated extract was then subjected to the PTP1B assay and the resulting percentage of inhibition was plotted against the chromatogram corresponding retention time, allowing to build a high-resolution inhibition profile.

Isolation of pure compounds

The dried crude extract was redissolved in a 1:1 ratio of solvent A and solvent B to a final concentration of 50 mg/mL for isolation of pure compounds using a semipreparative-scale HPLC. The experiments were performed on a Shimadzu instrument (Tokyo, Japan) consisting of a SIL-20A autosampler, an LC-20AD pump, a CTO-10AS column oven, an SPD-20A UV/VIS detector and an FRC-10A fraction collector. The separation was achieved using a Phenomenex Luna RP C₁₈(2) (10.0 mm Ø x 250 mm, 5 µm particle size) column and using the same solvent mentioned above and the

same elution gradient reported in table 47, but with a flow rate of 4 mL/min.

For each consecutive injection of 90 μ L of the extract, 14 different fractions were collected and then evaporated to dryness. Three out of these were pure and they led to the isolation of compound **2** (Rt 6.1 min, 0.96 mg), compound **9** (Rt 16.3 min, 3 mg) and compound **10** (Rt 21.9 min, 18.44 mg). The material eluted with fractions 1, 3, 4, 5, 6, 7, 8, 11, 12, 13, 14 were further purified using the analytical-scale HPLC set up described above but equipped with a Phenomenex Kinetex PFP (4.6 mm $\varnothing$ x 150 mm, 2.6 μ m particle size) column.

All the fractions were redissolved with methanol to a concentration between 10 and 20 mg/mL and fractioned following different elution programs and injection volumes as reported in table 52.

Table 52. Gradient elution programs for HPLC analysis.

Time (min)	A%	B%	Time (min)	A%	B%
Fraction 1 (10- μ L injection)			Fraction 3 (10- μ L injection)		
0	85	15	0	80	20
20	80	20	25	73	27
22	0	100	27	20	80
25	0	100	30	0	100
26	85	15	33	0	100
34	85	15	34	80	20
			40	80	20
Fraction 4 (10- μ L injection)			Fraction 5 and 8 (10- μ L injection)		
0	75	25	0	100	0
20	70	30	20	0	100
22	0	100	25	0	100
26	0	100	27	100	0
27	75	25	35	100	0
35	75	25			
Fraction 6 (5- μ L injection)			Fraction 7 (5- μ L injection)		
0	63	37	0	100	0
20	60	40	20	0	100
22	0	100	25	0	100
25	0	100	27	100	0
26	63	37	35	100	0
34	63	37			
Fraction 11 (10- μ L injection)			Fraction 12, 13, 14 (10- μ L injection)		
0	80	20	0	65	35
20	0	100	20	30	70
25	0	100	25	0	100
27	80	20	32	0	100
35	80	20	35	65	35
			43	65	35

The purified fraction led to the isolation of compounds **1a** (Rt 12.0 min, 1.73 mg), **1b** (Rt 12.9 min, 0.80 mg), **3a** (Rt 15.0 min, 0.80 mg and Rt 15.05 min, 0.80 mg for its epimer), **3b** (Rt 15.1 min, 0.60 mg), **4a** (Rt 17.8 min, 0.26 mg), **4b** (Rt 18.4 min, 1.17 mg), **4c** (Rt 19.2 min, 0.60 mg and Rt 19.9, 0.50 mg for its epimer), **5** (Rt 16.0 min, 0.24 mg), **6a** (Rt 12.0 min, 0.70 mg), **6b** (Rt 12.5 min, 0.53 mg), **6c** (Rt 12.7 min, 0.55 mg), **7** (Rt 16.1 min, 2.07 mg),

8 (Rt 21.7 min, 0.30 mg), **11** (Rt 16.9 min, 3.00 mg), **12** (Rt 25.1 min, 1.25 mg), **13** (Rt 25.0 min, 0.63 mg) and the inseparable mixture of peak **14** (Rt 27.3 min, 1.12 mg). The purity of each of the sample was analysed using an analytical-scale HPLC with the same chromatographic conditions already mentioned above.

NMR experiments

The NMR experiments were performed on a Bruker Avance III 600 MHz NMR spectrometer equipped with a Bruker SampleJet sample changer and a cryogenically cooled gradient inverse triple-resonance 1.7 mm TCI probe-head (Bruker Biospin, Karlsruhe, Germany) at 300 K.

The operating frequencies for recording ^1H and ^{13}C spectra were 600.13 and 150.90 MHz, respectively and the chemical shift were calibrated based on the residual solvent signals, so δ_{H} 3.31 ppm/ δ_{C} 49.00 ppm for methanol- d_4 , δ_{H} 7.26 ppm/ δ_{C} 77.16 ppm for chloroform- d and δ_{H} 2.50 ppm/ δ_{C} 39.52 ppm for DMSO- d_6 . NMR data of **1a**, **1b**, **2**, **3a**, **3b**, **4a**, **4b**, **4c**, **6a**, **6b**, **6c**, **7**, **8**, **10**, **11**, and **12** were acquired in methanol- d_4 , **14** in chloroform- d , and **5**, **9** and **13** in DMSO- d_6 .

The ^1H NMR data were acquired with 30° pulses, an acquisition time of 2.72 s, a relaxation delay of 1.0 s, a spectral width of 20 ppm, and 64 k data points. The ^{13}C NMR data were acquired with 30° pulses, an acquisition time of 0.90 s, a relaxation delay of 2.0 s, a spectral width of 240 ppm, and 64k data points. Multiplicity edited HSQC and HMBC spectra were acquired with 12 ppm spectral width for ^1H and 170 ppm and 240 ppm for the latter for ^{13}C . The relaxation delay for both was of 1.0 s. The HMBC, phase-

sensitive DQF-COSY and ROESY spectra were all recorded with 2048 whereas the HSQC spectra with 1730 data points in the direct dimension. The HSQC and ROESY spectra were both acquired with 256, whereas the HMBC and COSY spectra with 512 data points in the indirect dimension. Automated NMR data acquisition was controlled by Icon NMR (version 4.2, Bruker Biospin, Karlsruhe, Germany), and the obtained NMR data were processed using Topspin (version 4.0.6, Bruker Biospin).

Chapter 3 – *Set-up of novel chemical assays for preliminary biological investigation*

The final part of the projects consisted in the set-up of novel and alternative chemical assays by using hyphenated techniques to rapidly screen the preliminary biological activity of natural and semisynthetic compounds.

The development of preliminary screening methods is important to limit the number of molecules to be further investigated, reducing cost and time when working on the evaluation of the biological efficacy on a specific target.

3.1 Novel approach for assessing the GPx-like activity of natural and semisynthetic compounds: A revised version of the Iwaoka's assay.

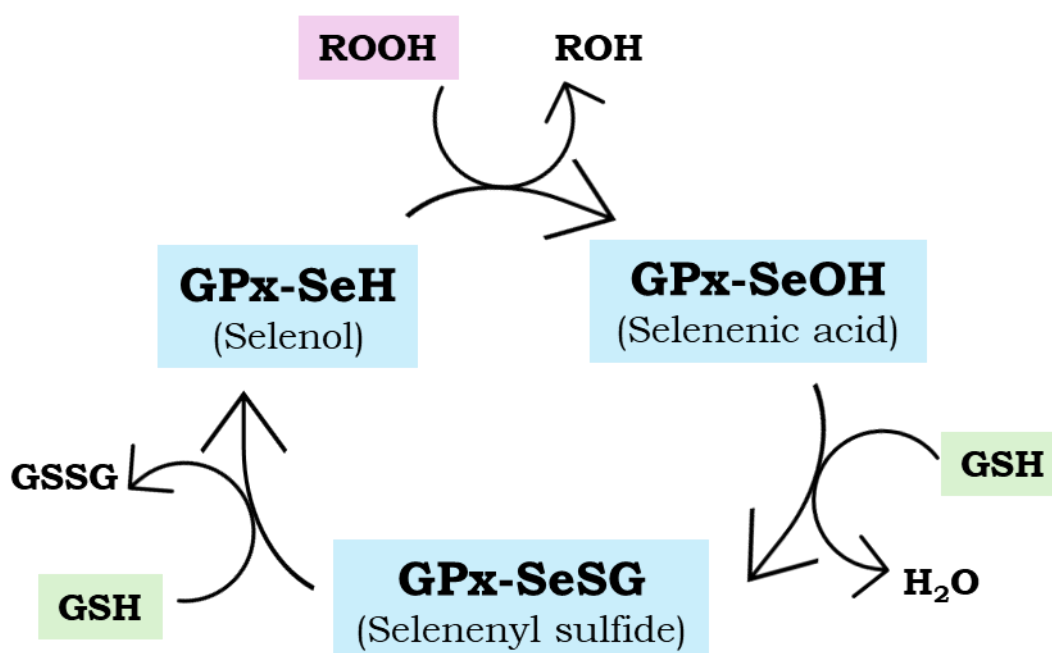
Reactive oxygen species (ROS) are normal products of the aerobic metabolism and are formed because of the ability of O_2 to accept electrons [211]. This is what ultimately leads to the production of species like superoxide anions ($\cdot O_2^-$), hydrogen peroxides (H_2O_2), hydroxyl radicals ($OH\cdot$) and singlet oxygen ($^1O_2$) [212]. Other than in the mitochondria, where the mitochondrial respiration is responsible for the production of a large number of ROS, they can be formed by multiple enzymes, like NADPH oxidases or nitric oxide synthase, but also by different types of radiations, drugs and other exogenous sources [213]. ROS species are fundamental to maintain redox homeostasis in a normal functioning cell, but their excessive production is the key for the development of oxidative stress [214-215]. Oxidative damage can be executed through lipids peroxidation, proteins oxidation and DNA and RNA direct damage, preventing the cells normal functions to be carried out, thus leading to the development of multiple diseases and degenerative conditions such as cancer, cardiovascular diseases, neurological disorders, inflammation, and so on [216-217-218-219].

One way through which the organism can counteract oxidative stress is with the action of antioxidants, specifically enzymatic and non-enzymatic antioxidants [220].

Among the first group, glutathione peroxidases (GPXs) are a family of oxidoreductases able to limit oxidative damage by catalysing the reductions

of various hydroperoxides and H_2O_2 to the respective alcohols and/or to H_2O using glutathione (GSH) as a reducing agent. GPxs are tetrads, where redox-active selenocysteine (Sec) or cysteine (Cys) are linked through hydrogen bonds with asparagine (Asn), glutamine (Gln) and tryptophane (Trp) residues [221]. They exist as 8 differently distributed isoforms: as Sec-enzymes in isoforms 1 – 4, while from 5 – 8 the amino acid in the active site is a Cys [222]. Among those, only GPX8 is a triad, in which the Gln residue is substituted with glutamic acid (Glu) or Glycine (Gly) [223]. The redox catalytic cycle of a selenocysteine GPx is represented in figure 43.

Figure 43. Catalytic cycle of a selenocysteine GPx.



In the active site, the selenocysteine residue, selenol, undergoes oxidation to selenenic acid by reacting with hydroperoxide. Two subsequent reactions with GSH are necessary to firstly reduce selenenic acid to selenenyl sulfide and then back to selenol again, producing an oxidized form of glutathione (GSSG) [224].

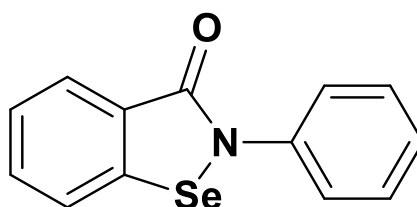
GPx1, the highest expressed Sec-enzyme, is found in all cell's mitochondria and cytoplasm and has an important role as an antioxidant system against oxidative stress in the organism [225-226]. On the other hand, in the cells of the gastrointestinal tract, GPx2, also known as GI-GPx, can be found. Its main role seems to be involved in preventing the absorption of hydroperoxides released during food digestion by acting as a first barrier [227-228]. GPx3 is responsible of scavenging ROS in plasma but also in the cells of the proximal tubule, suggesting a possible role as an antioxidant system in the kidneys. After its secretion by the basolateral membrane of the renal proximal convoluted tubule, GPx3 can circulate in the plasma, thus reaching other tissues such as heart, lung, liver and adipose tissue [229-230]. Lipid hydroperoxides reduction is performed mainly by GPx4, which exists in three different isoforms: CGPx4, a cytoplasmic form ubiquitous in all cells, MGPx4, a mitochondrial form, and SNGPx4, a spermatic form, the last two largely expressed in the testis [231-232]. Similarly, GPx5 can be found in epididymis, limiting ROS activity thus regulating sperm maturation [233-234]. GPx6 is believed to be connected to the transmission of olfactory signals since it's highly expressed olfactory epithelium cells [235].

GPx7 is mainly found in the endoplasmic reticulum, but it can also be secreted to the Golgi apparatus, and it's responsible for the limitation of oxidative stress [236-237]. Alike GPx7, GPx8 is highly expressed in the endoplasmic reticulum, reducing ROS damages [236]. Therefore, considering their important roles throughout all the organism, it's clear how polymorphism or downregulation of these enzymes can lead to excessive peroxide formation and oxidative stress, which is strictly involved with the

pathogenesis of numerous diseases and conditions such as diabetes and obesity, inflammation, cancer, infertility, neurological disorders and so on [238-239-240-241-242-243-244].

Considering how easily GPxs can get overwhelmed in these degenerative circumstances, it is important to note that small molecules can mimic their activity in counteracting peroxides damages and thus restoring redox balance [245]. For this aim, numerous compounds have been synthesized to mimic the GPx peroxidase activity and reduce oxidative stress, making them a valid pharmacological alternative for curing the above-mentioned diseases [246]. Among these molecules, Ebselen (2-Phenyl-1,2-benzoselenazol-3(2H)-one) is the most studied organoselenium drug (figure 44).

Figure 44. Ebselen structure.



After its first synthesis in 1924, Ebselen was tested in multiple clinical trials for its promising pharmacological activity in the treatment of multiple conditions such as hearing impairment, ischemic heart diseases and strokes, neurodegenerative and bipolar disorders, cancer, diabetes and for antibacterial, antiviral and antimycotic properties [247-248-249-250-251-252-253-254-255-256].

To this day, Ebselen antioxidant GPx mimicking mechanism hasn't been completely elucidated yet, still requiring further studies. Two of the main

pathways that were proposed to explain its catalytic activity are, firstly, the selenol pathway, where, after a rapid thiolysis of the compound to the selenenyl analogue, a reaction with a second thiolic substrate results in the formation of the reductant for peroxides. The second pathway happens through a disproportionation (methatesis) of the selenenyl sulfide to a diselenide, acting as the reductant agent [257]. One of the main problems associated with the use of Ebselen as a drug candidate, it's the compound low solubility in aqueous solution, which could be limiting both for intravenous delivery and for when rapid and urgent administration is required. Nonetheless, Ebselen promising biological effects led to the development of numerous analogues aiming to maintain a similar activity. Those can be divided in three main categories: cyclic selenenyl amides, diaryl diselenides and aromatic or aliphatic monoselenides. Organotellurium compounds were also considered as possible analogues [258].

For what concerns the toxicity profile of organochalcogen molecules, ebselen has been linked with some disadvantages, mainly due to thiol depletion, through which it acts as pro-oxidant [259]. This toxic behaviour could also be linked to its analogues, associating them with limitations for their potential use as drug candidates. Thus, new compounds having GPx-like activity similar to Ebselen, but with less toxic effects and solubility problems, still need to be studied. Two main characteristics need to be kept in considerations, regardless of the presence of Selenium and Tellurium in their structures: these compounds should be able to increase both the rate of oxidation phase of the catalytic reaction, mainly by an electronic effect

carried out by a polarizing group, and of the reduction step and regeneration of the compound itself, acting as a catalyst [260-261-262].

To screen GPxs mimicking effects of already known or new molecules, natural, semisynthetic or synthetic compounds, a rapid chemical assay could be especially useful. In this context, the so called Iwaoka's assay was developed.

In the original version of this test, Iwaoka and his Coworkers were able to monitor the oxidation of a thiol substrate in the presence of H₂O₂ and a catalyst, for instance with DHS^{red} (*trans*-3,4-dihydroxyselenolane), a water-soluble cyclic selenide and its linear analogues. Among the different ways through which the GPx-like activity could be recorded, the easiest and fastest consisted in the addition of a dithiol substrate, 1,4-dithiothreitol (DTT^{red}), in methanol-*d*₄ and by monitoring its oxidation rate to 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) in the presence of H₂O₂ through ¹H NMR spectroscopy.

Recording the ¹H NMR spectrum at different time, with and without the catalysts allowed them to evaluate the antioxidant and GPx-mimicking activity of each of the compounds under investigation [263-264-265].

While this assay appeared to be useful and efficient for the screening of the peroxidase activity of multiple compounds, some limitations can be highlighted. Firstly, the need of specific and expensive instrumentation such as an NMR spectrometer. Indeed, other than the difficult tuning, calibration and limited sensibility, the instrument could not be readily available in every laboratory. Other than that, the need to use deuterated solvents, and specifically methanol-*d*₄, which allowed better recording of the spectra,

could further elevate the cost of the overall procedure, limiting also the possibility to test more apolar compounds. All these factors come together to lower the applicability of the Iwaoka's assay.

Thus, the aim of this project was to try and extend the applications of Iwaoka's assay using cheaper and widespread hyphenated techniques, with easier tuning, calibration and availability respect to NMR spectrometer, like HPLC-DAD and GC-MS. In addition, the category of GPx-mimicking agents to be assessed was widened by performing the test in lower polarity and not deuterated solvents, lowering the cost of the procedure. Similarly to the original test, both the GC-MS based and HPLC-DAD based methodologies were utilized to monitor the oxidation rate of DTT^{red} to DTT^{ox} in the presence of H₂O₂. To validate the effectiveness of these alternative methods, the test was firstly conducted with with and without Ebselen as a reference catalyst, because of its well established GPx-like activity [266].

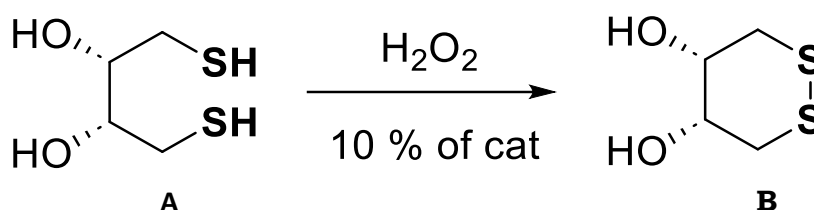
This was followed by the application of these revised versions of the Iwaoka's assay for the characterization of the GPx-like effects of known and novel natural and semisynthetic compounds.

3.1.1 Sample preparation procedures

The assay was performed with a very similar protocol to that reported by Iwaoka and his Coworkers, but with a few differences in the sample preparation [263]. All the assay solutions were prepared in a 4 mL dark vial by adding 0.103 mmol of DTT^{red} (16 mg), 0.103 mmol of H₂O₂ 30% (12 μ L) and with or without a 0.011 mmol of catalyst (in a 0.1 molar ratio to 1,4-dithiotreitol) all dissolved in 0.8 mL of an appropriate solvent, EtOAc for GC-

MS and MeOH for HPLC-DAD analysis. The reaction mixtures were sampled at fixed time (0 min, 30 min, 1 h, 2 h, 3 h, 4 h, and 24 h) and before each injection they were diluted 400-fold with the corresponding solvent. The involved reaction is reported in figure 45.

Figure 45. Oxidation of 1,4-dithiotreitol (**A**, DTT^{red}) to 4,5-dihydroxy-1,2-dithiane (**B**, DTT^{ox}) in the presence of H₂O₂.



The use of EtOAc as the reaction solvent for the GC-MS assay was necessary to obtain an optimum peak shape in the chromatograms.

Indeed, this choice did not compromise the rate of the reaction and other solvents such as MeOH, Et₂O or EtOH did not perform better than EtOAc. H₂O was excluded due to an excessive increase in the reaction rate that did not allow a correct quantification of the oxidation product.

3.1.2 HPLC-DAD and GC-MS methods validation

HPLC-DAD analysis

An Agilent 1100 (Santa Clara, CA, USA) series instrument equipped with an autosampler, a binary solvent pump, a diode array detector (DAD) and a fluorescence detector (FLD), was used to accomplish HPLC analysis. The qualitative and quantitative analysis were achieved employing a Kromasil RP C18 (4.6 mm Ø x 150 mm, 5 µm particle size) column. The column

temperature was 25 °C and the injection volume was 20 µL, while the wavelength set for the acquisition was at 209 nm. The mobile phase consisted of H₂O as solvent A and of CH₃CN as solvent B, following a gradient elution program as depicted in table 53 at a flow rate of 1.0 mL/min, for a total run time of 17 min.

Table 53. Gradient elution program for HPLC analysis.

Time (min)	A %	B %
0	95	5
3	95	5
5	76	24
15	73	27
17	95	5

It has been previously described how the mobile phase for the analysis of both 1,4-dithiothreitol and 4,5-dihydroxy-1,2-dithiane does not require any pH modification [267]. Indeed, when adding acid or bases buffers, the peak shape of the two analytes did not appear ideal.

By using H₂O and CH₃CN as solvent A and B, the retention times recorded for 1,4-dithiothreitol (DTT^{red}) and 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) were 7.09 ± 0.04 and 7.82 ± 0.03 min, respectively.

The HPLC-DAD method was validated according to ICH recommendations, and all the relevant analytical parameters are reported in table 54 [86].

The intra-day precision was determined by the injection of the 4,5-dihydroxy-1,2-dithiane standard mixture solution five times a day. For the inter-day precision, measurements were recorded once a day on three consecutive days. All these were expressed as relative standard deviations (RSDs) and they were considered acceptable when having a value of ≤ 2.0 %.

Precision was calculated at three concentration levels for quality control (QC) samples, namely $QC_{Low} = 0.5 \mu\text{g/mL}$, $QC_{Medium} = 25.0 \mu\text{g/mL}$, and $QC_{High} = 100.0 \mu\text{g/mL}$. Accuracy was calculated by spiking samples deriving from the assay reaction with Ebselen with three concentrations of the standard compound (low, medium, and high spikes) in three replicates. LODs and LOQs were determined by injecting serial dilutions of the same solutions of the calibration standards, having a signal-to-noise (S/N) ratio of 3 and 10 as the reference, respectively.

The calibration curve, drawn on eight concentrations values and plotted using weighted ($1/x^2$) linear least-squares regression analyses, was linear over the applied concentrations range, providing r^2 values of 0.9996.

The stability of both 1,4 dithiothreitol and 4,5-dihydroxy-1,2- dithiane was determined under different storage conditions using the QC samples, analysing them immediately after preparation and after the applied storage conditions. By comparing them to the nominal concentration, stock solutions were stable at room temperature for 48 h and at 4 °C for 9 days. No degradations were recorded after three cycles of freezing and thawing.

The carry over effect ($<0.14\%$), was not observed [268].

Table 54. HPLC and GC method validation parameters.

	Analytical method			
	HPLC		GC	
Slope	64,932		52,232	
Intercept	-1807		-2970	
r ²	0.9996		0.9992	
SD _s (slope)	3156		804	
SD _i (intercept)	88		112	
LOD (µg/mL)	0.16		0.05	
[3.3 X SD _s /S]				
LOQ (µg/mL)	0.48		0.15	
[10 X SD _s /S]				
Precision				
Intra-day	% RSD (n = 3)		% RSD (n = 3)	
QC _{low}	100.2 / 0.989		100.1 / 0.992	
QC _{medium}	99.8 / 0.993		100.1 / 0.997	
QC _{high}	100.3 / 1.012		99.9 / 1.035	
Inter-day	% RSD (n = 3)		% RSD (n = 3)	
QC _{low}	99.7 / 1.023		100.4 / 0.975	
QC _{medium}	99.9 / 0.997		100.1 / 1.004	
QC _{high}	100.2 / 1.003		99.6 / 0.988	
Accuracy				
Intra-day	% Recovery (n = 3)		% Recovery (n = 3)	
QC _{low}	102.3 / 1.002		102.4 / 0.898	
QC _{medium}	101.4 / 0.996		104.6 / 1.372	
QC _{high}	107.6 / 0.932		107.6 / 0.895	
Inter-day	% Recovery (n = 3)		% Recovery (n = 3)	
QC _{low}	101.8 / 1.012		101.9 / 0.922	
QC _{medium}	103.6 / 0.902		103.8 / 1.322	
QC _{high}	106.5 / 0.914		106.4 / 0.925	
	DTT ^{red}	DTT ^{ox}	DTT ^{red}	DTT ^{ox}
Capacity factor	1.2 ± 0.01	1.6 ± 0.01	3.2 ± 0.02	3.7 ± 0.03
Resolution	1.4 ± 0.01	1.2 ± 0.01	0.8 ± 0.003	0.9 ± 0.005
Peak symmetry	0.9 ± 0.01	1.0 ± 0.02	1.0 ± 0.01	1.0 ± 0.01
Tailing	1.1 ± 0.03	1.0 ± 0.01	1.0 ± 0.02	1.0 ± 0.01
Theoretical plate numbers	7693 ± 75	8122 ± 132	8544 ± 92	9112 ± 112

GC-MS analysis

GC-MS analyses were carried out using an Agilent 8860 GC apparatus equipped with a 5977B GC/MSD Agilent detector. Matrices components

were separated on a J&W DB-5 ms Ultra Inert GC Column 30 m length $\times$ 0.25 mm i.d. capillary column coated with a 1 μ m film thickness stationary phase (Agilent Technologies). The sample volume of 0.2 μ L was injected using an autoinjector. The injection port was set at 250 $^{\circ}$ C, with a constant flow (1.2 mL/min) of He as the carrier (pressure 34 kPa), in a pulsed splitless mode. The initial temperature was set at 80 $^{\circ}$ C with a 3 min hold, then increased by 10 $^{\circ}$ C/min until 300 $^{\circ}$ C (with 5 min hold). Solvent delay was set at 6 min while run time was 30 min. The ion source temperature in the MS was set at 230 $^{\circ}$ C and the interface at 280 $^{\circ}$ C. Total Ion Chromatogram (TIC) was created for the m/z range 40–450.

GC peaks were identified by comparing their mass spectra to those of the database of the National Institute of Standards and Technology (NIST) NIST11 Mass Spectral Library (2011/EPA/NIH). The retention times recorded for 1,4-dithiothreitol and 4,5-dihydroxy-1,2-dithiane were 13.31 ± 0.03 and 14.82 ± 0.02 min respectively. The method was validated according to the provision of the ICH international guidelines in terms of system suitability and precision, linearity, limit of detection (LOD), limit of quantification (LOQ), accuracy, and robustness [269] (table 54). For system suitability and precision, parameters were calculated accomplishing 3 replicate injections of 4,5-dihydroxy-1,2-dithiane and in terms of intra-day precision and inter-day one, this latter once a day for three consecutive days. The relative standard deviation (RSD) of these latter was considered as acceptable when having a value $\leq 2.0\%$. The calibration curve, drawn on eight concentrations values and plotted using weighted ($1/x^2$) linear least-squares regression analyses, was linear over the applied concentrations

range, providing r^2 values of 0.9992. LODs and LOQs were calculated based on the standard deviation (SD) of the slope of the regression line. The accuracy was calculated by spiking samples deriving from the assay reaction with Ebselen with three concentrations of the standard compound (low, medium, and high spikes) in three replicates. Finally, robustness of the set-up method was evaluated investigating the influence of multiple variations: differences of ± 5 kPa of the pressure of the carrier gas He, ± 10 °C of the original oven temperature and $\pm 20\%$ of the split ratio. For each set of variations three replicates of the 4,5-dihydroxy-1, 2-dithiane standard solution were used (table 55).

Table 55. Effects of GC experimental conditions changes on system suitability.

Variations			
Pressure (kPa)	Initial T (° C)	Split flow (mL/min)	DTT^{ox} (µg/mL $\pm$ SD)*
34	80	1.2	7.08 $\pm$ 0.04
34	90	1.2	7.05 $\pm$ 0.02
34	70	1.2	7.06 $\pm$ 0.03
39	80	1.2	7.08 $\pm$ 0.01
29	80	1.2	7.04 $\pm$ 0.03
34	80	0.96	7.01 $\pm$ 0.02
34	80	1.44	7.04 $\pm$ 0.01

*Data obtained for the reaction carried out in the presence of Ebselen after 30 min.

These results clearly indicate how the analytical method can be defined as precise, accurate, free of systematic errors and robust.

3.1.3 GPx-like activity quantification

Once the two new hyphenated methodologies were successfully developed, the rate of the reaction, and thus the GPx-like effects of ebselen, was observed by quantifying the formation of DTT^{ox} and comparing it to that of

the reaction without any catalyst. The reaction mixtures were prepared, sampled and analysed at fixed times (0 min – 30 min – 1 h – 2 h – 3 h – 4 h – 24 h) in GC-MS and HPLC-DAD. The original protocol with $^1\text{H-NMR}$ spectroscopy was also performed for comparison [263].

The quantification results for DTT^{ox} are reported in table 56 for each of the three methodologies.

Table 56. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications ($\mu\text{g/mL}$)* by GC-MS, HPLC-DAD and $^1\text{H-NMR}$.

Time (h)		GC-MS	HPLC-DAD	$^1\text{H-NMR}$
0	+ Ebselen	ND	ND	ND
	No catalyst	ND	ND	ND
0.5	+ Ebselen	7.08 ± 0.04	7.02 ± 0.06	6.86 ± 0.04
	No catalyst	2.52 ± 0.06	2.44 ± 0.12	2.22 ± 0.10
1	+ Ebselen	12.26 ± 0.06	12.14 ± 0.08	11.88 ± 0.04
	No catalyst	6.08 ± 0.01	6.00 ± 0.05	5.88 ± 0.06
2	+ Ebselen	16.08 ± 0.07	15.96 ± 0.12	15.46 ± 0.10
	No catalyst	8.28 ± 0.02	8.16 ± 0.06	7.86 ± 0.04
3	+ Ebselen	18.04 ± 0.02	17.92 ± 0.04	17.44 ± 0.06
	No catalyst	9.04 ± 0.03	8.94 ± 0.04	8.48 ± 0.02
4	+ Ebselen	19.92 ± 0.11	19.78 ± 0.06	19.32 ± 0.08
	No catalyst	9.82 ± 0.06	9.62 ± 0.12	9.20 ± 0.06
24	+ Ebselen	19.98 ± 0.06	19.96 ± 0.01	19.36 ± 0.08
	No catalyst	10.28 ± 0.04	10.02 ± 0.06	9.46 ± 0.01

*Values expressed as mean $\pm$ SD (n = 3).

The single factor ANOVA analysis showed no noteworthy differences between the $\mu\text{g/mL}$ of 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) detected after each analysis with the three methodologies. For all of them, the reaction came to completion between 3 and 4 h when Ebselen was used, also shown by the GC-MS and HPLC-DAD chromatograms reported in figure 46 and 47 respectively. This further highlighting and confirmed Ebselen GPx-like mimicking effect.

Figure 46. Representative GC-MS chromatograms of the Iwaoka's assay with and without Ebsen as a catalyst.

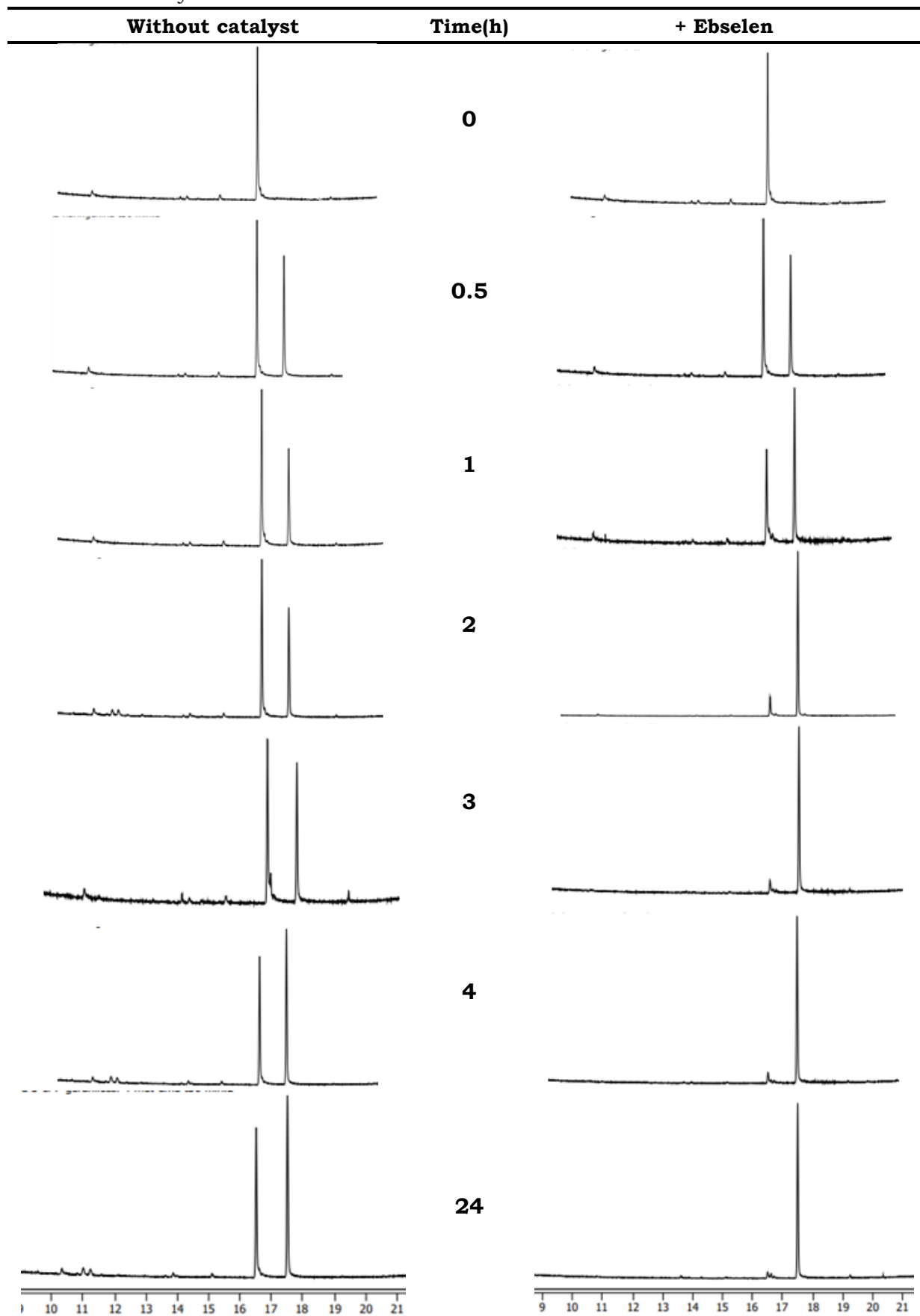
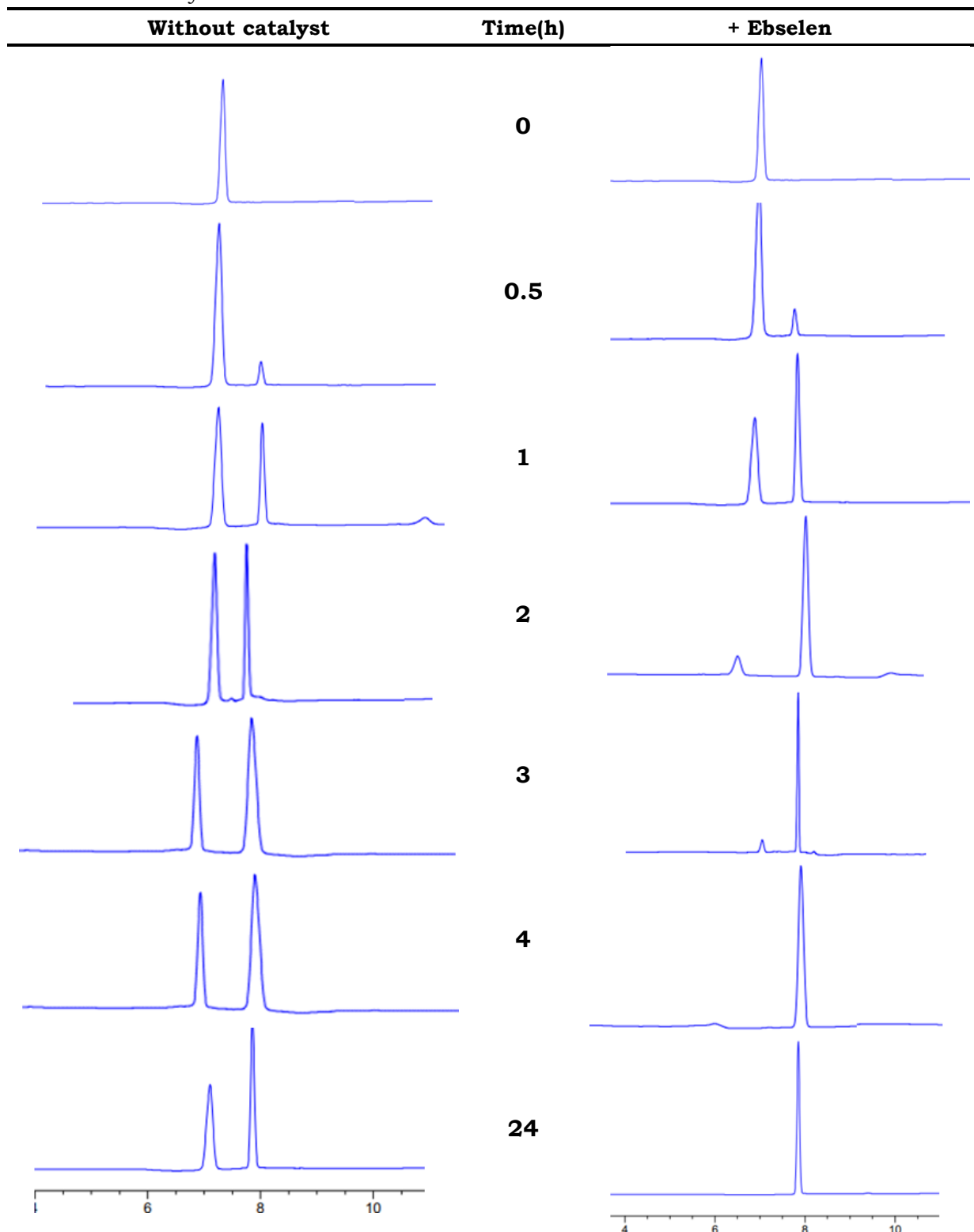


Figure 47. Representative GC-MS chromatograms of the Iwaoka's assay with and without Ebsen as a catalyst.



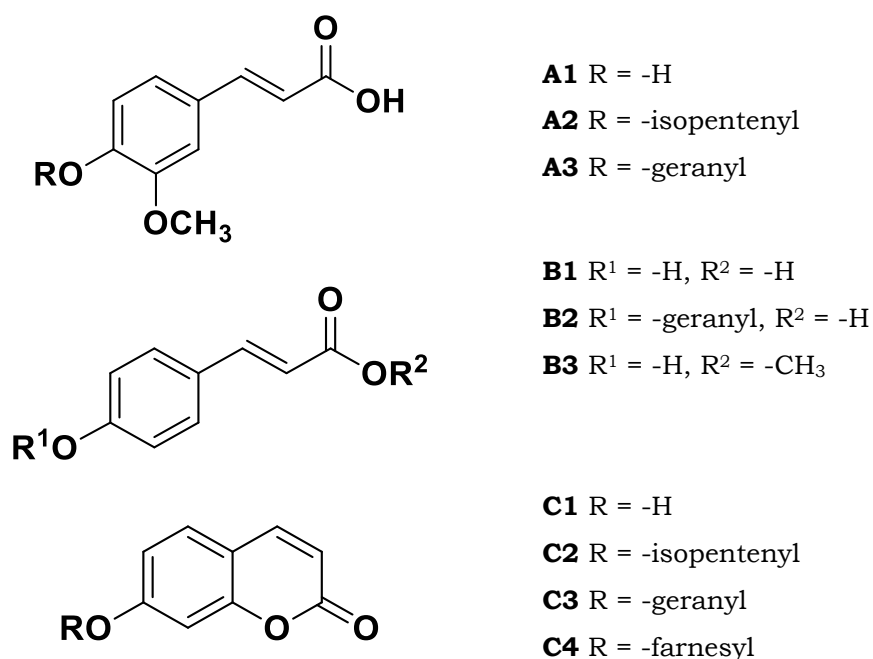
Once the two hyphenated methodologies were successfully validated, the next step of the project consisted in the application of the revised version of the Iwaoka's assay to screen the GPx-like effects of natural and semisynthetic compounds.

3.1.4 Application of the revised version of Iwaoka's assay: Evaluation of phenylpropanoids and oxyprenylated derivatives GPx-like effects

Phenylpropanoids are important secondary metabolites having a C6-C3 carbon backbone pattern deriving from the shikimate pathway. They are ubiquitous in plants, and they are well known for their numerous biological properties, including a strong antioxidant capacity [270]. To see if part of this effect could be ascribed to a GPx-like mimicking effect, three naturally occurring phenylpropanoids, ferulic acid (**A1**), *p*-coumaric acid (**B1**) and umbelliferone (**C1**) and some of their semisynthetic derivatives were tested (figure 48).

The test solutions were prepared as previously mentioned and then analysed at fixed times following both the GC-MS and HPLC-DAD analytical approach.

Figure 48. Chemical structure of ferulic acid (**A1**), boropinic acid (**A2**), 4'-geranyloxyferulic acid (GOFA) (**A3**), *p*-coumaric acid (**B1**), geranyloxy *p*-coumaric acid (**B2**), *p*-coumaric acid methyl ester (**B3**), umbelliferone (**C1**), 7-isopentenylxycoumarin (**C2**), auraptene (**C3**), umbelliprenin (**C4**).



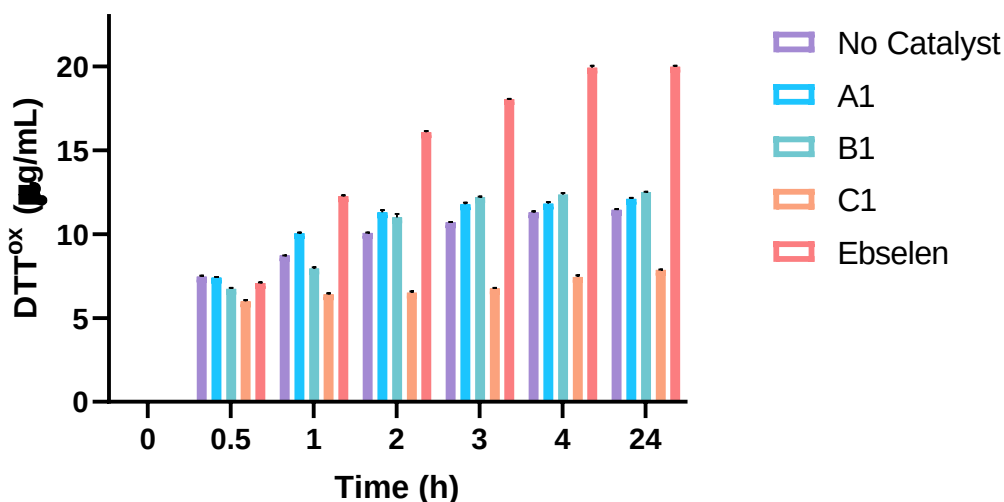
The results of the revised assays are reported in table 57, together with the quantification of DTT^{ox} obtained in the absence of a catalyst and with ebselen, as reference. To better visualize this data, a graph of the formation of the oxidated substrate is reported in figure 49, specifically in regards of the values obtained with the GC-MS analysis. As shown from the resulting data, compound **A1** and **B1** increased the formation of DTT^{ox} in contrast to the reaction without the catalyst, but still not comparable with the rate obtained in the presence of Ebselen. Between the two, compound **A1** seemed to catalyse the process in a slightly faster manner in the first hour, but at the end of the 24 h they both resulted in a similar outcome, signifying a not fundamental role of the methoxy moiety in position 3 of the phenyl ring.

Table 57. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (µg/mL)* with no catalyst and with ferulic acid (**A1**), *p*-coumaric acid (**B1**), umbelliferone (**C1**) and ebselen.

Time (h)		GC-MS	HPLC-DAD
0	+ Ebselen	ND	ND
	A1	ND	ND
	B1	ND	ND
	C1	ND	ND
	No catalyst	ND	ND
0.5	+ Ebselen	7.08 ± 0.04	7.02 ± 0.06
	A1	7.40 ± 0.05	7.34 ± 0.23
	B1	6.73 ± 0.07	6.87 ± 0.45
	C1	5.98 ± 0.09	6.05 ± 0.29
	No catalyst	2.52 ± 0.06	2.44 ± 0.12
1	+ Ebselen	12.26 ± 0.06	12.14 ± 0.08
	A1	10.05 ± 0.04	9.46 ± 0.42
	B1	7.95 ± 0.08	8.02 ± 0.32
	C1	6.41 ± 0.05	6.23 ± 0.14
	No catalyst	6.08 ± 0.01	6.00 ± 0.05
2	+ Ebselen	16.08 ± 0.07	15.96 ± 0.12
	A1	11.30 ± 0.13	11.74 ± 0.32
	B1	11.00 ± 0.21	11.13 ± 0.16
	C1	6.51 ± 0.07	6.48 ± 0.13
	No catalyst	8.28 ± 0.02	8.16 ± 0.06
3	+ Ebselen	18.04 ± 0.02	17.92 ± 0.04
	A1	11.78 ± 0.10	12.01 ± 0.43
	B1	12.19 ± 0.05	12.23 ± 0.52
	C1	6.75 ± 0.05	6.82 ± 0.18
	No catalyst	9.04 ± 0.03	8.94 ± 0.04
4	+ Ebselen	19.92 ± 0.11	19.78 ± 0.06
	A1	11.82 ± 0.09	12.12 ± 0.15
	B1	12.36 ± 0.08	12.27 ± 0.19
	C1	7.45 ± 0.10	7.39 ± 0.23
	No catalyst	9.82 ± 0.06	9.62 ± 0.12
24	+ Ebselen	19.98 ± 0.06	19.96 ± 0.01
	A1	12.11 ± 0.05	12.30 ± 0.43
	B1	12.50 ± 0.02	12.44 ± 0.32
	C1	7.86 ± 0.03	7.96 ± 0.54
	No catalyst	10.28 ± 0.04	10.02 ± 0.06

*Values expressed as mean ± SD (n = 3).

Figure 49. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (μg/mL)* with no catalyst and with ferulic acid (**A1**), *p*-coumaric acid (**B1**), umbelliferone (**C1**) and ebselen.



On the other hand, the closed coumarin structure of compound **C1** did not lead to an improvement of the mimicking effect, not even in comparison to the reaction without a catalyst. Additionally, it is worth mentioning that, even with the introduction of different catalyst, the results obtained with the two hyphenated techniques are still completely analogous.

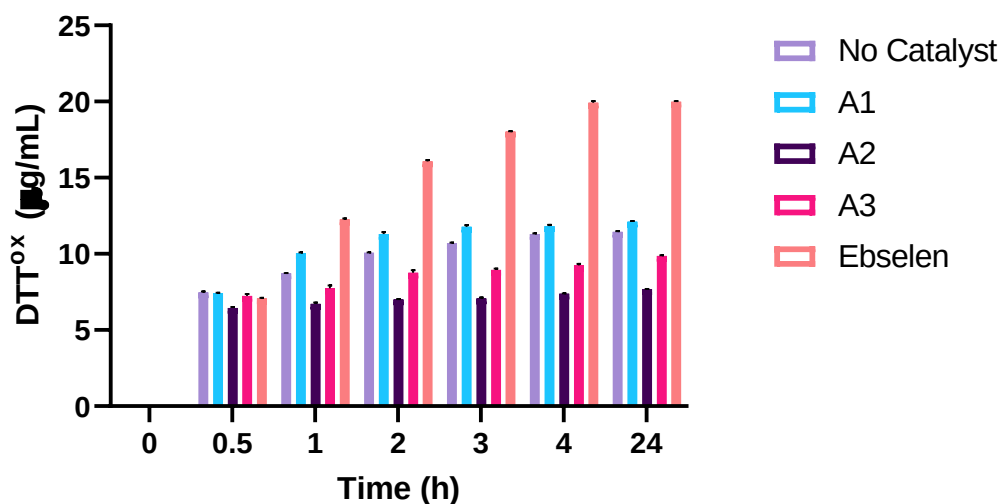
To further understand the structure-activity relationship of each of these compounds, different derivatives, both naturally occurring and semisynthetic, were tested in the same manner. The first set of molecules considered were in relation to **A1**, specifically boropinic acid (**A2**) and 4'-geranyloxyferulic acid (GOFA) (**A3**) (figure 48). The results are reported in 58 and a graphical summary regarding the GC-MS analysis is reported in figure 50.

Table 58. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (µg/mL)* with no catalyst and with ferulic acid (**A1**), boropinic acid (**A2**), 4'-geranyloxyferulic acid (GOFA) (**A3**) and ebselen.

Time (h)		GC-MS	HPLC-DAD
0	+ Ebselen	ND	ND
	A1	ND	ND
	A2	ND	ND
	A3	ND	ND
	No catalyst	ND	ND
0.5	+ Ebselen	7.08 ± 0.04	7.02 ± 0.06
	A1	7.40 ± 0.05	7.34 ± 0.23
	A2	6.45 ± 0.05	6.56 ± 0.25
	A3	7.24 ± 0.13	7.17 ± 0.43
	No catalyst	2.52 ± 0.06	2.44 ± 0.12
1	+ Ebselen	12.26 ± 0.06	12.14 ± 0.08
	A1	10.05 ± 0.04	9.46 ± 0.42
	A2	6.72 ± 0.08	6.87 ± 0.23
	A3	7.74 ± 0.19	7.65 ± 0.21
	No catalyst	6.08 ± 0.01	6.00 ± 0.05
2	+ Ebselen	16.08 ± 0.07	15.96 ± 0.12
	A1	11.30 ± 0.13	11.74 ± 0.32
	A2	7.01 ± 0.03	6.98 ± 0.46
	A3	8.76 ± 0.16	8.84 ± 0.34
	No catalyst	8.28 ± 0.02	8.16 ± 0.06
3	+ Ebselen	18.04 ± 0.02	17.92 ± 0.04
	A1	11.78 ± 0.10	12.01 ± 0.43
	A2	7.08 ± 0.05	7.02 ± 0.54
	A3	8.95 ± 0.09	8.91 ± 0.32
	No catalyst	9.04 ± 0.03	8.94 ± 0.04
4	+ Ebselen	19.92 ± 0.11	19.78 ± 0.06
	A1	11.82 ± 0.09	12.12 ± 0.15
	A2	7.39 ± 0.10	7.43 ± 0.54
	A3	9.24 ± 0.10	9.17 ± 0.32
	No catalyst	9.82 ± 0.06	9.62 ± 0.12
24	+ Ebselen	19.98 ± 0.06	19.96 ± 0.01
	A1	12.11 ± 0.05	12.30 ± 0.43
	A2	7.67 ± 0.02	7.73 ± 0.23
	A3	9.86 ± 0.05	9.93 ± 0.14
	No catalyst	10.28 ± 0.04	10.02 ± 0.06

*Values expressed as mean ± SD (n = 3).

Figure 50. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (μg/mL)* with no catalyst and with **A1**, **A2**, **A3** and ebselen.



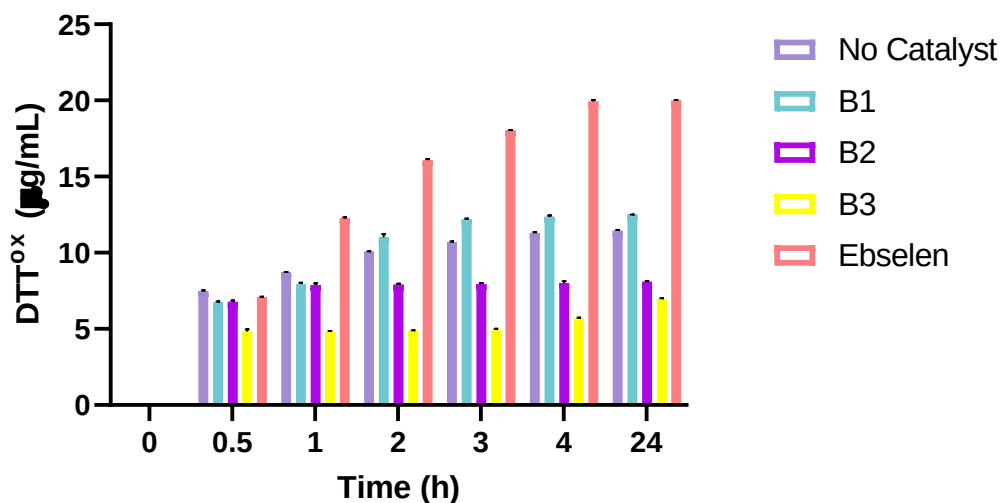
Although the oxyprenylated molecule with the geranylic chain seemed to act as a better catalyst, neither **A2** nor **A3**, performed better than **A1**. Thus, the prenylation appeared to cause a reduction of the GPx-like activity. This was further confirmed by performing the assay on additional oxyprenylated compounds of **B1** and **C1**, namely, geranyloxy *p*-coumaric acid (**B2**), *p*-coumaric acid methyl ester (**B3**), 7-isopentenylloxycoumarin (**C2**), auraptene (**C3**) and umbelliprenin (**C4**) (figure 48). The results of these analysis are reported in table 59-60, while the GC-MS data are plotted in figure 51-52.

Table 59. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (µg/mL)* with no catalyst and with *p*-coumaric acid (**B1**), geranyloxy *p*-coumaric acid (**B2**), *p*-coumaric acid methyl ester (**B3**) and ebselen.

Time (h)		GC-MS	HPLC-DAD
0	+ Ebselen	ND	ND
	B1	ND	ND
	B2	ND	ND
	B3	ND	ND
	No catalyst	ND	ND
0.5	+ Ebselen	7.08 ± 0.04	7.02 ± 0.06
	B1	6.73 ± 0.07	6.87 ± 0.45
	B2	6.78 ± 0.09	6.70 ± 0.19
	B3	4.82 ± 0.15	4.90 ± 0.23
	No catalyst	2.52 ± 0.06	2.44 ± 0.12
1	+ Ebselen	12.26 ± 0.06	12.14 ± 0.08
	B1	10.05 ± 0.04	9.46 ± 0.42
	B2	7.88 ± 0.10	7.83 ± 0.14
	B3	4.83 ± 0.03	4.91 ± 0.08
	No catalyst	6.08 ± 0.01	6.00 ± 0.05
2	+ Ebselen	16.08 ± 0.07	15.96 ± 0.12
	B1	11.00 ± 0.21	11.13 ± 0.16
	B2	7.90 ± 0.07	7.88 ± 0.16
	B3	4.88 ± 0.02	4.94 ± 0.32
	No catalyst	8.28 ± 0.02	8.16 ± 0.06
3	+ Ebselen	18.04 ± 0.02	17.92 ± 0.04
	B1	12.19 ± 0.05	12.23 ± 0.52
	B2	7.94 ± 0.07	7.91 ± 0.44
	B3	4.92 ± 0.09	4.96 ± 0.12
	No catalyst	9.04 ± 0.03	8.94 ± 0.04
4	+ Ebselen	19.92 ± 0.11	19.78 ± 0.06
	B1	12.36 ± 0.08	12.27 ± 0.19
	B2	8.02 ± 0.11	7.99 ± 0.25
	B3	5.67 ± 0.07	5.63 ± 0.22
	No catalyst	9.82 ± 0.06	9.62 ± 0.12
24	+ Ebselen	19.98 ± 0.06	19.96 ± 0.01
	B1	12.50 ± 0.02	12.44 ± 0.32
	B2	8.10 ± 0.04	8.05 ± 0.54
	B3	6.97 ± 0.03	7.01 ± 0.15
	No catalyst	10.28 ± 0.04	10.02 ± 0.06

*Values expressed as mean ± SD (n = 3).

Figure 51. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (μg/mL)* with no catalyst and with *p*-coumaric acid (**B1**), geranyloxy *p*-coumaric acid (**B2**), *p*-coumaric acid methyl ester (**B3**) and ebselen.



In the case of **B1** derivatives, the geranylation process observed with compound **B2** did not lead to any improvement of the activity, instead appearing even lower than the reaction monitored without a catalyst. The same can be said for **B3**, where the esterification of the carboxylic moiety lowered even more the GPx-mimicking effect. This seemed to suggest a significant role of the carboxylic acid for the catalysation process.

Table 60. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (μg/mL)* with no catalyst and with umbelliferone (**C1**), 7-isopentenylxycoumarin (**C2**), auraptene (**C3**), umbelliprenin (**C4**) and ebselen.

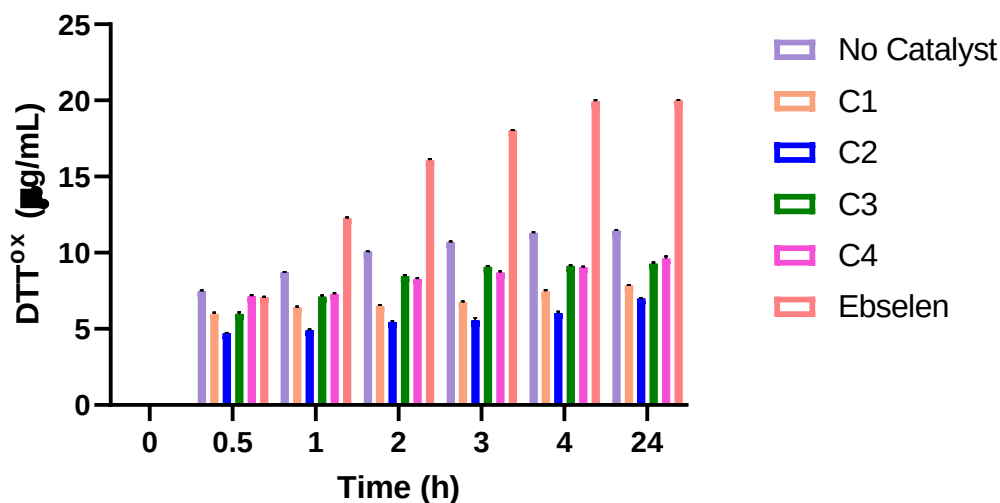
Time (h)		GC-MS	HPLC-DAD
0	+ Ebselen	ND	ND
	C1	ND	ND
	C2	ND	ND
	C3	ND	ND
	C4	ND	ND
	No catalyst	ND	ND

Table 60 (continued). 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (µg/mL)* with no catalyst and with umbelliferone (**C1**), 7-isopentenylloxycoumarin (**C2**), auraptene (**C3**), umbelliprenin (**C4**) and ebselen.

Time (h)		GC-MS	HPLC-DAD
0.5	+ Ebselen	7.08 ± 0.04	7.02 ± 0.06
	C1	5.98 ± 0.09	6.05 ± 0.29
	C2	4.70 ± 0.04	4.68 ± 0.14
	C3	5.98 ± 0.12	6.02 ± 0.13
	C4	7.13 ± 0.08	7.09 ± 0.15
	No catalyst	2.52 ± 0.06	2.44 ± 0.12
1	+ Ebselen	12.26 ± 0.06	12.14 ± 0.08
	C1	6.41 ± 0.05	6.23 ± 0.14
	C2	4.90 ± 0.07	4.87 ± 0.41
	C3	7.12 ± 0.08	7.08 ± 0.17
	C4	7.28 ± 0.06	7.30 ± 0.16
	No catalyst	6.08 ± 0.01	6.00 ± 0.05
2	+ Ebselen	16.08 ± 0.07	15.96 ± 0.12
	C1	6.51 ± 0.07	6.48 ± 0.13
	C2	5.44 ± 0.06	5.38 ± 0.19
	C3	8.46 ± 0.07	8.39 ± 0.33
	C4	8.25 ± 0.10	8.29 ± 0.20
	No catalyst	8.28 ± 0.02	8.16 ± 0.06
3	+ Ebselen	18.04 ± 0.02	17.92 ± 0.04
	C1	6.75 ± 0.05	6.82 ± 0.18
	C2	5.55 ± 0.16	5.61 ± 0.25
	C3	9.08 ± 0.06	8.99 ± 0.13
	C4	8.69 ± 0.09	8.70 ± 0.18
	No catalyst	9.04 ± 0.03	8.94 ± 0.04
4	+ Ebselen	19.92 ± 0.11	19.78 ± 0.06
	C1	7.45 ± 0.10	7.39 ± 0.23
	C2	6.03 ± 0.10	6.09 ± 0.17
	C3	9.13 ± 0.05	9.08 ± 0.36
	C4	9.05 ± 0.04	9.07 ± 0.16
	No catalyst	9.82 ± 0.06	9.62 ± 0.12
24	+ Ebselen	19.98 ± 0.06	19.96 ± 0.01
	C1	7.86 ± 0.03	7.96 ± 0.54
	C2	6.98 ± 0.05	7.05 ± 0.14
	C3	9.29 ± 0.08	9.23 ± 0.21
	C4	9.62 ± 0.13	9.70 ± 0.26
	No catalyst	10.28 ± 0.04	10.02 ± 0.06

*Values expressed as mean ± SD (n = 3).

Figure 52. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (μg/mL)* with no catalyst and with umbelliferone (**C1**), 7-isopentenylloxycoumarin (**C2**), auraptene (**C3**), umbelliprenin (**C4**) and ebselen.



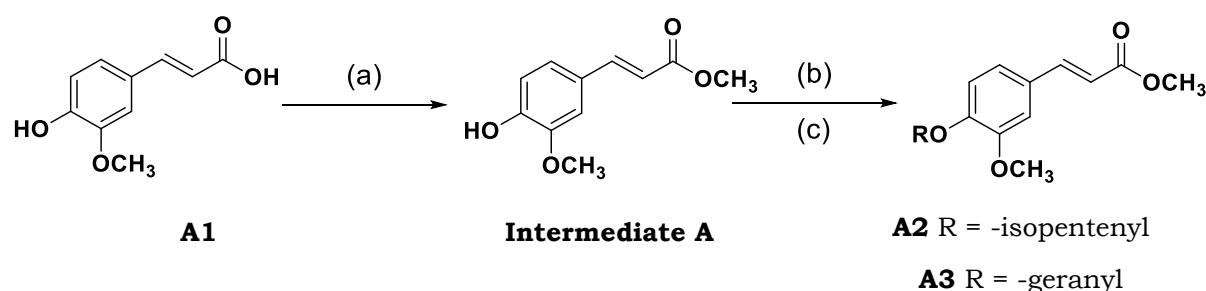
Even for **C1** derivatives, the oxyprenylated catalysts did not show any activity improvement.

In conclusion, all the compounds, even the less active, increased the velocity of the reaction in the first half hour, but after 24 h the effect remained similar to that in the absence of a catalyst. The two most active molecules were **A1** and **B1**, while a closed ring structure lowered the activity. This could be attributed to a better mimicking effect of the di-substituted *p*-phenyl ring. More studies are being made by testing compound with a different substitution pattern to confirm this theory. In addition, the hydroxy group in position 4 seemed fundamental for the activity. Indeed, both **A1** and **B1** could be considered weak GPx-mimicking agents.

3.1.5 Synthetic scheme of the oxyprenylated phenylpropanoids

The synthesis of compound **A2** (boropinic acid) and **A3** (4'-geranyloxyferulic acid, GOFA) was accomplished from commercially available ferulic acid (**A1**) following a previously validated route [271]. Ferulic acid (**A1**) was converted into the corresponding methyl ester by treatment with a solution of concentrated H₂SO₄ in refluxing MeOH for 24 h. This was followed by an acid-base workup to purify the reaction intermediate. Then the alkylation was achieved with the corresponding alkyl bromide, 3,3-dimethylallyl bromide for **A2** and geranyl bromide for **A3**, in acetone at 80 °C for 2 h and in the presence of dry K₂CO₃ as the base. Finally, a basic hydrolysis of the methyl ester was performed for 3 h at the same temperature with NaOH 2N. The purification of the two final products required an acid-base work up followed by a crystallization in *n*-hexane. A representation of the synthesis of compounds **A2** and **A3** is reported in scheme 1.

Scheme 1. Synthetic scheme of ferulic acid (**A1**) oxyprenylated derivatives boropinic acid (**A2**) and 4'-geranyloxyferulic acid (**A3**).

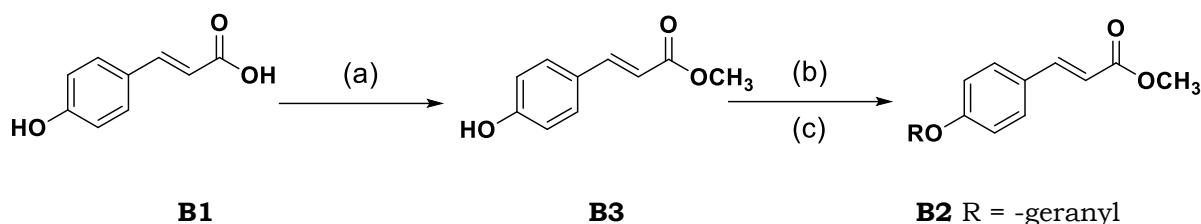


Reagents and conditions: a) MeOH, H₂SO₄ conc., reflux, 24 h; b) alkyl bromide, K₂CO₃, acetone, 80 °C, 2 h; c) basic hydrolysis, NaOH 2N, reflux, 3h.

The same exact procedure was performed for the synthesis of molecules **B2** and **B3**, this time starting from commercially available *p*-coumaric acid (**B1**).

A schematic representation of their synthesis is reported in scheme 2. In this case, the reaction intermediate **B3**, was also collected and tested in the revised version of the Iwaoka's assay.

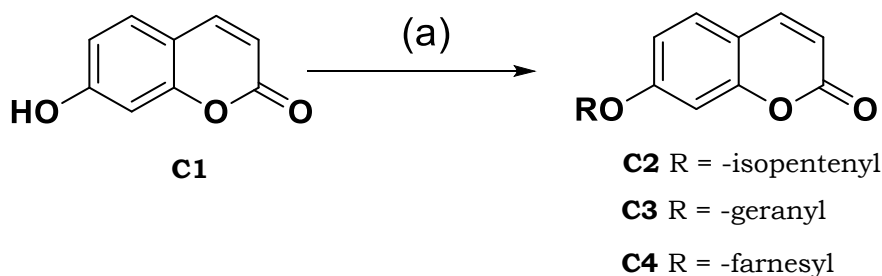
Scheme 2. Synthetic scheme of *p*-coumaric acid (**B1**) oxyprenylated derivatives geranyloxy *p*-coumaric acid (**B2**) and *p*-coumaric acid methyl ester (**B3**).



Reagents and conditions: a) MeOH, H₂SO₄ conc., reflux, 24 h; b) geranyl bromide, K₂CO₃, acetone, 80 °C, 2 h; c) basic hydrolysis, NaOH 2N, reflux, 3h.

Oxyprenylated coumarins have been similarly obtained from commercially available umbelliferone (**C1**) in a single step of a Williamson's reaction by alkylation with the respective alkyl bromide in acetone and in the presence of dry K₂CO₃ as the base at 80 °C for 2 h [272]. Specifically, the alkyl bromide selected were 3,3-dimethylallyl bromide for **C2**, geranyl bromide for **C3** and farnesyl bromide for **C4**. In scheme 3 a representation of the synthetic steps is shown.

Scheme 3. Synthetic scheme of umbelliferone (**C1**) oxyprenylated derivatives 7-isopentenylcoumarin (**C2**), auraptene (**C3**) and umbelliprenin (**C4**).

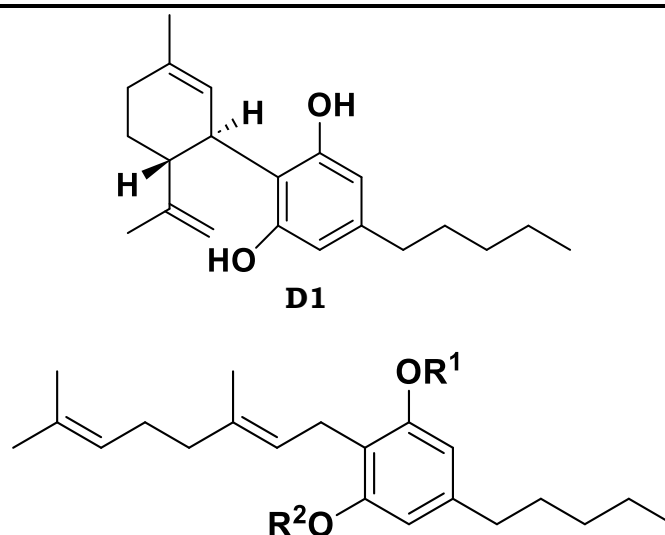


Reagents and conditions: a) alkyl bromide, K₂CO₃, acetone, 80 °C, 2 h

3.1.6 Application of the revised version of Iwaoka's assay: Evaluation of cannabinoids and semisynthetic derivatives GPx-like effects

Among the numerous secondary metabolites that can be isolated from *Cannabis sativa* L., non-psychoactive cannabinoids are the most well-known and studied thanks to their multiple beneficial impacts on human health [273]. In particular, cannabidiol (CBD, **D1**) and cannabigerol (CBG, **E1**) exert several biological activities, including a strong antioxidant effect (figure 53) [274-275]. As for the previous compounds, to see if part of their activity could be ascribed to a GPx-like effect, **D1** and **E1** were tested with the revised version of the Iwaoka's assay.

Figure 53. Chemical structure of cannabidiol (**D1**), cannabigerol (**E1**), 1-ethoxy-cannabigerol (**E2**), 1,3-diethoxy-cannabigerol (**E3**), 1-propoxy-cannabigerol (**E4**), 1,3-dipropoxy-cannabigerol (**E5**), 1-butoxy-cannabigerol (**E6**), 1,3-dibutoxy-cannabigerol (**E7**), 1-cannabigerol-dimethylacryloyl ester (**E8**), 1,3-cannabigerol-di-dimethylacryloyl ester (**E9**), cannabigerol-1-benzoate (**E10**), cannabigerol-1,3-dibenzoate (**E11**).



E1 R¹ = -H, R² = -H

E2 R¹ = -CH₂CH₃, R² = -H

E3 R¹ = -CH₂CH₃, R² = -CH₂CH₃

E4 R¹ = -CH₂CH₂CH₃, R² = -H

E5 R¹ = -CH₂CH₂CH₃, R² = -CH₂CH₂CH₃

E6 R¹ = -CH₂CH₂CH₂CH₃, R² = -H

E7 R¹ = -CH₂CH₂CH₂CH₃,

R² = -CH₂CH₂CH₂CH₃

E8 R¹ = -C=OCH=C(CH₃)₂, R² = -H

E9 R¹ = -C=OCH=C(CH₃)₂, R² = -C=OCH=C(CH₃)₂

E10 R¹ = -C=OPh, R² = -H

E11 R¹ = -C=OPh, R² = -C=OPh

The test solutions were prepared in the same way as mentioned before and then analysed at fixed times in both GC-MS and HPLC-DAD.

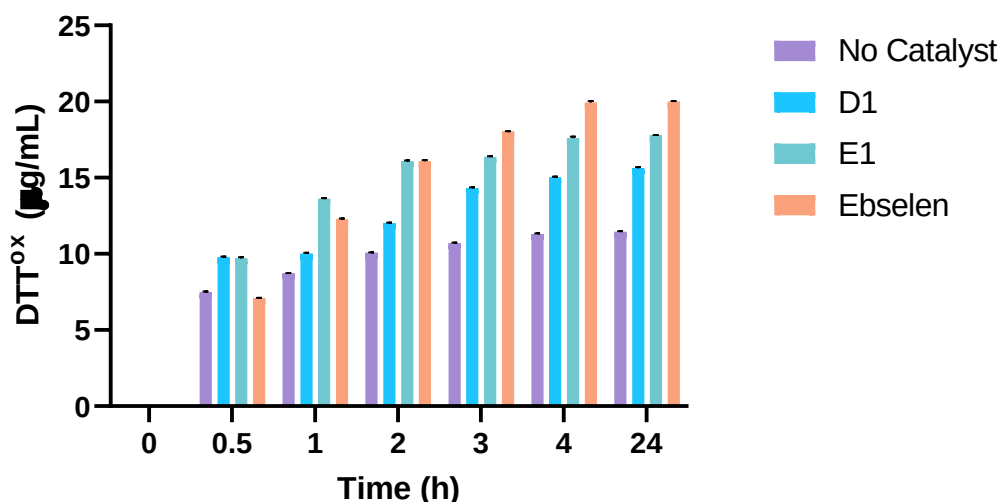
The results of the revised assays are reported in table 61, together with the quantification of DTT^{ox} obtained in the absence of a catalyst and with ebselen, for reference. The values obtained with the GC-MS analysis are plotted in the graph in figure 54.

Table 61. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (µg/mL)* with no catalyst and with cannabidiol (**D1**), cannabigerol (**E1**) and ebselen.

Time (h)		GC-MS	HPLC-DAD
0	+ Ebselen	ND	ND
	D1	ND	ND
	E1	ND	ND
	No catalyst	ND	ND
0.5	+ Ebselen	7.08 ± 0.04	7.02 ± 0.06
	D1	9.76 ± 0.07	9.80 ± 0.13
	E1	9.70 ± 0.08	9.67 ± 0.25
	No catalyst	2.52 ± 0.06	2.44 ± 0.12
1	+ Ebselen	12.26 ± 0.06	12.14 ± 0.08
	D1	10.02 ± 0.05	9.99 ± 0.24
	E1	13.62 ± 0.04	13.65 ± 0.42
	No catalyst	6.08 ± 0.01	6.00 ± 0.05
2	+ Ebselen	16.08 ± 0.07	15.96 ± 0.12
	D1	12.02 ± 0.03	11.98 ± 0.21
	E1	16.08 ± 0.06	16.10 ± 0.18
	No catalyst	8.28 ± 0.02	8.16 ± 0.06
3	+ Ebselen	18.04 ± 0.02	17.92 ± 0.04
	D1	14.32 ± 0.04	14.29 ± 0.31
	E1	16.37 ± 0.03	16.41 ± 0.25
	No catalyst	9.04 ± 0.03	8.94 ± 0.04
4	+ Ebselen	19.92 ± 0.11	19.78 ± 0.06
	D1	15.00 ± 0.08	14.97 ± 0.27
	E1	17.06 ± 0.09	17.11 ± 0.19
	No catalyst	9.82 ± 0.06	9.62 ± 0.12
24	+ Ebselen	19.98 ± 0.06	19.96 ± 0.01
	D1	15.62 ± 0.08	15.59 ± 0.33
	E1	17.78 ± 0.02	17.82 ± 0.22
	No catalyst	10.28 ± 0.04	10.02 ± 0.06

*Values expressed as mean ± SD (n = 3).

Figure 54. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (μg/mL)* with no catalyst and with cannabidiol (**D1**), cannabigerol (**E1**) and ebselen.



Compound **D1** and **E1** increased the formation of DTT^{ox}, and for the latter, in a manner almost comparable to the rate obtained in the presence of Ebselen as catalyst. Indeed, the reaction was boosted quicker by **E1** in the first hour, but then it did not reach completion at the 24 h, unlike Ebselen. Still, among all the natural compound tested, cannabigerol (**E1**) was the most active and thus can be considered a moderate GPx-like mimicking agent. This weaker activity could be advantageous in therapies where the collateral effects of Ebselen are too great. At the same time compound **D1** can be considered a weak moderate GPx-like mimicking agent, since it increased the rate of the reaction, but still not as much as Ebselen or **E1**. Utilising **E1** as lead compound, different derivatives were synthesized to test their structure-activity relationship (figure 53).

The first batch of compounds that were subjected to the revised version of the Iwaoka's assay were ether analogues of **E1**, namely 1-ethoxy-cannabigerol (**E2**), 1,3-diethoxy-cannabigerol (**E3**), 1-propoxy-cannabigerol

(**E4**), 1,3-dipropoxy-cannabigerol (**E5**), 1-butoxy-cannabigerol (**E6**) and 1,3-dibutoxy-cannabigerol (**E7**). The results of these analysis are reported in table 62, while the GC-MS data are plotted in figure 55. In both cases the reactions without a catalyst and in the presence of ebselen and **E1**.

Table 62. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (µg/mL)* with no catalyst and with cannabigerol (**E1**), 1-ethoxy-cannabigerol (**E2**), 1,3-diethoxy-cannabigerol (**E3**), 1-propoxy-cannabigerol (**E4**), 1,3-dipropoxy-cannabigerol (**E5**), 1-butoxy-cannabigerol (**E6**), 1,3-dibutoxy-cannabigerol (**E7**) and ebselen.

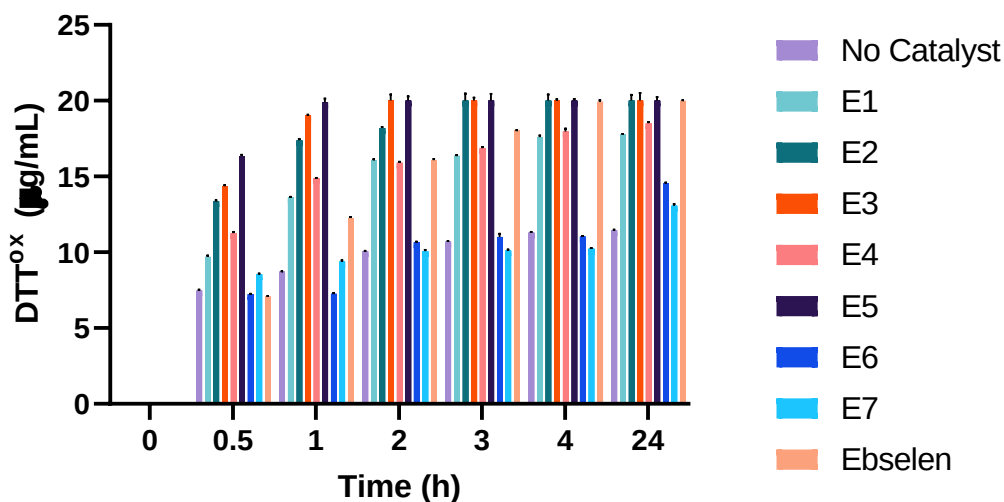
Time (h)		GC-MS	HPLC-DAD
0	+ Ebselen	ND	ND
	E1	ND	ND
	E2	ND	ND
	E3	ND	ND
	E4	ND	ND
	E5	ND	ND
	E6	ND	ND
	E7	ND	ND
	No catalyst	ND	ND
0.5	+ Ebselen	7.08 ± 0.04	7.02 ± 0.06
	E1	9.70 ± 0.08	9.67 ± 0.25
	E2	13.36 ± 0.06	13.68 ± 0.14
	E3	14.36 ± 0.07	14.40 ± 0.21
	E4	11.26 ± 0.08	11.23 ± 0.25
	E5	16.34 ± 0.08	16.40 ± 0.34
	E6	7.23 ± 0.04	7.19 ± 0.31
	E7	8.55 ± 0.04	8.62 ± 0.15
	No catalyst	2.52 ± 0.06	2.44 ± 0.12
1	+ Ebselen	12.26 ± 0.06	12.14 ± 0.08
	E1	13.62 ± 0.04	13.65 ± 0.42
	E2	17.38 ± 0.09	17.41 ± 0.31
	E3	19.04 ± 0.04	18.98 ± 0.27
	E4	14.88 ± 0.02	14.90 ± 0.26
	E5	19.88 ± 0.25	19.91 ± 0.32
	E6	7.25 ± 0.06	7.22 ± 0.24
	E7	9.38 ± 0.08	9.43 ± 0.16
	No catalyst	6.08 ± 0.01	6.00 ± 0.05

Table 62 (continued). 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (µg/mL)* with no catalyst and with cannabigerol (**E1**), 1-ethoxy-cannabigerol (**E2**), 1,3-diethoxy-cannabigerol (**E3**), 1-propoxy-cannabigerol (**E4**), 1,3-dipropoxy-cannabigerol (**E5**), 1-butoxy-cannabigerol (**E6**), 1,3-dibutoxy-cannabigerol (**E7**) and ebselen.

Time (h)		GC-MS	HPLC-DAD
2	+ Ebselen	16.08 ± 0.07	15.96 ± 0.12
	E1	16.08 ± 0.06	16.10 ± 0.18
	E2	18.20 ± 0.06	18.18 ± 0.21
	E3	19.99 ± 0.41	19.98 ± 0.31
	E4	15.90 ± 0.06	15.88 ± 0.24
	E5	19.99 ± 0.28	19.98 ± 0.22
	E6	10.65 ± 0.04	10.70 ± 0.43
	E7	10.08 ± 0.07	10.11 ± 0.21
	No catalyst	8.28 ± 0.02	8.16 ± 0.06
3	+ Ebselen	18.04 ± 0.02	17.92 ± 0.04
	E1	16.37 ± 0.03	16.41 ± 0.25
	E2	19.98 ± 0.47	19.99 ± 0.32
	E3	19.99 ± 0.19	19.98 ± 0.23
	E4	16.86 ± 0.08	16.89 ± 0.14
	E5	19.99 ± 0.44	19.99 ± 0.25
	E6	11.00 ± 0.22	10.97 ± 0.33
	E7	10.12 ± 0.05	10.15 ± 0.17
	No catalyst	9.04 ± 0.03	8.94 ± 0.04
4	+ Ebselen	19.92 ± 0.11	19.78 ± 0.06
	E1	17.06 ± 0.09	17.11 ± 0.19
	E2	19.98 ± 0.40	19.99 ± 0.22
	E3	19.99 ± 0.09	19.98 ± 0.25
	E4	17.96 ± 0.19	18.00 ± 0.25
	E5	19.99 ± 0.11	19.99 ± 0.35
	E6	11.03 ± 0.05	11.01 ± 0.23
	E7	10.23 ± 0.05	10.26 ± 0.32
	No catalyst	9.82 ± 0.06	9.62 ± 0.12
24	+ Ebselen	19.98 ± 0.06	19.96 ± 0.01
	E1	17.78 ± 0.02	17.82 ± 0.22
	E2	19.98 ± 0.38	19.99 ± 0.41
	E3	19.99 ± 0.42	19.98 ± 0.28
	E4	18.52 ± 0.07	18.61 ± 0.35
	E5	19.99 ± 0.23	19.99 ± 0.31
	E6	14.55 ± 0.05	14.61 ± 0.13
	E7	13.08 ± 0.09	13.11 ± 0.25
	No catalyst	10.28 ± 0.04	10.02 ± 0.06

*Values expressed as mean ± SD (n = 3).

Figure 55. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (μg/mL)* with no catalyst and with cannabigerol (**E1**), 1-ethoxy-cannabigerol (**E2**), 1,3-diethoxy-cannabigerol (**E3**), 1-propoxy-cannabigerol (**E4**), 1,3-dipropoxy-cannabigerol (**E5**), 1-butoxy-cannabigerol (**E6**), 1,3-dibutoxy-cannabigerol (**E7**) and ebselen.



When ether analogues of **E1** were added as a catalyst of the reaction, an interesting trend could be observed. Di-substituted compounds **E3** and **E5** were the most efficient in boosting the formation of DTT^{ox}, indeed the process came to completion between 1 and 2 h, even faster than in the case of ebselen. On the other hand, the cannabigerol mono-ether derivative **E2** showed a GPx-like activity quite similar to that of the original catalyst.

Considering how all the **E1** ether analogues improved the reaction rate, the di-substitution, and to a lesser extent, the mono-substitution, of the hydroxy groups in position 1 and 3 with small alkyl moieties (C2-C3) can be considered the cause of improvement of the GPx-mimicking activities. In fact, compounds **E6** and **E7**, having a slightly longer chain (C4), had a much lower response, like that obtained with the reference reaction.

The high GPx-mimicking effect of **E3** and **E5** may be linked to the hydroxyl substitutions with electron-donor groups (EDGs), having a stabilizing on the electron doublet on the oxygen atom adjacent to the π system. The increase of the electronic density in the aromatic ring quickens the H_2O_2 reduction, working as a better reducing agent than the original natural molecule **E1**. From here a mild oxidation is enough to cause the formation of the disulfide bond, and thus the conversion of DTT^{red} to DTT^{ox} . This effect is doubled in the case of di-alkoxy analogues.

On the other hand, an opposite behaviour should be witnessed when the substitution is performed with esters, which act as electron-withdrawing groups (EWGs). To confirm this theory the test was performed with a second batch of **E1** mono and di-esters derivatives, namely 1-cannabigerol-dimethylacryloyl ester (**E8**), 1,3-cannabigerol-di-dimethylacryloyl ester (**E9**), cannabigerol-1-benzoate (**E10**) and cannabigerol-1,3-dibenzoate (**E11**). The results of these experiments are reported in table 63, while the data of the GC-MS analysis is plotted on a graph in figure 56.

Table 63. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (µg/mL)* with no catalyst and with cannabigerol (**E1**), 1-cannabigerol-dimethylacryloyl ester (**E8**), 1,3-cannabigerol-dimethylacryloyl ester (**E9**), cannabigerol-1-benzoate (**E10**), cannabigerol-1,3-dibenzoate (**E11**) and ebselen.

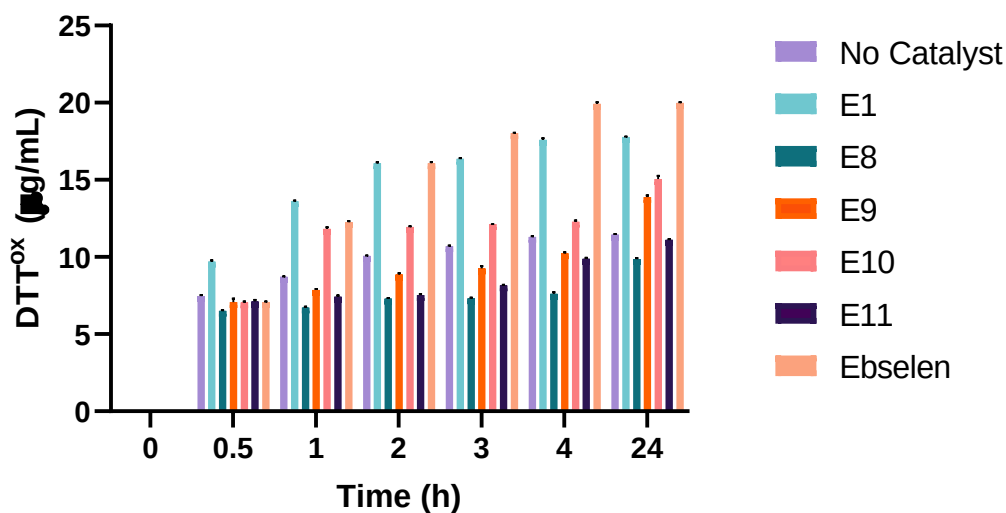
Time (h)		GC-MS	HPLC-DAD
0	+ Ebselen	ND	ND
	E1	ND	ND
	E8	ND	ND
	E9	ND	ND
	E10	ND	ND
	E11	ND	ND
	No catalyst	ND	ND
0.5	+ Ebselen	7.08 ± 0.04	7.02 ± 0.06
	E1	9.70 ± 0.08	9.67 ± 0.25
	E8	6.52 ± 0.04	6.58 ± 0.24
	E9	7.08 ± 0.22	7.04 ± 0.31
	E10	7.05 ± 0.06	7.06 ± 0.23
	E11	7.12 ± 0.10	7.09 ± 0.24
	No catalyst	2.52 ± 0.06	2.44 ± 0.12
1	+ Ebselen	12.26 ± 0.06	12.14 ± 0.08
	E1	13.62 ± 0.04	13.65 ± 0.42
	E8	6.72 ± 0.06	6.79 ± 0.11
	E9	7.86 ± 0.07	7.91 ± 0.17
	E10	11.83 ± 0.10	11.79 ± 0.22
	E11	7.43 ± 0.07	7.38 ± 0.13
	No catalyst	6.08 ± 0.01	6.00 ± 0.05
2	+ Ebselen	16.08 ± 0.07	15.96 ± 0.12
	E1	16.08 ± 0.06	16.10 ± 0.18
	E8	7.31 ± 0.04	7.37 ± 0.23
	E9	8.87 ± 0.08	8.92 ± 0.11
	E10	11.95 ± 0.03	11.90 ± 0.27
	E11	7.54 ± 0.06	7.51 ± 0.25
	No catalyst	8.28 ± 0.02	8.16 ± 0.06
3	+ Ebselen	18.04 ± 0.02	17.92 ± 0.04
	E1	16.37 ± 0.03	16.41 ± 0.25
	E8	7.33 ± 0.03	7.38 ± 0.33
	E9	9.26 ± 0.14	9.30 ± 0.22
	E10	12.13 ± 0.01	12.09 ± 0.24
	E11	8.16 ± 0.04	8.08 ± 0.15
	No catalyst	9.04 ± 0.03	8.94 ± 0.04

Table 63 (continued). 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (µg/mL)* with no catalyst and with cannabigerol (**E1**), 1-cannabigerol-dimethylacryloyl ester (**E8**), 1,3-cannabigerol-di-dimethylacryloyl ester (**E9**), cannabigerol-1-benzoate (**E10**), cannabigerol-1,3-dibenzoate (**E11**) and ebselen.

Time (h)		GC-MS	HPLC-DAD
4	+ Ebselen	19.92 ± 0.11	19.78 ± 0.06
	E1	17.06 ± 0.09	17.11 ± 0.19
	E8	7.62 ± 0.09	7.69 ± 0.42
	E9	10.23 ± 0.07	10.18 ± 0.35
	E10	12.29 ± 0.07	12.33 ± 0.25
	E11	9.89 ± 0.05	9.94 ± 0.31
	No catalyst	9.82 ± 0.06	9.62 ± 0.12
24	+ Ebselen	19.98 ± 0.06	19.96 ± 0.01
	E1	17.78 ± 0.02	17.82 ± 0.22
	E8	9.87 ± 0.05	9.91 ± 0.18
	E9	13.89 ± 0.10	13.86 ± 0.27
	E10	15.06 ± 0.20	14.99 ± 0.28
	E11	11.12 ± 0.03	11.17 ± 0.11
	No catalyst	10.28 ± 0.04	10.02 ± 0.06

*Values expressed as mean ± SD (n = 3).

Figure 56. 4,5-dihydroxy-1,2-dithiane (DTT^{ox}) quantifications (µg/mL)* with no catalyst and with cannabigerol (**E1**), 1-cannabigerol-dimethylacryloyl ester (**E8**), 1,3-cannabigerol-di-dimethylacryloyl ester (**E9**), cannabigerol-1-benzoate (**E10**), cannabigerol-1,3-dibenzoate (**E11**) and ebselen.



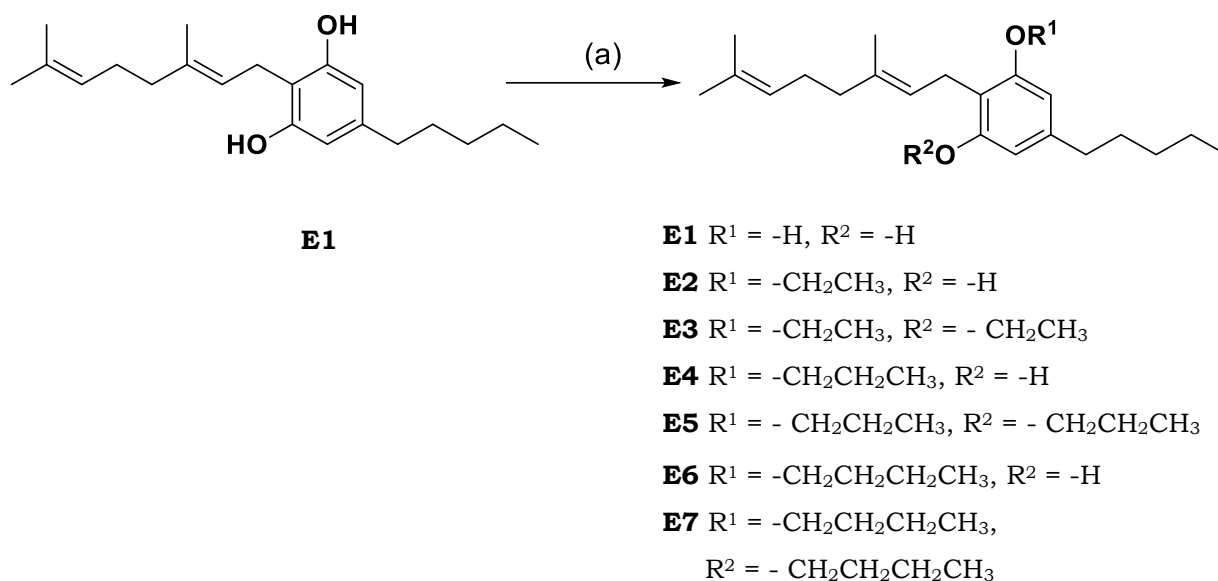
Indeed, these results showed how the ester derivatives were less efficient as catalyst, even less than the lead compound cannabigerol (**E1**).

In conclusion, the two most active molecules were **E3** and **E5**, the di-alkoxy derivatives of cannabigerol (**E1**), which can be considered strong mimicking agents of GPxs. More studies are still required to test additional semisynthetic derivatives of both compound **D1** and **E1** with a different substitution pattern.

3.1.7 Synthetic scheme of cannabigerol derivatives

The synthesis of the ether derivatives 1-ethoxy-cannabigerol (**E2**), 1,3-diethoxy-cannabigerol (**E3**), 1-propoxy-cannabigerol (**E4**), 1,3-dipropoxy-cannabigerol (**E5**), 1-butoxy-cannabigerol (**E6**) and 1,3-dibutoxy-cannabigerol (**E7**) was accomplished from commercially available cannabigerol (**E1**). The alkylation of the hydroxy moieties in position 1 and 3 was performed by reaction of **E1** with the corresponding alkyl iodide, in acetone at 80 °C for 16 h and in the presence of dry K₂CO₃ as the base. Specifically, the alkyl iodide selected were iodoethane for **E2** and **E3**, 1-iodopropane for **E4** and **E5** and 1-iodobutane for **E6** and **E7**. A representation of the reaction steps is reported in scheme 4. The reaction mixture was diluted with H₂O (100 mL) and extracted two times with CH₂Cl₂ (100 mL). The organic phase was then collected and evaporated under vacuum. Finally, the purification of the mono- and di-alkoxy derivatives was performed through silica gel column chromatography.

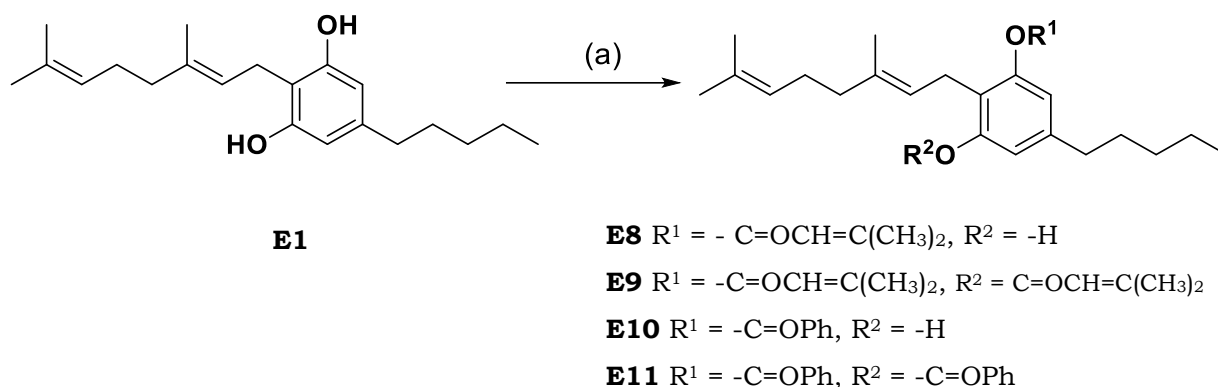
Scheme 4. Synthetic scheme of cannabigerol (**E1**) ether derivatives 1-ethoxy-cannabigerol (**E2**), 1,3-diethoxy-cannabigerol (**E3**), 1-propoxy-cannabigerol (**E4**), 1,3-dipropoxy-cannabigerol (**E5**), 1-butoxy-cannabigerol (**E6**) and 1,3-dibutoxy-cannabigerol (**E7**).



Reagents and conditions: a) alkyl iodide, K_2CO_3 , acetone, 80 °C, 16 h.

Similarly, the esterification of the same hydroxy moieties to obtain 1-cannabigerol-dimethylacryloyl ester (**E8**), 1,3-cannabigerol-dimethylacryloyl ester (**E9**), cannabigerol-1-benzoate (**E10**) and cannabigerol-1,3-dibenzoate (**E11**) was accomplished starting from commercially available cannabigerol. In this case, **E1** was left to react with an acyl chloride, 3,3-dimethylacryloyl chloride for **E8** and **E9** and benzoyl chloride for **E10** and **E11**, in Et_2O at 80 °C for 16 h and in the presence of dry Et_3N as the base. The same purification steps as the previous products were performed. A schematic representation of the synthetic steps is shown in scheme 5.

Scheme 5. Synthetic scheme of cannabigerol (**E1**) ester derivatives 1-cannabigerol-dimethylacryloyl ester (**E8**), 1,3-cannabigerol-di-dimethylacryloyl ester (**E9**), cannabigerol-1-benzoate (**E10**) and cannabigerol-1,3-dibenzoate (**E11**).



Reagents and conditions: a) acyl chloride, Et_3N , Et_2O , 80 °C, 16 h.

3.1.8 Conclusions

Easy to handle chemical process coupled to versatile and widespread analytical hyphenated techniques like GC-MS and HPLC-DAD was used to develop a revised version of the Iwaoka's assay, a test aiming to screen the preliminary GPx-like activity of different natural, semisynthetic and purely synthetic compounds. This was done by proposing an overall less expensive and more accessible procedure, which also allowed to extend the category of chemicals to be screened as potential GPx-mimicking agents.

The revised version of the Iwaoka's assay was used to test a wide array of natural and semisynthetic compounds, and among them the di-ethylic and di-propylic ethers of cannabigerol (**E3**, **E5**) were the most promising mimetics. Additional derivatives are still being tested to further understand the structure-relationship activity of multiple classes of compounds.

3.1.9 Experimental procedures

Chemicals

Ebselen (purity degree $\geq 99.0\%$), 1,4-dithiothreitol (purity degree $\geq 99.0\%$), copper (II) nitrate trihydrate (purity degree $\geq 99\%$), H_2O_2 30%, umbelliferone (purity degree $\geq 99\%$), *p*-coumaric acid (purity degree $\geq 99\%$), ferulic acid (purity degree $\geq 99\%$), cannabidiol (purity degree $\geq 99\%$), cannabigerol (purity degree $\geq 99\%$), all the reagents employed in the synthesis, EtOAc, Et_2O , acetone, n-hexane, CH_2Cl_2 , H_2O , CH_3CN , and MeOH (all HPLC grade) were purchased from Honeywell Research Chemicals (Charlotte, North Carolina, USA) and from Merck Sigma Aldrich (Darmstadt, Germany).

Boropinic acid (A2)

Concentrated H_2SO_4 (500 μL , 9.38 mmol) was added to a solution of ferulic acid (**A1**) (700 mg, 3.6 mmol) in refluxing MeOH (22 mL). The mixture was left to react under magnetic stirring at 80 °C for 24 h. 200 mL of a 3% solution of NaHCO_3 were used to dilute the reaction mixture which was then extracted with CH_2Cl_2 (2 x 200 mL). The organic phases were collected and dried over anhydrous Na_2SO_4 and then evaporated to dryness with a rotary evaporator to obtain **intermediate A**, ferulic acid methyl ester.

K_2CO_3 (193.5 mg, 1.44 mmol) was added to a 5 mL solution of **intermediate A** (200 mg, 0.96 mmol) in acetone. The resulting mixture was left under magnetic stirring at room temperature for about 10 min. This solution was then made to react with 3,3-dimethylallyl bromide (166 μL , 1.44 mmol) in refluxing acetone at 80 °C for 2 h. 400 mg of NaOH were then added to the same solution to perform a basic hydrolysis of the methyl ester, leaving it under magnetic stirring for 3 h and at the same temperature. The resulting

mixture was then diluted to 70 mL with H₂O and extracted with Et₂O (2 x 70 mL). The organic phases were discarded, while the aqueous solution was acidified to pH 1 with 10% HCl and then extracted again with CH₂Cl₂ (2 x 70 mL). The organic phases were collected and dried over anhydrous Na₂SO₄ and then evaporated to dryness with a rotary evaporator. A crystallization with *n*-hexane was enough to obtain the product (white solid) in 95 % yield and 97.8% purity. The spectroscopic data of **A2** corresponds to those already reported in literature for the same compound [276].

4'-geranyloxyferulic acid (GOFA) (A3)

Compound **A3** was obtained following the same general procedure pursued for **A2**. In this case the only difference consisted in the alkylation step, which was performed by adding geranyl bromide (286 µL, 1.44 mmol) as the reagent. After crystallization with *n*-hexane the product (white solid) was obtained in 92.0% yield and 97.8% purity. The spectroscopic data of **A2** corresponds to those already reported in literature for the same compound [277].

***p*-coumaric acid methyl ester (B3)**

Concentrated H₂SO₄ (500 µL, 9.38 mmol) was added to a solution of *p*-coumaric acid (**B1**) (700 mg, 4.2 mmol) in refluxing MeOH (22 mL). The mixture was left to react under magnetic stirring at 80 °C for 24 h. 200 mL of a 3% solution of NaHCO₃ were used to dilute the reaction mixture which was then extracted with CH₂Cl₂ (2 x 200 mL). The organic phases were collected and dried over anhydrous Na₂SO₄ and then evaporated to dryness with a rotary evaporator. This led to obtain **B3**, *p*-coumaric acid methyl ester, in 99.71 % yield.

^1H -NMR (300 MHz, CDCl_3) δ_{H} : 7.80 (d, 1H, alkenyl CH), 7.52 (d, 2H, aromatic CH), 6.95 (d, 2H, aromatic CH), 6.35 (d, 1H, alkenyl CH), 3.83 (s, 3H, CH_3).

^{13}C -NMR (300 MHz, CDCl_3) δ_{C} : 115.3, 115.8, 115.8, 125.3, 130.1, 130.1, 144.2, 159.6, 168.0, 52.0.

Geranyloxy *p*-coumaric acid (B2)

K_2CO_3 (170 mg, 1.23 mmol) was added to a 5 mL solution of **B3** (200 mg, 1.1 mmol) in acetone. The resulting mixture was left under magnetic stirring at room temperature for about 10 min. This solution was then made to react with geranyl bromide (250 μL , 1.25 mmol) in refluxing acetone at 80 $^\circ\text{C}$ for 3 h. 400 mg of NaOH were then added to the same solution to perform a basic hydrolysis of the methyl ester, leaving it under magnetic stirring for 3 h and at the same temperature. The resulting mixture was then diluted to 70 mL with H_2O and extracted with Et_2O (2 x 70 mL). The organic phases were discarded, while the aqueous solution was acidified to pH 1 with 10% HCl and then extracted again with CH_2Cl_2 (2 x 70 mL). The organic phases were collected and dried over anhydrous Na_2SO_4 and then evaporated to dryness with a rotary evaporator. A crystallization with *n*-hexane was enough to obtain the product (white solid) in 90.49% yield and 96.7% purity.

^1H -NMR (300 MHz, CDCl_3) δ_{H} : 7.80 (d, 1H, alkenyl CH), 7.52 (d, 2H, aromatic CH), 6.95 (d, 2H, aromatic CH), 6.35 (d, 1H, alkenyl CH), 5.48 (t, 1H, alkenyl CH), 4.59 (d, 2H, CH_2), 2.79 (d, 6H, CH_3).

^{13}C -NMR (300 MHz, CDCl_3) δ_{C} : 18.6, 24.6, 64.9, 115.3, 115.8, 115.8, 119.6, 125.3, 130.1, 130.1, 138.4, 144.2, 159.6, 168.0.

7-isopentenylloxycoumarin (C2)

K₂CO₃ (281 mg, 2.03 mmol) was added to a 10 mL solution of umbelliferone (**C1**) (300 mg, 1.85 mmol) in acetone. The resulting mixture was left under magnetic stirring at room temperature for about 10 min. This solution was then made to react with 3,3-dimethylallyl bromide (236 µL, 2.03 mmol) in refluxing acetone at 80 °C for 2 h. The resulting mixture was then diluted to 70 mL with H₂O and extracted with CH₂Cl₂ (2 x 70 mL). The organic phases were collected and dried over anhydrous Na₂SO₄ and then evaporated to dryness with a rotary evaporator. After crystallization with *n*-hexane the product (white solid) was obtained in 94.4% yield and 98.4% purity. The spectroscopic data of **C2** corresponds to those already reported in literature for the same compound [278].

Auraptene (C3)

Compound **C3** was obtained following the same general procedure pursued for **C2**. In this case the only difference consisted in the alkylation step, which was performed by adding geranyl bromide (403.9 µL, 2.03 mmol) as the reagent. After crystallization with *n*-hexane the product (white solid) was obtained in 94.5% yield and 97.8% purity. The spectroscopic data of **C3** corresponds to those already reported in literature for the same compound [278].

Umbelliprenin (C4)

Compound **C4** was obtained following the same general procedure pursued for **C2**. In this instance the alkylation step was performed by adding *trans,trans*-farnesyl bromide (550.4 µL, 2.03 mmol) as the reagent. After crystallization with *n*-hexane the product (white solid) was obtained in

98.5% yield and 97.8% purity. The spectroscopic data of **C4** corresponds to those already reported in literature for the same compound [278].

1-ethoxy-cannabigerol (E2) and 1,3-diethoxy-cannabigerol (E3)

K₂CO₃ (140 mg, 1.03 mmol) was added to a 5 mL solution of cannabigerol (**E1**) (300 mg, 0.94 mmol) in acetone. The resulting mixture was left under magnetic stirring at room temperature for about 10 min. This solution was then made to react with iodoethane (151 μ L, 1.88 mmol) in refluxing acetone at 80 °C for 16 h. The completion of the reaction was monitored by TLC, with 100 % CH₂Cl₂ as the mobile phase. The resulting mixture was then diluted to 100 mL with H₂O and extracted with CH₂Cl₂ (2 x 100 mL). The organic phases were collected and dried over anhydrous Na₂SO₄ and then evaporated to dryness with a rotary evaporator. The purification of the two products was performed through silica gel column chromatography with 100 % CH₂Cl₂ as the mobile phase.

(E2) Yield = 50 %; R_f = 0.35; ¹H-NMR (300 MHz, CDCl₃) δ _H 6.295-6.311 (dd, 2H, aromatic CH), 5.251-5.257 (t, 1H, alkenyl CH), 5.153-5.179 (t, 1H, alkenyl CH), 3.999-4.091 (q, 2H, O-CH₂-CH₃), 3.389-3.412 (d, 2H, Carom-CH₂-CH), 2.469-2.522 (t, 2H, Carom-CH₂-CH₂), 2.053-2.084 (q, 2H, CH₂), 1.801 (s, 3H, CH₃), 1.672 (s, 3H, CH₃), 1.586 (s, 3H, CH₃), 1.373-1.392 (t, 2H, CH₂), 1.336-1.356 (t, 3H, CH₃), 1.229-1.313 (m, 4H, CH₂), 0.865-0.889 (t, 3H, CH₃).

¹³C-NMR (300 MHz, CDCl₃) δ _C 156.6, 155.1, 142.4, 136.5, 132.0, 123.5, 122.3, 111.9, 109.9, 106.5, 64.9, 39.7, 36.3, 31.4, 30.9, 26.4, 24.6, 22.7, 22.2, 18.6, 16.4, 14.8, 14.1.

(E3) Yield = 55 %; R_f = 0.65; $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ_{H} 6.332 (s, 2H, aromatic CH), 5.234-5.238 (t, 1H, CH), 5.072-5.076 (t, 1H, alkenyl CH), 3.971-4.041 (q, 4H, O- $\text{CH}_2\text{-CH}_3$), 3.324-3.348 (d, 2H, Carom- $\text{CH}_2\text{-CH}$), 2.496-2.549 (t, 2H, Carom- $\text{CH}_2\text{-CH}_2$), 1.951-1.967 (q, 2H, CH_2), 1.770 (s, 3H, CH_3), 1.570 (s, 6H, CH_3), 1.666-1.632 (t, 2H, CH_2), 1.416-1.369 (t, 6H, $\text{CH}_2\text{-CH}_3$), 1.293-1.340 (m, 4H, CH_2), 0.867-0.891 (t, 3H, CH_3).

$^{13}\text{C-NMR}$ (300 MHz, CDCl_3) δ_{C} 155.8, 141.6, 136.5, 132.0, 123.5, 122.3, 110.4, 108.9, 64.9, 39.7, 36.3, 31.4, 30.9, 24.6, 24.4, 22.7, 22.5, 18.6, 16.4, 14.8, 14.1.

1-propoxy-cannabigerol (E4) and 1,3-dipropoxy-cannabigerol (E5)

Compounds **E4** and **E5** were obtained following the same general procedure pursued for **E2** and **E3**. In this instance the alkylation step was performed by adding 1-iodopropane (183 μL , 1.88 mmol) as the reagent. The resulting mixture was then diluted to 100 mL with H_2O and extracted with CH_2Cl_2 (2 x 100 mL). The organic phases were collected and dried over anhydrous Na_2SO_4 and then evaporated to dryness with a rotary evaporator. The purification of the two products was performed through silica gel column chromatography with 100 % CH_2Cl_2 as the mobile phase.

(E4) Yield = 50 %; R_f = 0.55; $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ_{H} 6.225-6.375 (dd, 2H, aromatic CH), 5.275-5.210 (t, 1H, alkenyl CH), 5.038-5.062 (t, 1H, alkenyl CH), 3.386-3.937 (t, 2H, O- $\text{CH}_2\text{-CH}_3$), 3.375-3.425 (d, 2H, Carom- $\text{CH}_2\text{-CH}$), 2.452-2.527 (t, 2H, Carom- $\text{CH}_2\text{-CH}_2$), 2.05-2.1 (m, 2H, CH_2), 2.00-2.06 (m, 2H, CH_2), 1.750-1.850 (q, 4H, CH_2), 1.662 (s, 6H, CH_3), 1.550 (s, 3H, CH_3), 1.247-1.372 (m, 4H, CH_2), 1.000-1.075 (t, 3H, CH_3), 0.865-0.915 (t, 3H, CH_3).

^{13}C -NMR (300 MHz, CDCl_3) δ_{C} 157.2, 155.3, 142.4, 137.5, 132.0, 123.944, 122.357, 112.426, 109.9, 106.5, 69.6, 39.73, 36.3, 31.4, 30.7, 26.457, 24.6, 22.7, 22.6, 22.2, 18.6, 16.4, 14.1, 10.4.

(E5) Yield = 60 %; R_f = 0.61; ^1H -NMR (300 MHz, CDCl_3) δ_{H} 6.324 (s, 2H, aromatic CH), 5.254-5.200 (t, 1H, alkenyl CH), 5.057-5.091 (t, 1H, alkenyl CH), 3.874-3.916 (t, 4H, O- CH_2 - CH_3), 3.334-3.357 (d, 2H, Carom- CH_2 -CH), 2.496-2.547 (t, 2H, Carom- CH_2 - CH_2), 2.004-2.055 (t, 2H, CH- CH_2 - CH_2), 1.906-1.955 (t, 2H, CH- CH_2 - CH_2), 1.832 (s, 3H, CH_3), 1.809 (s, 3H, CH_3), 1.740-1.756 (m, 4H, CH_2), 1.639 (s, 3H, CH_3), 1.509-1.563 (m, 2H, CH_2), 1.365-1.340 (m, 2H, CH_2), 1.330-1.309 (m, 2H, CH_2), 1.014-1.063 (t, 6H, CH_2 - CH_3), 0.871-0.912 (t, 3H, CH_3).

^{13}C -NMR (300 MHz, CDCl_3) δ_{C} 155.8, 141.6, 136.5, 132.0, 123.5, 122.3, 110.4, 108.9, 69.6, 39.7, 36.3, 31.4, 30.9, 24.6, 24.4, 22.7, 22.6, 22.5, 18.6, 16.4, 14.1, 10.4.

1-butoxy-cannabigerol (E6) and 1,3-dibutoxy-cannabigerol (E7)

Compounds **E6** and **E7** were obtained following the same general procedure pursued for **E2** and **E3**. In this instance the alkylation step was performed by adding 1-iodobutane (610 μL , 2.33 mmol) as the reagent. The resulting mixture was then diluted to 100 mL with H_2O and extracted with CH_2Cl_2 (2 x 100 mL). The organic phases were collected and dried over anhydrous Na_2SO_4 and then evaporated to dryness with a rotary evaporator. The purification of the two products was performed through silica gel column chromatography with 100 % CH_2Cl_2 as the mobile phase.

(E6) Yield = 67 %; R_f = 0.58; ^1H -NMR (300 MHz, CDCl_3) δ_{H} 6.297-6.309 (dd, 2H, aromatic CH), 5.230-5.258 (t, 1H, alkenyl CH), 5.064-5.068 (t, 1H,

alkenyl CH), 3.918-3.959 (t, 2H, O-CH₂-CH₃), 3.387-3.410 (d, 2H, Carom-CH₂-CH), 2.475-2.525 (t, 2H, Carom-CH₂-CH₂), 2.012-2.106 (m, 4H, CH₂), 1.801 (s, 3H, CH₃), 1.717-1.758 (m, 2H, CH₂), 1.672 (s, 3H, CH₃), 1.467-1.541 (m, 4H, CH₂), 1.293-1.342 (m, 4H, CH₂), 1.590 (s, 3H, CH₃), 0.951-1.000 (t, 3H, CH₃), 0.871-0.916 (t, 3H, CH₃).

¹³C-NMR (300 MHz, CDCl₃) δ_C 157.2, 155.3, 142.4, 137.5, 132.0, 123.9, 122.3, 112.4, 108.6, 104.4, 68.0, 39.7, 36.0, 31.5, 31.4, 31.0, 26.4, 25.6, 22.5, 22.1, 19.3, 17.6, 16.1, 14.0, 13.9.

(E7) Yield = 60 %; R_f = 0.72; ¹H-NMR (300 MHz, CDCl₃) δ_H 6.324 (s, 2H, aromatic CH), 5.195-5.219 (t, 1H, alkenyl CH), 5.053-5.080 (t, 1H, alkenyl CH), 3.912-3.953 (t, 4H, O-CH₂-CH₃), 3.318-3.342 (d, 2H, Carom-CH₂-CH), 2.496-2.549 (t, 2H, Carom-CH₂-CH₂), 2.004-2.057 (t, 2H, CH-CH₂-CH₂), 1.906-1.955 (t, 2H, CH-CH₂-CH₂), 1.818-1.768 (m, 4H, CH₂), 1.750 (s, 3H, CH₃), 1.643 (s, 3H, CH₃), 1.565 (s, 3H, CH₃), 1.509-1.563 (m, 2H, CH₂), 1.463-1.537 (m, 4H, CH₂), 1.363-1.309 (m, 2H, CH₂), 1.330-1.309 (m, 2H, CH₂), 0.938-1.010 (t, 6H, CH₂-CH₃), 0.869-0.891 (t, 3H, CH₃).

¹³C-NMR (300 MHz, CDCl₃) δ_C 157.2, 142.4, 137.5, 132.0, 123.9, 122.3, 110.4, 108.6, 68.0, 39.7, 36.0, 31.6, 31.5, 31.0, 26.7, 22.5, 19.3, 16.1, 14.0.

1-cannabigerol-dimethylacryloyl ester (E8) and 1,3-cannabigerol-dimethylacryloyl ester (E9)

Et₃N (187 μL, 1.35 mmol) was added to a 5 mL solution of cannabigerol (**E1**) (300 mg, 0.94 mmol) in Et₂O. The resulting mixture was left under magnetic stirring at room temperature for about 10 min. This solution was then made to react with 3,3-dimethylacryloyl chloride (200 μL, 1.88 mmol) in refluxing Et₂O at 80 °C for 3 h. The completion of the reaction was monitored by TLC,

with CH₂Cl₂ and MeOH in a ratio of 99:1 as the mobile phases. The resulting mixture was then diluted to 100 mL with H₂O and extracted with CH₂Cl₂ (2 x 100 mL). The organic phases were collected and dried over anhydrous Na₂SO₄ and then evaporated to dryness with a rotary evaporator. The purification of the two products was performed through silica gel column chromatography with CH₂Cl₂ and MeOH in a gradient as the mobile phases.

(E8) Yield = 60 %; R_f = 0.58; ¹H-NMR (300 MHz, CDCl₃) δ_H 6.465-6.549 (dd, 2H, aromatic CH), 5.916 (s, 1H, alkenyl CH), 5.020-5.047 (t, 2H, alkenyl CH), 3.215-3.238 (d, 2H, Carom-CH₂-CH), 2.477-2.529 (t, 2H, Carom-CH₂-CH₂), 2.221 (s, 3H, CH₃), 1.981 (s, 3H, CH₃), 1.758 (s, 6H, (CH₃)₂), 1.580 (s, 3H, CH₃), 2.027-2.090 (m, 4H, CH₂), 1.533-1.602 (m, 2H, CH₂), 1.272-1.320 (m, 4H, CH₂), 0.854-0.899 (t, 3H, CH₃).

¹³C-NMR (300 MHz, CDCl₃) δ_C 170.3, 160.1, 154.5, 151.5, 142.2, 136.5, 132.0, 123.5, 122.4, 119.1, 115.9, 115.4, 111.7, 39.7, 36.3, 31.4, 30.9, 26.4, 24.6, 22.7, 22.1, 20.4, 18.6, 16.2, 13.9.

22.5, 22.1, 19.3, 17.6, 16.1, 14.0, 13.9.

(E9) Yield = 80 %; R_f = 0.72; ¹H-NMR (300 MHz, CDCl₃) δ_H 6.783 (s, 2H, aromatic CH), 5.914 (s, 2H, alkenyl CH), 5.025-5.075 (t, 2H, alkenyl CH), 3.145-3.168 (d, 2H, Carom-CH₂-CH), 2.543-2.596 (t, 2H, Carom-CH₂-CH₂), 2.223 (s, 12H, (CH₃)₂), 1.975 (s, 3H, CH₃), 1.979-2.050 (t, 2H, CH₂), 2.075-2.125 (t, 2H, CH₂), 1.652 (s, 3H, CH₃), 1.572 (s, 3H, CH₃), 1.575-1.650 (m, 2H, CH₂), 1.305-1.338 (m, 4H, CH₂), 0.865-0.910 (t, 3H, CH₃).

¹³C-NMR (300 MHz, CDCl₃) δ_C 170.3, 160.1, 150.5, 141.2, 136.5, 132.0, 124.8, 123.5, 122.4, 120.1, 115.4, 39.7, 35.3, 31.4, 30.9, 26.3, 24.6, 23.9, 22.4, 20.4, 18.6, 16.2, 13.9.

Cannabigerol-1-benzoate (E10) and cannabigerol-1,3-dibenzoate (E11)

Compounds **E10** and **E11** were obtained following the same general procedure pursued for **E8** and **E9**. In this instance the esterification step was performed by adding benzoyl chloride (234 μ L, 1.53 mmol) as the reagent and leaving the reaction on for 16 h. The completion of the reaction was monitored by TLC, with CH_2Cl_2 and *n*-hexane in a ratio of 80:20 as the mobile phases. The resulting mixture was then diluted to 100 mL with H_2O and extracted with CH_2Cl_2 (2 x 100 mL). The organic phases were collected and dried over anhydrous Na_2SO_4 and then evaporated to dryness with a rotary evaporator. The purification of the two products was performed through silica gel column chromatography with CH_2Cl_2 and *n*-hexane in a ratio of 95:5 as the mobile phases.

(E10) Yield = 40 %; R_f = 0.65; $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ_{H} 8.191-8.219 (d, 2H, benzoate aromatic CH), 7.632-7.672 (t, 1H, benzoate aromatic CH), 7.531-7.582 (t, 2H, benzoate aromatic CH), 6.586-6.614 (dd, 2H, aromatic CH) 5.401 (s, 1H, OH), 5.201-5.302 (t, 1H, alkenyl CH), 5.024-5.031 (t, 1H, alkenyl CH), 3.288-3.312 (d, 2H, Carom- CH_2 -CH), 2.756-2.705 (t, 2H, Carom- CH_2 - CH_2), 2.018-2.074 (m, 4H, CH_2), 1.807 (s, 3H, CH_3), 1.660 (s, 3H, CH_3), 1.570 (s, 3H, CH_3), 1.572-1.588 (m, 2H, CH_2), 1.307-1.315 (m, 4H, CH_2), 0.861-0.902 (t, 3H, CH_3).

$^{13}\text{C-NMR}$ (300 MHz, CDCl_3) δ_{C} 165.2, 154.9, 151.5, 142.2, 136.5, 133.9, 132.0, 130.3, 130.2, 128.6, 123.5, 122.3, 119.1, 115.9, 111.7, 39.7, 36.0, 31.4, 30.9, 26.4, 24.6, 22.7, 22.1, 18.6, 16.4, 14.1.

(E11) Yield = 55 %; R_f = 0.82; $^1\text{H-NMR}$ (300 MHz, CDCl_3) δ_{H} 8.230-8.258 (d, 4H, benzoate aromatic CH), 7.263-7.672 (t, 2H, benzoate aromatic CH),

7.498-7.549 (t, 4H, benzoate aromatic CH), 7.002 (s, 2H, aromatic CH), 5.140-5.196 (t, 1H, alkenyl CH), 5.023-5.031 (t, 1H, alkenyl CH), 3.311-3.332 (d, 2H, Carom-CH₂-CH), 2.643-2.695 (t, 2H, Carom-CH₂-CH₂), 1.805-1.940 (m, 4H, CH₂), 1.652 (s, 3H, CH₃), 1.563 (s, 3H, CH₃), 1.385 (s, 3H, CH₃), 1.301-1.361 (m, 2H, CH₂), 1.307-1.315 (m, 4H, CH₂), 0.916-0.932 (t, 3H, CH₃).

¹³C-NMR (300 MHz, CDCl₃) δ_c 165.2, 150.5, 141.2, 136.5, 133.9, 132.0, 130.3, 130.2, 128.6, 124.8, 123.5, 122.3, 120.1, 39.7, 35.7, 31.4, 30.9, 26.4, 22.7, 22.3, 16.4, 14.1.

Calibration curve preparation

Two solutions, A and B, were firstly prepared and then mixed in a 2:1 v/v ratio. Solution A consisted in 6 mg/mL of copper (II) nitrate trihydrate in H₂O, while solution B in 3 mg/mL of 1,4-dithiotreitol in H₂O. The resulting 9 mL solution was then left to react at room temperature for around 90 min, diluted with 20 mL of H₂O and extracted two times with 10 mL of EtOAc. After anhydrification with Na₂SO₄, EtOAc was dried with a rotary evaporator. This allowed to obtain a solid white crystalline product that was confirmed to be 4,5-dihydroxy-1,2-dithiane by its MS fragmentation pattern and with comparison to the NIST library database [279]. The solid was re-suspended in EtOAc for the GC-MS analysis, while in MeOH for the HPLC-DAD ones, in both cases to a final concentration of 1 mg/mL. This solution was diluted with the corresponding solvent to obtain eight concentrations values for the construction of the calibration curve, namely 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, and 90.0 µg/mL.

Conclusions

Plants and their secondary metabolites have always been used in the treatment of numerous diseases and conditions. Indeed, natural products are at the base of modern medicine [280]. Moreover, plant-derived molecules often exhibit great biological activities, making them valuable lead compounds in drugs development [281]. Thus, the development of innovative methods for the extraction, isolation, and biological screening of plant-based natural products and of their semisynthetic analogues it's a topic of great interest nowadays, especially in the contest of "Green Chemistry".

This Ph.D. projects had the aim of focusing on all these points. Firstly, the development of extraction techniques that could easily pre-concentrate active metabolites from complex plant matrices, using easy to handle, cheap, non-toxic and green procedures. At the same time the use of solid phase adsorption with biocompatible lamellar solid to selectively isolate secondary metabolites could potentially allow to use the same sorbents directly for the formulation of pharmaceuticals, nutraceuticals and cosmeceuticals products. All was done keeping in mind threatened species, and also applying this methodology for the removal of unwanted synthetic dyes from the environment.

A new procedure, combining bioactivity-guided fractionation, isolation and structural elucidation of secondary metabolites was performed, leading to the discovery of new and potential drug leads for type 2 diabetes management.

Finally, the development and validation of new chemical assays, which are more advantageous and rapid, to preliminary reveal the pharmacological activity of natural and semisynthetic compounds. Specifically focusing on the application of hyphenated techniques to test the GPx mimetic activity of multiple molecules.

References

- [1] Teoh, E.S. (2019), Secondary Metabolites of Plants. *Medicinal Orchids of Asia*, 59-73.
- [2] Chaachouay, N.; Zidane, L. *Drugs and drugs candidates*, **2024**, 3, 184-207.
- [3] Chemat, F.; Vian, M. A.; Cravotto, G. *International Journal of Molecular Sciences*, **2012**, 13 (7), 8615-8627.
- [4] Calvano, C. D.; Tamborrino, A. *Foods*. **2012**, 11, 768.
- [5] Liu, R.; Mathieu, C.; Berthelet, J.; Zhang, W.; Dupret, J.; Lima, F. R. *International Journal of Molecular Sciences*. **2012**, 23(13), 7027.
- [6] Huang, H. W.; Hsu, C. P.; Yang, B. B.; Wang, C. Y. *Trends in Food Science & Technology*, **2013**, 33, 54 -62.
- [7] Zhang, Q. W.; Lin, L. Q.; Ye, W. C. *Chinese Medicine*. **2018**, 20.
- [8] Bitwell, C.; Indra, S. S.; Luke, C.; Kakoma, M. K. *Scientific African*. **2023**, 19, e01585.
- [9] Azwanida, N. N. *Medicinal & Aromatic Plants*, **2015**, 4:3.
- [10] Rathi, B. S.; Bodhankar, S. L.; Baheti, A. M. *Indian Journal of Experimental Biology*, **2006**, 44, 898-901.
- [11] Rezvankhah, A.; Emam-Djomeh, Z.; Safari, M.; Askari, G.; Salami, M. *Journal of Food Science and Technology*. **2019**, 56, 4198-4210.
- [12] Kumar, K.; Srivastav, S.; Sharanagat, V. S. *Ultrasonics Sonochemistry*, **2021**, 70, 105325.
- [13] Iqbal, A.; Schulz, P.; Rizvi, S. H. S. *Food Bioscience*, **2021**, 44, 101384.

- [14] Delazar, A.; Nahar, L.; Hamedeyazdan, S.; Sarker, S. D. *Natural products Isolation: Methods in Molecular Biology*, **2012**, 864.
- [15] Vijaya Bhaskar Reddy, A.; Moniruzzaman, M.; Madhavi, V.; Jaafar, J. *Studies in Natural Products Chemistry*, **2020**, 66, 197-223.
- [16] Ibanez, E.; Mendiola, J. A.; Castro-Puyana, M. *Encyclopedia of Food and Health*, **2016**, 227-233.
- [17] Ozkan, K.; Bayaram, Y.; Karasu, S.; Karadag, A.; Sagdic, O. *Saffron*, **2021**, 99-141.
- [18] Patil, S. B. S.; Wakte, P. S.; Shinde, D. B. *Journal of Advanced Research*. **2013**, 5, 629-635.
- [19] Vagi, E.; Balazs, M.; Komoczi, A.; Mihalovits, M.; Szekely, E. *Journal of Supercritical Fluids*. **2020**, 164, 104898.
- [20] Curren, M. S. S.; King, J. W. *Journal of Agricultural and Food Chemistry*, **2001**, 49, 2175-2180.
- [21] Bajer, T.; Bajerová, P.; Kremr, D.; Eisner, A.; Ventura, K. J. *Food Compos. Anal.* **2015**, 40, 32-38.
- [22] Calderon-Oliver, M.; Ponce-Alquicira, E. *Molecules*, **2021**, 26, 1869.
- [23] Chemat, F.; Vian, M. A.; Cravotto, G. *International Journal of Molecular Science*, **2012**, 13(7), 8165-8627.
- [24] Billiard, K. M.; Dershem, A. M.; Gionfriddo, E. *Molecules*, **2020**, 25, 5297.
- [25] Fiorito, S.; Epifano, F.; Preziuso, F.; Palumbo, L.; Marchetti, L.; Bastianini, M.; Cardellini, F.; Spogli, R.; Genovese, S.; *Food Anal. Methods*, **2021**, 14, 1133-1139.

- [26] Badawy, M. E. I.; El-Nouby, M. A. M.; Kimani, P. K.; Lim, L. W.; Rabea, E. I. *Analytical Sciences*, **2022**, 38(12), 1457-1487.
- [27] Pebdani, A. A.; Dadfarnia A.; Shabani, A. M.; Khodadoust, S.; Haghgoo, S. *Journal of Chromatography A*, **2016**, 1437, 15-24.
- [28] Shi, P. Z.; Ye, N. S. *Talanta*, **2015**, 143, 219-225.
- [29] Rajabi, M.; Mahanpoor, K.; Moradi, O. *RCS Advances*, **2017**, 7(74), 47083-47090.
- [30] Ganan, J.; Perez-Quintanilla, D.; Morante Zarcero, S.; Sierra, I. *Journal of Hazardous Materials*, **2013**, 260, 609.
- [31] Wasti, A.; Awan, M. A. *Journal of the Association of Arab Universities for Basic and Applied Sciences*, **2016**, 20, 26-31.
- [32] Plotka-Wasyłka, J.; Marc, M.; Szczepanska, N.; Namiesnik, J. *Critical Reviews in Analytical Chemistry*, **2017**, 47, 373-383.
- [33] Choy, J. H.; Choi, S. J.; Oh, J. M.; Park, T. *Applied Clay Science*, **2007**, 36, 122-132.
- [34] Genovese, S.; Epifano, F.; Palumbo, L.; Pulito, G.; Bastianini, M.; Cardellini, F.; Spogli, R.; Fiorito, S. *Journal of Pharmaceutical and Biomedical Analysis*, **2020**, 187, 113348.
- [35] www.prolabintefarm.com
- [36] Khurana, I. S.; Kaur, S.; Kaur, H.; Khurana, R. K. *Future Science OA*, **2015**, 1, FS06.
- [37] Claverie, M.; Dumas, A.; Carême, C.; Poirier, M.; Le Roux, C.; Micoud, P.; Martin, F.; Aymonier, C. *Chemistry – A European Journal*. **2018**, 24, 519 – 542.

- [38] Moreno, J.; Peinado, R. Chapter 19 - Wine Colloids. *Enological Chemistry, Academic Press*, **2012**, 323-354.
- [39] Ra, H.; Kim, S.; Kim, T.; Bae, J.; Shim, W. *Materials Matters*, **2022**, 17.1.
- [40] Schieber, M.; Chandel, N. S. *Current Biology*, **2014**, 24(10).
- [41] Alam, M. N.; Bristi, N. J.; Rafiquzzaman, M. *Saudi Pharmaceutical Journal*, **2013**, 21(2), 143-152.
- [42] Kinnula, V. L.; Crapo, J. D. *Free Radical Biology and Medicine*, **2004**, 36(6), 718-744.
- [43] Singh, U.; Jialal, I. *Pathophysiology*, **2006**, 13(3), 129-142.
- [44] Sas, K.; Robotka, H.; Toldi, J.; Vécsei, L. *Journal of the Neurological Sciences*. **2007**, 257(1-2), 221-239.
- [45] Khlebnikov, A. I.; Schepetkin, I. A.; Domina, N. G.; Kirpotina, L. N.; Quinn, M. T. *Bioorganic & Medicinal Chemistry*, **2007**, 15, 1749-1770.
- [46] Halliwell, B. *Free Radical Research*, **1990**, 9, 1-32.
- [47] Pisoschi, A. M.; Pop, A.; Cimpeanu, C.; Predoi, G. *Oxidative Medicine and Cellular Longevity*, **2016**, 9130976.
- [48] Carocho, M.; Ferreira, I. C. F. R. *Food and Chemical Toxicology*, **2013**, 51, 15-25.
- [49] Puri, V.; Nagpal, M.; Singh, I.; Singh, M.; Dhingra, G. A.; Huanbutta, K.; Dheer, D.; Sharma, A.; Sangnim, T. *Nutrients*, **2022**, 14, 4637.
- [50] Valero-Mendoza, A. G.; Meléndez-Rentería, N. P.; Chavez-Gonzales, M. I.; Flores-Gallegos, A. C.; Wong-Paz, J. E.; Govea-Salas, M.; Zugasti-Cruz, A.; Ascacio-Valdes, J. A. *Food Chemistry Advances*, **2023**, 2, 100153.

- [51] Gimenez-Bastida, J. A.; Avila-Galvez, M. A.; Espin, J. C.; Gonzales-Sarrias, A. *Trends in Food Science & Technology*, **2021**, *114*, 410-423.
- [52] Fahmy, H.; Hegazi, N.; El-Shamy, S.; Farag, M. A. *Food & Function*, **2020**, *11*, 5768-5781.
- [53] Gosh, N.; Chakraborty, T.; Malick, S.; Mana, S.; Singha, D.; Gosh, B.; Roy, S. *Spectrochimica Acta A Molecular Biomolecular*, **2015**, *151*, 807-813.
- [54] Chandra, S.; Khan, S.; Avula, B.; Lata, H.; Yang, M. H.; El Sohy, M. A.; Khan, I. A. *Evidence-Based Complementary and Alternative Medicine*, **2014**, 253875.
- [55] Gardeli, C.; Varela, K.; Krokida, F.; Mallouchos, A. *Medicines (Basel)*, **2019**, *6*, 90.
- [56] Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, M. *Free Radical Biology & Medicine*, **1999**, *26*, 1231-1237.
- [57] Khattab, T. A.; Abdelrahman, M. S.; Rehan, M. *Environmental Science and Pollution Research*, **2020**, *27*(4), 3803-3818.
- [58] Rawat, D.; Mishra, V.; Sharma, R. S. *Chemosphere*, **2016**, *155*, 591-605.
- [59] Hossain, M. S.; Das, S. C.; Islam, J. M.; Al Mamun, M. A.; Khan, M. A. *Radiation and Physics and Chemistry*, **2018**, *151*, 77-83.
- [60] Lellis, B.; Favaro-Polonio, C. Z.; Pamphile, J. A.; Polonio, J. C. *Biotechnology Research and Innovation*. **2019**, *3*(2), 275-290.
- [61] Amchova, P.; Kotolova, H.; Ruda-Kucerova, J. *Regulatory Toxicology and Pharmacology*, **2015**, *73*, 914-922.
- [62] Setiadi, T.; Andriani, Y.; Erlania, M. *The First Onternational Symposium on Southeast Asian Water Environment*. **2006**, 159-166.

- [63] Hassan, M. M.; Carr, C. M. *Chemosphere*. **2018**, *209*(1), 201–219.
- [64] Imran, M.; Crowley, D. E.; Khalid, A.; Hussain, S.; Mumtaz, M. W.; Arshad, M. *Reviews in Environmental Science and Biotechnology*, **2015**, *14*(1), 73–92.
- [65] Khatri, J.; Nidheesh, P. V; Singh, T. A.; Kumar, M. S. *Chemical Engineering Journal*, **2018**, *348*, 67–73.
- [66] Cheng, L.; Wei, M.; Huang, L.; Pan, F.; Xia, D.; Li, X.; Xu, A. *Industrial & Engineering Chemistry Research*, **2014**, *53*, 3478–3485.
- [67] Dasgupta, J.; Sikder, J.; Chakraborty, S.; Curcio, S.; Drioli, E. *Journal of Environmental Management*, **2015**, *147*, 55–72.
- [68] Candia-Onfray, C.; Thiam, A.; Salazar, C.; Martinez-Huitle C. A.; Salazar R. *Journal of The Electrochemical Society*, **2017**, *164*(13), 440–447.
- [69] Robinson, T.; McMullan, G.; Marchant, R.; Nigam, P. *Bioresource Technology*, **2001**, *77*, 247–255.
- [70] Shi, P. Z.; Ye, N. S. *Talanta*, **2015**, *143*, 219–225.
- [71] Ganan, J.; Perez-Quintanilla, D.; Morante Zarcero, S.; Sierra, I. *Journal of Hazardous Materials.*, **2013**, *260*, 609.
- [72] Wasti, A., Awan, M. A. *Journal of the Association of Arab Universities for Basic and Applied Science*, **2016**, *20*, 26–31.
- [73] Zhang, L.; Sellaoui, L.; Franco, D; Dotto, G. L.; Bajahzar, A; Belmabrouk, H; Bonilla-Petriciolet, A.; Oliveira, M. L. S; Li, Z. *Chemical Engineering Journal*, **2020**, *382*, 122952.
- [74] El-Menshawy, A. M.; Kenawy, I. M.; El Asmy, A. A. *Journal of Hazardous Materials*, **2010**, *173*, 523–527.

- [75] Liu, X. Y.; Yin, J. J.; Zhu, L.; Zhao, G. H.; Zhang, H. X. *Talanta*, **2011**, 85, 2451-2457.
- [76] Sharma, G.; Kumar, A.; Naushad, M.; Thakur, B.; Vo, D. V. N.; Gao, B.; Al-Khatani, A. A.; Stadler, F. J. *Journal of Hazardous Materials*, **2021**, 416, 125714.
- [77] Perveen, S.; Nadeem, R.; Nosheen, F.; Asjad, M. I.; Awrejcewicz, J.; Anwar, T. *Nanomaterials*, **2022**, 12, 2828.
- [78] Sharma, A. K.; Choudhary, B. P. *Journal of Critical Reviews*, **2020**, 7(3), 2437–2441.
- [79] Khayyat, L.; Essawy, A.; Sorour, J.; Soffar, A. *PeerJ*, **2017**, 5, e3041.
- [80] Ouassif, H.; Moujahid E. M.; Lahkale, R.; Sadik, R.; Bouragba, F. Z.; Sabbar, E. M.; Diouri, M. *Surfaces and Interfaces*, **2020**, 18, 100401.
- [81] Costantino, U.; Ambroggi, V.; Nocchetti, M.; Perioli, L. *Microporous and Mesoporous Materials*, **2008**, 107, 149–160.
- [82] Xiao, H.; Liu, S. *Materials and Design*, **2018**, 155, 19-35.
- [83] Zhang, Q.; Du, W.; Pan, B.; Zhang, W.; Zhang, Q.; Xu, Z. *Journal of Hazardous Materials*, **2008**, 152, 469–475.
- [84] Vu, A. T.; Vu, V. T. *Indian Journal of Science and Technology*, **2020**, 11(41), 1-15.
- [85] Sahnoun, S.; Boutahala, M.; Tiar, C.; Kahoul, A. *COMPTES Rendus Chimie*, **2018**, 21(3-4), 391 – 398.
- [86] Bhavyasri, K.; Vshnumurthy, K. M.; Rambabu, D.; Sumakanth, M. *GSC Biological and Pharmaceutical Sciences*, **2019**, 6, 89-106.
- [87] fda.gov.
- [88] Silva, M. M.; Reboredo, F. H.; Lidon, F. C. *Foods*, **2022**, 11, 379.

- [89] Sharma, A. K.; Choudhary, B. P. *Journal of Critical Reviews*. **2020**, 7(03), 2437-2441.
- [90] Silva, M. M.; Reboredo, F. H.; Lidon, F. C. *Foods*, **2022**, 11(3), 379.
- [91] Inietanbor, J. E.; Ykubu, J. M.; Ezeonu, S. C.; *Asian Journal of Science and Technology*, **2015**, 6, 1118-1135.
- [92] Vojdani, A.; Vojdani, C. *Alternative Therapies in Health & Medicine*. **2015**, 21, 52-62.
- [93] Rajan, J.; Simon, R.; Bosso, J. *The Journal of Allergy and Clinical Immunology: In Practice*, **2014**, 2(2), 168-171.
- [94] Miller, M. D.; Steinmaus, C.; Golub, M. S.; Castorina, R.; Thilakartne, R.; Bradman, A.; Marty, M. A. *Environmental Health*. **2022**, 21, 45.
- [95] McCann, D.; Barrett, A.; Cooper, A.; Crumpler, D.; Dalen, L.; Grimshaw, K.; Kitchin, E.; Lok, K.; Porteous, L.; Prince, E. *Lancet*, **2007**, 370, 1560-1567.
- [94] El-Rabey, H. A.; Al-Seen, M. N.; Al-Sieni, A. I.; Al-Hamed, A. M.; Zamzami, M. A.; Almutairi, F. M. *Journal of Food Biochemistry*. **2019**, 43, e12780.
- [95] Saxena, B.; Sharma, S. *Toxicology International*, **2015**, 22, 152-157.
- [96] Mpountoukas, P.; Pantazaki, A.; Kostareli, E.; Christodoulou, P.; Kareli, D.; Poliliou, S.; Lialiaris, T. *Food and Chemical Toxicology*, **2010**, 48, 2934-2944.
- [97] Kobylewski, S.; Jacobson, M. F. *International Journal of Occupational & Environmental Health*, **2012**, 18, 220-246.
- [98] EFSA Panel in Food Additives and Nutrient Sources added to Food (ANS). *EFSA Journal*, **2009**, 7, 1331.

- [99] EFSA Panel in Food Additives and Nutrient Sources added to Food (ANS). *EFSA Journal*, **2010**, 8, 1853.
- [100] Goreishi, S. M.; Behpour, M.; Golestaneh, M. *Analytical Methods*, **2011**, 3, 2842-2847.
- [101] Wang, W.; Chen, Y.; Zhang, J.; Wang, X.; Chen, Z. *Journal of AOAC International*, **2015**, 98, 817- 821.
- [102] Scigalski, P.; Kosobucki, P. *Molecules*. **2020**, 25, 4869.
- [103] Fernández-Andrade, K. J.; Gonzáles-Vargas, M. C.; Rodriguez-Rico, I. L.; Ruiz-Reyes, E.; Quiroz-Fernández, L. S.; Baquerizo-Crespo, R. J.; Rodriguez-Diaz, J. M. *Results in Engineering*. **2022**, 14, 100449.
- [104] Fernández-Andrade, A.; Avila-Toro, B.; Baquerizo-Crespo, R. J.; Rodriguez-Diaz, J. M. *Communication, Smart Technologies and Innovation for Society: Proceedings of CITIS 2021*. **2022**, 41-51.
- [105] Zhang, L.; Sellaoui, L.; Franco, D.; Dotto, G. L.; Bajahzar, A.; Belmabrouk, H.; Li, Z. *Chemical Engineering Journal*. **2020**, 382, 122952.
- [106] Awogbemi, O.; Von Kallon, A. V. *Case Studies in Chemical and Environmental Engineering*. **2022**, 6, 100229.
- [107] Koul, B.; Yakoob, M.; Shah, M. P. *Environmental Research*. **2022**, 206, 112285.
- [108] Food and Agriculture Organization of the United Nations. The State of Food and Agriculture 2016 (SOFA): Climate Change, Agriculture and Food Security. *Food and Agriculture Organization of the United Nations*, **2016**.
- [109] Lynch, J.; Cain, M.; Frame, D.; Pierrehumbert, R. *Frontiers in Sustainable Food Systems*, **2021**, 4, 300.

- [110] Reshmy, R.; Philip, E.; Madhavan, A.; Sirohi, R.; Pugazhendhi, A.; Binoid, P.; Awashti, M. K.; Vivek, N.; Kumar, V; Sindhu, R. *Bioresource Technology*, **2022**, 344, 126241.
- [111] Chojnacka, K.; Gorazda, K.; Witek-Krowiak, A.; Moustakas, K. *Renewable and Sustainable Energy Reviews*, **2019**, 110, 485-498.
- [112] Dwivedi, S.; Tanveer, A.; Yadav, S.; Anand, G.; Yadav, D. Agro-Wastes for Cost Effective Production of Industrially Important Microbial Enzymes: An Overview. **2022**, 435-460.
- [113] Buckland, G.; Gonzalez, C. Olives and Olive Oil in Health and Diseases Prevention. **2010**, 689-698.
- [114] Cappelletti, G. M.; Grilli, L.; Nicoletti, G. M.; Russo, C. *Journal of Cleaner Production*. **2017**, 159, 95-105.
- [115] Brahmi, F.; Flamini, G.; Mechri, B.; Hammami, M. Virgin olive oil. Production, composition, uses and benefits for man, *Nova Science Publishers, Inc.* **2014**, 69-78.
- [116] Boskou, D. Specialty Oils and Fats in Food and Nutrition, *Woodhead Publishing Series in Food Science, Technology and Nutrition*. **2015**, 3-38.
- [117] Rapa, M.; Ciano, S. *Sustainability*, **2022**, 14(2), 654.
- [118] Calvano, C. D.; Tamborrino, A. *Foods*, **2022**, 11(6), 768.
- [119] Selim, S.; Albqmi, M.; Al-Sanea, M. M; Alnusaire, T. S.; Almuhayawi, M. S.; Abdelgawad, H.; Al Jaouni, S. K.; Elkelish, A.; Hussein, S.; Warrad, M.; El-Saadony, M. *Frontiers in Nutrition*, **2022**, 9, 1008349.
- [120] Briante, R.; Patumi, M.; Terenziani, S.; Bismuto, E.; Febbraio, F. *Journal of Agricultural and Food Chemistry*, **2002**, 50, 4934-4940.

- [121] Abaza, L.; Youssef, N. B.; Manai, H.; Haddada, F. M.; Methenni, K.; Zarrouk, M. *Grasas Aceites*, **2011**, 62, 96-104.
- [122] Özcan, M. M.; Matthäus, B. *European Food Research and Technology*, **2016**, 243, 89-99.
- [123] Barbaro, B.; Toietta, G.; Maggio, R.; Arciello, M.; Tarocchi, M.; Galli, A.; Balsano, C. *International Journal of Molecular Science*, **2014**, 15(10), 18508-18524.
- [124] Savournin, C.; Baghdikian, B.; Elias, R.; Dargouth-Kesraoui, F.; Boukef, K.; Balansard, G. *Journal of Agricultural and Food Chemistry*, **2001**, 49, 618-621.
- [125] Monteleone, J. I.; Sperlinga, E.; Siracusa, L.; Spagna, G.; Parafati, L.; Todaro, A.; Palmeri, R. *Agronomy*, **2014**, 11, 465.
- [126] Safarzadeh Markhali, F.; Teixeira, J. A.; Rocha, C. M. R. *Processes*, **2020**, 8, 1177.
- [127] Antoniadis, L.; Angelis, A.; Stathopoulos, P.; Bata, E. M.; Papoutsaki, Z.; Halabalaki, M.; Skaltsounis, L. A. *Planta Medica*, **2022**, 88, 805-813.
- [128] Abbattista, R.; Losito, I.; De Ceglie, C. M.; Castellaneta, A.; Calvano, C. D.; Palmisano, F.; Cataldi, T. R. I. *Talanta*, **2019**, 205, 120107.
- [129] Chatzikonstantinou, A. V.; Giannakopoulou, A.; De Spyrou, S.; Simos, Y. V.; Kontogianni, V. G.; Peschos, D.; Katapodis, P.; Polydera, A. C.; Stamatis, H. *Environmental Science and Pollution Research*, **2022**, 29, 29624-29637.
- [130] Yuan, J. J.; Wang, C. Z.; Ye, J. Z.; Tao, R.; Zhang, Y. S. *Molecules*, **2015**, 20, 2903-2921.

- [131] Salamanca, A.; Almodovar, P.; Jarama, I.; Gonzalez-Hedström, D.; Prodanov, M.; Inarejos-Garcia, A. M. *Antiviral Chemistry & Chemotherapy*, **2021**, 29, 20402066211063391.
- [132] Wang, Y.; Wu, Y.; Wang, A.; Alkhalidy, H.; Helm, R.; Zhang, S.; Ma, H.; Zhang, Y.; Gilbert, E.; Xu, B.; Liu, D. *Frontiers in Nutrition*, **2022**, 9, 1051452.
- [133] Habibi, E.; Baati, T.; Njim, L.; M'Rabet, Y.; Hosni, K. *Food Science & Nutrition*, **2021**, 9, 6818-6830.
- [134] Kaur, M.; Sharma, S.; Bedi, P. M. S. *Chinese Journal of Chemistry*, **2015**, 36, 520-549.
- [135] Ponticelli, M.; Lela, L.; Moles, M.; Mangieri, C.; Bisaccia, D.; Faraone, I.; Falabella, R.; Milella, L. *Phytochemistry*, **2023**, 206, 113518.
- [136] Buchwald, W.; Mikolajczak, P. L. Gentianae radix. In: The Gentianaceae- Volume 2: Biotechnology and Applications. Ed. Springer, **2015**, 403-419.
- [137] Kusar, A.; Zupancic, A.; Sentjurc, M.; Baricevic, D. *Human & Experimental Toxicology*, **2006**, 25(10), 599-604.
- [138] Cappelletti, E. M.; Trevisan, R.; Caniato, R. *Journal of Ethnopharmacology*, **1982**, 6(2), 161-190.
- [139] Jiang, M.; Cui, B. W.; Wu, Y. L.; Nan, J. X.; Lian, L. H. *Journal of Ethnopharmacology*, **2021**, 264, 113391.
- [140] Amin, A. *Experimental and Toxicologic Phatology*, **2008**, 59(6), 377-384.
- [141] Weckesser, S.; Engel, K.; Simon-Haarhaus, B.; Wttmer, A.; Pelz, K.; Schempp, C. *Phytomedicine*, **2007**, 14(7-8), 508-516.

- [142] Cafaro, T.; Carnicelli, V.; Caprioli, G.; Maggi, F.; Celenza, G.; Perilli, M.; Bozzi, A.; Amicosante, G.; Brisdelli, F. *Advances in Traditional Medicine*, **2020**, *20*(4), 619-630.
- [143] Potunuru, U. R.; Priya, K. V.; Varsha, M. S.; Mehta, N.; Chandel, S.; Manoj, N.; Raman, T.; Ramar, M.; Gromiha, M. M.; Dixit, M. *Biochimica et Biophysica Acta*, **2019**, *1863*(8), 1270-1282.
- [144] Park, E.; Kim, J.; Yeo, S.; Kim, G.; Ko, E. H.; Lee, S. W.; Li, W. Y.; Choi, C. W.; Jeong, S. Y. *Molecules*, **2018**, *23*(7), 1663.
- [145] De Vita, S.; Chini, M. G.; Saviano, G.; Finamore, C.; Festa, C.; Lauro, G.; De Marino, S.; Russo, R.; Avagliano, C.; Casapullo, A.; Calignano, A.; Bifulco, G.; Iorizzi, M. *Biomolecules*, **2021**, *11*(10), 1490.
- [146] Arino, A.; Arberas, I.; Leiton, M. J.; De Renobales, M.; Dominguez, J. B. *Zeitschrift für Lebensmitteluntersuchung und -Forschung A*, **1997**, *205*, 295-299.
- [147] Idolo, M.; Motti, R.; Mazzoleni, S. *Journal of Ethnopharmacology*, **2010**, *127*(2), 379-395.
- [148] Takino, Y.; Koshioka, M.; Kawaguchi, M.; Miyahara, T.; Tanizawa, H.; Ishi, T.; Higashino, M.; Hayashi, T. *Planta Medica*, **1980**, *38*, 352-355. [149] Kumar, V.; Sood, H.; Chauhan, R. S. *Natural Product Research*, **2015**, *29*, 1449-1455.
- [150] Fiorito, S.; Epifano, F.; Marchetti, L.; Palumbo, L.; Mascioli, F.; Bastianini, M.; Cardellini, F.; Spogli, R.; Genovese, S. *Food Chemistry*, **2021**, *364*, 130383.
- [151] Pălinkő, I.; Sipos, P.; Berkesi, O.; Varga, G. *The Journal of Physical Chemistry*, **2022**, *126*, 15254-15262.

- [152] Rehman, M. U.; Khan, F.; Niaz, K. *Recent Advances in Natural Products Analysis*, **2020**, 3-15.
- [153] Mendoza, N.; Silva, E. M. E. *Phytochemicals—Source of Antioxidants and Role in Disease Prevention*, **2018**.
- [154] War, A. R.; Paulraj, M. G.; Ahmad, T.; Buhroo, A. A.; Hussain, B.; Sharma, H. C. *Plant Signaling & Behavior*, **2012**, 7, 1306-1320.
- [155] Abdel-Nasser, A.; Hathout, A. S.; Badr, A. N.; Barakat, O. S.; Fathy, H. M. *Biotechnology Reports*, **2023**, 38, e00799.
- [156] Veeresham, C. *Journal of advanced Pharmaceutical Technology and Research*, **2012**, 3, 200.
- [157] Ngueyem, T. A.; Brusotti, G.; Marrubini, G.; Grisoli, P.; Dacarro, C.; Vidari, G.; Vita Finzi, P.; Caccialanza, G. *Journal of Ethnopharmacology*, **2008**, 120, 13-16.
- [158] Khalid, H.; Abdalla, W. E.; Abdelgadir, H.; Opatz, T.; Efferth, T. *Natural Products and Bioprospecting*, **2012**, 2, 92-103.
- [159] Kitajima, M. *Journal of Traditional Medicine*, **2012**, 29, 41-45.
- [160] Brusotti, G.; Cesari, I.; Gilardoni, G.; Tosi, S.; Grisoli, P.; Picco, A. M.; Caccialanza, G. *Journal of Ethnopharmacology*, **2012**, 142, 657-662.
- [161] Zhang, L.; Ravipati, A. S.; Koyyalamudi, S. R.; Jeong, S. C.; Reddy, N.; Smith, P. T.; Bartlett, J.; Shanmugam, K.; Munch, G.; Wu, M. J. *Journal of Agricultural and Food Chemistry*, **2011**, 59, 12361-12367.
- [162] Atanasov, A. G.; Zotchev, S. B.; Dirsch, V. M.; the International Natural Product Sciences Taskforce; Supuran, C. T. *Nature Reviews Drug Discovery*, **2021**, 20, 200-216.

- [163] Chinnok, R. J. *Eremophila* and allied genera: A monograph of the plant family Myoporaceae. *Rosenburg Publishing Pty Ltd*, **2007**.
- [164] Ebach, M. C.; Murphy, D. J. *Australian Journal of Botany*, **2020**, 68(3).
- [165] Richmond, G. S.; Ghisalberti, E. L. *Economic Botany*, **1994**, 48, 35-59.
- [166] Judd, W. S. Plant systematics: A phylogenetic approach. *Sunderland: Sinauer Associates*, **2008**.
- [167] Ghisalberti, E. L. *Journal of Ethnopharmacology*, **1994**, 44, 1-9.
- [168] Ghisalberti, E. L.; Jefferies, P. P.; Sheppard, P. *Journal of Ethnopharmacology*, **1975**, 16, 1775-1778.
- [169] Gericke, O.; Hansen, N. L.; Pedersen, G. B.; Kjaerulff, L.; Luo, D.; Staerk, D. *BMC Plant Biology*, **1994**, 44, 1-9.
- [170] Pedersen, H. A.; Ndi, C.; Semple, S. J.; Buirchell, B.; Moller, B. L.; Staerk, D. *Journal of Natural Products*, **2020**, 83(5), 1598-1610.
- [171] Tahtah, Y.; Wubshet, S. G.; Kongstad, K. T.; Heskes, A. M.; Pateraki, I.; Moller, B. L.; Jager, A. K.; Staerk, D. *Fitoterapia*, **2016**, 110, 52-58.
- [172] Liu, Q.; Harrington, D.; Kohen, J. L.; Vemulpad, S.; Jamie, J. F. *Phytochemistry*, **2006**, 67, 1256-1261.
- [173] Ndi, C. P.; Semple, S. J.; Griesser, H. J.; Pyke, S. M.; Barton, M. D. *Phytochemistry*, **2007**, 68, 2684-2690.
- [174] Semple, S. J.; Reynolds, G. D.; O'Leary, M. C.; Flower, R. L. *Journal of Ethnopharmacology*, **1998**, 60, 163-172.
- [175] Mijajlovic, S.; Smith, J.; Watson, K.; Parsons, P.; Jones, G. *Journal of Australian Traditional-Medicine Society*, **2006**, 2, 129-132.

- [176] Kumar, R.; Duffy, S.; Avery, V. M.; Carroll, A. R.; Davis, R. A. *Journal of Natural Products*, **2018**, *81*, 1079-1083.
- [177] Youssef, F. S.; Ashour, M. L.; Ebada, S. S.; Sobeh, M.; El-Beshbishy, H. A.; Singab, A. N.; Wink, M. *Journal of Pharmacy and Pharmacology*, **2017**, *69*, 733-742.
- [178] Mon, H. H.; Christo, S. N.; Ndi, C. P.; Jasieniak, M.; Rickard, H.; Hayball, J. D.; Griesser, H. J.; Semple, S. J. *Journal of Natural Products*, **2015**, *78*, 3031-3040.
- [179] von Mueller, F. J. H.; Tate, R. *Transactions, Proceedings and Reports of the Royal Society of South Australia*, **1890**, *13*, 94-109.
- [180] Chinnok, R. J. *Journal of the Adelaide Botanic Garden*, **2007**, *21*, 1-3.
- [181] Curtis, A. L.; Grierson, P. F.; Batley, J.; Naaykens, J.; Fowler, R. M.; Severn-Ellis, A.; Thiele, K. R. *Australian Systematic Botany*, **2022**, *35(1)*, 1-18.
- [182] Orgurtsova, K.; Guariguata, L.; Barengo, N. C.; Ruiz, P. L. D.; Sacre, J. W.; Karuranga, S.; Sun, H.; Boyko, E. J.; Magliano, D. J. *Diabetes Research and Clinical Practice*, **2022**, *183*, 109118.
- [183] Galicia-Garcia, U.; Benito-Vicente, A.; Jebari, S.; Larrea-Sebal, A.; Siddiqi, H.; Uribe, K. B.; Ostolaza, H.; Martin, C. *International Journal of Molecular Sciences*, **2020**, *21(17)*, 6275.
- [184] Ahmad, E.; Lim, S.; Lamptey, R.; Webb, D. R.; Davies, M. J. *Seminar*, **2022**, *400*, 1803-1820.
- [185] Garcia-Molina, L.; Lewis-Michael, A. M.; Riquelme-Gallego, B.; Cano-Ibanez, N.; Oliveras-Lopez, M. J.; Bueno-Cavanillas, A. *European Journal of Nutrition*, **2020**, *59*, 1313-1328.

- [186] Davies, M. J.; Aroda, V. R.; Collins, B. S.; Gabbay, R. A.; Green, J.; Maruthur, N. M.; Rosas, S. E.; Del Prato, S.; Mathieu, C.; Mingrone, G.; Rossing, P.; Tankova, T.; Tsapas, A.; Buse, J. B. *Diabetes Care*, **2022**, *45*(11), 2753-2786.
- [187] Liu, Z.; Gao, H.; Zhao, Z.; Huang, M.; Wang, S.; Zhan, J. *Biomedicine & Pharmacotherapy*, **2023**, *157*, 113990.
- [188] Villamar-Cruz, O.; Loza-Mejía, M. A.; Arias-Romero, L. E.; Camacho-Arroyo, I. *Bioscience Reports*, **2021**, *41*, BSR20211994.
- [189] Bence, K. K.; Delibegovic, M.; Xue, B.; Gorgun, C. Z.; Hotamisligil, G. S.; Neel, B. G.; Khan, B. B. *Nature Medicine*, **2006**, *12*(8), 917-924.
- [190] Bandyopadhyay, D.; Kusari, A.; Kenner, K. A.; Liu, F.; Chernoff, J.; Gustafson, T. A.; Kusari, J. *The Journal of Biochemical Chemistry*, **1997**, *272*(3), 1639-1645.
- [191] Liu, R.; Mathieu, C.; Berthelet, J.; Zhang, W.; Dupret, J. M.; Lima, F. R. *International Journal of Molecular Sciences*, **2022**, *23*, 7027.
- [192] Rasmussen, L. F.; Anton, J.; Kjaerulff, L.; Zhao, Y.; Semple, S. J.; Ndi, C.; Buirchell, B.; Moller, B. L.; Staerk, D. *Phytochemistry*, **2022**, *203*, 113408.
- [193] Petersen, M. J.; Liang, C.; Kjaerulff, L.; Ndi, C.; Semple, S. J.; Buirchell, B.; Coriani, S.; Moller, B. L.; Staerk, D. *Phytochemistry*, **2022**, *196*, 113072.
- [194] Liang, C.; Ndi, C.; Semple, S. J.; Buirchell, B.; Coriani, S.; Moller, B. L.; Staerk, D. *Phytochemistry*, **2024**, *219*, 113972.
- [195] Pedersen, H. A.; Ndi, C.; Semple, S. J.; Buirchell, B.; Moller, B. L.; Staerk, D. *Journal of Natural Products*, **2020**, *83*, 1598-1610.

- [196] Yam, M. F.; Lim, V.; Salman, I. M.; Ameer, O. Z.; Ang, L. F.; Rosidah, N.; Abdulkarim, G. Z.; Basir, R.; Sadikun, A.; Asmawi, M. Z. *Molecules*, **2010**, *15*(6), 4452-4466.
- [197] Martinez, V.; Barbera, O.; Sanchez-Parareda, S.; Marco, J. A. *Phytochemistry*, **1987**, *26*(9), 2619-2624.
- [198] Wu, T. S.; Tien, H. J.; Arisawa, M.; Shimizu, M.; Morita, N. *Phytochemistry*, **1980**, *19*, 2227-2228.
- [199] Barua, A. K.; Chakrabarti, P.; Dutta, S. P.; Mukherjee, D. K.; Das, B. C. *Tetrahedron*, **1971**, *6*, 1141-1147.
- [200] Irungu, B. N.; Orwa, J. A.; Gruhonjic, A.; Fitzpatrick, P. A.; Landberg, G.; Kimani, F.; Midiwo, J.; Erdelyi, M.; Yenesew, A. *Molecules*, **2014**, *19*(9), 14235-14246.
- [201] Ibrahim, A.; Khalifa, S. I.; Khafagi, I.; Youssef, D. T.; Khan, S.; Mesbah, M.; Khan, I. *Chemical and Pharmaceutical Bulletin*, **2008**, *56*(9), 1253-1258.
- [202] Bai, C. L.; Wang, Q. H.; Xu, Y. H.; Han, J. S.; Bao, Y. P. *Zeitschrift für Naturforschung B*, **2018**, *73*(6), 377-379.
- [203] Gericke, O.; Hansen, N. L.; Pedersen, G. B.; Kjaerulff, L.; Luo, D.; Staerk, D.; Moller, B. L.; Pateraki, I.; Heskes, A. M. *BMC Plant Biology*, **2020**, *20*, 1-15.
- [204] Ghisalberti, E. L.; Jefferies, P. R.; Mori, T. A. *Australian Journal of Chemistry*, **1984**, *37*, 635-647.
- [205] Ghisalberti, E. L. *Studies in Natural Products Chemistry*, **1995**, *15*(C), 225-287.

- [206] Liang, C.; Ndi, C.; Semple, S. J.; Buirchell, B.; Coriani, S.; Moller, B. L.; Staerk, D. *Phytochemistry*, **2024**, 219, 113972.
- [207] Hansen, C. C.; Nelson, D. R.; Moller, B. L.; Werck-Reichhart, D. *Molecular Plant*, **2021**, 14, 1244-1265.
- [208] Petersen, M. J.; Liang, C.; Kjaerulff, L.; Ndi, C.; Semple, S. J.; Buirchell, B.; Coriani, S.; Moller, B. L.; Staerk, D. *Phytochemistry*, **2022**, 196, 113072.
- [209] Wang, L.; Chen, K.; Zhang, M.; Ye, M.; Qiao, X. *Critical Reviews in Biotechnology*, **2021**.
- [210] Tahtah, Y.; Wubshet, S. G.; Kongstad, K. T.; Heskes, A. M.; Pateraki, I.; Moller, B. L.; Jager, A. K.; Staerk, D. *Fitoterapia*, **2016**, 110, 52-58.
- [211] Collin, F. *International Journal of Molecular Science*, **2019**, 20, 2407.
- [212] Juan, C. A.; Perez de la Lastra, J. M.; Plou, F. J.; Perez-Lebena, E. *International Journal of Molecular Science*, **2021**, 22(9), 4642.
- [213] Magnani, F.; Mattevi, A. *Current Opinions in Structural Biology*, **2019**, 59, 91-97.
- [214] Sies, H.; Jones, D. P. *Nature Reviews Molecular Cell Biology*, **2020**, 21, 363-383.
- [215] Heid, M. E.; Keyel, P. A.; Kamga, C.; Shiva, S.; Watkins, S. C.; Russel, D. S. *The Journal of Immunology*, **2013**, 191(10), 5230-5238.
- [216] Ito, F.; Sono, Y.; Ito, T. *Antioxidants*, **2019**, 8(3), 72.
- [217] Hawkins, C. L.; Davies, M. J. *Journal of Biological Chemistry*, **2019**, 294(51), 19683-19708.
- [218] Yan, L. L.; Zaher, H. S. *Journal of Biological Chemistry*, **2019**, 294(41), 15158-15171.

- [219] Dichi, I.; Bregano, J. W.; Simao, A. N. C.; Cecchini, R. *CRC Press*, **2014**.
- [220] Carocho, M.; Ferreira, I. C. F. R. *Food and Chemical Toxicology*, **2013**, *51*, 15-25.
- [221] Tosatto, S. C. E.; Bosello, V.; Fogolari, F.; Mauri, P.; Roveri, A.; Toppo, S.; Flohé, L.; Ursini, F.; Maiorino, M. *Antioxidants & Redox Signaling*, **2008**, *10*(9).
- [222] Brigelius-Flohé, R.; Maiorino, M. *Biochimica et Biophysica Acta – General Subjects*, **2013**, *1803*(5), 3289-32303.
- [223] Toppo, S.; Vanin, S.; Bosello, V.; Tosatto, S. C. E. *Antioxidants & Redox Signaling*, **2008**, *10*(9).
- [224] Petrovic, M. *ChemTexts*, **2021**, *7*, 11.
- [225] Handy, D. E.; Loscalzo, J. *Free Radical Biology and Medicine*, **2022**, *188*(1), 146-161.
- [226] Liu, C.; Yan, Q.; Gao, C.; Lin, L.; Wei, J. *International Journal of Biological Macromolecules*, **2021**, *170*, 503-513.
- [227] Esworthy, R. S.; Doroshov, J. H.; Chu, F. F. *Free Radical Biology and Medicine*, **2022**, *188*, 419-433.
- [228] Florian, S.; Wingler, K.; Schmehl, K.; Jacobasch, G.; Kreuzer, O. J.; Meyerhof, W.; Brigelius-Flohé, R. *Free Radical Research*, **2001**, *35*(6), 655-663.
- [229] Nirgude, S.; Choudhary, B. *Biochemical Pharmacology*, **2021**, *184*, 114365.
- [230] Zhao, L.; Zong, W.; Zhang, H.; Liu, R. *Toxicological Sciences*, **2019**, *168*(1), 201-208.

- [231] Jia, M.; Qin, D.; Zhao, C.; Chai, L.; Yu, Z.; Wang, W.; Tong, L.; Lv, L.; Wang, Y.; Rehwinkel, J.; Yu, J.; Zhao, W. *Nature Immunology*, **2020**, *21*, 727-735.
- [232] Bersurek, K.; Hendricks, J. M.; Li, Z.; Magtanong, L.; Ford, B.; Tang, P. H.; Roberts, M. A.; Tong, B.; Maimone, T. J.; Zoncu, R.; Bassik, M. C.; Nomura, D. K.; Dixon, S. J.; Olzmann, J. A. *Nature Immunology*, **2019**, 575, 688-692.
- [233] Taylor, A.; Robson, A.; Houghton, B. C.; Jepson, C. A.; Ford, W. C. L.; Frayne, J. *Human Reproduction*, **2013**, *28*(9), 2332-2342.
- [234] Luan, Z.; Fan, X.; Song, H.; Li, R.; Zhang, W.; Zhang, J. *In Vitro Cellular & Developmental Biology - Animal*, **2019**, *55*, 677-685.
- [235] Tan, S.; Liu, Q.; Yang, J.; Cai, J.; Yu, M.; Ji, Y. *Biological Trace Element Research*, **2023**, *201*, 368-376.
- [236] Raykhel, I.; Alanen, H.; Salo, K.; Jurvansuu, J.; Nguyen, V. D.; Latva-Ranta, M.; Ruddock, L. *Journal of Cell Biology*, **2007**, *179*(6), 1193-1204.
- [237] Chen, Y. I.; Wei, P. C.; Hsu, J. L.; Su, F. Y.; Lee, W. H. *American Journal of Translational Research*, **2016**, *8*(4), 1626-1640.
- [238] Hariharan, S.; Dharmaraj, S. *Inflammopharmacology*, **2020**, *28*, 667-695.
- [239] Huang, J. Q.; Zhou, J. C.; Wu, Y. Y.; Ren, F. Z.; Lei, X. G. *Free Radical Biology and Medicine*, **2018**, *127*, 108-115.
- [240] Tinkov, A. A.; Ajsuvakova, O. P.; Filippini, T.; Zhou, J. C.; Lei, X. G.; Gatiatulina, E. R.; Michalke, B.; Skalnaya, M. G.; Vinceti, M.; Aschner, M.; Skalny, A. V. *Biomolecules*, **2020**, *10*, 658.

- [241] Zhang, M. L.; Wu, H. T.; Chen, W. J.; Xu, Y.; Ye, Q. Q.; Shen, J. X.; Liu, J. *Journal of Translational Medicine*, **2020**, *18*, 247.
- [242] Yu, B.; Huang, Z. *BioMed Research International*, **2015**, 513196.
- [243] Sharma, G.; Shin, E. J.; Sharma, N.; Nah, S. Y.; Mai, H. N.; Nguyen, J. H.; Jeong, X.; Lei, G.; Kim, H. C. *Food and Chemical Toxicology*, **2021**, *148*, 111945.
- [244] Pei, J.; Pan, X.; Wei, G.; Hua, Y. *Frontiers in Pharmacology*, **2023**, *14*.
- [245] Sands, K. N.; Back, T. G. *Tetrahedron*, **2018**, *74*, 4959-4967.
- [246] Jain, V. K.; Priyadarsini, K. I. *RSC Publications*, **2018**.
- [247] Lesser, R.; Weiss, R. *Chemische Berichte*, **1924**, *57*, 1077-1082.
- [248] Yamaguchi, T.; Sano, K.; Takakura, K.; Saito, I.; Shinohara, Y.; Asano, T.; Hajime, Y. *Stroke*, **1998**, *29(1)*, 12-17.
- [249] Moussaoui, S.; Obinu, M. C.; Daniel, N.; Reibaud, M.; Blanchard, V.; Imperato, A. *Experimental Neurology*, **2000**, *166(2)*, 235-245.
- [250] Singh, N.; Sharpley, A. L.; Emir, U. E.; Masaki, C.; Herzallah, M. M.; Gluck, M. A.; Sharp, T.; Harmer, C. J.; Vasudevan, S. R.; Cowen, P. J.; Churchill, G. C. *Neuropsychopharmacology*, **2016**, *41*, 1768-1778.
- [251] Kil, J.; Lobarinas, E.; Spankovich, C.; Griffiths, S. K.; Antonelli, P. J.; Lynch, E. D.; Le Prell, C. G. *The Lancet*, **2017**, *390*, 969-979.
- [252] Lynch, E. D.; Gu, R.; Pierce, C.; Kil, J. *Anti-Cancer Drugs*, **2005**, *16(5)*, 569-579.
- [253] Wang, X.; Yun, J. W.; Lei, X. G. *Antioxidants & Redox Signalling*, **2014**, *20(2)*, 191-203.

- [254] Gustafsson, T. N.; Osman, H.; Werngren, J.; Hoffner, S.; Engman, L.; Holmgren, A. *Biochimica et Biophysica Acta (BBA) – General Subjects*, **2016**, 1860(6), 1265-1271.
- [255] Nozawa, R.; Arai, M.; Kurutu, R.; Motohashi, T.; Masayasu, H. *Journal of Pharmacy and Pharmacology*, **1996**, 48, 64-67.
- [256] Thenin-Houssier, S.; De Vera, I. M. S.; Pedro-Rosa, L.; Brady, A.; Richard, A.; Konnick, B.; Opp, S.; Buffone, C.; Fuhrmann, J.; Kota, S.; Billack, B.; Pietka-Ottlik, M. *Antimicrobial Agents and Chemotherapy*, **2016**, 60(4), 2195-2208.
- [257] Sands, K. N.; Back, T. G. *Tetrahedron*, **2018**, 74, 4959-4967.
- [258] Bhabak, K. P.; Mugesh, G. *Accounts of Chemical Research*, **2010**, 43, 1408-1419.
- [259] Yang, C. F.; Shen, H. M.; Ong, C. N. *Archives of Biochemistry and Biophysics*, **2000**, 374, 142-152.
- [260] Iwaoka, M.; Tomoda, S. *The Journal of American Chemical Society*, **1994**, 116, 2557-2561.
- [261] Mugesh, G.; Du Mont, W. W. *Chemistry – A European Journal*, **2001**, 7, 1365-1370.
- [262] Pearson, J. K.; Boyd, R. J. *The Journal of Physical Chemistry A*, **2008**, 112, 1013-1017.
- [263] Kumakura, F.; Mishra, B.; Priyadarsini, K. I.; Iwaoka, M. *European Journal of Organic Chemistry*, **2010**, 2010(3), 440-445.
- [264] Arai, K.; Tashiro, A.; Osaka, Y.; Iwaoka, M. *Molecules*, **2017**, 22(3), 354.

- [265] Iwaoka, M.; Kumakura, F. *Phosphorus, Sulfur, and Silicon and the Related Elements*, **2008**, 183(4), 1009-1017.
- [266] Santi, C.; Scimmi, C.; Sancineto, L. *Molecules*, **2008**, 26, 4230.
- [267] Cindric, M.; Cepo, T.; Marinc, S.; Paskvan, I.; Mijic, I.; Bindila, L.; Katalinic, J. P. *Journal of Separation Science*, **2008**, 31, 3489-3496.
- [268] Zhou, W.; Yang, S.; Wang, P. G. *Bioanalysis*, **2017**, 9, 1839-1844.
- [269] Bhardwaj, S. K.; Dwivedi, K.; Argarwal, D. D. *International Journal of Analytical and Bioanalytical Chemistry*, **2016**, 6, 1-7.
- [270] Neelam; Khatkar, A.; Sharma, K. K. *Critical Reviews in Food Science and Nutrition*, **2020**, 60(16), 2655-2675.
- [271] Gargaro, M.; Epifano, F.; Fiorito, S.; Taddeo, V. A.; Genovese, S.; Pirro, M.; Turco, A.; Pucetti, P.; Schmidt-Weber, C.; Fallarino, F. *Journal of Natural Products*, **2017**, 80(6), 1939-1943.
- [272] Preziuso, F.; Genovese, S.; Marchetti, L.; Sharifi-Rad, M.; Palumbo, L.; Epifano, F.; Fiorito, S. *Molecules*, **2020**, 25(24), 5923.
- [273] Amar, M. B. *Journal of Ethnopharmacology*, **2006**, 105, 1-25.
- [274] Li, S.; Li, W.; Malhi, N. K.; Huang, J.; Li, Q.; Zhou, Z.; Wang, R.; Peng, J.; Yin, T.; Wang, H. *Molecules*, **2024**, 29(22), 5471.
- [275] Casedas, G.; de Yarza-Sancho, M.; Lopez, V. *Pharmaceuticals*, **2024**, 17(11), 1438.
- [276] Ito, C.; Itoigawa, M.; Otsuka, T.; Tokuda, H.; Nishino, H.; Furukawa, H. *Journal of Natural Products*, **2000**, 63, 1344-1348.
- [277] Bruyere, C.; Genovese, S.; Lallemand, B.; Ionescu-Motatu, A.; Curini, M.; Kiss, R.; Epifano, F. *Bioorganic & Medicinal Chemistry Letters*, **2011**, 21(14), 4174-4179.

- [278] Fiorito, S.; Epifano, F.; Preziuso, F.; Cacciatore, I.; Di Stefano, A.; Taddeo, V. A.; de Medina, P.; Genovese, S. *European Journal of Medicinal Chemistry*, **2018**, 152, 274-282.
- [279] Wang, J.; Wang, P.; Dong, C.; Zhao, Y.; Zhou, J.; Yuan, C.; Zou, L. *Future Medicinal Chemistry*, **2020**, 12(23), 2141-2160.
- [280] Najmi, A.; Javed, S. A.; Al Bratty, M.; Alhazmi, H. A. *Molecules*, **2022**, 27, 349.
- [281] Balunas, M.J.; Kinghorn, A.D. *Life Science*. **2005**, 78, 431–441.

List of publications

1. Fiorito, S.; Palumbo, L.; Epifano, F.; Fraternale, D.; **Collevecchio, C.**; Genovese, S.; “Modulation of the biosynthesis of oxyprenylated coumarins in calli from *Ferulago campestris* elicited by ferulic acid”, *Biomass Conversion and Biorefinery*, **2022**, DOI:10.1007/s13399-022-03309-z.
Impact Factor: 4.0
2. Fiorito, S.; Epifano, F.; Palumbo, L.; **Collevecchio, C.**; Genovese, S.; “A revised version of the Iwaoka’s assay: Application of hyphenated techniques” *Journal of Pharmaceutical and Biomedical Analysis*, **2022**, 212, 114652.
Impact Factor: 3.4
3. Fiorito, S.; Palumbo, L.; Epifano, F.; **Collevecchio, C.**; Cardellini, F.; Bastianini, M.; Spogli, R.; Genovese, S.; “An easy way for the hydrolysis, pre-concentration, and chemical stabilization of crocetin from saffron powder”, *Food Chemistry*, **2022**, 377, 132040.
Impact Factor: 8.8
4. Fiorito, S.; Epifano, F.; Palumbo, L.; **Collevecchio, C.**; Bastianini, M.; Cardellini, F.; Spogli, R.; Genovese, S.; “Efficient removal of tartrazine from aqueous solutions by solid sorbents”, *Separation and Purification Technology*, **2022**, 290, 120910.
Impact Factor: 8.6
5. Genovese, S.; Epifano, F.; Palumbo, L.; **Collevecchio, C.**; Cardellini, F.; Bastianini, M.; Spogli, R.; Fiorito, S.; “A novel and efficient

concentration of pomegranate juice with enhanced antioxidant activity”, *Food Chemistry*, **2022**, 387, 132901.

Impact Factor: 8.8

6. Fiorito, S.; Epifano, F.; Palumbo, L.; **Collevecchio, C.**; Genovese, S.; “A subcritical butane-based extraction of non-psychoactive cannabinoids from hemp inflorescences”, *Industrial Crops and Products*, **2022**, 183, 114955.

Impact Factor: 5.9

7. Fiorito, S.; Epifano, F.; Palumbo, L.; **Collevecchio, C.**; Mascioli, F.; Spogli, R.; Genovese, S.; “Leaves of Yellow Gentian (*Gentiana lutea*) as an Alternative Source of Bitter Secoiridoid Glycosides”, *Journal of Natural Products*, **2022**, 85(9), 2232-2235.

Impact Factor: 5.1

8. Fiorito, S.; **Collevecchio, C.**; Epifano, F.; Genovese, S.; Palumbo, L.; “Oxyprenylated secondary metabolites: a survey of their innovative extraction methodologies”, *Phytochemistry Reviews*, **2023**, 22(1), 73-84.

Impact Factor: 7.7

9. Epifano, F.; Genovese, S.; Palumbo, L.; **Collevecchio, C.**; Fiorito, S.; “Protection of Mitochondrial Potential and Activity by Oxyprenylated Phenylpropanoids”, *Antioxidants*, **2023**, 12(2), 259.

Impact Factor: 7.0

10. Palumbo, L.; Fiorito, S.; Epifano, F.; Sharifi-Rad, M.; Genovese, S.; **Collevecchio, C.**; “Solid-phase adsorption methodologies of

naturally occurring anthraquinones: A review”, *Phytochemistry Analysis*, **2023**, 34(2), 153-162.

Impact Factor: 3.3

11. Palumbo, L.; Genovese, S.; **Collevecchio, C.**; Epifano, F.; Fiorito, S.; “Novel insights into the biomolecular mechanism of action of 4'-geranyloxyferulic acid, a colon cancer chemopreventive agent”, *Phytochemistry*, **2023**, 211, 113706.

Impact Factor: 3.8

12. Fiorito, S.; Epifano, F.; Palumbo, L.; **Collevecchio, C.**; Spogli, R.; Genovese, S.; “Separation and quantification of Tartrazine (E102) and Brilliant Blue FCF (E133) in green colored foods and beverages”, *Food Research International*, **2023**, 172, 113094.

Impact Factor: 8.1

13. Genovese, S.; Epifano, F.; Palumbo, L.; **Collevecchio, C.**; Fiorito, S.; “An easy way for the removal of residual hydrocarbon fractions from crystallized cannabigerol and cannabidiol”, *Food and Bioproducts Processing*, **2023**, 142, 50-58.

Impact Factor: 4.6

14. Fiorito, S.; Genovese, S.; Epifano, F.; **Collevecchio, C.**; “Prolidase activity assays. A survey of the reported literature methodologies”, *Analytical Biochemistry*, **2024**, 689, 115506.

Impact Factor: 2.9

15. Fiorito, S.; Genovese, S.; Epifano, F.; **Collevecchio, C.**; “Two additional oxyprenylated phenylpropanoids in *Citrus paradisi* Macfad.

Seeds extract”, *Biochemical Systematics and Ecology*, **2024**, 114, 104819.

Impact Factor: 1.6

16. Fiorito, S.; **Collevecchio, C.**; Spogli, R.; Epifano, F.; Genovese, S.; “Novel procedures for olive leaves extracts processing: Selective isolation of oleuropein and elenolic acid”, *Food Chemistry*, **2024**, 447, 139038.

Impact Factor: 8.8

17. Fiorito, S.; **Collevecchio, C.**; Epifano, F.; Genovese, S.; “Targeting prolidase. A survey of the literature data to depict a structure-activity relationship frame and to address future studies for drug development”, *Bioorganic & Medicinal Chemistry*, **2024**, 110, 117833.

Impact Factor: 3.3

18. **Collevecchio, C.**; Genovese, S.; Epifano, F.; Marchetti, L.; Fiorito, S.; “Albumin as an effective auxiliary agent for the enriched extraction of anthraquinones and curcumin from plant matrices”, *molecules*, **2025**, 30, 249.

Impact Factor: 4.6

Acknowledgements

Giunta alla fine di questo percorso, vorrei rendere omaggio a tutti coloro che ne hanno fatto parte.

Il primo grazie va al Prof. Salvatore Genovese, senza il quale non avrei intrapreso questa avventura e ancor prima, la tesi di laurea. Il giorno dell'esame di Analisi dei Medicinali II sarà un ricordo che porterò per sempre con me. Spaventata da un esame così pesante e un professore così esigente, lei mi ha fatto sentire a mio agio e ha capito che c'era del potenziale in me. Mi piace pensare al fatto che la sua prima domanda per una me studentessa sia stata l'interpretazione di uno spettro protonico, e mi sono poi ritrovata circa 3 anni dopo a risolvere spettri NMR in danimarca durante il dottorato. Grazie per la fiducia che mi ha dato e che mi sta continuando a dare.

L'altro ringraziamento speciale va al Prof. Francesco Epifano. Lei è un esempio di quello che ogni professore dovrebbe essere e riesce a trasmettere la sua passione per quello che fa a tutti noi. Molte delle nuove esperienze fatte in 4 anni di lavoro nel suo gruppo sono state grazie a lei, e le sono estremamente grata per tutte le opportunità di crescita che mi ha dato.

Un'altra persona speciale che ho conosciuto è stata Serena. Il primo anno, durante la tesi, ti ho conosciuto come un tutor, una figura di riferimento per ogni mio dubbio o incertezza. Dopo questi 4 anni mi piace pensarti come molto di più, un'amica, una sorella. Grazie di cuore per tutto quello che fai per me.

Oltre alle soddisfazioni accademica, il dottorato a portato con se tante conoscenze, persone che mi hanno totalmente cambiato la vita.

Lucia (also known as Lusilla Plumbagina!!), è la prima della lista. Che dire, siamo diventate inseparabili (condividevamo gli stessi germi in laboratorio) ed ogni giorno era meglio del precedente. Quando sei andata via è stata difficile, passare da vederti ogni giorno all'averti così lontano è stata dura. Ma ogni volta mi rendi sempre più orgogliosa del percorso che hai intrapreso, con un lavoro completamente nuovo e diverso che hai iniziato. Sei una vera e propria bussiness woman, anche se mi manchi tanto. Molte delle cose conosco e so fare oggi, sono grazie a te (anche le colonne!!) e di questo te ne sarò eternamente grata.

Ovviamente non c'è due senza tre, e quindi devo ringraziare anche Federica, la zia americana e la mia compagna di pazzie. Piercing, bungee jumping e chi più ne ha più ne metta. Siamo persone che esprimono raramente i loro sentimenti, eppure ci siamo trovate in pieno. Anche se non ce lo diciamo mai, ti voglio un mondo di bene. Finisce sempre per capitare nei momenti meno opportuni, tipo dal nulla in mezzo a Pescara Centro.

Con loro anche Lorenza, che è rimasta qui con me nel grigiame chietino. Per pochissimo non ci siamo dottorate lo stesso giorno, ma forse meglio così, doppi festeggiamenti!

A big thank you goes to all the wonderful people I met back in Denmark, mainly Prof. Dan, Louise, Jianzhong, Chao, Uraiwan, Helle and all the master students, especially Simona and Cornelia who spent their vacation with me here in Pescara. You made me feel welcome and at home even though I was in a foreign country.

Oltre alle nuove conoscenze, un grazie speciale va anche a chi ci è sempre stato.

Inanzitutto le mie Chiare, Cav e Pierfi. Sono più di 10 anni che ci conosciamo, e il fatto che siamo rimaste così legate anche dopo aver preso strade diverse mi fa tanto piacere. In più nel giro di 20 giorni abbiamo tutte raggiunte un grande traguardo e sono estremamente fiera di voi.

Valeria, che, nonostante la lontananza, rimane sempre un punto fermo della mia vita. Questa tua avventura bi-settimanale qui a Montesilvano ci ha almeno permesso di vederci più spesso.

Un grazie speciale ai miei compagni di viaggio autisti autunnali, Davide e Sara. Mi fate ridere come pochi, ma allo stesso tempo mi capite più di tanti altri. *immagine dell'emoji che dà un bacio con le labbra rosse + sticker di Renato*. Subito dopo di voi un'altro grazie speciale va a Meli, Moli e aihmè anche Mali, perchè la gioia che mi danno quegli esserini è incommentabile. *sticker di Michele pazzo*

Ai miei genitori, che mi danno ogni giorno un supporto infinito. Avete appoggiato ogni mia scelta, senza fare domande e pronti a supportarmi in ogni dettaglio. Un grazie infinito perchè senza di voi non sarei arrivata qui.

Un ringraziamento speciale va anche ai nonni, Bianca, Antonio e Lina che vorranno esserci nel giorno della discussione anche se sarà in inglese. Vi voglio bene. Anche a te Nonno Gualtiero.

Infine agli zii, Galliano e Carla, e Riccardo e Valentina, con il mio cuginetto numero uno Alessio.