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Hortense Plainfossé
Université Côte d'Azur, CNRS, ICN, Parc Valrose, Cedex 2, 06108 Nice, France

Pauline Burger
Niss Active, Pépinière Innovagrasse, Espace Jacques-Louis Lions, 4 traverses Dupont, 06130 Grasse, France

Gaëtan Herbette
Aix-Marseille Université, CNRS, Centrale Marseille, FSCM, Spectropole, Campus de St Jérôme-Service 511, 13397 Marseille, France

Samuel Bertrand
Groupe Mer, Molécules, Santé-EA 2160, Faculté des Sciences pharmaceutiques et biologiques, Université de Nantes, 9 rues Bias, BP 53508, F-44035 Nantes Cedex 01, France
Thalass OMICS Metabolomics Facility, Plateforme Corsaire, Biogenouest, 44035 Nantes, France

Grégory Verger-Dubois
Niss Active, Pépinière Innova grasse, Espace Jacques-Louis Lions, 4 traverses Dupont, 06130 Grasse, France

Stéphane Azoulay
Université Côte d'Azur, CNRS, ICN, Parc Valrose, Cedex 2, 06108 Nice, France

Anne Landreau
Université Côte d'Azur, CNRS, ICN, Parc Valrose, Cedex 2, 06108 Nice, France
Faculté de Santé, Université d'Angers, 16 Boulevard Daviers, 49045 Angers Cedex 01, France

Nicolas Papaiconomou
Université Côte d'Azur, CNRS, ICN, Parc Valrose, Cedex 2, 06108 Nice, France

Xavier Fernandez
Université Côte d'Azur, CNRS, ICN, Parc Valrose, Cedex 2, 06108 Nice, France

Corresponding Author:
Nicolas Papaiconomou
Université Côte d'Azur, CNRS, ICN, Parc Valrose, Cedex 2, 06108 Nice, France

Anti-inflammatory and anti-aging potential of extracts and constituents from *Teucrium lucidum* L. aerial parts

Hortense Plainfossé, Pauline Burger, Gaëtan Herbette, Samuel Bertrand, Grégory Verger-Dubois, Stéphane Azoulay, Anne Landreau, Nicolas Papaiconomou and Xavier Fernandez

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Abstract
The property of hydroalcoholic extract of aerial part of *Teucrium lucidum* L. and some constituents were evaluated as antioxidant and as inhibitors of enzymes involved in inflammation using *in vitro* bioassays. Bioguided fractionation led to the characterization of 8 compounds, including 5 unknown ones in the Teucrium genus, and to their structural elucidation (NMR and HRMS experiments). Among them, 5,3',5'-trihydroxyflavone-7-O-β-xylosyl-(1'''→6'')-β-glucopyranoside is reported here for the first time. With the help of GC-MS analysis, 23 volatile compounds were identified and quantified in the *T. lucidum* extract, and a total of 38 compounds were finally identified through this study.

Keywords: *Teucrium lucidum*, Lamiaceae, HPLC-DAD-ELSD, GC-MS, *in vitro* cosmetic bioassays, wound healing

1. Introduction
Facing the urgent need to protect the environment and to preserve resources, consumers are profoundly changing their perception about the products they use daily and are notably rethinking the nature of cosmetics. In the last few decades, "Neutrality" has emerged as a crucial principle in most aspects of everyday life, to the point where consumers are now looking for natural, ethical, and sustainable ingredients that meet high quality standard as well as favour products integrating locally sourced products. The "natural" cosmetic industry is on the rise and is expected to reach USD 54.4 billion by 2027 because consumers reject synthetic ingredients in favour of sustainable alternatives, better for them and the environment [1, 2]. Apart from neutrality, consumers have also shifted their attention towards efficient cosmetics products, driving ingredient manufacturers to scientifically substantiate cosmetic allegations [3]. In 2018, the World Health Organization (WHO) reported proportion of worldwide population over 60 years will nearly double over the 2015-2050 period [4]. Added to this, consumers raise concerns about the impacts of their hectic lifestyle and their daily exposition to pollution on their skin. This explains why the "anti-aging skincare" segment occupies a premium position on the global cosmetic market. The anti-aging market has recently been determined to reach USD 271 billion by 2024 [5].
Skin is composed of three major layers. First, the epidermis constituted of keratinocytes is topped by the stratum corneum, a layer of dead cells that are exposed to the environment, preventing the penetration of exogeneous substances (microorganisms, etc.) and the dehydration of underlying tissues. It also supplies a mechanical protection against abrasion for those deeper layers. The epidermis interacts via the dermal-epidermal junction (DEJ) with a underlying richly vascularized and highly innervated dermis [6]. This structure is composed of fibroblasts and extracellular matrix (ECM) constituted notably of collagen and elastin fibres that provides strength and elasticity to the skin [7]. Finally, the hypodermis links the dermis to underlying structures, contains adipose tissue for fat storage and protecting cushioning [8].
Skin aging is a natural and very complex phenomenon that depends on several factors. Chronic skin aging implies functional changes and destructuration of ECM's components (elastin, collagen), mainly due to increased activity of matrix metalloproteinase or MMP [7], together with epidermal thinning, leading to the apparition of wrinkles, age spots and dryness [8, 9]. Other ECM components (Glycosaminoglycans, proteoglycans) are also affected during the aging process, ultimately leading to decreased amounts of individual functional dermal

Constituents [7]. Factors including skin's exposition to UV or pollutants, diet, lifestyle, trauma, cigarettes or alcohol consumption influence significantly the way a person ages [10], notablyvia the production of Reactive Oxygen Species (ROS) that induce oxidative stress. High levels of ROS may cause further degradative changes, notably via the activation of enzymes such as hyaluronidases, collagenases and elastases that specifically degrade structural cutaneous building blocks, hence further contributing to skin aging. Nowadays, numerous assays, assessing the cosmetic ingredients' bioactivities can be efficiently performed *in vivo* or *in vitro*. However, *in vivo* assays are often highly complex to implement and may raise ethical questioning due to the use of living organisms. *In vitro* bioassays, on the contrary, are cost effective, less time consuming and do not imply the participation of volunteers. Even if a bit less effective as the previous models, *in vitro* assays and notably enzymatic tests are very convenient for high-throughput screening and bioguided development of novel cosmetic ingredients. Hence, assessing the free radical scavenging (DPPH assay) and anti-inflammatory (Lipoxygenase assay) activities of plant extracts together with their abilities to modulate skin pigmentation (Tyrosinase) and to inhibit enzymes implied in the maintenance of skin structure (Elastase, collagenase and hyaluronidase assays) enables the identification of the ones potentially interesting for the development of natural anti-aging ingredients [7, 8].

Biodiversity is a rich source of highly diverse molecules that can be tapped for the development of novel natural active ingredients intended for the pharmaceutic, food, cosmetics, and personal care (PC) industries [11–13].

Mediterranean basin represents only 2% of the world's surface, but houses 20% of the world's total floristic richness [16]. Therefore, and because of a high rate of endemic species, it is therefore recognized as one biodiversity hotspot among the 34 world's most prominent ones [14, 15].

For the present study, 77 plants from the mediterranean basin were selected over 500 plants available at the laboratory. Selection was based on how accessible these plants are and the fact that they was no published data on their anti-aging activity. Starting from that selection, 91 aqueous and hydroalcoholic extracts obtained from these plants were investigated using *in vitro* bioassays for their ability to slow down skin aging. *In vitro* bioactivity screening was privileged because it enables the rapid and cost-effective examination of a large number of natural extracts without implying the participation of volunteers [17]. A total of 9 of these 91 extracts display interesting anti-aging bioactivities; among those hits, the extract of *Teucrium lucidum* L. aerial parts was selected for further analysis for both its interesting bioactivities and its novelty and endemicity to the Mediterranean basin. In fact, the phytochemistry of *T. lucidum* extracts was only poorly studied previously, mainly given the assess toxicity of many species in the Teucrium genus. To our knowledge, only one article mentions the identification of 5 ent-clerodanes diterpenoids (or neo-clerodanes) – teucvidin, Tuflin, teucrin F, teucrin G and 6α-hydroxyteuscordin – from an acetone extract of *T. lucidum* aerial parts collected in the Maritime Alps [18].

Teucrium lucidum L. (syn. *Chamaedrys lucida* (L.) Moench) is commonly known as shining germander because of its gleaming leaves (that why the epithet "lucida" is implicated to this species) [19, 20]. It is an orophilous species of shrub belonging to the Lamiaceae family, found at altitudes ranging from 200 to 2200 m, and sometimes even to 3000 m [21]. Its native range is South-Western Alps (South-East France and Northern Italy); common in Mediterranean climates, it is also distributed in Spain [22]. The vegetative plant can reach 20-60 cm high. This perennial species is characterized by dark green leaves with dented edges and pink to purple flowers by pairs (the flowering period lasts from July to August). Contrary to other Teucrium species, and notably to *T. chamaedrys* L., *T. polium* L. and *T. capitatum* L. which are known for their hepatotoxicity, *T. lucidum* is not toxic for human nor for animals [23].

To evidence links between phytochemical composition and bioactivities, analytical and semi-preparative HPLC-DAD-ELSD (High-Performance Liquid Chromatography coupled to Diode Array and Evaporative Light Scattering Detectors), GC-FID (Gas Chromatography with a Flame Ionization Detector), GC-MS (Gas Chromatography coupled to Mass Spectrometry), LC-HRMS and NMR (Nuclear Magnetic Resonance) were used to characterize this hydroalcoholic *T. lucidum* extract. These analyses enabled the identification of new compounds, never identified in the genus Teucrium before, the quantitation and the assessment of the bioactivities of some of its main constituents.

2. Materials and Methods

All chemicals were obtained from Merck (Darmstadt, Germany) unless otherwise stated.

2.1 Plant material

T. lucidum was identified by the botanists F. Boilot and J.-L. Polidori: aerial parts were collected in 2016 from Valdebore-La Colmiane, Provence-Alpes-Côte d'Azur, France, at an altitude of 1500 m. A voucher has been deposited in the botanical collection at the Natural History Museum, Nice under the reference number NICE-D-4445.

2.2 Plant extraction

After air drying a plant material, it was crushed into a fine powder. Extraction thereof was carried out by maceration using a mixture of water-ethanol (H_2O:EtOH 50:50 w/w), and a dry plant material / solvent ratio of 1/5 at room temperature (RT). Solvent and plant material were then muixed together in an erlemeyer using a magnetic stirrer (500 rpm) for 2 h. Resulting extract was filtered using a filter paper of 8-12 µm and concentrated until it was found to be dry using a rotary evaporator. At the end of the experiment, 13,7 g of extract were obtained, corresponding to an extraction yield of 17,6%.

2.3 Bioassays

Bioassays were carried out as reported presented [24, 25]. Procedure is briefly described in table 1. Experimental protocols were precisely described in supplementary information.

Table 1: Experimental conditions for bioactivities assays.

Activity	Enzyme/Reactive	Substrate	Positive controls	Ref.
Antioxydant assay – DPPH radical scavenging	Ethanol and acetate buffer (0.1 M pH 5.4, 50: 50)	386.25 µM DPPH in EtOH	3607.8 µM Trolox in DMSO with 3.433 mg/ml of a commercial extract of *Rosmarinus officinalis* L. in DMSO	[26]
Lipoxygenase assay	Soybean lipoxygenase in phosphate buffer (50 mM pH 8, 686.66 U/ml)	Linoleic acid in phosphate buffer at pH 8	1000 µM Quercetin hydrate in DMSO with 3.433 mg/ml of a commercial extract of *Arnica montana* L. in DMSO	[27]
Elastase assay	Porcine pancreatic elastase in Tris buffer (50 mM pH 8, 0.171 U/ml)	2.06 mM N-succinyl-Ala-Ala-Ala-p-nitroanilide in Tris buffer	8583.33 µM quercetin hydrate in DMSO with a 3.433 mg/ml commercial extract of *Rubus idaeus* L. in DMSO	[28]
Hyaluronidase assay	Hyaluronidase in hyaluronidase buffer (pH 7, 13.3 U/ml)	150 µg/ml of hyaluronic acid prepared in buffer at pH 5.35. After a 2nd incubation, solution containing 40 mM CTAB (cetyltrimethylammonium bromide) prepared in 2% NaOH solution	0.435 mg/mL tannic acid in DMSO with 3.433 mg/ml of a commercial extract of *Rubus idaeus* L. in DMSO	[29]
Collagenase assay	Collagenase in tricine buffer (pH 7.5, 53 U/ml)	5.15 mM 2-furanacryloyl-L-leucylglycyl-L-prolyl-L-alanine (FALGPA) in tricine buffer	3.433 mg/ml betulinic acid in DMSO	[17, 30]
Tyrosinase assay	Mushroom tyrosinase in phosphate buffer (pH = 6.8, 171.66 U/ml)	1 mM L-tyrosine (monophenolic activity assay) or L-DOPA (diphenolic activity assay) in phosphate buffer (1 mM)	3.433 mM kojic acid in DMSO and 3.433 mM phenylethyl resorcinol in DMSO	[31]

2.4 Analysis of the non-volatile fraction of the *T. lucidum* extract

2.4.1 High Performance Liquid Chromatography

HPLC-DAD-ELSD analyses

First, a solution containing 10 mg.mL^{-1} of each crude extract and fraction was prepared in chromatographic grade methanol (MeOH) and then filtrated over 0.45 µm PTFE syringe filter. Solution was then analysed using an HPLC Agilent 1200 system (Courtaboeuf, Ile-de-France, France) equipped with DAD (Diode Array Detector) and ELSD (Evaporative Light Scattering Detector). Operating conditions are as follows: 20 µL injection volume, 1.0 ml/min flow rate. Separations were carried out on a C18 column (Phenomenex, Le Pecq, Ile-de-France, France; Luna® 5 µm, 250 mm × 4.6 mm i.d.) equipped with a pre-column. The mobile phase consisted in a multistep gradient of water (A), acetonitrile (B) and 2-propanol (C), all acidified with 0.1% acid formic. Solvent gradient was as follows: 0–5 min, 5% B; 5–40 min, 5–55% B; 40–50 min, 55-100% B; 50–55 min, 100% B; 55–68 min, 100% C, 68–70 min, 100% C. DAD was set atwavelngths of 220, 254, 280, 330 and 366 nm. ELSD was used under the following conditions: 3.7 bars of nebulizer gas pressure, evaporative tube set at a temperature of 40 °C and a gain of 4.

Quantitation of non-volatile compounds

Quantitation of betulinic and oleanolic acids was carried out using and external calibration on the same HPLC instrument equipped with the same column using the same gradient of solvents acidified with 0.1% formic acid as detailed in the previsou paragraph. The quantitation was performed using calibration curves obtained using methanolic solutions of a standard prepared at six concentrations ranging from 0.05 mg/ml to 5.00 mg/ml. Each solution was injected in triplicate.

Semi-preparative HPLC

Fractions TL_FR3, TL_FR4 and TL_FR6 obtained by fractionation of the crude extract were evaporated until dry. Each fraction was then dissolved in methanol at a concentration of 50 mg/mL. Semi-preparative HPLC was carried out using a C18 column (Phenomenex, Le Pecq, Ile-de-France, France; Luna® 5 µm, 250 mm × 10 mm i.d.) and under the following conditions:: 30 µL injection volume, 3.0 ml/min flow rate. Multiple injections were carried out and the resulting sub-fractions were pooled together.

Elution was performed using solvents A, B and C detailed above and the three following solvent gradient conditions. Gradient 1 conditions: 35–50% B, 0–40 min; 50–100% B, 40–45 min; 100% B, 45–50 min. This gradient was used to further fractionate TL_FR3 recovered into sub-fractions TL_FR3A and TL_FR3B corresponding to the main peaks noticeable around 30 min on the HPLC-DAD-ELSD chromatogram of TL_FR3. Gradient 2 conditions: 15–20% B, 0–5 min; 20–30% B, 5–23 min; 30–42% B, 23–34 min; 42% B, 34–36 min; 42–100% B 36–42 min. This gradient was used to isolate some 4 mg of the main compound observed in fraction TL_FR4. Gradient 3 conditions: 15–15.5% B, 0–40 min; 15.5–25% B, 40–58 min; 25–100% B, 58–70 min. This gradient enabled the recovery of 6 sub-fractions were isolated from fraction TL_FR6 (3 mg of each sub-fractions TL_FR6A, TL_FR6B and TL_FR6F were recovered, 4 mg of sub-fractions TL_FR6C and TL_FR6D respectively and only 1 mg of fraction TL_FR6E).

2.4.2 High performance thin-layer chromatography

High performance thin-layer chromatography (HPTLC) analysis was carried out using 10 cm x 20 cm HPTLC silica gel 60 FR254 pre-coated plates (Merck, Darmstadt, Germany). Standards and samples were first applied either as bands 8 mm form the left edge and 10.3 mm apart or 12 mm from the left edge and 9.2 mm apart when 18 or 20 samples were deposited on the plate, accordingly. Application of these standards and samples was carried out using an automated ATS4 sampler (Camag, Muttenz, Switzerland). 2 µL of each standard and sample was applied on the plate. Solvent systems and revelation reagents were chosen according to phytochemical molecules to be analysed. On plate separation was performed in an automatic ADC2 developing chamber (Camag, Muttenz, Switzerland) with a tank previously saturated with the developing solvent system. Developing distance was 7 cm. Only for derivatisation reagent aniline diphenylamine o-phosphoric acid, developing distance was 6 cm according experimental conditions described by Ruppel and Morlock, 2015. After developing and drying for 5 min, plates were then dipped in detection reagent with the help of dedicated Camag immersion device, dried in a stream of warm air and finally sprayed with a specific revelation reagent. Bands were observed under visible and UV (254 and

366 nm) light using the TLC Visualizer (Camag, Muttenz, Switzerland).
For quantitative measurements, 15 samples were applied on the plate as 8 mm bands, 20 mm from the external edges and 11.4 mm apart). Five different volumes of standards (1.0, 2.0, 4.0, 7.0 and 9.0 µl) and 3 different volumes of extract (0.5, 1.0 and 2.0 µl) were deposited on the plates. The developed plates were scanned every 20 nm from 300 to 500 nm at 10 mm/s, with data resolution of 100 µm/step and slit size of 4.0 mm x 0.1 mm.
Data analysis was carried out using WinCATS Planar Chromatography Manager software (Camag, Muttenz, Switzerland).

Amino acids
Development system used in order to identify amino acids consisted in an ACN:H_2O 75:25 (v/v) system. Plate was then treated with a solution of 0.6 g ninhydrin in 190 ml of isopropanol and 10 ml of glacial acetic acid, as recommended by CAMAG [32]. Amino acid bands were detedcted using visible light after heating the plate at 120 °C for 8-10 min and using visible light [33].

Sugars
Sugars were revealed using a solvent system consisting in Butanol: Isopropanol: Boric acid: Formic acid 10:26:5:0.2 (v/v/v/v) and aniline diphenylamine o-phosphoric acid as post-chromatographic derivatisation reagent (prepared as recommended by CAMAG: 4 ml of diphenylamine are dissolved in 160 ml acetone, and 4 ml of aniline are added, followed by 30 ml of o-phosphoric acid; the mixture is then gently shaken to dissolve the precipitate before use) [32]. The plate is then heated at 110 °C for 10 min and observed under visible light.

Polyphenols and flavonoids
Development system used in order to identify polyphenols and flavonoids consisted in Ethyl acetate: Formic acid: Acetic acid: Water 100:11:11:26 (v/v/v/v) system. The plate is heated at 100 °C for 3 min and dipped while still hot in NP reagent (prepared as recommended by CAMAG: 1 g of 2-aminoethyl diphenylborinate is dissolved in 200 ml of ethyl acetate). The derivatisation process continues using PEG reagent (prepared as recommended by CAMAG by dissolution of 5 g of polyethylene glycol 400 in 100 ml of ethanol) [32]. The plate is then observed at 366 nm.

Less polar compounds
A solvent system constituted of $CHCl_3$:MeOH:H_2O 90:20:1.5 (v/v/v) is used as the development system to identify less polar compounds [34]. Sulfuric anisaldehyde was prepared as described in the literature [35]. 10 ml of sulfuric acid (10 ml) were carefully added to an ice-cold solution of MeOH (170 ml) and acetic acid (20 ml), followed by the addition of of p-methoxybenzaldehyde (1 ml).

Proanthocyanidins, *alkaloids, and anthocyanins*
Toluene: Acetone: Formic acid 45:45:10 (v/v/v) is used as the development system to identify proanthocyanidins, alkaloids, and anthocyanins [37]. Fast blue salt B reagent was prepared as previously described [33]. Fast Blue Salt B (500 mg) was dissolved in water (100 mL).

2.4.3 LC-MS analysis
HPLC-HRMS analysis
Fractions prepared in UPLC grade MeOH (10 mg/ml), were analysed by injection 2 µl on a from Shimadzu (Prominence Ultra-Fast Liquid Chromatography coupled to High Resolution Electrospray Ionization Mass Spectrometry (UFLC-HRESIMS) combining Ion trap and Time of Flight analysers (IT-TOFMS). Elution was carried out using a C18 column (100 x 2.1 mm, 2.6 µm) from Kinetex™ maintained at 40 °C using mobile phases (flow rate: 0.3 ml/min) consisting in water (solvent A) and acetonitrile (solvent B), both acidified with 0.1% formic acid, used in multistep gradient mode.
Gradient used for the analysis of fractions TL_FR3, TL_FR4 and TL_FR6: 5% B, 0 min; 5 - 45% B; 0 – 11.63 min; 45 - 100% B, 11.63 – 14.6 min; 100% B, 14.6 – 15.89 min; 100 - 5% B, 15.89 – 15.9 min; 5% B, 15.9 – 22.0 min.
Gradient used for the analysis of fraction TL_FR3B: 30 - 50% B, 0-8, 34 min; 50 - 100% B, 8.34 – 12.5 min; 100% B 12.5 – 13.89 min; 100 - 30% B, 13.9 - 20 min.
Detection using electron spray ionisation was carried out in positive and negative modes with the help of a coupled ion trap and TOFMS analyser within mass range *m/z* 100–1000, with a mass accuracy of 5 ppm and a resolution of 10,000 at *m/z* 500 [36]. MS2 acquisition was added in the protocol in the positive mode for a selection of samples to improve compounds identification.

HPLC-MS analysis
Subfractions prepared in methanol (chromatography grade, 10 mg/ml) were filtrated over 0.45 µm PTFE syringe filter and analysed using an HPLC 1100 system from Agilent (Courtaboeuf, Ile-de-France, France). HPLC was equipped with a DAD and coupled to an Electrospray-Ionization Mass Spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Separation was performed on a Luna 5 µm C18 column (250 mm × 4.6 mm i.d.) from Phenomenex (Le Pecq, Ile-de-France, France). Mobile phase consisted in a multistep gradient of water (A) and acetonitrile (B), both acidified with 0.1% acid formic with a flow rate: 0.3 ml/min. Gradient was 0–15 min, 10-100% B; 15-17 min, 100-10% B; 17–20 min, 10% B.
The detection using ESI was performed both in positive and negative modes with the following parameters: the capillary temperature was set at 275 °C, the capillary voltage at ± 4.5 kV and the cone voltage at ± 10 V. Nitrogen was used as nebulization gas with a cone flow rate of 50 L/h and a solvation flow rate of 10 L/h. Scans collected over the range *m/z* 50 to 1000 and MS/MS experiments were carried using a collision energy of ± 35 eV to fragment the most intense ions. The DAD was set at 214, 254 and 280 nm.

2.5. Analysis of the volatile fraction of the *T. lucidum* extract
2.5.1. Gas Chromatography
GC-MS analysis was performed using an Agilent 6890 gas chromatograph (Palo Alto, California, USA) equipped with a multifunction automatic sampler (Combi-Pal, CTC Analytics, Zwingen, Switzerland) and an Agilent MSD5973N mass selective detector. An HP-1 capillary column (100% poly dimethyl polysiloxane; 0.2 mm × 50 m; film thickness, 0.33 µm) and a split-type inlet liner packed with 10% OV-1 on Chromosorb W HP (Supelco TM Analytical-Merck, Darmstadt, Germany) were used.
Analysis is carreid out by first dissolving extract is dissolved in MeOH at a concentration of 0.1 mg/ml and filtered over PTFE membrane with a porosity of 0.45 µm. 1 µL is then

injected in splitless mode. Injector temperature was set at 250 °C. Carrier gas was helium with a constant flow mode at 1 ml/min. Oven temperature was programed to increase from 60 °C up to 180 °C at 2 °C/min, then from 180 °C to 300 °C at 6 °C/min and finally kept at 300 °C for 5 min. Acquisition was performed in scan mode (35–500 A.M.U. (Atomic mass unit)/s; scan rate: 3.15 scans/s) and mass spectra were genusted at 70 eV.

Identification of compounds was carried out comparing first mass spectra with literature, commercial libraries (NIST, Wiley, Indianapolis, Indiana, USA) and laboratory MS libraries built up from pure substances and second by comparing GC linear retention index (LRI) [37, 38]. Retention index was determined with a series of linear alkanes C_8-C_{24} analysed in the same conditions and used as reference.

GC-FID analysis was carried out under similar conditions. Carrier gas was hydrogen. Extract was dissolved in MeOH (at 0.1 g/ml) and filtered over PTFE membrane (porosity: 0.45 µm), before being injected (1 µl) in triplicates in splitless mode.

Quantitation of volatile compounds identified in *T. lucidum* extract and fractions thereof was carried out by external calibration in GC-FID using calibration curves obtained using seven concentrations of guaiacol or palmitic acid (Merck, Darmstadt, Germany) dissolved in a mixture of 2:1 (v/v) methanol and dichlormetahen. R^2 coefficient were in both cases considered as acceptable. Data acquisition and previously mentioned modules were monitored by a computer equipped with the Agilent Chemstation software.

2.6. Solid Phase Extraction (SPE) fractionation

Oasis HLB 6cc-500 mg (Waters, Milford, Massachusetts, USA) SPE cartridges composed of a copolymer of divinylbenzene and vinylpyrrolidinone were selected for the separation of polar compounds from phenolic ones. Cartridges were fitted in stopcocks and connected to a vacuum manifold (Bioblock Scientific, Illkirch, France). The sorbent was conditioned with MeOH (10 ml) and equilibrated with water (10 ml) as recommended by the supplier. The raw *T. lucidum* extract is diluted in water to reach a final concentration (5 mg/ml) as recommended by the cartridge manufacturer. To avoid the saturation of the sorbent during the loading step and the improper elimination in the dead-volume of molecules normally retained on the stationary phase, the quantity of extract loaded has to be selected carefully. The loading capacity of the cartridge used being of 10-15% of the sorbent mass (50-75 mg of sample can hence be loaded on this HLC 6cc – 500 mg cartridge), and as, as previously stated, polar compounds represent over 17% of the overall mass of the extract analysed, the extract (50 mg) were loaded on the cartridge, meaning that maximum 41.5 mg are really retained on the sorbent. The sample is then loaded on the cartridge and the dead-volume consisting in solvent and molecules that are not retained on the stationary phase, i.e. polar compounds and notably sugars corresponding to the group A, constitutes Fraction 1. Metabolites retained on the stationary phase are then eluted using respectively 4 solvents/systems of solvents (10 ml): MeOH:H_2O 1:1 (v/v), MeOH, MeOH: DCM 1:1 (v/v) and DCM to collect Fractions 2 to 5.

Fractionation of the *T. lucidum* extract

To identify the active fractions, *T. lucidum* extract was then fractionated over silica gel (normal phase; Geduran Si 60, 40 - 63 µm, Merck, Darmstadt, Germany) using 7 solvents/systems of solvents of increasing polarity and leading to the recovery of seven distinct fractions: FR1 (Cyclohexane: Diethyl ether 50:50 (v/v); extraction yield: 0.3%), TL_FR2 (Diethyl ether; extraction yield: 0.9%), TL_FR3 (Diethyl ether: Methanol 80:20 (v/v); extraction yield: 1.4%), TL_FR4 (Diethyl ether: Methanol 50:50 (v/v); extraction yield: 4.2%), TL_FR5 (Diethyl ether: Methanol 20:80 (v/v); extraction yield: 32.5%), TL_FR6 (methanol; extraction yield: 26.1%) and TL_FR6 (Methanol: Water 50:50 (v/v); extraction yield: 12.1%). Bioactivities of the resulting fractions were then evaluated, and their respective compositions were analysed using HPLC.

NMR

1D (^{1}H, ^{13}C) and 2D (COSY, HSQC, HMBC, NOESY) NMR spectra were recorded for all compounds isolated by semi-preparative HPLC in $CDCl_3$, in D_2O+TMSP or DMSO-d_6 at 25 °C on either 400 MHz or 500 MHz Bruker® Avance NMR spectrometers, or on 600 MHz Bruker® Avance II+ spectrometer equipped with TCI cryoprobe ^{1}H,^{15}N,^{13}C (Bruker, Wissembourg, France). Chemical shifts (δ) were expressed in Hz relative to $CDCl_3$, D_2O+TMSP or DMSO-d_6.

5,3',5'-trihydroxyflavone-7-O-β-D-xylosyl-(1'''→6'')-β-D-glucopyranoside 3. 1H-NMR (500MHz, DMSO-d6, Figure S1): 7.41 (brs, 2H, H2' and H6'); 6.93 – 6.83 (m, 1H, H4'); 6.77 (brs, 1H, H8); 6.71 (s, 1H, H3); 6.47 (brs, 1H, H6); 5.05 (d, J = 6.7, 1H, H1''); 4.16 (d, J = 7.6, 1H, H1'''); 3.97 – 3.91 and 3.64 – 3.61 (m, 2H, H6''), 3.71 – 3.66 and 3.02 – 2.99 (m, 2H, H5'''). ^{13}C-NMR (125 MHz, DMSO-d_6): 119.8 (2CH, C2' and C6'); 116.4 (CH, C4'); 104.6 (CH, C1'''); 103.3 (CH, C3); 100.4 (CH, C1''); 99.8 (C, C6); 95.1 (C, C8); 68.8 (CH_2, C6''); 66.3 (CH_2, C5'''). HR-MS: *m/z* 581.1502 ([M+H]+, $C_{29}H_{29}O_{15}$+; calc. 581.1501).

Experimental data of other compounds were precisely described in supplementary information: Figure S2 ^{1}H-NMR spectrum of trans-chlorogenic acid 1, (Z)-and (E)-p-4-O-β-D-glucopyranoside coumaric acids 37 & 38; Figure S3 ^{1}H-NMR spectrum of of angoroside A 4; Figure S4 ^{1}H-NMR spectrum of diosmetin-7-O-β-D-xylosyl-(1'''→6'')-β-D glucopyranoside 5. Figure S5 ^{1}H-NMR spectrum of Troponin 36. Figure S6 ^{1}H-NMR spectrum spectrum of Tuflin 29; Figure S7 ^{13}C NMR spectrum spectrum of Tuflin 29.

3. Results and discussions

In order to identify the molecules present in the extracts of *T. lucidum* and reveal what compounds are responsible for the bioactivity of such extracts, as well as isolate bioactive molecules, extracts were first analysed with several techniques, including HPLC, HPTLC, and GC-MS, as detaield below, and then fractionated.

3.1 Chemical analysis of raw *T. lucidum* extract

3.1.1 High Pressure Liquid Chromatography

The hydroalcoholic extract of *T. lucidum* was obtained by maceration of the species' aerial parts in 50:50 w/w mixture of H_2O and EtOH, respectively. An extraction yield of 17.6% was obtained. Extract displays excellent antioxidant activity, as well as promising anti-hyaluronidase and anti-inflammatory properties (Figure 1), even if it appears to be slightly less active as a reference commercial plant extract. No whitening, nor anti-collagenase and anti-elastase activities were evidenced for this extract. This latter activity was already reported for a dichloromethane-extract of *T. lucidum* [24].

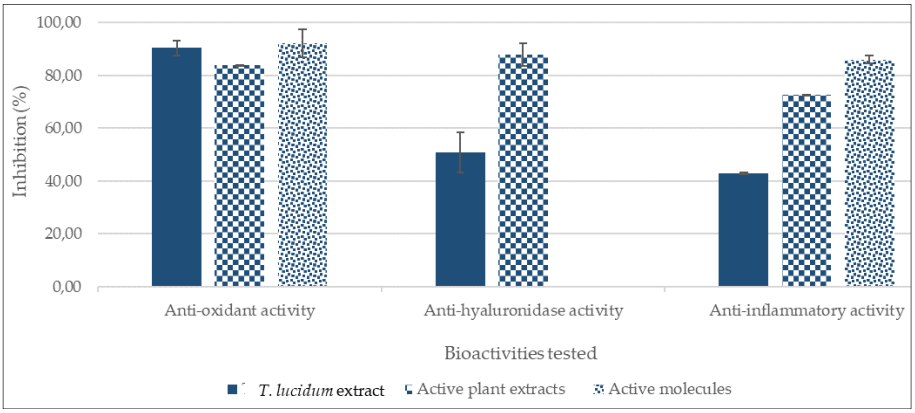

Fig 1: Bioactivities of the hydroalcoholic extract from *T. lucidum* aerial parts compared to the bioactivities of natural extracts and of individual active molecules renown in cosmetics for their respective activities (antioxidant activity: Rosmarinus officinalis extract and Trolox; anti-hyaluronidase activity: *Rubus idaeus* extract and anti-inflammatory activity: Arnica montana extract and quercetin hydrate).

HPLC-DAD-ELSD fingerprint of this extract (Figure 2) reveals the presence of two groups of compounds in the extract: group A is constituted of polar molecules displaying retention times shorter than 10 min, that correspond to sugars, amino acids, etc. Group B is composed of more lipophilic molecules eluting between 16 and 48 min. Corresponding UV spectra indicate a polyphenolic nature for the compounds f group B. Among those, five compounds tagged 1-5, were chosen to be characterized.

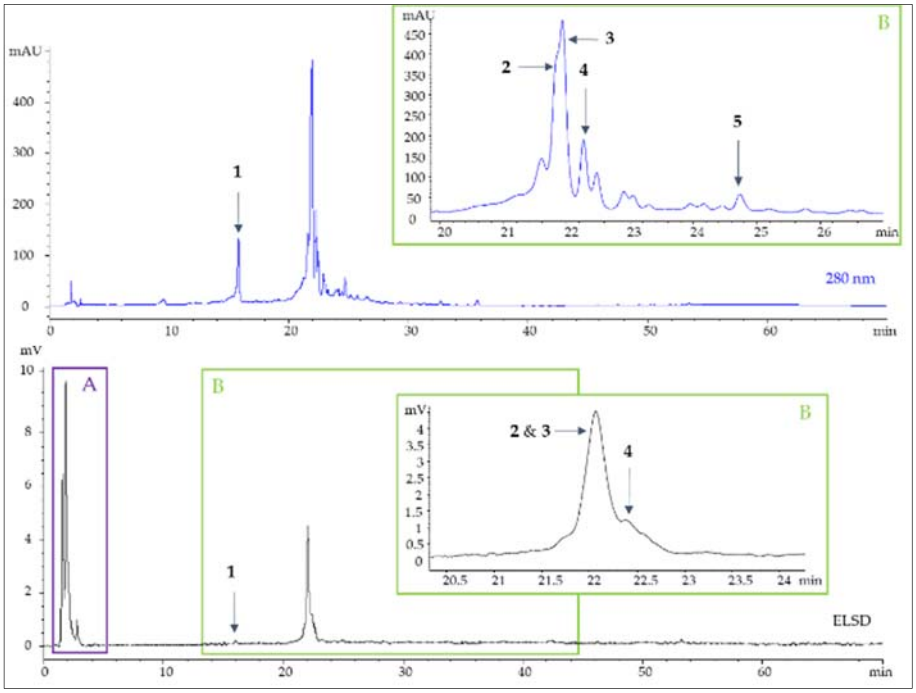

Fig 2: HPLC chromatograms obtained on a Luna® C18 column (150 × 4.6 mm; 5 µm) with a DAD at 280 nm and an ELSD. Major families of compounds identified in *T. lucidum* hydroalcoholic extract are presented (group A: polar compounds; group B: more lipophilic compounds).

High-Performance Thin Lyaer Chromatogrphy

High-Performance Thin Layer Chromatography (HPTLC) analyses was used in order to get phytochemical information and an insight into the nature of constituents present in the crude extract of *T. lucidum* aerial parts [25]. Results are shown in Figure 3. Evidence of amino acids have been reported previously using ACN: H_2O (75:25 (v/v)) as mobile phase and ninhydrin as reagent [26]. Some sugars have been revealed using Butanol: Isopropanol: Boric acid: Formic acid (100:11:11:26 (v/v/v/v) as elution system and aniline diphenylamine o-phosphoric acid as post-chromatographic derivatisation reagent [27, 28]. Under our present experimental conditions, glucose 6 (blue bland), saccharose 7 (dark blue band) and fructose 8 (brown band) were identified, as the main sugars constituting this *T. lucidum* extract by comparison of its Rf with the one of the corresponding standards (Figure 3a) [29]. HPTLC quantification revealed that the extract contained 80±1 mg/gextract, 46±3 mg/gextract and 50±3 mg/gextract glucose 6, saccharose 7 and fructose 8, respectively, representing a total of 17.6±0.7 % of the overall extract. Amino acids and sugars constitute the majority of the compounds of group A.

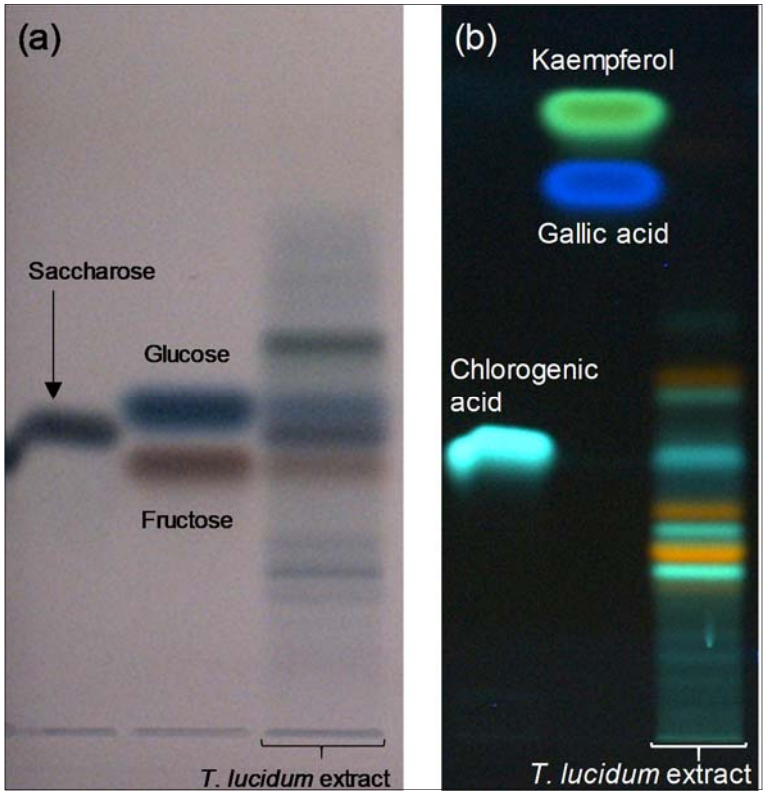

Fig 3: (a) HPTLC plate illuminated under visible light to visualize sugars present in the *T. lucidum* extract after derivatization with aniline diphenylamine o-phosphoric acid reagent. Tracks: 1: saccharose; 2: glucose and fructose; 3: *T. lucidum* extract. (b) HPTLC plate illuminated at 366 nm to visualize polyphenols and flavonoids present in the *T. lucidum* extract after derivatizations with NP and PEG reagents. Tracks: 1: chlorogenic acid; 2: kaempferol and gallic acid; 3: *T. lucidum* extract.

In order to reveal the presence of polyphenols and flavonoids in group B, the latter was analysed by HPTLC using NP/PEG 400 reagent [30]. Boric salt contained in the NP reagent forms a complex with phenolic compounds present in natural extracts, yielding a fluorescence depending on the nature of polyphenols [25, 31]. A blue color is characteristic of phenolic acids and/or coumarins. Similarly, using PEG 400 brings further insight into the type of flavonoids (flavones, flavonols, etc.) present in the extract [25]. Orange and green colors observed when exposing the plate at 366 nm indicate the presence of flavones and flavonols, respectively. Among phenolic compounds revealed by a light blue color on the HPTLC plate for group B, presence of chlorogenic acid has been confirmed based on a band coloration in blue and a Rf value similar to that of the corresponding standard (Figure 3b). Such a compound has already been identified in species belonging to the Teucrium genus, notably in *T. montanum* [32] and *T. chamaedrys* [33]. By co-injecting this standard with crude *T. lucidum* extract by HPLC-DAD-ELSD, compound 1 in Figure 2 appears to correspond to chlorogenic acid and was estimated to represent 1.0% of the total extract by external standard calibration. This compound was tested alone under the same conditions as that for the total extract and displayed significant antioxidant (94.38±0.01%; positive control (natural extract): 84.03±0.01%) and anti-inflammatory activities (76.19±0.01%; positive cosmetic control: 72.80±0.02%), in agreement with previous reports, [34]. Furthermore, to the best of our knowledge, we are the first to measure a very promising anti-hyaluronidase activity of 100.00±1.00% with a positive cosmetic control of 87.88±4.38%, for chlorogenic acid. Therefore, compound 1 significantly contributes to the total extract's bioactivities.

The presence and nature of less polar compounds was investigated using Solvent $CHCl_3$: MeOH: H_2O (90:20:1.5 (v/v/v). A red band was observed upon exposition of the track at 366 nm, characteristic of chelorophyll derivatives. Sulfuric anisaldehyde was further used as a derivatisation reagent in order to assess the occurrence of monoterpenes, triterpenes and steroids, yielding respectively blue, purple and grey colors under white light exposition [35]. No such band was observed, confirming absence or very low amounts of such compounds in the extract.

Finally, Toluene: Acetone: Formic acid (45:45:10 (v/v/v) and Fast blue salt B reagent were used in order to reveal the presence of proanthocyanidins and alkaloids, exhibiting red band under white light and black bands at 254 nm, respectively [36, 37]. This development system also enables the identification of anthocyanins as red bands under white light without derivatisation [36]. From HPTLC results, occurrence of alkaloids and of anthocyanins can be ruled out. Trace amounts of proanthocyanidins were evidenced.

3.1.3 Gas Chromatography

Analysis of volatile and semi-volatile compounds constituting *T. lucidum* extract was performed by GC-MS. Four compounds were identified: guaiacol 9, hydroquinone 10, 4-vinyl guaiacol 11 (which was already identified in the essential oil of *T. flavum*,) [38] and *pyrocatechol* 12. Proportion of these compounds in *T. lucidum* extract were assessed by external calibration in GC-FID, and their individual bioactivities were evaluated under the same experimental conditions as those used in order to evaluate raw *T. lucidum* extract (Table 1).

Table 1: Percentages and bioactivities of the volatile compounds identified in the *T. lucidum* extract determined by quantitation in GC-FID (for bioactivities: mean of percentage values of enzyme inhibition ± SEM, n=3).

Volatile compounds	RI[a,b] HP-1/RI literature	Proportion (%) in the total extract	Occurrence in Teucrium species	Bioactivities
Guaiacol 9	1086/1089	0.20	Never reported in the literature concerning the Teucrium genus	Antioxidant (75.48±0.01%)
Pyrocatechol 12	1198/1197	0.20	Never reported in the literature concerning the Teucrium genus	No Activity Evidenced Using The Bioassays Used
Hydroquinone 10	1271/1241	0.03	Never reported in the literature concerning the Teucrium genus	N.E. [C]
4-Vinyl Guaiacol 11	1300/1328	0.08	identified in the essential oil of *T. flavum* [38]	Anti-Inflammatory (58.58 ± 0.01%)

\[a] Compounds eluted from a HP-1 column. [b] RI = retention indices determined using the homologous series of n-alkanes from C_8 to C_{24}). [c] n.e.= not evaluated

3.2. Separation of molecules from groups A and B

Starting from our results, separation molecules from groups A and B was carried out in order to assess their respective contribution to the overall bioactivity of the total extract. Solid Phase Extraction (SPE) is a widely used method for separating compounds based on their differential affinities for a given stationary phase and was selected in this step of our work. Using a polymeric stationary phase (HLB cartridge), group A molecules, namely polar and highly hydrophilic molecules were not retained on the stationary phase. On the contrary, compounds from group B were found to share affinities with the stationary phase. The latter were thus retained on the stationary phase of a HLB cartridge and further eluted by use of organic solvents of appropriate polarity. Five separate fractions were collected. The first one corresponding to group A, not retained on the HLB cartridge, while the 4 other fractions corresponded to elution of compounds remaining in the cartridge and eluted using varying organic solvents. Details can be found in the experimental section.

Resulting fractions were then analysed by HPLC and bioactivities of Fractions 1 to 3 were assessed in the same analytical conditions as those used in order to evaluate raw *T. lucidum* extract. Fractions 4 and 5 were not tested because of low amounts collected. Fraction 1, composed of molecules belonging to group A, displayed no bioactivity. This implies that molecules belonging to group A are not involved in the bioactivity of the raw extract. Fraction 2 is composed by the remaining molecules belonging to group A, but is largely enriched in molecules from group B. This fraction displayed anti-hyaluronidase and anti-inflammatory activities similar to those of the raw extract. An antioxidant activity lower than that observed for the total extract was however observed. Remaining molecules of group B were collected in Fraction 3. Another group of molecules eluting between 45 and 65 min, named group C, not identified previously because of their very low proportions, was also found in fraction 3. This fraction displayed antioxidant and anti-hyaluronidase activities like those of the raw extract, as well as an anti-inflammatory activity higher than those of raw extract. This SPE fractionation enabled to reach the conclusion that molecules belonging to groups B and C are responsible for the raw extract's bioactivities. Hence the strategy adopted consisting in obtaining a larger amounts of groups B and C to characterize their phytochemical composition using HPTLC, GC-MS, GC-FID, HPLC-MS and HPLC-DAD-ELSD.

3.3. Bio guided fractionation of the raw extract and characterisation of major compounds

In order to characterize more precisely compounds constituting groups B and C, a bigger quantity of raw extract was fractionated on a silica gel column using 7 solvents/systems of solvents of increasing polarity. Fractionation yielded recovery of seven distinct fractions: TL_FR1 (Cyclohexane: Diethyl ether 50:50 (v/v)), TL_FR2 (Diethyl ether), TL_FR3 (Diethyl ether Methanol 80:20 (v/v)), TL_FR4 (Diethyl ether: Methanol 50:50 (v/v)), TL_FR5 (Diethyl ether: Methanol 20:80 (v/v)), TL_FR6 (Methanol) and TL_FR7 (Methanol: Water 50:50 (v/v)). The resulting fractions TL_FR1 to TL_FR7 were analysed using HPLC and bioactivities evaluated. Results of these bioassays are presented in Table 2. None of the fractions display any whitening nor anti-collagenase activity. Because of very low recovery yield for TL_FR1 and no remarkable bioactivity was observed, this fraction was excluded from further investigations. Recovery yields for TL_FR2 and TL_FR3 were low and revealed interesting anti-elastase and anti-inflammatory activities and no significant anti-oxidant and anti-hyaluronidase activities. Fraction TL_FR4 displayed moderate antioxidant and anti-inflammatory activities. Fraction TL_FR5 was obtained with the best yield but was found to exhibiting poor biological activities. Fraction TL_FR6 was recovered with a good yield as well and was found to exhibit moderate anti-inflammatory and promising anti-antioxidant and anti-hyaluronidase activities. Finally, Fraction TL_FR7 was recovered with a moderate but acceptable yield and was found to exhibit significant antioxidant properties and moderate anti-hyaluronidase and anti-inflammatory activities.

Table 2: Fractionation parameters and bioactivities of the fractions TL_FR1-TL_FR7 resulting from the fractionation of the *T. lucidum* extract.

Fractionation parameters and property	TL_FR1	TL_FR2	TL_FR3	TL_FR4	TL_FR5	TL_FR6	TL_FR7	Positive controls [a]	
								Active plant extract	Active molecule
Solvent system (400 ml of each)	Cyclohex: Ether 50:50 (v/v)	Ether	Ether: MeOH 80:20	Ether: MeOH 50:50 (v/v)	Ether: MeOH 20:80 (v/v)	MeOH	MeOH: H_2O 50:50 (v/v)	/	/
Fractions' mass	38 mg	116 mg	170 mg	526 mg	4068 mg	3259 mg	1510 mg	/	/
Yield	0.3%	0.9%	1.4%	4.2%	32.5%	26.1%	12.1%	/	/
Antioxidant activity	-	-	-	++	+	++++	++++	+++	++++
Anti-hyaluronidase activity	-	-	-	-	-	++++	++	+++	/
Anti-inflammatory activity	-	+++	+++	+	-	++	+	+++	+++
Anti-elastase activity	+	+++	+++	-	-	-	-	++	+++

(−): inhibition < 30%; (+): 30% < inhibition < 50%; (++): 50% < inhibition < 70%; (+++): 70% < inhibition < 90%; (++++): inhibition > 90%.

[a] Anti-inflammatory activity: *A. montana* L. extract and quercetin hydrate respectively; anti-elastase activity: *R. idaeus* L. extract and quercetin hydrate; antioxidant activity: *R. officinalis* L. and Trolox; anti-hyaluronidase activity: *R. idaeus* L. extract.

All seven fractions were further characterised using GC-MS, GC-FID, HPTLC and HPLC-DAD-ELSD (see section 2.4).

3.4. Chemical characterization of the resulting *T. lucidum* fractions

Chemical analysis of fractions from *T. lucidum* extracts were analyzed using gas chromatography, high pressure liquid chromatography and nuclear magnetic resonance techniques.

3.4.1. GC-MS and GC-FID analysis of T. lucidum fractions

Expect for the fraction TL_FR1, analysis of volatile and semi-volatile compounds constituting each fraction was performed by GC-MS: a total of 23 compounds was identified and when possible, their individual bioactivities were evaluated under the same experimental conditions as those used in order to evaluate *T. lucidum* extract (Table 3). of all 23 coumpounds, only guaiacol 9 identified by GC-MS was characterised by GC-FID as representing more than 0.1% of the total extract and was evaluated in terms of biological activities. As shown in Table 3, it was found to exhibit significant antioxidant activity, thus contributing significantly to the antioxidant activity of the total extract. Only 4-vinyl guaiacol11 was found to exhibit some anti-inflammatory properties but was found in very low proportions in the extract.

Table 3: Percentages and bioactivities of the volatile compounds identified in fractions of *T. lucidum* extract determined by quantitation in GC-FID (grey cells: compounds identified and directly quantified in the total extract, for bioactivities: mean of percentage values of enzyme inhibition ± SEM, n=3).

Ti	Volatile compounds	RI [a, b] HP-1/ RI lit.	Proportion (%) in total extract	Occurrence in Teucrium species	Bioactivity
TL_FR5	benzylic alcohol 13	1040/1033	n.d.[d]	Identified in essential oil of *T. polium* and *T. montanum* [39]	n.e.[e]
TL_FR4 TL_FR5 TL_FR6 TL_FR7	guaiacol 9	1086/1089	0.2	Never reported in *Teucrium* genus	Antioxidant (75.48±0.02%)
TL_FR2	nonanal 14	1101/1102	<0.1	Identified in *T. chamaedrys* [40]	Anti-elastase (52.08±0.02%)
TL_FR6 TL_FR7	*pyrocatechol* 12	1198/1197	0.2	Never reported in *Teucrium* genus	No activity using bioassays used
TL_FR5 TL_FR6	hydroxymethylfurfural 15	1221/1224	<0.1	Never reported in *Teucrium* genus	No activity using bioassays used
TL_FR7	hydroquinone 10	1221/1241	<0.1	Never reported in *Teucrium* genus	n.e.
TL_FR2	(E)-2-decenal 16	1252/1255	<0.1	identified in essential oil of *T. flavum* subspecies [41]	Anti-inflammatory (100.00±0.10%)
TL_FR6 TL_FR7	4-vinyl guaiacol 11[c]	1300/1328c	<0.1	Identified in *T. flavum* EO [38]	Anti-inflammatory (58.58±0.01%)
TL_FR4	tyrosol 17	1421/1427	<0.1	Identified in *T. polium* extract [42]	n.e.
TL_FR3	4-hydroxybenzene ethanol 18	1423/1425	<0.1	Never reported in *Teucrium* genus	n.e.
TL_FR1	ethyl 9-oxononanoate 19	1495/1502	n.d.	Never reported in *Teucrium* genus	n.e.
TL_FR2	lauric acid 20	1571/1567	<0.1	Identified in several varieties of *T. orientale* EO [43]	No activity using bioassays used
TL_FR2	myristic acid 21	1767/1770	<0.1	Identified in several varieties of *T. orientale* EO [43]	No activity using bioassays used
TL_FR1	octadecene 22	1790/1794	n.d.	Identified in *T. flavum subsp. flavum* EO [44]	Anti-elastase (65, 29±0, 01%)
TL_FR5	neophytadiene 23	1846/1829 c	n.d.	Never reported in *Teucrium* genus	n.e.
TL_FR1 TL_FR2 TL_FR3 TL_FR5	methyl palmitate 24	1923/1919	<0.1	Identified in *T. hyrcanicum* EO [45]	Anti-inflammatory (30.73±0,01%)
TL_FR2 TL_FR3 TL_FR5	palmitic acid 25	1978/1970	<0.1	Identified in *T. chamaedrys* EO [40] and *T. orientale* EO [43]	Anti-hyaluronidase (52.00±12.00%)
TL_FR5	methyl linoleate 26	2089/2069 c	n.d.	Never reported in *Teucrium* genus	n.e.
TL_FR5	ethyl linoleate 27	2147/2139 c	n.d.	Never reported in *Teucrium* genus	n.e.
TL_FR5	stearic acid 28	2168/2152 c	n.d.	Identified in *T. labiosum* [46]	Anti-elastase (73.45±0.01%)
TL_FR4	tuflin 29	2258/-c	<0.1	Identified in *T. lucidum* extract [18]	n.e.
TL_FR1	4,8,12,16-tetramethyl-hepta-decan-4-olid 30	2345/2364	n.d.	Never reported in *Teucrium* genus	n.e.
TL_FR4	1,2-epi-teucvin 31	2348/-c	<0.1	Identified in *T. lucidum* extract [18]	n.e.

[a] Compounds listed as a function of elution time using a HP-1 column; [b] RI = retention indices are determined on HP-1 column using the homologous series of n-alkanes (C8-C24); [c] Tentative identification; [d] n.d.= not determined; [e] n.e.= not evaluated.

3.4.2. HPLC-DAD-ELSD analysis of the resulting T. lucidum fractions

HPLC chromatograms for all 6 fractions obtained here are presented in Figure 4. All chemical compounds assigned to specific peaks have been elucidated here following a two-step methodology: the whole fraction was first characterized using HPLC-HRMS analysis, and major compounds were then isolated by semi-preparative HPLC to pursue identification and to assess their bioactivities. Further NMR characterisation and HPLC-MS/MS analyses were performed in order to identify those isolated compounds. Because fractions TL_FR3, TL_FR4 and TL_FR6 displayed significant bioactivities and were available in appropriate amounts, they were selected for further chemical characterization of *T. lucidum* extract, as detailed below.

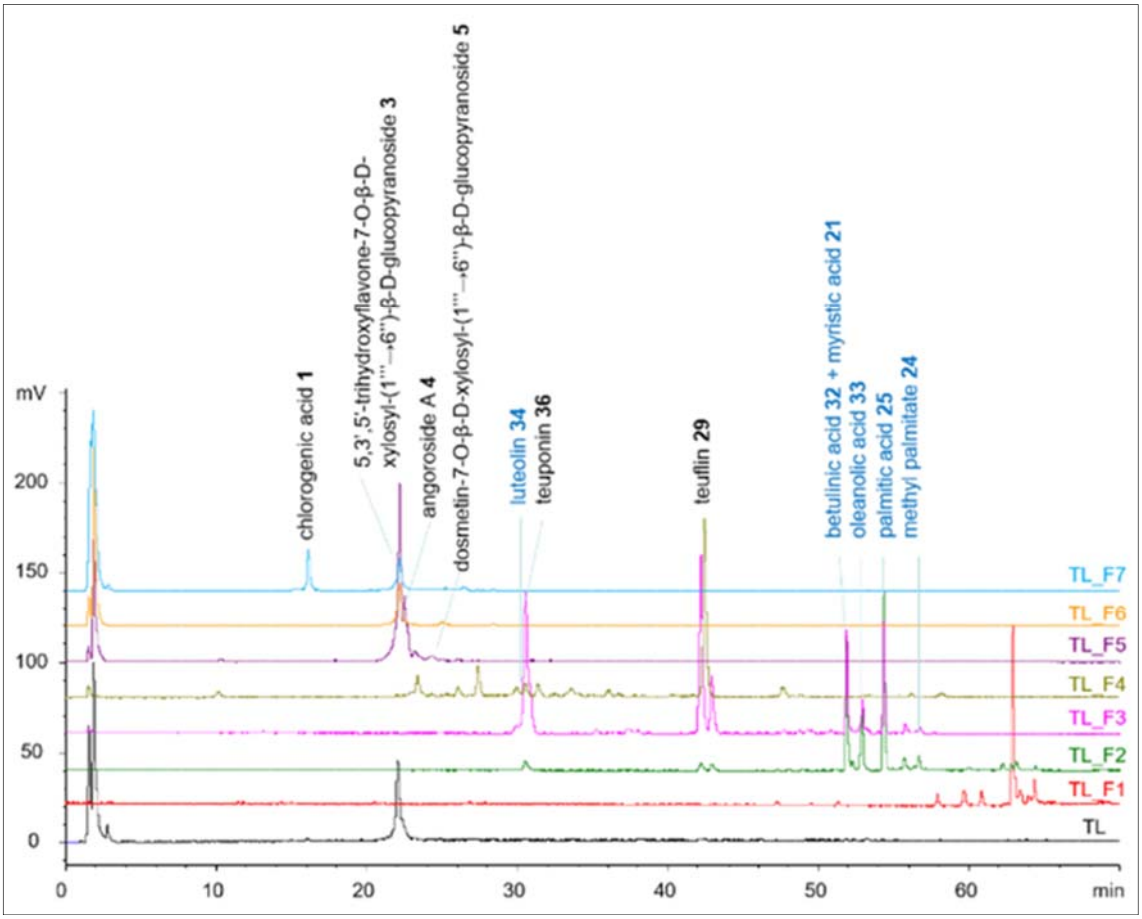

Fig 4: HPLC-ELSD chromatograms of the *T. lucidum* hydroalcoholic extract and of the seven fractions resulting from its fractionation (Luna® C18 column:150 × 4.6 mm; 5 µm) and compounds identified following isolation by semi-preparative HPLC (black) or by comparison with the corresponding analytical standards (blue).

HPLC-DAD-ELSD analysis of fraction TL_FR3 reveals group C, namely compounds eluting between 45 and 65 min, include terpenoids and fatty acids. The three major compounds were found to elute between 51 and 55 min. By co-injection of analytical standards, they were found to correspond to betulinic and oleanolic acids 32-33, two triterpenes already identified in Teucrium species [47, 48], and to palmitic acid 25, formerly identified by GC-MS. Betulinic and oleanolic acids 32-33 were quantified by external standard calibration and were foud to represent respectively 1.5 and 0.1% of this fraction. Bioactivities of these triterpenes were assessed *in vitro*: oleanolic acid 33 displays anti-inflammatory (53,93±0,01%) and anti-elastase (49,09±0,02%) activities, in agreement with previous reports, [49, 50] whereas betulinic acid 32 contributes to the anti-elastase (77,14±0,01%), anti-hyaluronidase (51,00±3,00%) [51, 52] and anti-collagenase (47,00±1,00%) activities of this fraction [52]. Occurrence of these 3 acids in fraction TL_FR2 was also deduced considering its HPLC chromatographic profile was found to be similar with that of TL_FR3. This was further confirmed by co-injections of standards for methyl palmitate and myristic acid previously identified by GC-MS.

The HPLC-DAD-ELSD analysis of fraction TL_FR4 reveals mainly occurrence of compounds from group B; among them, compound eluting at 31.1 min was identified as luteolin 34, a flavone already described in other Teucrium species, including *T. polium* [53]. The occurrence of this molecule was already suggested during the HPTLC analysis of this fraction using NP/PEG 400 reagents. It was thus confirmed by HPLC co-injection of the standard molecule together with fraction TL_FR4.

3.4.3. HPLC-HRMS analysis of fractions TL_FR3, TL_FR4 and TL_FR6

HPLC-HRMS analyses of the three selected fractions led to the identification of two compounds. Among all ionized compounds in TL_FR3, the one eluting at 11.48 min in HPLC-HRMS is characterised by a *m/z* 273.0762 [M+H]+ and a *m/z* 271.0612 [M-H]-. This is consistent with the molecular formula (MF) C15H12O5, that could correspond to several "trihydroxyflavone" structures, among which naringenin 35 appears to be the most consistent one from a phylogenic point of view. In fact, this flavanone was already identified in species from the Lamiaceae family, notably in

other Teucrium sp., including *T. chamaedrys* [54] and *T. gnaphalodes* [55]. This elucidation was confirmed by co-injection of the analytical standard using HPLC-DAD-ELSD.
In fraction TL_FR6, compound eluting at 6.02 min in HPLC-HRMS characterised by a *m/z* 355.1025 [M+H]+ and a *m/z* 353.0880 [M-H]-, consistent with a MF C16H18O9 was identified as chlorogenic acid 1. Identification was confirmed by co-injection of the standard together with TL_FR6 in HPLC-DAD-ELSD.

3.4.4. Compounds isolation by semi-preparative HPLC and NMR characterisation

The main compounds of the fractions of interest were isolated by semi-preparative HPLC and the resulted subfractions were characterized by HPLC-MS, MS/MS and NMR. Please note that all RMN have been collected in the supplementary file. Structural identifications were elucidated by comparing experimental data obtained from analyses of isolated compounds and UFLC-HRMS analyses of the 3 selected fractions with spectroscopic NMR data reported in the literature. Isolated compounds identified in *T. lucidum* extract are presented in figure 5.

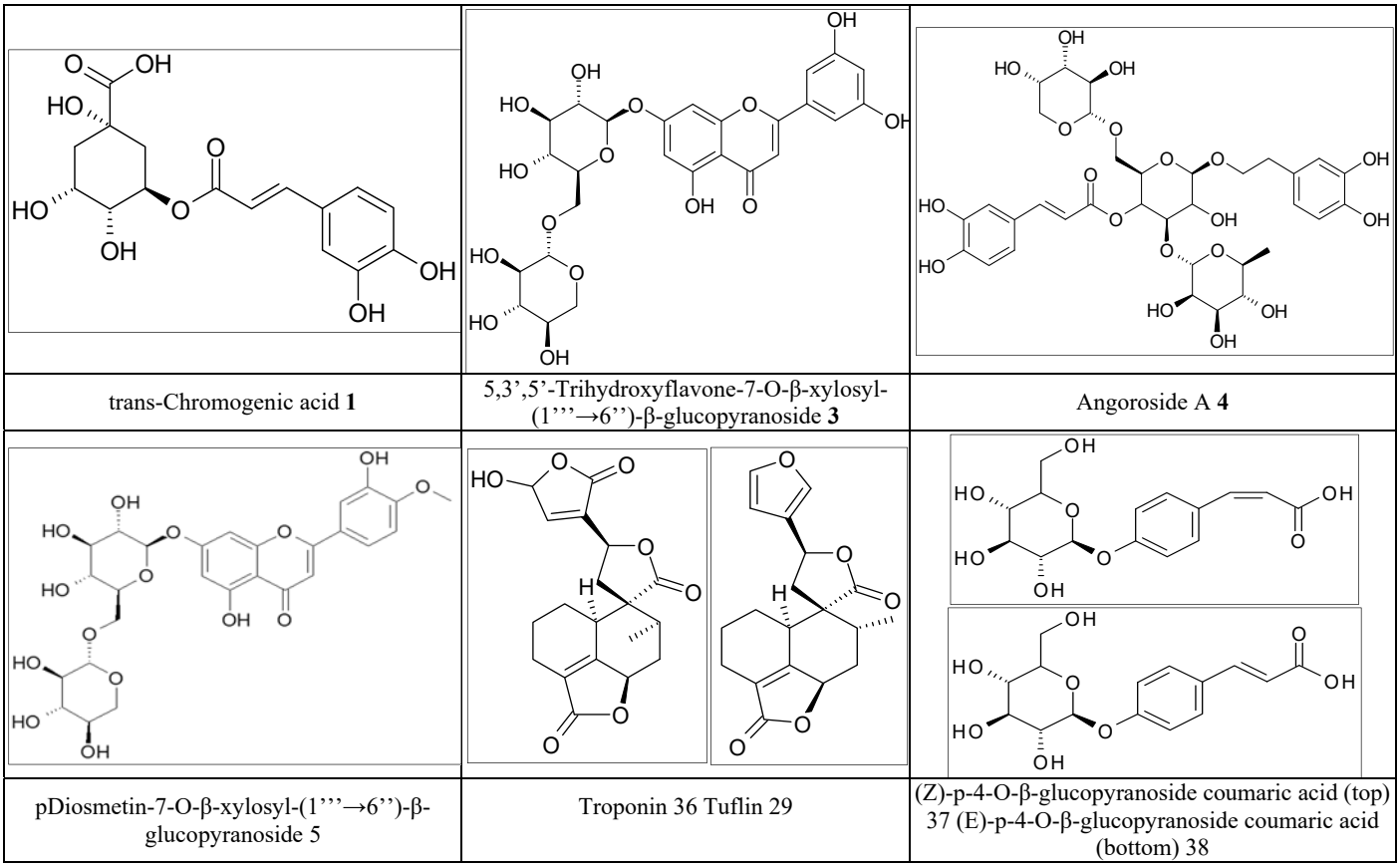

Fig 5: Chemical structures of compounds isolated from *T. lucidum* hydro alcoholic extract (compound **2**: isomer of compound 3).

Subfraction TL_FR3B isolated from fraction TL_FR3 corresponds to three compounds that co-elute on the semi-preparative HPLC column, but that are properly separated during the HPLC-HRMS analysis. Among them, one molecule is characterised by *m/z* 377.1219 [M+H] + and *m/z* 375.2746 [M-H] - and is therefore consistent with the formula C19H20O8.The two other compounds also observed in positive mode are characterised by *m/z* 361.1271 (ESI+) and *m/z* 359.1128 (ESI-). They could correspond to isomers characterised by the MF C19H20O7. The ^{1}H-NMR spectra highlighted one major compound: it corresponds to Troponin, a neo-clerodane diterpene already identified in an ethanolic extract of aerial parts of *T. japonicum* [56], referred to as compound 36. This compound could not be quantified by external standard calibration, and its bioactivities were not assessed as the standard was not available.
The major compound of fraction TL_FR4, eluting at 42 min in HPLC-DAD-ELSD, is characterised by a protonated molecule at *m/z* 329 [M+H]+ and a fragment at *m/z* 311 [M+H-H_2O]+. It corresponds to the formula C19H20O5 determined by the exact mass of the fragment [M+H-H_2O]+ (*m/z* 311.1287) obtained from the HRMS analyses of fraction TL_FR4. Together with GC-MS analysis of fraction TL_FR4, and in agreement with previous reports [57–60], NMR analysis enabled the characterisation of this compound as Tuflin 29 (or 6-epi-teucvin), a compound already described in *T. lucidum*, [18] and other Teucrium species [59, 61, 62]. This compound could not be quantified by external standard calibration as the corresponding analytical standard was not available.
In order to assess the bioactivity properties of Tuflin 29, the latter was isolated from TL_FR4 by column chromatography on silica gel. Results revealed that this compound displays some interesting anti-inflammatory activity (59.74 ± 0.01%).
The composition of the fraction TL_FR6 is more complex: 6 subfractions were isolated by semi-preparative HPLC. Because subfraction TL_FR6F helped in identifying compounds in subfraction TL_FR6C, structural characterisation of subfractions is not presented in the elution order, but according to the process used to identify them.
Subfraction TL_FR6A appeared to be composed of 2 molecules FR6A1 and FR6A2 according to the HPLC-MS analyses. Molecule FR6A1 was characterised by a deprotonated molecule at *m/z* 353 [M-H]- and by the adduct ion of *m/z* 707 [2M-H]-. Under its protonated form, it

corresponds to a molecule at *m/z* 355.1025 [M+H]+ as identified with the HPLC-HRMS analysis of fraction TL_FR6 and assigned to chlorogenic acid 1. Chlorogenic acid 1 is a polyphenolic compound evidenced earlier in the manuscript (paragraph 3.2.3). Its trans- configuration was determined by NMR analyses of the subfraction [63, 64]. Numerous studies described its bioactivities, as compiled in the review by Naveed *et al*. [34] The evaluation of the analytical standard's bioactivities confirmed its antioxidant and anti-inflammatory activities (respectively 94.38±0.01% and 76.19±0.01%) and highlighted for the first time its very interesting anti-hyaluronidase activity (100.00±1.00%).

Molecule FR6A2 was characterised by a deprotonated molecule at *m/z* 325 [M-H]- and by an adduct ion of *m/z* 371 [M+HCOO]-. The presence of fragment ion of *m/z* 163 indicates the occurrence of a hexose moiety linked to an aglycone. NMR spectra indicated the occurrence of two isomers for the molecule FR6A2, identified as the (Z)- and (E)- isomers of the 4-O-β-D-glucopyranoside coumaric acid by comparison with the literature. They were never described previously in the genus Teucrium [65] and are further referred to as compounds 37 and 38. HPLC-HRMS analyses could not discriminate between these two isomeric forms, hence justifying the fact that only 2 compounds FR6A1 and FR6A2 were observed. Given the amounts of both isomers recovered, no bioactivity evaluation could be undertaken. (E)- isomer was previously stated as displaying some antioxidant activity. [66]

^{1}H-NMR integrations of the ethylenic function of chlorogenic acid and coumaric acid part enabled to estimate the relative contents of each compound in the subfraction TL_FR6A: trans-chlorogenic acid 1 represents 43% of the mixture, while (Z)-4-O-β-D-glucopyranoside coumaric acid 37 represents 35%, and isomer (E)- 38, 22%.

Subfraction TL_FR6F consisted in one major compound FR6F, corresponding to a flavone compound according to the UV spectrum (data not shown). It was characterised by a protonated molecule at *m/z* 595.1653 [M+H]+ composed of two glycosylated moieties, one pentose (*m/z* 463) and one hexose (*m/z* 301), linked to an aglycone moiety of *m/z* 300. It was consistent with the MF C27H30O15 and with the MF C16H12O6 for its aglycone (fragment ion of *m/z* 301.0710 – ESI+) by correlation with HPLC-HRMS analysis of the fraction TL_FR6. By comparison with the literature, experimental NMR signals allowed to identify compound FR6F as diosmetin-7-O-β-D-xylosyl-(1'''→6'')-β-D-glucopyranoside, referred to as compound 5 identified in the hydroalcoholic extract in Figure 2 [67, 68]. This compound bioactivities were not assessed yet due to the low amount of compound recovered. Quantification using external standard calibration were not possible because corresponding analytical standard was not available.

Subfractions TL_FR6B and TL_FR6C, recovered at very close retention times, displayed UV spectra characteristic for flavones. They correspond to isomers displaying a protonated molecules at *m/z* 581.1502 [M+H]+ composed by two glycosylated moieties, one pentose (*m/z* 449) and one hexose (*m/z* 287), linked to an aglycone moiety of *m/z* 286. By comparison with the HPLC-HRMS analysis of the fraction TL_FR6, these compounds are consistent with the MF C26H28O15. NMR spectra exhibited similarities with diosmetin-7-O-β-D-xylosyl-(1'''→6'')-β-D-glucopyranoside 5 (compound FR6F), specifically considering carbon atoms C3, C6 and C8 in the aglycone moiety and the signals of protons H2', H4' et H6' (multiplicity and correlation observed on the COSY spectrum) suggested the aglycone is a 5,7,3',5'-tetrahydroxyflavone. The chemical shifts corresponding to the glycosylated moieties also appear to be similar to those observed for the corresponding moieties in diosmetin-7-O-β-D-xylosyl-(1'''→6'')-β-D-glucopyranoside 5 (FR6F), especially for carbons 1'', 6'', 1''' and 5''' and of the more discriminant protons. All these data led to the tentative identification of compound FR6C as 5,3',5'-trihydroxyflavone-7-O-β-D-xylosyl-(1'''→6'')-β-D-glucopyranoside, a new compound never described in the literature. It corresponds to compound 3 in Figure 2.

NMR data obtained for the molecule FR6B, corresponding to the compound 2 (Figure 2) suggests it would be an isomer of the compound 3, characterized by the same aglycone 5,7, 3',5'-tetraydroxyflavone and glycosylated moieties (one pentose and one hexose according to the HRMS analyses).

Subfraction TL_FR6D consisted mainly in compound FR6D, and in traces of compound FR6E correspond to flavonols according to their UV spectra (data not shown). These compounds display similar mass spectra characterised by a deprotonated molecule at *m/z* 755.2328 [M-H]-, the occurrence of one caffeic moiety (*m/z* 593) and two glycosylated moieties, namely one pentose (*m/z* 461) and one rhamnose (*m/z* 315), linked to an aglycone moiety of 316 g/mol. HRMS analysis of fraction TL_FR6 led a siggested formula C34H44O19 for compound FR6D, which is consistent with a fragment ion of *m/z* 593.1564 [M-H-caffeic moiety]- (ESI-). The NMR data compared with the literature [69] enabled its characterisation as angoroside A, never described previously in the Teucrium genus. It corresponds to compound 4 in Figure 2. It is known to display anti-inflammatory activity, inhibitory capacity against lipopolysaccharides, as well as positive influence on thromboxane B2 release [70]. It also exhibits cytostatic and cytotoxic activities against several cell lines [70, 71]. No assessment of its bioactivities was possible because of the low amount of compound recovered. Similarly, because the corresponding analytical standard was not available, its quantification by external standard calibration was not achieved.

Overall, a total of 36 molecules, divided in four categories of compounds, namely volatile/semi-volatiles, groups A, B and C were identified in *T. lucidum* extract. Their structure and contribution to *T. lucidum* biological activities is collected in Figure 6. Among these 36 molecules, 14 of them appear to exhibit biological activities on their own. Finally, as shown in Figure 4 and 6, results allowed to identify most compounds found on the chromatogram of *T. lucidum* extract.

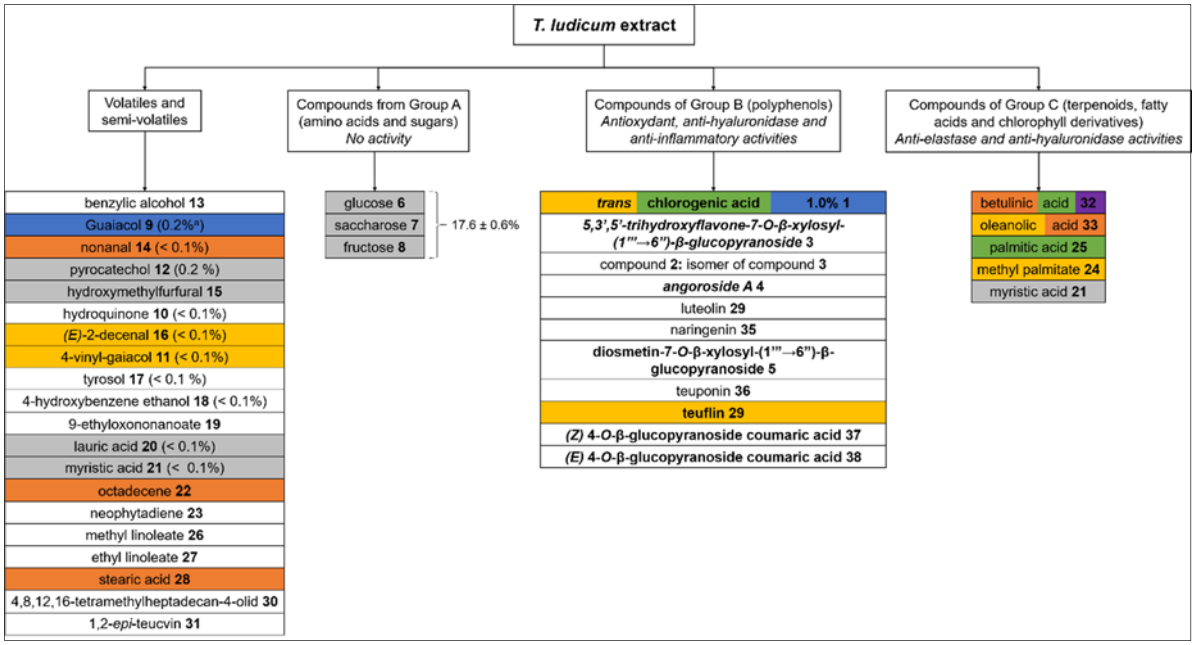

Fig 6: Main volatile and non-volatile constituents identified in the *T. lucidum* extract. A Proportion in the total extract (no percentage indicated means it could not be determined); Italic: tentative identification; Bold: described for the first time in the Teucrium genus; Blue cell: contributes to the antioxidant activity of the whole extract; Orange cell: contributes to the anti-elastase activity of the whole extract; Yellow cell: contributes to the anti-inflammatory activity of the whole extract; Green cell: contributes to the anti-hyaluronidase activity of the whole extract; Purple cell: contributes to the anti-collagenase activity of the whole extract; Grey cell: contributes to none of the assessed activity; White cell: bioactivities not determined.

4. Conclusions

A hydroalcoholic extract obtained by maceration of *T. lucidum* aerial parts exhibited excellent antioxidant activity, as well as promising anti-hyaluronidase and anti-inflammatory potencies. Phytochemical profile of this extract was successfully analysed in order to establish relations between molecular composition and bioactivities. Fractionation of the extract and subsequent HPTLC and HPLC-DAD-ELSD analyses revealed presence of three groups of compounds, named A, B and C in the extract. Group A was composed with polar molecules (sugars, amino acids, etc.) and represented approximately 17% of the total extract and was proven to play no noticeable role in the bioactivity properties of the extract. Groups B and C, composed with lipophilic moelcuels such as polyphenolic compounds, triterpenes, fatty acids, etc. These compounds were found to be associated to anti-hyaluronidase and antioxidant activities of the extract. A total of 38 compounds were revealed in this study and four compounds were isolated and structurally characterised: trans-chlorogenic acid 1, 5,3',5'-trihydroxyflavone-7-O-β-D-xylosyl-(1'''→6'')-β-D-glucopyranoside 3, angoroside A 4 and diosmetin-7-O-β-D-xylosyl-(1'''→6'')-β-D-glucopyranoside 5. Trans-chlorogenic acid 1 was estimated to represent 1.0% of the total extract and to contribute to the antioxidant, anti-inflammatory and anti-hyaluronidase activities of the total extract. Compounds 3,4 and 5 were identified for the first time in the Teucrium genus, but their bioactivities could not be assessed due to the low amounts of compound recovered.

Four additional compounds were identified from fractions of interest, and further identified by NMR. Troponin 36 and Tuflin 29 two neo-clerodanes typical of the Teucrium genus displayed moderate anti-inflammatory activity, as well as both (Z)- and (E)- isomers of 4-O-β-D-glucopyranoside coumaric acid 37 and 3, that were described for the first time in the Teucrium genus.

The bioguided fractionation also allowed to identify six other compounds already identified in the Teucrium genus; they contribute to the bioactivities of the *T. lucidum* extract.

We have demonstrated here that hydroalcoholic extract of *Teucrium lucidum* L. aerial parts is a promising antiaging ingredient. In order to isolate the most active ingredient, it would be necessary to mainly extract compounds from groups B and C, identified as the most bioactive groups in *T. lucidum* extract after phytochemical characterisation. The extraction procedure can be implemented to develop a corresponding proficient cosmetic ingredient via the use of appropriate extraction solvent system or via the use of activation techniques to assist the extraction, such as microwaves or ultrasounds. Based on the activities found in this study, hydroalcoholic extract of aerial parts of *T. lucidum* and its fractions, can be considered as potential natural substances appropriated to *in vivo* experiments related to inflammation and anti-aging processes.

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