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TITOLO TESI

MEDICINAL PLANTS FROM ANCIENT TRADITION AS A SOURCE FOR MATRIX PROTEASES INHIBITORS

STUDY OF CORRELATION BETWEEN BIOLOGICAL ACTIVITY AND PHYTOCHEMICAL PROFILE

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1. Introduction

1.1 The Extracellular Matrix: functions and homeostasis

The extracellular matrix (ECM) is defined as an organized and dynamic network of insoluble macromolecules surrounding cells. It is composed of biochemically distinct constituents, mainly proteins (e.g. collagens and elastin), proteoglycans, matricellular proteins (dynamically expressed extracellular non-structural proteins), polysaccharides and hyaluronic acid, which is an anionic non sulphureted glycosaminoglycan (without protein core).[1]

Structurally these components make up different ECM types, namely: the basement membrane (surrounding epithelia, endothelia, muscle, adipose tissue and nerves) the stromal or interstitial matrix, the elastic fibers (surrounding the connective tissue: skin, lung, large blood vessels), the bone, tooth and cartilage ECMs and the tendon and ligament ECMs.[2]

The overall ECM forms an insoluble scaffold, which is essential to maintain organs positional homeostasis and to support tissue architecture, integrity and compartmentalization (e.g. epidermis vs. dermis). As well as, the ECM constitutes the cells **microenvironment** and provides structural support and hardness for cell organization and integrity.

Despite its evident structural role, the ECM serves many other functions and in recent years it has been increasingly recognized as a highly dynamic and interactive network, which represents one of the most important regulators of both individual and collective cellular behavior. As a matter of fact, cells continually adapt to their environment by perceiving its diverse physical, biochemical and biomechanical properties, which are directly modulated by ECM composition, structure and organization. In this sense, the ECM, far from being an inert scaffold, is rather than a dynamic structure accomplishing several and versatile functions in the organisms.

First of all, the **physical properties** of the ECM play a key role in the maintenance of tissue polarity, which is essential for correct cells behavior. Moreover, modifications in ECM physical properties, depending on the contexts, are able to block or facilitate cell migration serving as anchorage site, barrier or movement track (fig.1 stages 1-2-3), influencing, in this way, development, regenerative and healing processes.

Another ECM clue, involved in cell-ECM communication, is represented by its **biochemical properties**, which pertain to its indirect and direct signaling capabilities. Indirectly, through its highly charged protein network (rich in polysaccharide modifications) the ECM can act as a ‘sink’

for growth factors and cytokines, in fact, by preventing their otherwise free diffusion, it helps to shape a concentration gradient of this molecules (fig. 1 stage 4).[3] Moreover, certain ECM components, including heparan sulfate proteoglycans and the hyaluronic acid receptor CD44, can influence the direction of cell-cell communication, functioning as a signal coreceptor and/or as 'signal presenter', which selectively bind different growth factors (fig. 1 stages 5 and 6).[4]

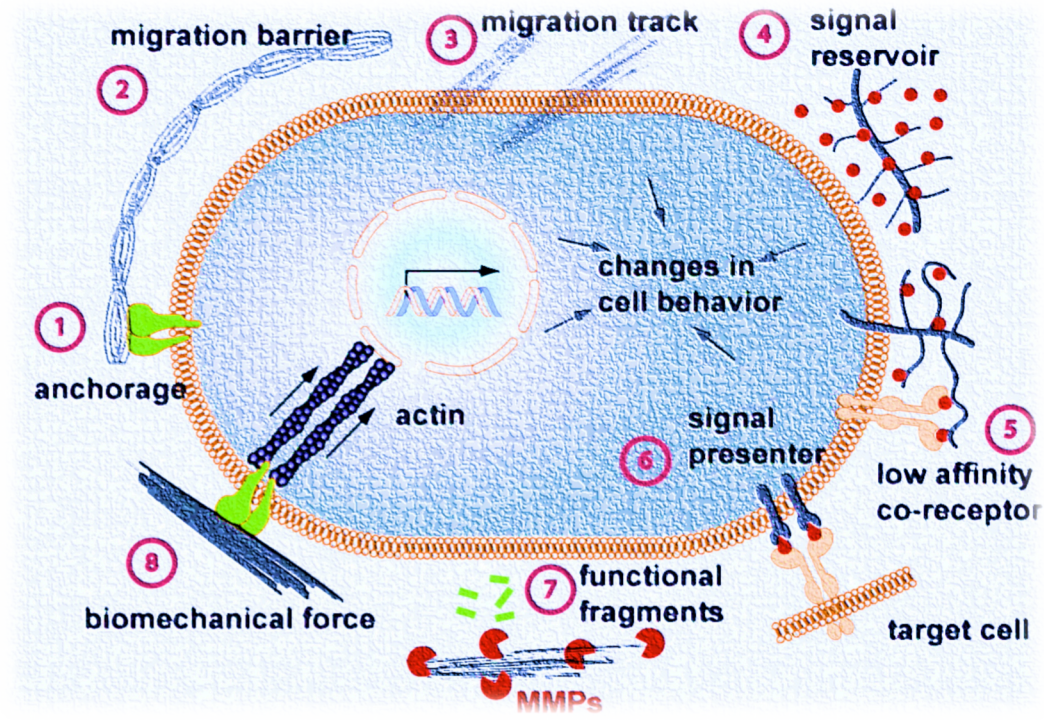


Fig.1 ECM-cell interactions. **Stages 1-2-3** show the importance of the physical property of the ECM in regulating cells behavior in particular during the migration processes. **Stage 4** shows the ability of ECM to acts as a sink for growth factor signaling molecules helping to shape a concentration gradient, by binding to these signals and preventing their otherwise free diffusion. **Stage 5** shows the possibility for certain ECM components to selectively bind different growth factors and function as a signal coreceptor or a presenter (**stage 6**) and help determine the direction of cell-cell communication. The ECM also directs signals to the cell by using its endogenous growth factor domains (**not depicted**) or functional fragment derivatives after being processed by proteases such as MMPs (**stage 7**). Finally, cells directly sense the biomechanical properties of the ECM, including its stiffness, and change a wide variety of behaviors accordingly (stage 8). (The figure is adapted form Pengfei Lu J. Cell Biol. 196, 395-406)

The ECM is also able to direct signals to the cell by its endogenous growth factor domains (not depicted in fig.1) or functional fragment generated after being processed by proteases (fig. 1 stage 7).

Lastly, cells directly sense the **biomechanical properties** of the ECM, for instance perturbations in ECM stiffness induces a wide variety of cells behaviors accordingly.

Considering all the possible ways for cell-ECM interaction, it results clear that ECM is involved in a huge number of physiological processes such as: development, morphogenesis, tissue repair and remodeling, cells adhesion, migration and proliferation, differentiation.

However, it's important to underline that cell-ECM interactions are definitely reciprocal, thus the ECM properties are, in turn, constantly influenced by intrinsic and extrinsic stimuli. This concept lead to figure out the ECM as a structure subjects to a continuous dynamic remodeling in its microenvironment, even when the macroscopic topology remains mostly unchanged (the latter changes only in response to growth or repair stimuli).

The dynamic nature of the ECM microenvironment is related to the concept of **ECM hemostasis**, which referred to the **correct balance** between the synthesis and degradation of ECM components.

Considering all the crucial physiological functions of the ECM, it results clear that perturbations in its homeostasis lead to the development and progression of several pathological conditions. For instance, the ECM remodeling is essential for normal wound healing processes but its excessive deposition, forming an aberrant ECM, determines fibrotic and degenerative diseases including, among the others, tumor cell invasion and metastasis. [5]

Several different factors are responsible for the tightly control of ECM homeostasis, most prominent among them are enzymes such as the **matrix metalloproteases (MMPs)**. As a consequence, the prolonged misregulation of these proteases is frequently observed in many multifactorial diseases.

1.2 Matrix Metalloproteases (MMPs): a valuable target for the drug discovery

1.2.1 Structural features

MMPs belong to the metzincins family and are multidomain calcium-dependent zinc (II)-containing endopeptidases.[6]

The majority of the MMPs are consisted of four distinct domains, namely: N-terminal pro-domain, catalytic domain, hinge region, and C-terminal hemopexin-like domain (fig. 2).

Regarding the membrane-type MMPs (MT-MMPs), they additionally contain a transmembrane domain, which anchors them in the cell surface.[7]

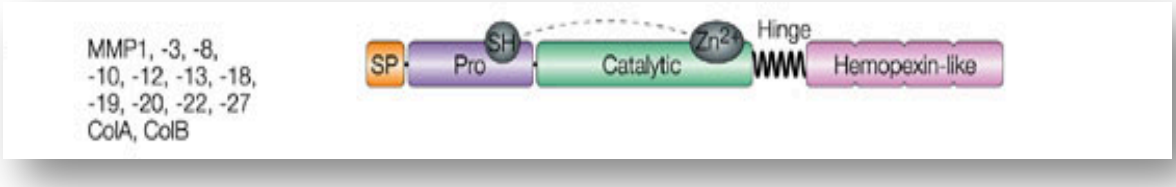


Fig.2 Structure of the MMP family basic domain.

The catalytic domain of this proteases superfamily is characterizes by two distinct regions: a groove centered on the catalytic zinc ion and an S1′ site, the latter plays a significant role in determining the **substrate specificity**.

Thus, characteristic of the active site grove is the presence of Zn (II) binding motif (HEXXHXXGXXH) in which the zinc atom is coordinated by three histidine residues (fig. 3); as well as a glutamic acid residue which facilitates catalysis and a strictly conserved methionine-containing tight 1,4 β-turn, forming a hydrophobic cleft for the catalytic zinc ion.[8]

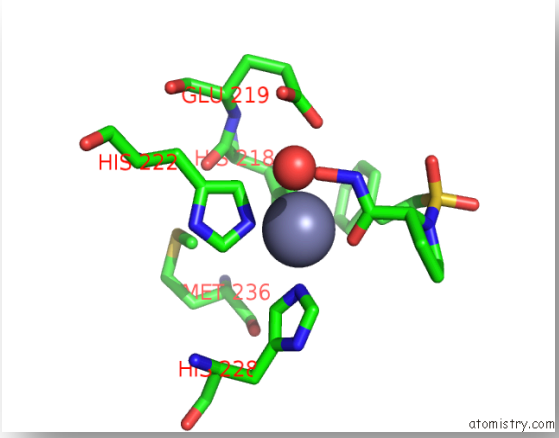


Fig.3 Characteristic catalytic domain of MMPs, the gray ball represents the Zn (II) ion.

Furthermore, the catalytic domain is characterized by the presence of a second ‘structural’ zinc (II) ion and two or three calcium ions, which stabilize the structure. The catalytic mechanism of MMPs (most probably) involves the carboxylate group of the highly conserved glutamate residue (in the

catalytic pocket), which draws a proton from a zinc-bound water molecule allowing its nucleophilic attack on the substrate's scissile amide bond.[9]

This process is facilitate by the positively charged zinc (II) catalytic ion, which allows to stabilize a negative charge at the carbon of the scissile amide and a conserved alanine residue, which helps to stabilize positive charge at the nitrogen of the scissile amide.

Since the catalytic domain is strictly conserved in this proteases family, the substrate specificity is often determine by non-catalytic ancillary domains.[10]

The C-terminal domain is present in almost all of the MMPs except MMP-7 and MMP-23, and seems to regulate the enzyme activity.[11]

1.2.2 biological role and regulation of MMPs

The earliest descriptions of MMPs were proposed in 1949 and they were defined as depolymerizing enzymes, which could be involved in tumor growth. After about 13 years, the first MMP was identified in 1962 as the protease responsible for the digestion of fibrillar collagen and was thus primarily named collagenase and subsequently MMP-1.[12] Nowadays, 23 different MMPs (17 secreted and 6 membrane bound) have been identified in humans and although heir catalytic domain results highly conserved, they differ for many other characteristics, in particular with respect to substrate specificity, cellular and tissue localization, membrane binding and regulation.

Regarding MMPs role in the organisms, they have been historically perceived as proteases responsible for the modulation and regulation of the ECM turnover by direct hydrolytic degradation of its components.[13] Nowadays, the foregoing appears not sufficient to provide a complete picture of MMPs biology, in fact the knowledge about substrate repertoires and physiological pathways under MMPs control result still inadequate. In fact, the dogma that MMPs are just matrix degraders has been resoundingly overturned, and more comprehensive **degradomics approaches** have reveled the possibility of MMPs to **process 'non-matrix' substrates** and therefore to play a 'multifunctional' role in the organisms.

Moreover, in order to better frame the MMPs biological relevance, it is crucial to focus on the consequences of their cleavage activity. The latter can, in fact, determine the liberation of biologically active proteins such as cytokines, growth factors and chemokines from their membrane-anchored proforms. Although degradomics is still in its 'infancy', this approach reveled that MMPs are effectors able to generate protein species with vastly differing activities from a single, original gene product. [14] These findings make the MMPs a highly versatile class of enzymes

responsible for a multitude of physiological roles, many of which, as above discussed, are still not fully understood.[15]

Some examples of physiological and pathological roles involving MMPs are reported in tab. 1; among them is noteworthy the intriguing activity of the MMPs in cellular apoptotic mechanisms, in fact, they can affect cell survival and proliferation both positively and negatively. These opposing roles played by the proteolytic activity of MMPs involve different mechanisms of actions. For instance, the interaction with cell surface receptors stimulates apoptosis and on the other hands the cleavage of other cell surface receptors, including proteinase-activated receptors (e.g. MMP-12), is involved in shedding of ICAM and CD44 during apoptosis of endothelial and epithelial cells (thus impeding apoptosis). [16]

Moreover, the MMPs can also promote a type of apoptosis induced by inadequate/altered cell-ECM interaction, the so called: **anoikis** (term coined in 1994).[17] The physiological role of the anoikis is related to processes such as: tissue remodeling and neoangiogenesis and it becomes a pathological event when determines decreased cancer-cell apoptosis influencing both tumor growth and angiogenesis.[18]

The excretion of the MMPs is due to different connective tissue and pro-inflammatory cells including: fibroblasts, osteoblasts, endothelial cells, macrophages, neutrophils, and lymphocytes.[17] Under normal physiological conditions, the activity of these enzymes is tightly regulated at multiple levels, first of all **transcriptional** controlled by: inflammatory cytokines, growth factors, hormones, cell-cell and cell-matrix interaction. Moreover, the MMPs are expressed as **inactive zymogens**, which are subsequently processed by other proteolytic enzymes (such as serine proteases, furin, plasmin, and others) in order to remove the prodomain, which limits entrance and catalysis of the substrate by blocking the catalytic zinc (II) ion via the so called 'cysteine switch' mechanism. The 'cysteine switch' motif PRCGXPD is a polypeptide segment, which lies in the substrate binding pocket, the -SH group of the cysteine in this propetide coordinate with the catalytic zinc ion keeping pro-MMPs inactive by preventing the binding to the zinc atom to the water molecule essential for catalysis. Thus, the MMPs are activated when the Cys-Zn (II) bond is disrupted and the active site is made available for the water molecule and the substrate.

Several agents, other than proteases, can also readily activate MMPs, including mercurial compounds, -SH reagents and chaotropic agents (this property is often used to activate pro-MMPs in the laboratorial assays). Moreover, oxidants such as HOCl and $ONOO^-$ activate pro-MMPs by 'cysteine switch' mechanism, this activation process might take place in the organism under inflammatory conditions. [19-20]

In addition to the activation of the zymogens, the MMPs activity is also regulated by inhibition of the active forms by two major types of **endogenous inhibitors**: α_2-macroglobulin (and related proteins) and various tissue inhibitors of MMPs named TIMPs, which are a group of four proteins of 21-30 kDa.

Human α_2-macroglobulin is a plasma glycoprotein (725 kDa) constituted of four identical subunits of 180 kDa. It inhibits especially MMPs in fluid phase through the formation of a complex, which is rapidly cleared by a receptor-mediated endocytosis.[21] The crystallographic structure of TIMP-MMP showed that the mechanism of inhibition involved the TIMPs N-terminal amino group and the carbonyl group of Cys1, which expels the water molecule bound to the zinc atom, bidentately chelate the MMPs catalytic zinc atom.[11]

In pathological conditions this controlling mechanisms are deregulated and the equilibrium is shifted toward increased MMP activity and consequent tissue degradation and the developing of a huge number of diseases (Tab. 1).

In addition to the genetic, several environmental, dietary and life-style related factors, including smoking,[22] lead to an altered level of MMPs in the tissues, serum and saliva, affecting the ECM turnover.

PHYSIOLOGICA PROCESSES	PATHOLOGICAL PROCESSES
Angiogenesis Apoptosis Blastocyst implantation Bone remodeling Cervical dilation Embryonic development Endometrial cycling Hair follicle cycling Immune response Inflammation Nerve growth Organ morphogenesis Ovulation Postpartum uterine involution Wound healing	Arthritis Alzheimer's disease Atherosclerosis Breakdown of blood-brain barrier Cancer Cardiovascular disease Central nervous system disorders Corneal ulceration Emphysema Fibrotic lung disease Gastric ulcer Guillian-Barre disease Liver cirrhosis Liver fibrosis Metastasis Multiple sclerosis Nephritis Neurological disease Osteoarthritis (OA) and Rheumatoid arthritis Periodontal disease Photoaging Skin ulceration and non healing wounds Sorby's fundus disease Vascular disease

Tab.1. MMPs involvement in some physiological and pathological processes (table is extracted form Verma P., 2007 [17]).

1.2.3. *MMPs as a factor of virulence of human pathogens infections*

For various human pathogens, including virus, bacteria, fungal and protozoa, the first step to invade and colonize the host organism is the attachment to host tissues, thus the interaction with ECM is of prime importance in this regard. Among other factors, it was reported that the invasive capacity of various microorganisms is linked to their ability to secrete serine proteases, collagenases and others MMPs that eventually degrade laminins and collagens. For instance, secreted proteases produced by invasive pathogens can breach tissue barriers such as the blood-brain and blood-cerebrospinal fluid (CSF) barriers and cause infections in the central nervous system. Moreover, during the infection, an increase in the ECM degradation is also due to the host MMPs activation as a consequence of the inflammatory responses. On the other hands, some pathogens can also manipulate this mechanism, inducing host MMPs expression during invasion in order to degrade ECM.[23-24] In addition, the partially degraded and exposed ECM components, generated during the ECM degradation process, are defiantly other attractive targets for adherence of pathogens.[25]

For this reasons, in recent years, MMPs inhibitors have been also considered as valuable antimicrobial therapeutics both to inhibit the invasive capacity (virulence) of pathogens and to control host ECM degradation.[26 27] In fact, controlling the microbial-dependent laminin/collagen interactions might be an attractive target for future antimicrobial therapeutics.

1.2.4. *Synthetic inhibitors of MMPs*

Considering their crucial role in several pathological events, the MMPs have been a highly active set of targets for the design of new therapeutic agents.

Some common features among the molecules endowed with MMPs inhibitory activity has been reported: i) a zinc-binding group (ZBG) as functional group capable to chelate the catalytic zinc (II) ion [e.g. hydroxamate ($CONH\text{-}O^{-}$), carboxylate (COO^{-}), thiolate (S^{-}), phosphinyl (PO_2^{-}), etc.]; ii); at least one functional group that provides a hydrogen bond interaction with the enzyme backbone in order to recognize the target enzyme and to achieve improved binding potency iii) one or more substrate-like hydrophobic fragment(s) able to determine MMPs specificity fitting the deeper S1'

subsite by van der Waals interactions.[28] On this base several synthetic MMPIs have been produced but although the first MMPs inhibitor **(MMPI)** was synthetized in the late 1970's, until now the only FDA medically approved MMPI (in the United States) still remains Periostat® (doxycycline hydrate, an analog of antibiotic tetracycline), which is prescribed (as MMPI) for the therapy of periodontal disease.[29] The scant success of the synthetics MMPI has largely been due to the disappointing results obtained in the clinical and preclinical studies. In particular they showed poor bioavailability and selectivity and several undesirable **side effects**, such as: tissue toxicity, promotion of liver metastasis and frequent occurring musculoskeletal syndrome.[30] For this reason plants and natural products can be considered an alternative valuable source in order to find active compounds able to modulate the activity of MMPs with an improved side-effect profile.
A large number of natural products and vegetal extracts have been investigated, as MMPI and some of them were found active.

1.3 Medicinal plants and natural products as MMPs inhibitors

A considerable amount of preliminarily investigations have been carried out on crude and refined extracts derived from plants or pure natural compounds in order to investigate their ability to modulate MMPs activity. The promising results of these studies have lead to define plants a 'gold mine' and 'green ally' for the maintenance of ECM homeostasis and to prevent and treat MMPs related diseases. Despite these increasing evidences, the potential of natural products in this field remains largely under-exploited. Objective of this paragraph is to provide an overview of some class of natural product found active in regulating MMPs activity. According to a research accrued out by www.scopus.com on December 2015, the most investigated and promising class of secondary metabolites are the flavonoids followed by the alkaloids. Concerning, terpenes and quinons the results obtained using the name of the general class are not really representative and it results more interesting to search directly using the name of their subclass (e.g. saponins, anthraquinons). The results obtained are depicted in fig. 4.

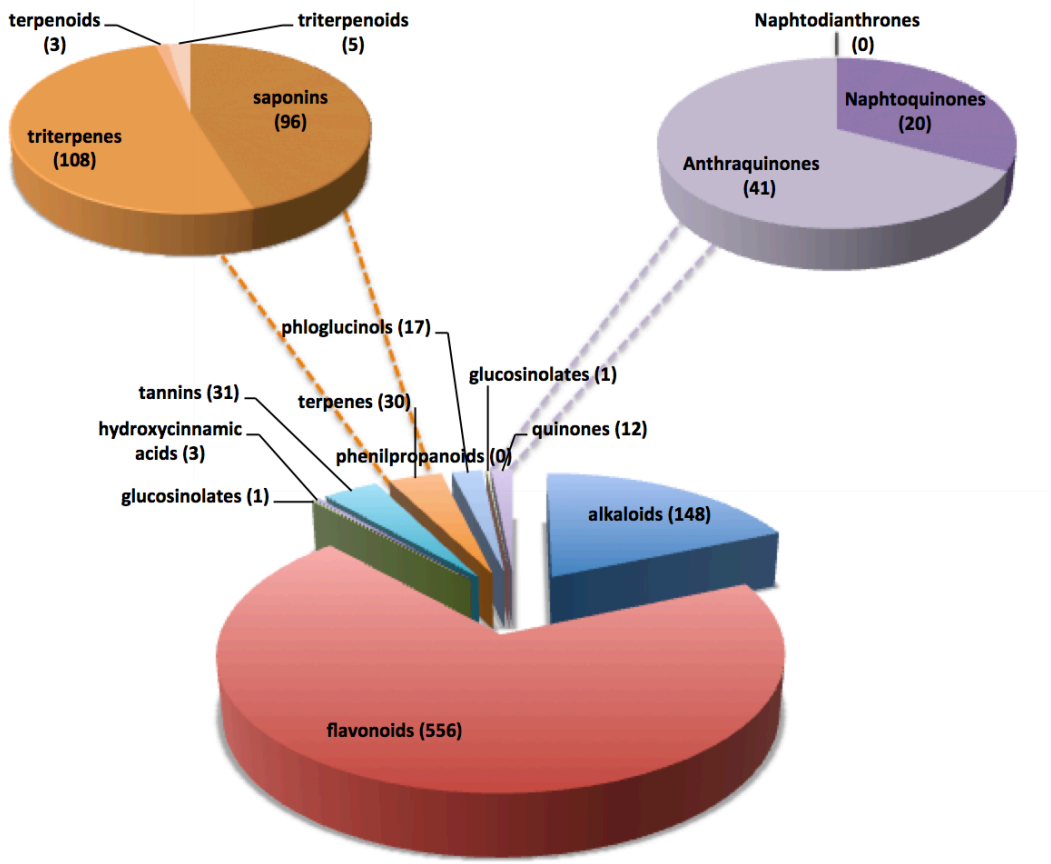


Fig.4 Number of articles (reported in brackets) found using www.scopus.com and searching in: Article Title, Abstract, Keywords: MMP and each reported class of secondary metabolites. In the case of terpenes and quinones a more specific research for subclass showed a highest number of articles.

Considering the highest number of publications found for the flavonoids, a more deep research targeted on the subclass of flavonoids and MMPs was carried on. As showed in fig. 5 the highest number of publication is focused on catechins and gallochatechins, which are particularly abundant in the *Camellia sinensis* (Tea).

The research was also extended to flavonoids and the specific MMPs targeted (fig. 6) and the highest number of publication involves MMP-2 (collagenase) and MMP-9 (gelatinase) followed by MMP-1 (fibroblast collagenase), while few results are reported for the modulation of other MMPs and no results were found for MMP-4-5-11-15 and MMPs from -17 to -23.

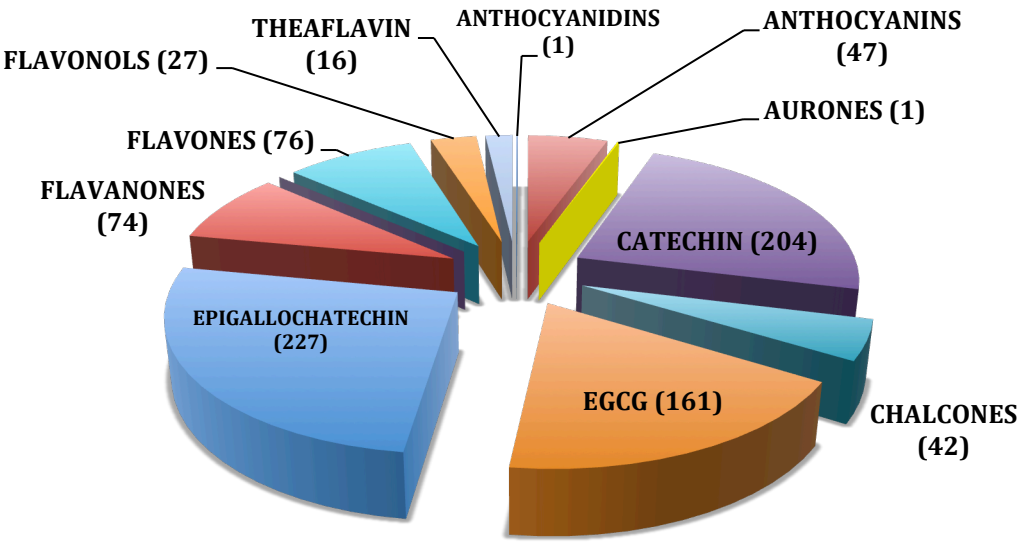


Fig. 5 Number of articles (reported in brackets) found using www.scopus.com and searching in Article Title, Abstract, Keywords for MMP and each reported class of flavonoids.

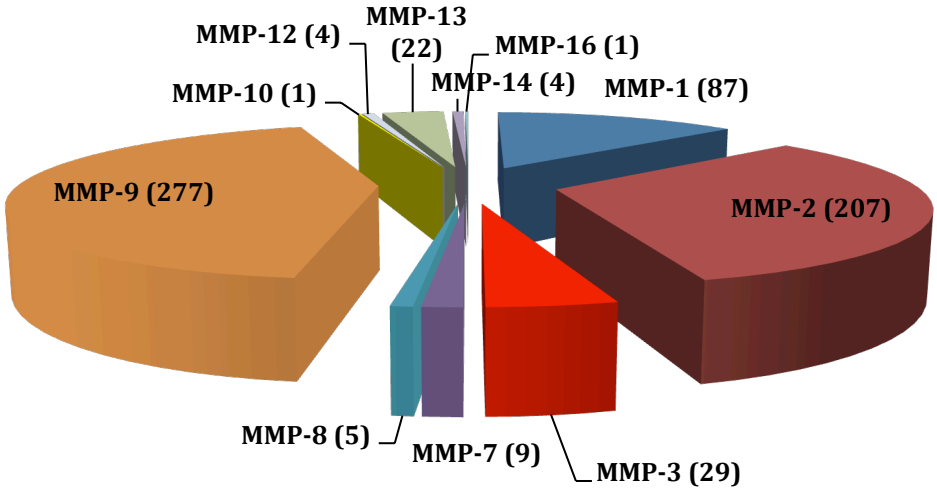


Fig. 6 Number of articles (reported in brackets) found using www.scopus.com and searching in Article Title, Abstract, Keywords: flavonoids and different MMP type. Searching for MMP-4-5-6-11-15-17-18-19-20-21-22-23 no results were found (not shown in the graphic).

1.4 Ethnobotany and 'reverse farmacology'

The use of medicinal plants for the prevention and treatment of diseases represents the cultural heritage handed down by traditional medicines, from which is possible to draw in order to identify plant sources as starting point for drug discovery studies.[31] The reevaluation of ancient traditions and the upgraded knowledge in this area is not only finalized to support the ethnobotanical use of these plants but also to discover new therapeutic potentials especially for the treatment of diseases of current interest such as complex **multifactiorial diseases**. In this context, the combination of traditional knowledge and modern science constitutes a significant innovation giving rise to the so called **'reverse pharmacology'**, which translates the canonical 'laboratory-to-clinic' approach to the 'clinics-to-laboratories' one, in which safety is awaited to be a starting point.[32-33]

The concept of "reverse pharmacology" was coined in India studying Ayurvedic medicines, and was also championed by the Chinese in the 1950s. The saving in time and cost coming from this approach is due to the substantial experience of traditional use, which increases the chances for a remedy to be found effective and safe. Certainly, before proceeding to clinical studies, it is important to establish that the remedy is unequivocally considered safe. In this context, WHO guidelines state that: *"If the product has been traditionally used without demonstrated harm, no specific restrictive regulatory action should be undertaken unless new evidence demands a revised risk-benefit assessment*." [34] WHO maintains the position that there is no requirement for pre-clinical toxicity testing and evidence of traditional use or recent clinical experience is sufficient. [35] Moreover, same plants are often traditionally used both as a food and medicine and clearly no toxicological tests are required for foods. Pre-clinical toxicity testing is only required for new medicinal herbal products which contain herbs with no traditional history of use. Therefore, if preliminary field studies have shown that the preparation is of common and ancient use and no known important side effects were reported, toxicological studies are unnecessary.[36]

1.5 Metabolomics: a new tool to analyze complex matrices

Metabolomic is defined as: '*the area of research which strives to obtain complete metabolic fingerprints (the metabolome), to detect differences between them and to provide hypothesis to explain those differences'*.[36] Metabolites are small molecules (molecular weight of 30-3000 Da), thus this category doesn't include polymeric biomolecules such as polysaccharides, lignin, peptides, proteins, DNA and RNA. The most suitable analytical strategy for metabolomics results, indeed, a **non-targeted approach**, which exclude an *a priori* knowledge of biologically interesting metabolites to detect. This approach allows to carry on an inductive (hypothesis generating) experiment instead of a hypothesis testing one.

Following this strategy, metabolomics can be figured out as a snapshot of an organism, showing the quali-quantitative composition of the metabolites at a given time point. It provides a platform from which it would be possible to answer various biological questions starting from an observation-based systems biology hypothesis, in which a broad spectrum of observations are possible (e.g. metadata). Thus, in order to extract information from a metabolomics analysis, it's required a large number of samples to compare in order to identify and underline the possible correlations between the observations.

In addiction to the other 'omics' (genomics, transcriptomics, proteomics and others), the study of the metabolome and the consequent database obtainment recently emerges as a tool for diagnosis of disease and evaluation of drug toxicity. Moreover, non-targeted metabolomic represents a valuable and innovative tool to analyze the complex mixture represented by plant extracts. However, it is important to remember that metabolomic is a tool useful to reveal slow changes related to lengthier processes (such as development and aging) but to measure and define faster metabolic processes it is necessary to study the fluxes (e.g. fluxomics) rather than the concentrations of metabolites, using labeled isotopes such as 13C.[37]

1.5.1 ***NMR-based metabolomics***

Among the different analytical technics used to perform a metabolomic analysis, nuclear magnetic resonance (NMR) spectroscopy has been used as a major analytical tool and NMR-based methabolomic protocols are validated for humans body fluids, microorganisms and plants

samples.[38-39] In particular, ^{1}H NMR has advantages over other techniques because it facilitates high-throughput analysis, in fact it is fast, simple, highly reproducible and avoids samples derivatization treatments. Moreover, NMR analysis provides information on the absolute quantity of metabolites because the signal intensity is only determined by the molar concentration and thus the ratio and amount of components in a mixture can be determined. Furthermore, in order to obtain comprehensive structural information, including stereochemical details, NMR is also the most suitable analytical tool.[40]

On the other hand, the weakness of NMR lies in its low sensitivity compared to other techniques (especially MS). However, recent technological developments including improvements in hardware (e.g. high-resolution NMR, cryoprobe, microprobe), pulse sequence and spectral acquisition protocols are going to ameliorate this aspect.[41] In some cases, another problem related to the 1H-NMR-metabolomics, is represented by signals overlapping (especially in the complex spectra obtained from plant extracts). This limitation can be solved in many cases using multidimensional-NMR spectroscopy, which allows the spread of the resonances in a second dimension, even if these methodologies are more time-consuming because of the long acquisition time required.

To date, numerous applications of NMR-based metabolomic have been reported. In particular, referring to plants, it has been used for: the quality control of botanicals or foods,[42 - 43] chemotaxonomy (classification[44] and characterization[45-46]), analysis of genetically modified plants [47-48] and interactions between plants and other organisms,[49] post-genomic study of plant models with respect to variations induced by perturbations including environmental changes and physical, biotic, abiotic or nutritional stress[50] or to identify biomarkers involved in plant resistance to pests and diseases.[51]

Moreover, recently, it was introduced the possibility to link the chemical profile of plant extracts to their bioactivity data using NMR-metabolomics combined with projection-based multivariate data analysis (PLS-DA, PLS).[52-53-54] This is a system biology approach in which bioassays data are combined with metabolomics and this represents an important innovation and a valid alternative to solve the major challenge in the studies of medicinal plants: **identify the active compounds into a complex biological matrix** (that includes also synergism and prodrugs). This new approach is now rapidly developing for studies on traditional medicine and is sometimes useful for studies of reversed drug discovery or reversed pharmacology, which start from an ethnobotany-based rational.

1.5.2 ***UV-Vis Spectroscopy based metabolomics***

The main drawbacks of the most common used techniques for the metabolomics (NMR, MS) are the expensive equipment, involving large operational or maintenance cost. For this reasons this kind of protocols may result prohibitive for some research group or not suitable for the industry to be applied in routine works. A valuable alternative can, sometimes, be provided by the use of ultraviolet-visible (UV-Vis) and near infrared (NIR) spectroscopy, which are based on inexpensive equipment and very common procedures saving economic resources. UV-Vis spectroscopy is one of the most common techniques used in routine analysis and therefore the possibility to develop metabolomics protocols using these instruments is always interesting.

In tab. 2 are reported some values of maximum absorbance for different class of molecules and natural products even if it is important to consider that absorption wave length other than the maximum may be crucial for the discrimination of the analyzed metabolites and the samples.

CATEGORY	SUBSTANCE	ABSORPTION (nm)*
ORGANIC COMPOUNDS	Ethylene (CH2=CH2) 1.3-butadiene Benzene Naphthalene Anthracene Naphthacene	180 217 255 286 375 477
	Betacyanins	530-554; 250-270
	Anthraquinones (yellow)	420-460; 220-290
FLAVONOIDS	Anthocyanins (mauve or red)	475-550; 275
	Chalcones and aurones (yellow)	365-430; 240-260
	flavonols (yellow)	365-390; 250-270
	Cathechins	380; 460
ALKALOIDS	Berberine	348

FAT-SOLUBLE NATURAL COMPOUNDS	Retinol	328
	β-carotene	450

*All the values are approximate, they can vary according to the pH and the solvent used

Tab. 2 Maximum of absorbition (nm) of some natural and organic compounds

Until now few results are published on the use of this technique for metabolomics and the majority of the protocols developed are proposed for food quality control. For instance, UV-based metabolomic analysis coupled with chemometrix techniques have been applied to the geographical and varietal classification of tea samples [55-56] as well as to determine simultaneously the content of several metabolite in a group of samples such as caffeine and theobromine in the tea,[57] beta-carboline derivatives in *Peganum harmala* seeds[58] and for the classification of coffees samples (type and conservation state),[59] to discriminate between species and subspecies of kiwi and pomelo fruit.[60] There are no works developing study of correlation between biological activity and UV-based metabolomics.

The limitations of this technique are due to its applicability to few class of chemical compound and the low selectivity, restrictions clearly connected with the UV-Vis spectroscopy itself.

1.5.3 *Chemometrix analysis*

In order to extract relevant information from the large metabolomics datasets is important to develop the appropriate automated data processing. Pre-processing and pre-treatment steps are required, followed by variable selection and modeling of the data and statistical validation. Dealing with 1H NMR spectra, the provided data are mono-dimensional and the intensity of the signals can be directly correlated to the abundance of the related metabolites.

For 1H NMR spectra pre-processing procedures usually involves: baseline correction, alignment, binning, normalization and scaling.

Baseline correction (fig. 7) must be applied to the spectra (usually by automatized procedure) in order to avoid problems in the statistical analysis and in the quantification of the metabolites. Moreover, it can happen that a peak shifts between different spectra for different reasons including: instrumental factors, changes of the pH or temperature, changes in salt concentration and so on, these variations, if occurring, obscure the discovery of patterns in the spectra. Thus, NMR spectra

are usually first aligned by spectral referencing, setting the internal reference signal of each spectrum to 0 ppm. Anyway this type of alignment removes only global shifts and may be not sufficient, because in NMR mostly local shifts are observable.

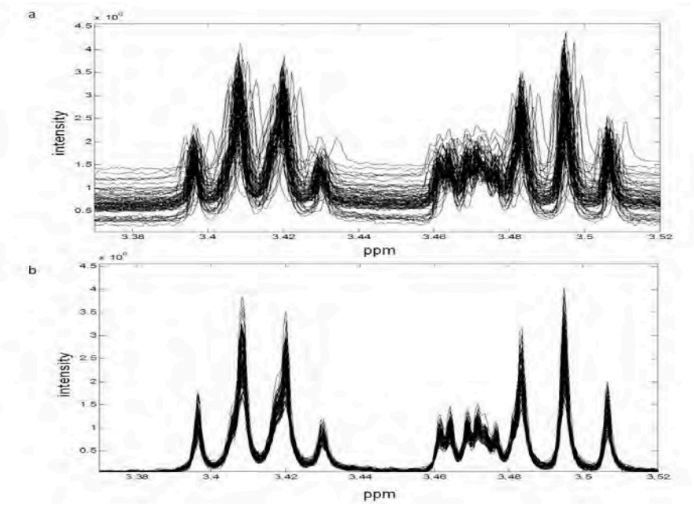


Fig. 7 Example of NMR spectra before (upper part of the figure) and after (bottom of the figure) baseline and alignment.

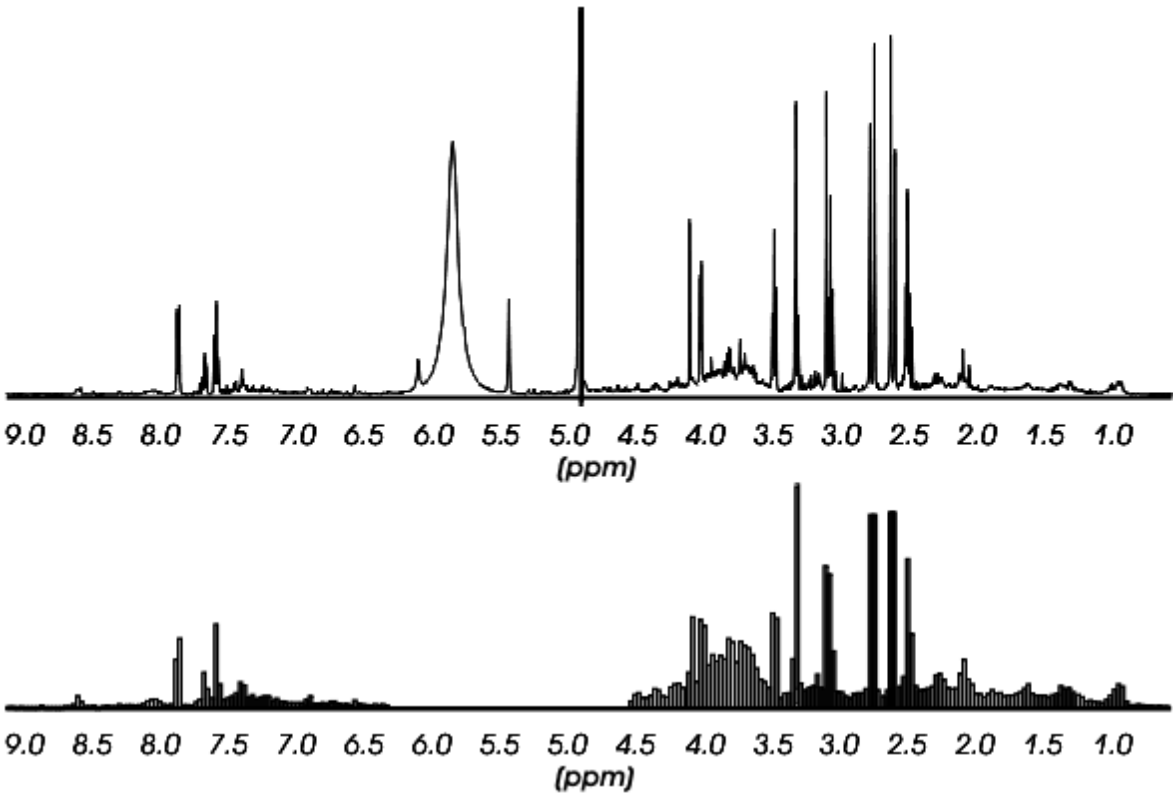


Fig. 8 Example of NMR binning, the spectrum after the binning procedure is represented in the down part of the figure

After the described first steps of pre-processing, it is required a procedure allowing the digitalization of NMR spectral data to numeric values for further statistical analysis. The objective is reached by binning procedure, by which multiple individuals can be summarized in a conventional data table and consequently classical chemometric tools such as PCA and PLS-based algorithms can be applied. This procedure operates dividing the NMR spectrum into a series of small bins (usually of 0.02 or 0.04 ppm) in which the sum of the signal intensities is then calculated (fig. 8). However, this process determines a decrease in the initial resolution of the spectra and the disadvantage of equal size binning is mainly the lack of flexibility of the boundaries that can determine peak splitting between two bins or peak collapsing within a bin. Therefore, if a peak is split between two bins, this peak frequency may significantly influence the data analysis and in this case more sophisticated methods are required in order to obtain segments of adjustable width sequentially aligned.

Another problem to afford, in order to obtain significant results, is that compounds present at lower concentrations are more difficult to measure and consequently more altered by the analytical noise than high abundance molecules. This makes the high amount metabolites have the highest influence on results of, for example, Principal Component Analysis (PCA) or Partial Least Squares (PLS). Thus, it is important to scale metabolites intensities before analysis to avoid selection of the most abundant metabolites as significant. For this reasons, usually, during the binning step, it is operated a scale to the sum of the total spectrum intensity (scale for total intensity). Anyway this first scaling procedure is not sufficient if in the data there are sub-populations with different variability (heteroscedastic data). Than, in order to make all the samples comparable with each other, a number of scaling methods are commonly applied to the overall set of results, namely: Unit Variance (UV) scaling, Meancentering, Autoscaling, Pareto scaling, Range scaling, Vast scaling, Level scaling. Each kind of scaling has its own limitation and should be carried out carefully. Pareto is a softer scaling and is one of the most suitable procedures for NMR and MS data, it uses weighting each variable with the inverse of the square root of its standard deviation (1/sqrt(SD)). This scaling method stays closer to real measurement but it is sensitive to large changes in the data.

After the pre-processing operations, the multivariate data model must be performed in order to extract the data mining. The first contact with the dataset can be provided by unsupervised model to facilitate the first understanding of the relationship between the samples and eventually the variables responsible for these relationships. Some of the most common unsupervised methods are Principal Component Analysis (PCA), Hierarchical Cluster Analysis (HCA) and the K-means clustering. Than, when a class attribute as a Y variable has to be predicted, more specific

information can be provided by supervised models like the PLS discriminant analysis (PLS-DA). Moreover the orthogonal PLS algorithm (OPLS) and O2-PLS have also recently been proposed in order to allow an easier interpretation of the models by separating the Y-predictive variability from the orthogonal one. Certainly, this methods need to be validated by using classic statistical additional methods like ANOVA or t-test or permutation test.

2. Aim of the thesis

Considering the crucial involvement of MMPs misregulated activity in the pathogenesis of several degenerative diseases, this class of proteases has been considered a highly active set of targets for the design of new therapeutic agents. However, the scant success of the synthetic MMPs inhibitors, largely due to the disappointing results obtained in the clinical and preclinical studies, makes medicinal plants a valuable source in order to find active principles able to modulate the MMPs activity. In this context, drawing from the knowledge inherited by the traditional medicine systems results an interesting starting point in order to increase the chances to select effective and safe plants.

This work is focused in particular on collagenase, which is the enzyme responsible for the cleavage of native fibrillar collagen and is considered the founding member of the MMPs family. Thus, on the base of Ayurveda, African Traditional Medicine (ATM) and Mediterranean traditional medicine, 49 plants were selected in order to find natural collagenase inhibitors.

In particular, the plants were selected on the basis of their ethnobotanical use to treat conditions possibly linked with MMPs related diseases, such as: wounds and burns healing, rheumatism, arthritis, skin diseases. Moreover, plants used for cosmetic purposes were included considering the involvement of collagenase in the wrinkles formation.

The selected plant material was extracted and the collagenase inhibitory activity of the obtained extracts was tested.

Thus, considering the complex mixture represented by a plant extract, a non-targeted 1H-NMR metabolomics approach was performed in order to obtain most comprehensive information. In particular, the metabolomics analysis was developed in order to investigate the possible correlation between the measured biological activity and the presence of common phytochemicals in the extracts. The NMR data were treated by a multivariate analysis (PLS and OPLS models) using the biological activity data as Y variable.

This procedure allowed to recognize tannins as high active collagenase inhibitors, and it allowed to select *Alchemilla vulgaris* as the most promising plant to study more in deep.

In fact, performing a tannin-removal procedure, the collagenase inhibitory activity of *Alchemilla vulgaris* extract resulted more or less unaltered. Thus, in order to identify active compounds other than tannins, it was performed a bio-assay guided fractionation of this plant and the procedure led to identify quercetin-3-O-β-glucuronide as the main active principle.

The second stage of this project was focused on different variety of tea (*Camellia sinensis* L.). Tea

is one of the most widely consumed beverages worldwide. Moreover, since the ancient time, the tea plant has been also used for medicinal purposes and nowadays several scientific evidences support its healthy-promoting effects. In particular, tea consumption is considered associated with the prevention of several illnesses as: cancer, metabolic and cardiovascular diseases, particularly of atherosclerosis and coronary heart disease. In addition, its anti-aging properties are also reported. The majority of the healthy effects of tea, including the potential anti-aging activity, can be associated with a possible effect on the maintenance of ECM homeostasis and green tea catechins were proved to be MMPs inhibitors.

Considering the existence of several variety of tea, aim of this work was to provide information on eventual significant differences in collagenase inhibitory activity between different tea samples. Moreover, since tea extracts are often used to develop cosmetics or food supplements endowed with a potential modulatory activity on collagenase, the work was focused on developing an UV-Vis based metabolomic protocol able to provide a simple and low-cost technique to select the most active tea on collagenase enzyme.

3. Methods and Materials

3.1 Biological activity screening and ^{1}H NMR metabolomic analysis of 49 plants

3.1.1 Plant Material

The Ayurvedic plants, dried and powdered, were kindly supplied by Maharishi Ayurveda Product Italy (Verona, Italy). They were collected during balsamic period in Ram Bagh (Rajasthan, India) and authenticated by Dr. MR Uniyal, Maharishi Ayurveda Product Ltd., Noida, India.

The African plants were collected from May to September 2007 and 2008, in six villages of Baskoure and Songretenga communes (Burkina Faso). Prof. Joseph Issaka Boussim did their identification, and voucher specimens were deposited in the herbarium of the Botanical Laboratory of the University of Ouagadougou.

The Mediterranean plants were kindly supplied by Byochima S.r.l Italy Località Mocaia, 44B 52031 Anghiari (AR).

All the details of the abovementioned plants are reported in Tab. 3.

TRADITIONAL MEDICIN	USED ACRONYM	PLANT NAME	FAMILY	PLANT PART USED
AYURVEDA	Ae	*Aconitum heterophyllum* Wall. Ex Royle	Apocynaceae	roots
	As	*Alstonia scholaris R.* Br.	Ranuncolaceae	bark
	Ar	*Asparagus racemosus* Willd.	Asparagaceae	tuberous root
	Ai	*Azadirachta indica* A. Juss.	Melicaceae	leaves
	Bd	*Bhoeravia diffusa* L.	Nictagynaceae	whole plant
	Cb	*Chlorophytum borivillanum*	Asparagaceae (subf. Agavoideae)	tuberous roots
	Cp	*Convolvulus pluricaulis* Choisy	Convulvolaceae	whole plant
	Cn	*Crataeva nurvala* Buch. Ham.	Capparidaceae	bark
	Co	*Curculigo orchioides* Gaertn	Epoxidaceae ex Amarillydaceae	tuberous root
	Er	*Embelia ribes* Burm. F.	Myrsinaceae	fruits

	Eo	*Emblica officnalis* Gaertn.	Phyllantaceae	pericarp
	Hi	*Hemidesmus indicus* L.	Apocynaceae	roots
	Mo	*Moringa oleifera* Lam.	Moringaceae	seeds
	Rc	*Rubia cordifolia* L.	Rubiaceae	roots
	Sc	*Swertia chirata* (Wall.) C.B. Clarke	Gentianaceae	whole plants
	Tb	*Terminalia bellerica* Roxb.	Combretaceae	fruits
	Tc	*Terminalia chebula* Retz.	Combretaceae	pericarp
	Tco	*Tinospora cordifolia* Willd.	Menispermaceae	stem
	Ws	*Withania somnifera (*L.) Dunal	Solanaceae	roots
AFRICAN (from Burkina Faso)	BpL	*Butyrospermum paradoxum* var. *pkii* C.F. Gaertn.	Sapotaceace	leaves
	BpR	*Butyrospermum paradoxum* var. *pkii* C.F. Gaertn	Sapotaceace	roots bark
	Csi	*Cassia sieberiana* D.C.	Fabaceae	roots bark
	Ci	*Chrysanthellum indicum* subsp. *afroamericanum* B.L. Turner.	Compositae	whole plant
	Cp	*Cochlospermum planchonii* Hook. Ef.	Cochlospermacea e	tuber
	Ct	*Cochlospermum tinctorium* A. Rich.	Cochlospermacea e	leaves
	Ep	*Euphorbia paganorum* A. Chev.	Euphorbiaceae	leaves and branches
	GsL	*Gardenia sokotensis* Hutch	Rubiaceae	leaves
	GsS	*Gardenia sokotensis* Hutch	Rubiaceae	stem bark
	Ks	*Khaya senegalensis* (Desr.) A. Juss.	Meliaceae	fruit
	Ps	*Panicum subalbidum* Kunth	Poaceae	roots
MEDITERRANEAN TRADITION	Ae	*Agrimonia eupatoria L.*	Rosaceae	aerial parts
	Av	*Alchemilla vulgaris L.*	Rosaceae	aerial parts
	Ao	*Althaea officinalis L.*	Malvaceae	roots
	Aso	*Asparagus officinalis L.*	Asparagaceae ex liliaceae	roots
	Bp	*Betula pendula Roth.*	Betulaceae	leaves
	Cao	*Calendula officinalis L.*	Asteraceae	petals
	Cs	*Camellia sinensis* L.	Teaceae	leaves
	Ce	*Centaurium erythraea Rafn.*	Gentianaceae	aerial parts
	Ea	*Equisetum arvense L.*	Equisetaceae	stem
	Gv	*Galium verum L.*	Rubiaceae	aerial parts
	Gl	*Gentiana lutea L.*	Gentianaceae	roots

	Mb	*Marrubium vulgare L.*	Lamiaceae	aerial parts
	Ms	*Medicago sativa L.*	Fabaceae	aerial parts
	Po	*Parietaria officinalis L.*	Urticaceae	aerial parts
	Ps	*Pinus sylvestris L.*	Pinaceae	gems
	Pv	*Primula veris L.*	Primulaceae	roots
	Ts	*Thymus serpyllum L.*	Lamiaceae	aerial parts
	Tv	*Thymus vulgaris L.*	Lamiaceae	leaves
	Vo	*Verbena officinalis L.*	Verbenaceae	aerial parts

Tab. 3 Details of the plants objects of the study

3.1.2 Extraction for the biological activity test

Fifty mg of dried and powdered plant material was extracted using 2 mL MeOH/H_2O (1:1) and sonication for 30 minutes. After this procedure the samples were centrifuged for 20 min and the surnatant was separated from the pellet and dried in to yield the extract.

3.1.3 Procedure of tannins removal

Extracts were solubilized in the minim amount of MeOH/H_2O and loaded on a polyamide column (10 x 1 cm) using a ratio of 0.5 g : 100 mg between resin and extract respectively. The elution was realized using 6 mL of EtOH. The collected EtOH eluent was dried in rotary evaporator to obtain the tannin-free extracts.

3.1.4 Extraction for the 1H NMR-Matabolomic analysis

Fifty mg of dried and powdered plant material was extracted using a 1mL of mixture (1:1) of phosphate buffer (Fluka Chemika; 90 mM; pH 6.0) in D_2O containing 0.1% trimethylsilylpropionic-2,2,3,3-d4 acid sodium salt (TMSP) and methanol-d4 by ultrasonication for 30 minutes. After this procedure, samples were centrifuged for 20 min and 350 µL of extract were transferred in NMR microtubes.

3.1.5 NMR measurement

1D-^{1}H NMR spectra, 2D J-resolved, ^{1}H-^{1}H homonuclear and inverse detected ^{1}H-^{13}C correlation experiments were recorded at 25 °C on a Bruker 600 MHz AVANCE II NMR spectrometer (600.13 MHz operating 1H frequency) equipped with TCI cryoprobe and Z-gradient system. CD_3OD was

used for internal lock purposes.

3.1.6 LC-MS analysis

LC-MS analysis was performed using Bruker microOTOF-Q ESI MS in negative mode with scan begin 150 m/z and scan end 800 m/z, capillary seted at 3800 V, End Plate offset -500 V, collision Cell RF 140.0 Vpp, Nebulized 2.0 Bar, Dry Heater 250 °C, Dry Gas 10.0 l/min.

3.1.7 Multivariate data analysis

The ^{1}H NMR spectra were automatically reduced to ASCII files. Spectral intensities were reduced to integrated regions of equal width (δ 0.04) corresponding to the region of d 0.0-10.0. The regions of δ 5-4.5 and d 3.34-3.30 were excluded from the analysis because of the residual signal of D_2O and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker) with scaling on total intensity. Principal component analysis (PCA), Projections to latent structures (PLS) and orthogonal PLS (OPLS) were performed with the SIMCA-P+ software (v. 12.0, Umetrics, Umeå Sweden). Scaling was based on UV for PLS and Pareto for PCA and OPLSDA.

3.1.7 Collagenase inhibitory assay

A concentration of 50 µg/mL of extract was tested. The collagenase assay was performed according to Van Wart and Steinbrink (1981)[61] with slight modifications. Collagenase (E.C. 3.4.24.3) from Clostridium histolyticum (type IA, ChC; specific activity 11.72U/mg) was purchased from Sigma–Aldrich Co. (Saint Louis, MO). 20 mU enzyme, prepared in Tris/HCl buffer (0.05 M, pH 7.5), containing 0.4 M NaCl and 0.01 M $CaCl_2$, were incubated for 10 min with test samples at different concentrations (from 10 to 300 µg/mL). The synthetic substrate N-(3-[2-Furyl]-acryloyl)-Leu- Gly-Pro-Ala (FALGPA), prepared in the same buffer solution, was added to start the reaction (final concentration 0.8 mM) in a final volume of 125 µL. The change in absorbance was monitored over a time interval of 3 min at 340 nm under the microplate reader at a constant temperature of 30 °C. The IC_{50} value was calculated by constructing a linear regression curve showing sample concentrations on the x-axis and percentage inhibition on the y-axis.

The percentage of inhibition of enzyme activity was calculated by the following formula:

$$(dAbs_{sample} - dAbs_{neg\ control})/dAbs_{neg\ control} \times 100$$

dAbs values were calculated in the time interval of 3 minutes.

A mixture of enzyme, substrate and water, instead of samples, was used as negative control. The positive control was represented by antibiotic doxycycline.

3.2. *Alchemilla vulgaris* bio-guided assay

3.2.1 Extraction and liquid/liquid partition

One Kg of dried and powdered plant material was extracted using 10 L of MeOH/H_2O (1:1). The extract was filtered in Bunker funnel and dried in a rotary evaporator.

The extract was than solubilized in H_2O and a liquid/liquid partition was performed using in order: hexane, $CHCl_3$ and *n*-butanol. The obtained extracts were anhydrified using Na_2SO_4 anhydrous, filtered, dried and tested for the collagenase inhibitory activity.

3.2.2 Column Chromatography

The first three steps of fractionation were performed by BUCHI MPLC equipped with Pump Module C-605, UV Photometer C-640 and Fraction collector C-660.

The *n*-butanolic extract (13.6 g) was fractionated using MPLC apparatus endowed with HP-20 resin column with flow rate of 50 mL/min and a maximum pressure of 40 bar and detection at λ 220, 250, 280 and 365 nm. Considering that the mobile phase was made by H_2O (solvent A) and EtOH (solvent B), the elution system performed is reported in Tab. 5. The performed column yielded 11 fractions (A1-A11).

Fraction A8 (930 mg) showed the highest activity value in the collagenase inhibitory assay, thus was fractionated in MPLC using a Buchi Sepacore Siliga-80g with flow rate of 20 mL/min and a maximum pressure of 50 bar and the abovementioned detection system. Considering that the mobile phase was made by $CHCl_3$ (solvent A) and MeOH/CH_3COOH 2% (solvent B), the elution system performed is reported in Tab. 6.

The performed column chromatography yielded 4 fractions (B1-B4).

Fraction B1 and B4 (the most active among the four) were in turn fractionated using MPLC and Buchi Sepacore C18-4 g column with flow rate of 10 mL/min and a maximum pressure of 16 bar and the abovementioned detection system. Considering that the mobile phase was made by MeOH/HCOOH 1% (solvent A) and H_2O/HCOOH 1% (solvent B), the elution system performed is reported in Tab. 7. Using this procedure fraction B1 yielded 8 fractions (from C1 to C8) and fraction B4 yielded 7 fractions (from D1 to D7).

Fraction D4 (26.55 mg) was purified on Sephadex LH-20 (GE healthcare) column, using MeOH as eluent and yielding 5 fractions.

The fraction after each column performed where previously analyzed using both H^1-NMR and TLC (mobile phase EFAW and visualized by UV-Vis lamp and anisaldehyde spry and heated).

Start B%	**End B%**	**min**	**s**
0	0	4	0
0	25	0	30
25	25	4	0
25	50	0	30
50	50	4	0
50	75	0	30
75	75	4	0
75	100	0	30
100	100	4	0

Tab. 5 Elution system used for the HP-20 column, solvent B is EtOH solvent A is H_2O

Start B%	**End B%**	**min**	**s**
90	90	15	0
90	70	5	0
70	70	20	0
50	50	5	0
50	50	10	0

Tab.6 Elution system used for the Silica-gel column, solvent B is MeOH/CH_3COOH 2% solvent A is $CHCl_3$

Start B%	**End B%**	**min**
90	90	10
90	80	1
80	80	20
80	70	1
70	70	10
70	50	1
50	50	10
50	30	1
30	30	5

Tab.7 Elution system used for the C18 column, solvent B is H_2O/HCOOH 1% and solvent A is MeOH/HCOOH 1%

3.3 Biological activity screening and ^{1}H NMR metabolomic analysis of 39 Tea varieties

3.3.1 Plant material, extraction and 1H NMR-based metabolomic analysis

The tea samples were purchased from Simon Lévelt Koffiebranderij en Theehandel A. (Hofmanweg 3 2031 BH Haarlem NL) and the details are reported in Tab. 8.

The extraction and the 1H NMR metabolomics analysis were performed as above described in chapter 3.1. PCA, PLS and OPLS models were also developed as described in chapter 3.1.

Tea	Type	Used acronym
Finest Oolong	Oolong	1
Grand Pouchong	Oolong	2
Puerh	Oolong	3
Jangwon Oolong	Oolong	4
Pai Mu Tan	White	5
White Moonlight	White	6
Ambootia Wit	White	7
Sencha Classic	Green	8
Gyokoru	Green	9
Ambootia Groen	Green	10
Blackwood Groen	Green	11
Lentedauw Mao Feng	Green	12
Chunmee Young Hyson	Green	13
Nilgiri Groen	Green	14
Dragonwell Xi HU Lung Ching	Green	15
Snow Bud Lu Xue Ya	Green	16
Yunnan Groen	Green	17
Nepal Groen	Green	18
Pi Lo Chun Groen	Green	19
Gunpowder	Green	20
Jangwon Groen	Green	21
Yunnan Gold	Black	22
Golden Lapsang Souchong	Black	23

Tarry Melange	Black	24
Mokalbari Zomerpluk	Black	25
Jangwon	Black	26
Dunsandle Jungle	Black	27
Ceylon Superieur	Black	28
Java Kertasari	Black	29
Sumatra Sidamanik	Black	30
Darjeeling Zomerpluk	Black	31
Ambootia Lentepluk	Black	32
Ambootia Regenpluk	Black	33
Ambootia summer	Black	34
Margareth's Hope Lentepluk	Black	35
Kenya Flowery Orange Pekoe	Black	36
Kukicha	Black	37
Huang Da Cha	Yellow	38
Kukicha	Green	39

Tab.8 The table reports all the details of the different tea variety subjected to the study

3.3.2 UV-Vis-based metabolomic analysis

The Tea extracts were solubilized in phosphate buffer (0.05M pH 7.5) at a concentration of 0.4% v/v. The UV-Vis spectra of the extracts were recorded in the spectrophotometer from 700 to 200 nm. The obtained data were exported to Microsoft excel and analyzed by SIMPCA-P+

3.3.3 Isolation of the active fractions

One hundred g of Tea 25 (Black tea Mokalbari Zomerpluk) was extracted in MeOH/H_2O (2 L) and sonicated for 1 hour. The procedure was repeated 2 times. After the extraction the extract was filtered and the pellet was discarded. The supernatant was dried in rotary evaporator.

The extract was purified by MPLC and Buchi Sepacore C18-4 g column with flow rete of 10 mL/min and a maximum pressure of 16 bar and the abovementioned detection system. Considering that the mobile phase was made by MeOH/HCOOH 1% (solvent A) and H_2O/HCOOH 1% (solvent B), the elution system performed is reported in Tab. 7 (see above).

4. Results and discussion

4.1 Correlation between collagenase inhibitory activity and chemical profile of 49 medicinal plants

In order to identify natural collagenase inhibitors, 49 medicinal plants were selected based on their ethnobotanical uses. In particular, it was given attention to plants traditionally used to promote wounds and burns healing and generally to treat skin related diseases as well as rheumatism, arthritis and bones diseases. In fact, since these illnesses have been associated to a misregulated collagenase activity, it was supposed that an inhibition of this enzyme might be involved in the beneficial effect attributed to the traditional remedy. Moreover, considering the involvement of collagenase in the wrinkles formation, plants used traditionally as anti-aging agents were also considered in the selection stage.

Then, the selected plants (see tab.3) were subjected to a proper extraction procedure and the obtained extracts were tested in the collagenase inhibitory assay at a concentration of 50 μg/mL. According to the obtained percentage of inhibition, the plants were divided in high activity (inhibition >60%), medium (inhibition between 60% and 30%) and low activity (inhibition <30%) (fig.9). Thus, in order to obtain a more comprehensive overview of the relations between the measured biological activity and the chemical profile of the extracts, it was performed a 1H-NMR metabolomic analysis. In particular, this procedure was followed to detect the eventual presence of a common class of metabolites in the most active plants. In order to easily extract this kind of information, the obtained 1H-NMR spectra were subjected to a multivariate data treatment (MVDA). In particular, two supervised models were used, namely PLS and OPLS considering as the y variable the percentage of collagenase inhibitory activity.

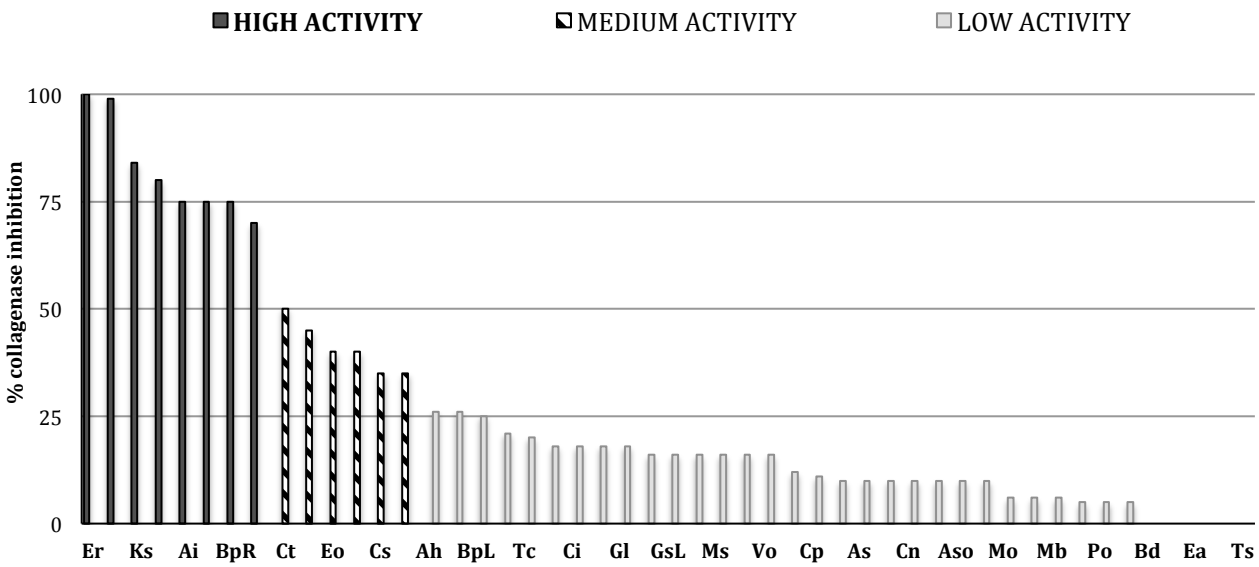
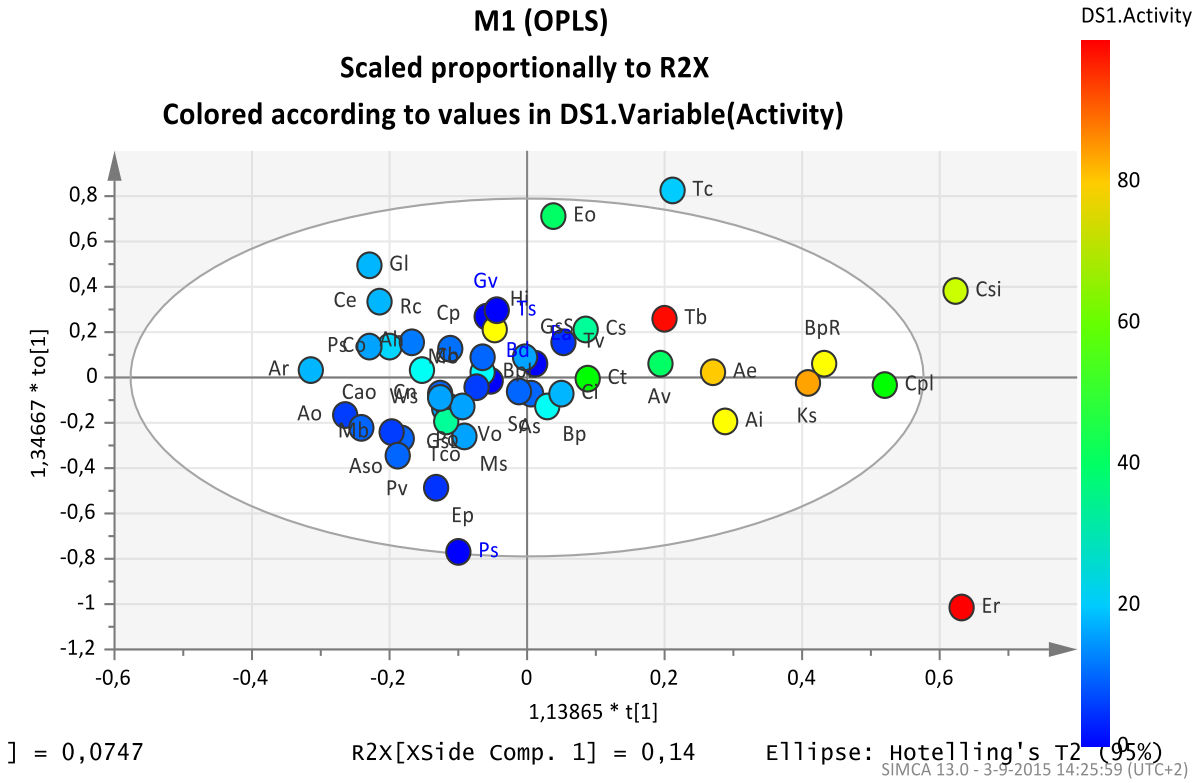
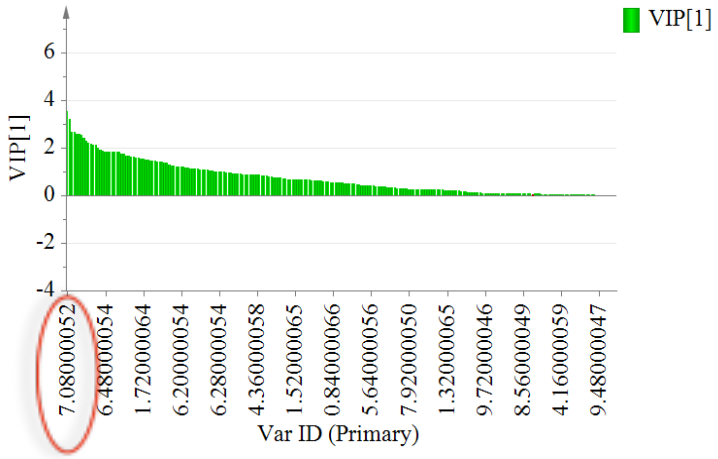


Fig. 9 Percentage of collagenase inhibitory activity measured for the 49 crude plant extracts at a concentration of 50 μg/mL. The plants were divided in high (activity>60%), medium (activity between 30% and 60%) and low activity (activity<30%).

The OPLS model (fig.10) showed the general importance of aromatic compounds in the collagenase inhibitory activity. In fact, the presence of several signals in the 1H-NMR spectral region from δ 7.4 to 6.4 was indicated as positively correlated to the biological activity. More in particular, the model identified a signal at δ 7.08 as the most correlated with the collagenase inhibitory activity. Dealing with plant material, spectral signals around δ 7.0 can be attributed to tannin-related compounds, especially gallotannins. The result is consistent with the data reported in literature, in fact, tannins were found capable to inhibit collagenase and other MMPs both *in vitro* and *in vivo* models.[62-63-64] However, to confirm the hypothesis of tannins involvement in the activity of the selected plants, a tannin-removal procedure was required. Moreover, the possibility to test tannin-free extracts was considered useful in order to investigate the eventual presence of other class of active metabolites taking also into account that some highly polymerized tannins might determine false positive results in the assay (especially in an *in vitro* test). In fact, this kind of compounds might precipitate proteins leading to an apparent enzyme inhibition.



A



B

Fig. 10 OPLS model results. **A.** Scatter plot colored according to the collagenase inhibitory activity

B. VIP plot in which is evident the importance for the activity of the signal at δ 7.08.

Several methods have been reported for tannins-removal but none of them is exempt from disadvantages. However, we elected filtration on polyamide columns as the best method, in fact, it is the most efficient and reproducible procedure, which shows also the advantage to avoid sample contamination with other substances.

Thus, the 14 most interesting extracts (high and medium activity) were eluted on polyamide columns (the yield after column is reported in fig. 11), then the biological activity of the obtained tannin-free extracts was tested.

After the tannins removal, only three out of 14 plants were found still able to inhibit the enzyme with an activity comparable with the one previously showed by the crude extract (fig. 12), namely: *Alchemilla vulgaris* (Av), *Tinospora cordifolia* (Tco) and *Emblica officinalis* (Eo).

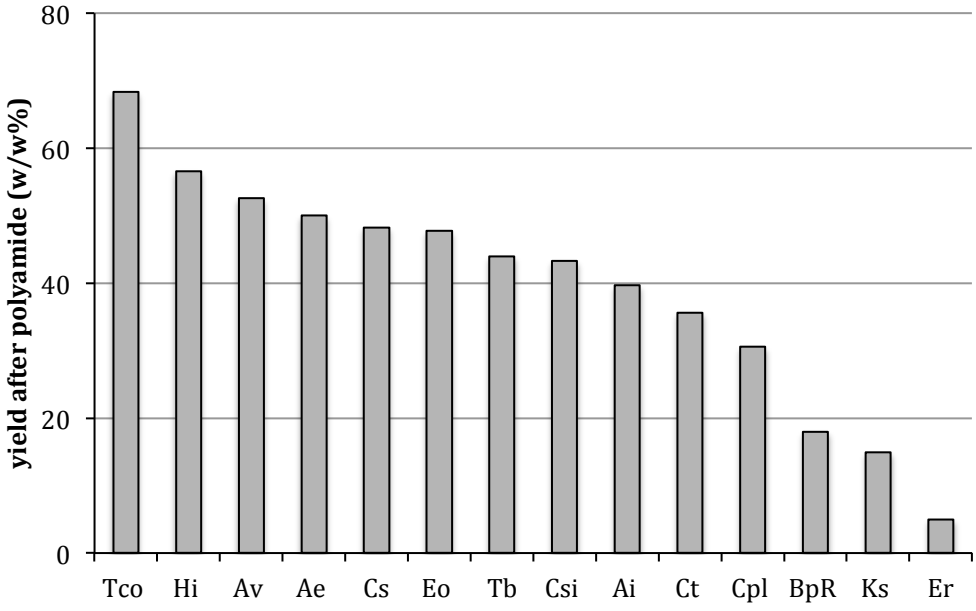


Fig. 11 Yield expressed in w/w% obtained after tannin removal procedure for the 14 active plants.

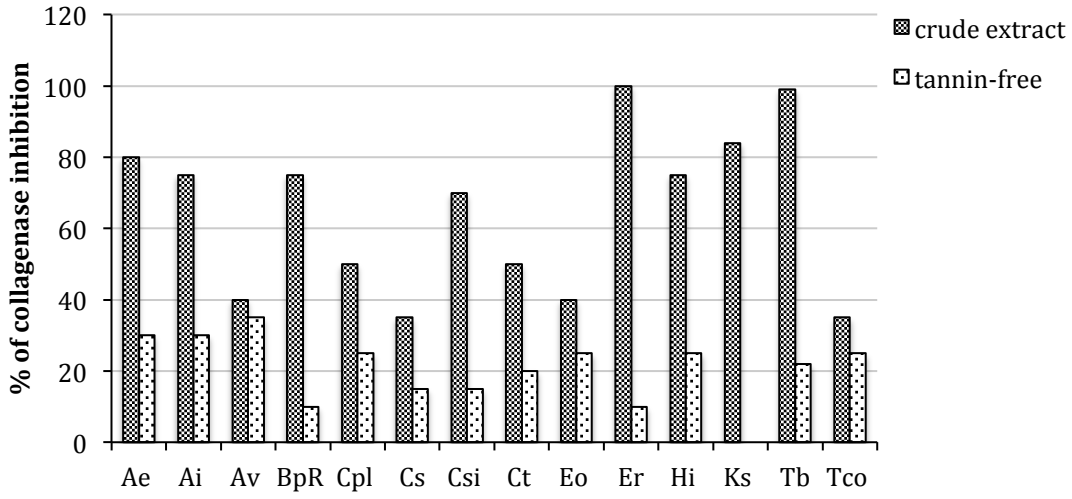


Fig. 12. Comparison in activity, expressed as percentage of collagenase inhibition, between the crude extracts and the respective tannin-free extracts of the 14 active plants. Only the activity of Av, Tco and Eo is almost unaltered after the tannin-removal procedure.

In order to check if the tannin-removal procedure was working efficiently, the extracts obtained after polyamide filtration were compared with the crude extracts by 1H NMR and HPLC-PDA. In most cases, only tannins were irreversibly retained on the polyamide resin, leaving the extracts composition almost unaltered in its other constituents. However, four cases represented an exception, in fact these four extracts were consistently modified in their composition using the polyamide column, namely: *Hemidesmus indicus* (Hi), *Khaya senegalensis* (Ks), *Butyrospermum paradoxum* (roots bark) (BpR) and *Embelia ribes* (Er).

Although unwanted, in the case of Hi, this inconvenient lead us to obtain useful information on the compounds responsible for its extract activity. In fact, two vanillin positional isomers are present in Hi, namely: 2-hydroxy-4-methoxy-benzaldheyde and 3-hydroxy-4-methoxy-benzaldheyde, and whereas both molecules are detectable in the 1H NMR spectrum of crude Hi extract, the corresponding tannin-free extract completely lost one of them: 2-hydroxy-4-methoxy-benzaldheyde (fig. 13).

The high amount of 2-hydroxy-4-methoxy-benzaldheyde found in the Hi extract and the lost in activity registered after its undesired removal after polyamide column. It suggest the crucial involvement of this compound in the collagenase inhibitory activity of Hi.

In the case of Ks, BPr and Er it was not possible to detect which compounds, other than tannins,

were missing after the polyamide filtration but the yield was noteworthy low compared to the other tannin-free extracts (fig. 11) and the characteristic red color of this three extracts was totally lost after column. In this case, it is important to consider that, as specified by the suppliers, the polyamide resin irreversibly retains quinones, therefore plants enriched in this class of compound (e.g. Er)[65] are not suitable to be treated with this procedure. Accordingly, it is not possible to completely excluded Ks, Bpr and Er from the group of interesting plants to investigate more in deep to found collagenase inhibitors other than tannins.

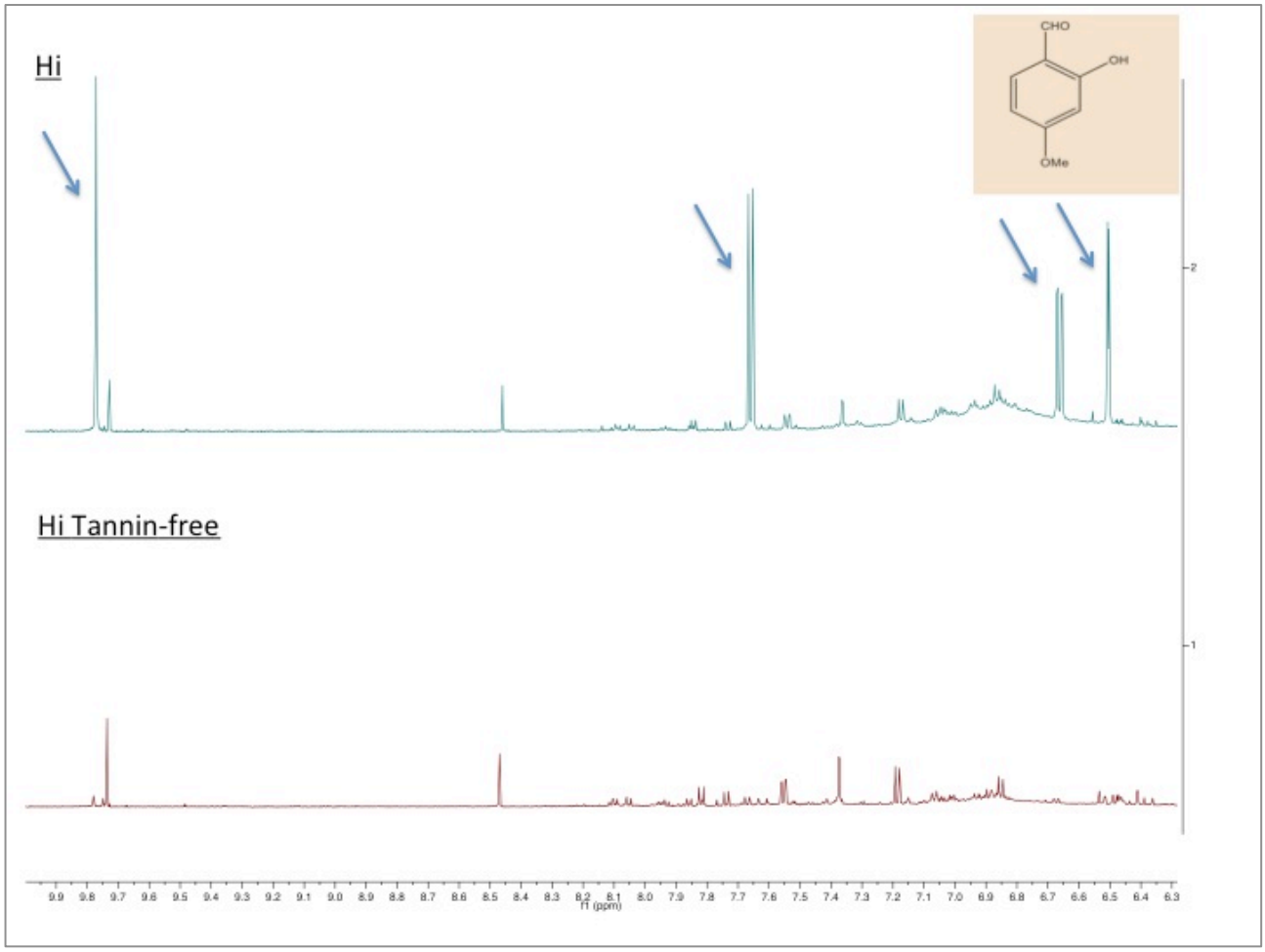


Fig. 13 *Hemidesmus indicus* (Hi) 1H-NMR. On the top part is reported the spectrum of the Hi extract and on the bottom the spectrum of the tannin-free Hi extract. The signals of 2-hydroxy-4-methoxy-benzaldheyde completely disappeared in the tannin-free extract. The blue arrows indicate the signals of the aldehyde and aromatic protons of this compound.

In the second stage of this work it was decided to select the plant less affected by the tannin removal procedure in term of activity, namely *Alchemilla vulgaris* (Av) and analyze it deeply by a bioassay-guided procedure in order to identify the compounds responsible for the biological activity.

First of all, it was performed a liquid-liquid partition using, in the following order: hexane, $CHCl_3$, *n*-butanol and H_2O to obtain four different fractions enriched in different polarity compounds. The activity of the four fractions was tested and since the *n*-butanolic one showed the highest activity it was subjected to further purification procedures.

Each fraction obtained from the *n*-butanolic one was tested for the collagenase inhibitory activity and the highest activity fractions were subjected to further purification steps (fig. 14).

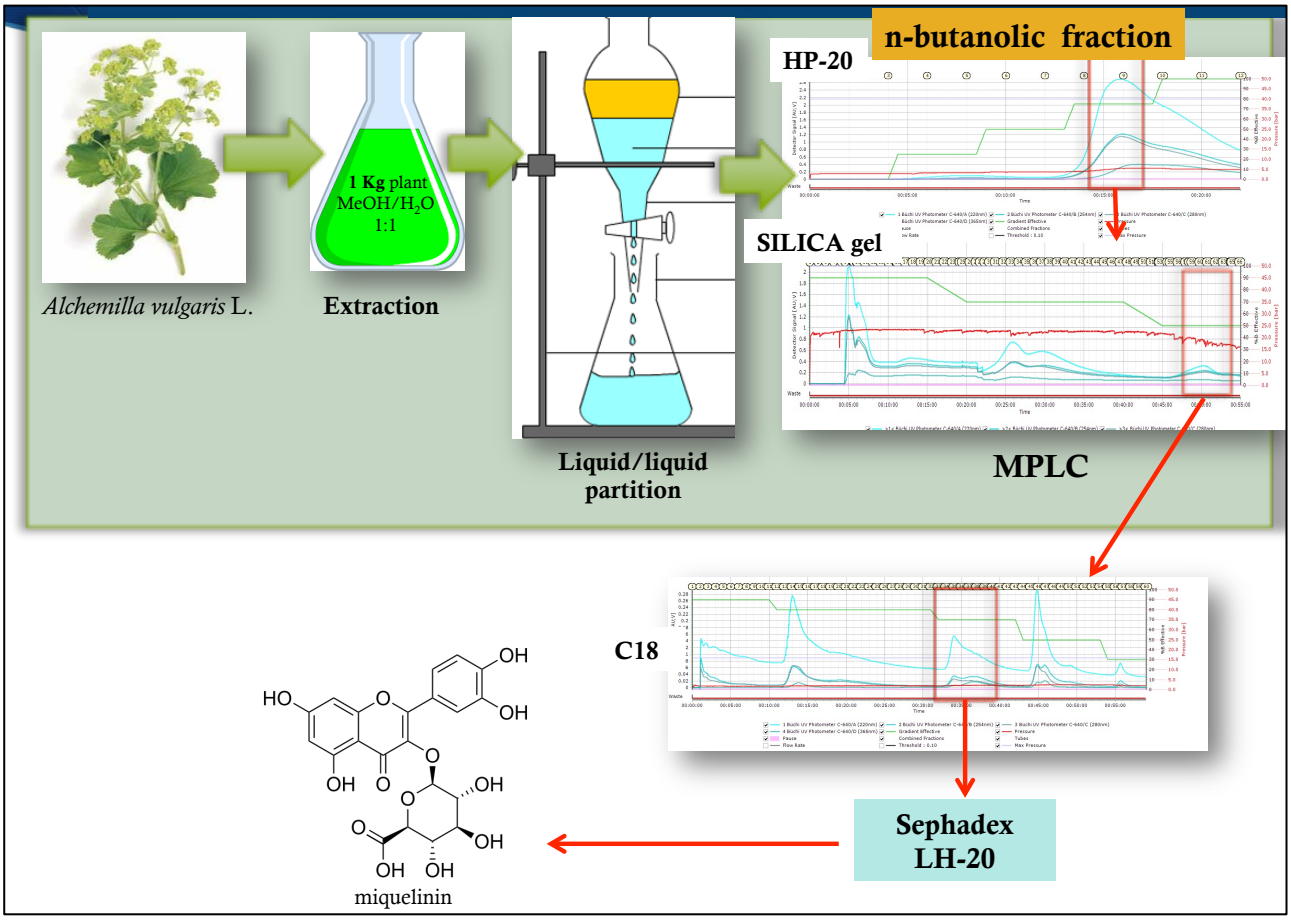


Fig. 14 Scheme of the bioassay-guided procedure performed on *Alchemilla vulgaris* and yielding miquelianin as the most active compound. The most active fractions are inserted in the red squares.

This procedure allowed to purify and identify: quercetin-3-O-β-glucuronide (miquelianin), which resulted the most active compound present in the plant extract (NMR details are reported in fig. 16). The molecule was identified by COSY, J-res, HMBC and HSQC experiments, the sugar configuration was confirmed by the coupling constants obtained by the J-res spectrum and the presence of glucuronic acid in the structure was evidenced by the HMBC correlation between the signal of the proton at δ 3.58 in the sugar moiety and the signal of the carbonylic carbon at δ 173.23. The position 3 of the glycoside was confirmed by HMBC experiment (fig. 15), which showed correlation between the anomeric proton at δ 5.36 and the carbon 3 of the aglycon at δ 135.84 (fig. 15). Miquelianin purity was confirmed by LC-MS, which allowed also to further confirm the structure by the given molecular weigh of 477.08 m/z.

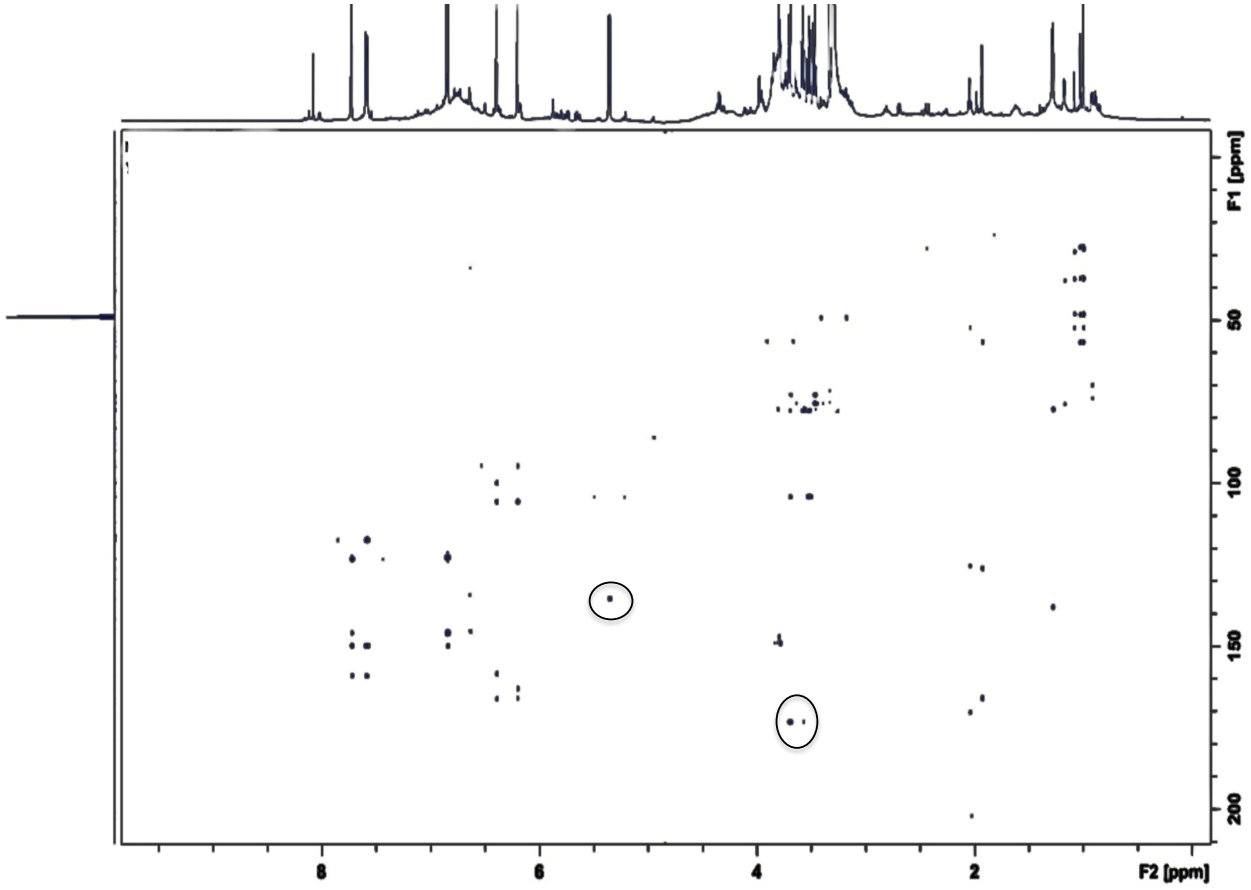
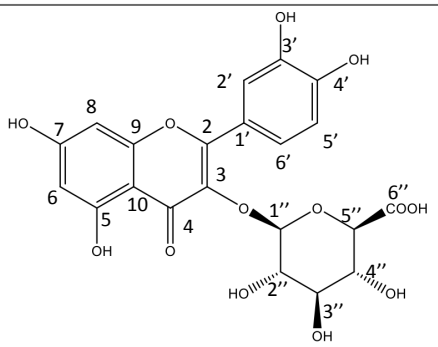


Fig. 15 HMBC spectrum of fraction D4 (containing miquelianin). The correlation between the anomeric proton (H1’’δ) and C3 (135 δ) is evidenced as well as the correlation between proton H4’’ and H5’’ with the carboxylic carbon C6’’ (173.23 δ).



(quercetin-3-O-β–glucuronid
or miquelian)

^{1}H	^{13}C
-	159.07
-	135.51
-	162.73
6.20 (d, *J* = 2.1 Hz, 1H)	99.54
-	165.97
6.39 (d, *J* = 2.1 Hz, 1H)	94.41
-	158.34
-	105.52
7.73 (d, *J* = 2.0 Hz, 1H)	117.07
-	145.92
-	149.92
6.85 (d, *J* = 8.4 Hz, 1H)	115.73
7.59 (dd, *J* = 8.5, 2.2 Hz, 1H)	122.96
5.37 (d, *J* = 7.50 Hz, 1H)	103.85
3.47 (t, J= 8.81 Hz, 1H)	75.30
3.47 (t, J=8.74 Hz, 1H)	77.60
3.574 (t, J=9.34 Hz, 1H)	72.50
3.68 (d, J=9.90 Hz, 1H)	77.10
-	173.32

Fig. 16 NMR table of miquelianin

The purified miquelianin was tested in the collagenase inhibition assay and compared with other two flavonoids glycosides with the same aglycone moiety (purchased form Sigma Aldrich) and the positive control doxycycline, the only drug approved by FDA as collagenase inhibitor.

The results (fig. 17) showed that miquelianin is interestingly more potent than the positive control and the other flavonoids, this let suppose the importance of the –COOH in position 6'', in fact this group close to the -OH of the B ring of the aglycone can promote metal chelation activity, interfering with both the enzyme active site (containing Zn II) and the environmental Ca(II) which is also essential for all the MMPs catalytic activity.

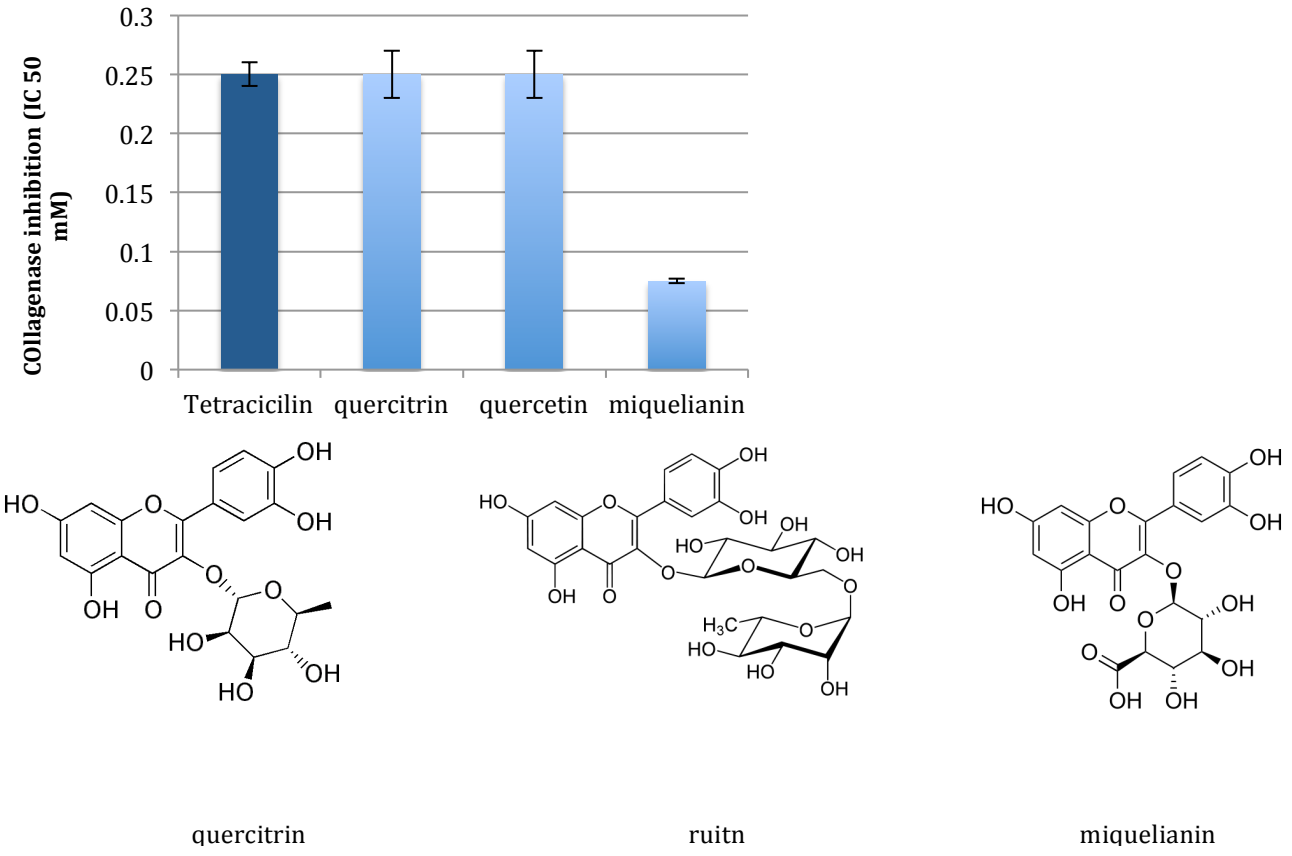


Fig 17. Collagenase inhibition (IC_{50}) of positive control doxycyline (deep blue) and miquelianin compared with other two flavonoids. The structure of the three flavonoids is reported below the figure.

4.2 Collagenase inhibitory activity of different variety of tea

Another stage of the project was focused on the tea pant (*Camellia sinensis* L.) as source of collagenase inhibitors.

Tea is one of the most widely consumed beverages worldwide and in addition, tea extracts and its constituents are often used as active ingredients for cosmetics and other supplements. In fact, since the ancient time, tea plant has been also used for medicinal purposes and nowadays several scientific evidences support its healthy-promoting effects. [66] In particular, tea consumption is considered associated with the prevention of several illnesses as: cancer, metabolic and cardiovascular diseases, particularly of atherosclerosis and coronary heart disease.[66] In addition, its anti-aging properties are also reported. In this context, the majority of the health promoting effects of tea, including the potential anti-aging activity, were supposed to be associated with a possible effect on the maintenance of ECM homeostasis and thus on MMPs. In the previous stage of this project an extract of green tea was tested among 49 plants and it was found effective as collagenase inhibitor. Therefore, in this other stage we considered different tea varieties in order to establish the occurrence of differences in activity among them and accordingly identify the most active tea and its features. In fact, although the plant source is the same, different teas varieties can be obtained depending on the manufacturing treatments.

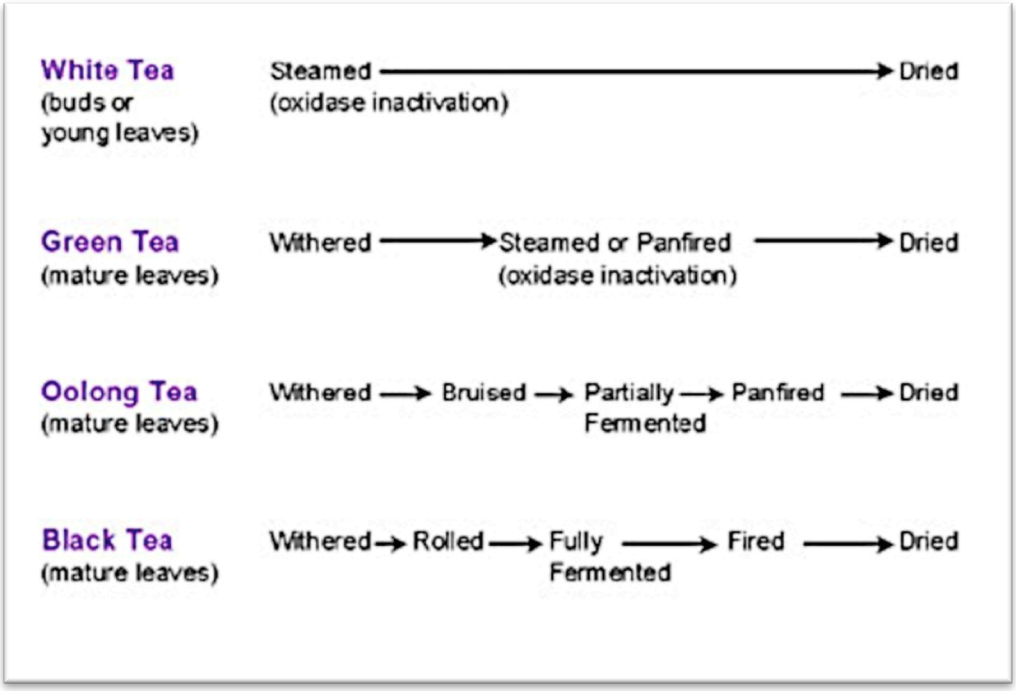


Fig 18. Scheme of different tea manufacturing processing

In fact, the plant material can be subjected to different drying and fermentation process, which, in particular, determine enzyme-catalyzed oxidation and partial polymerization of the contained catechins. The most commercialized tea varieties are: green, white, oolong and black (mentioned from the unfermented to the most fermented one (see fig. 18).

Thus, in this study, 39 different teas were analyzed showing a broad range of activity on collagenase (fig. 19). In particular black teas (represented in blue in the figure) were found generally endowed with the highest activity.

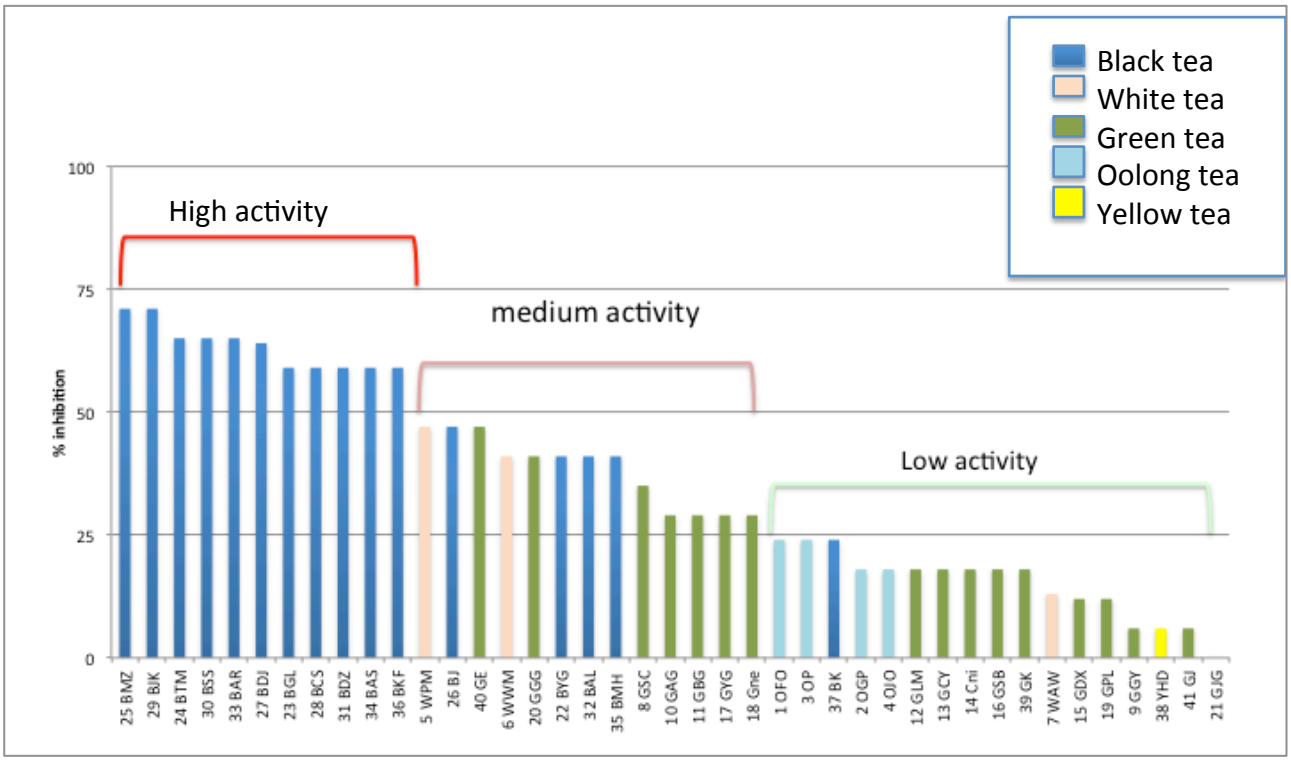


Fig. 19 Collagenase inhibitory activity of different tea variety. Samples were tested at a concentration of 50 µg/mL, black teas (blue) were found generally endowed with high activity.

One of the major differences, in terms of chemical composition, between the different tea varieties is represented by the catechins. In fact, these compounds are susceptible to chemical reactions of oxidation and polymerization during the different procedure to which the teas are subjected. According to this, it was supposed that some specific subclass of catechins, present only in the black teas, could be strongly active against collagenase. In order to prove this hypothesis the most common green tea catechins and theaflavin a characteristic catechin of the black tea were tested as collagenase inhibitors (fig. 20). In particular: catechin, epicatechin, gallocatechin, epigallocatechin,

catechingallate, epicatechingallate, gallocatechingallate, epigallocatechingallate and theaflavin in a concentration of 80 μM, which was reasonably consistent with the activity showed by the crude tea extracts.

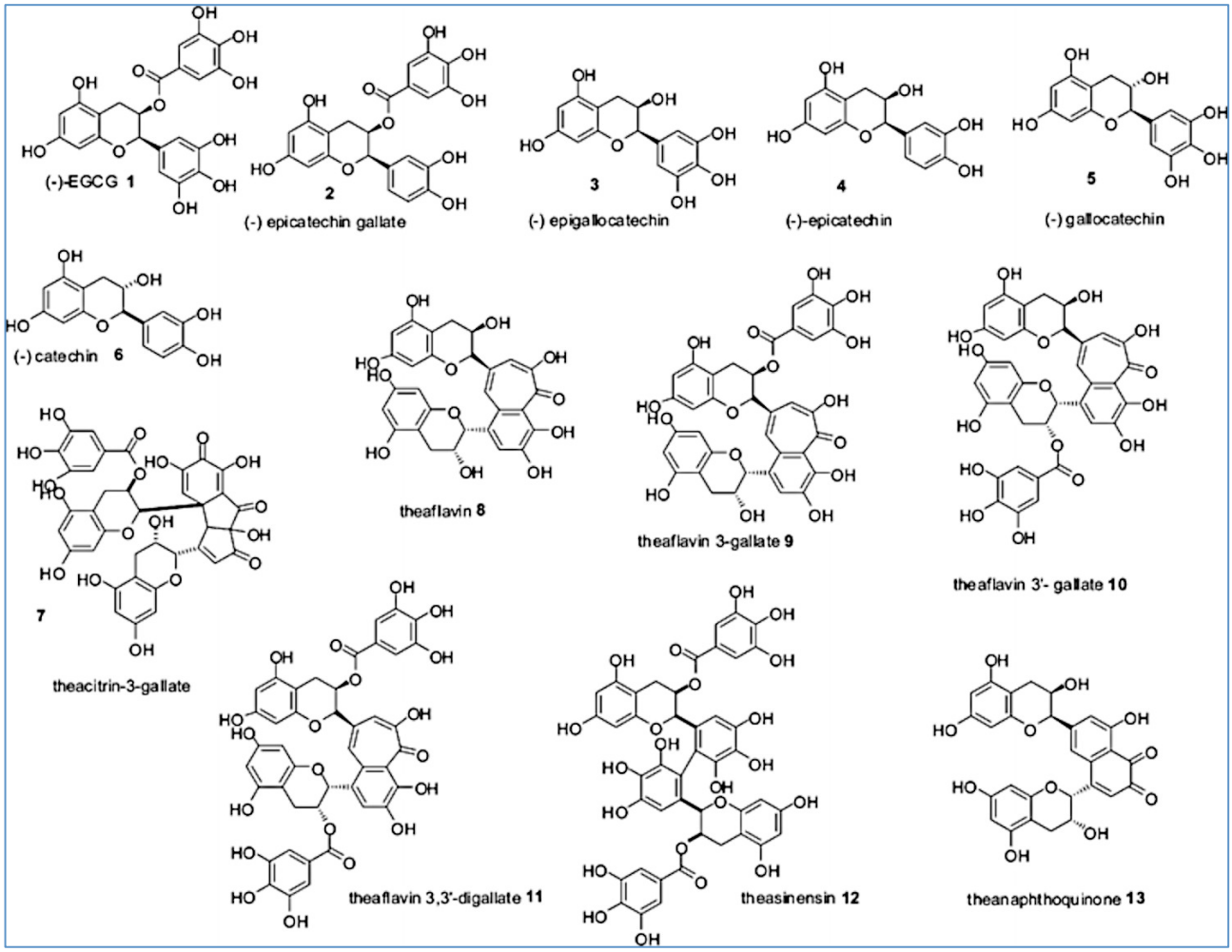


Fig. 20. Most common green tea cathechins (from1 to 6) and most common black teas catechins (from 7 to 13)

None of the tested catechins was active at the tested concentration, except theaflavin showing 60% of collagenase inhibition. This result corroborated the hypothesis that black tea catechins can be the responsible of the strong collagenase inhibitory activity of this variety of tea.

On the bases of these results we tried to provide an easy method, to screen tea samples in order to find strong anti-collagenase active teas.

In particular, the tea UV-visible spectra were measured in a proper buffer, processed and correlated with their collagenase inhibitory activity by performing an OPLS model (fig.21). The model indicated a strong correlation between increased activity and compounds absorbing in the region between 350-440 nm, which is usually attributed to black teas catechins.[56]

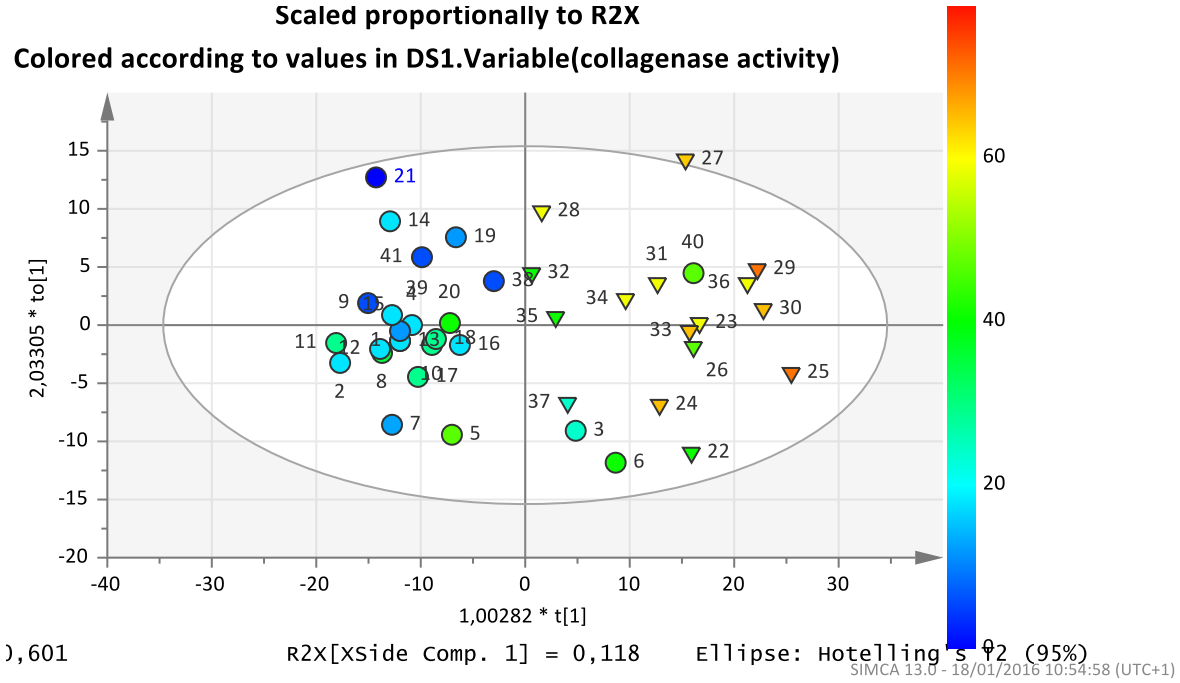


Fig. 21 OPLS model build using the UV-Vis spectra of teas and correlated to the collagenase inhibitory activity as y variable. The black teas are represented by the triangles in the plot.

In order to provide more information on the compounds possibly related to these absorbance signals, the ^{1}H-NMR metabolomics profile of the 49 teas was also measured and used to build another multivariate data model in order to correlate all the data obtained. In particular, it was performed an OPLS-DA model, in which samples were divided in two classes: active (activity >50%) and not active (activity <50%) and the ^{1}H-NMR signals were used as x variable to correlate with the absorbance value at 405 nm used as y variable.

The model showed a very good correlation between the absorbance value and the activity and the samples were clearly separated along the PC1 (fig 22).

The S-plot (fig. 23) showed a positive correlation between highest activity, absorption at 405 and

some aromatic signals in the H-NMR spectra (δ 7.90; 7.04; 6.84), which can be related to black teas catechins.

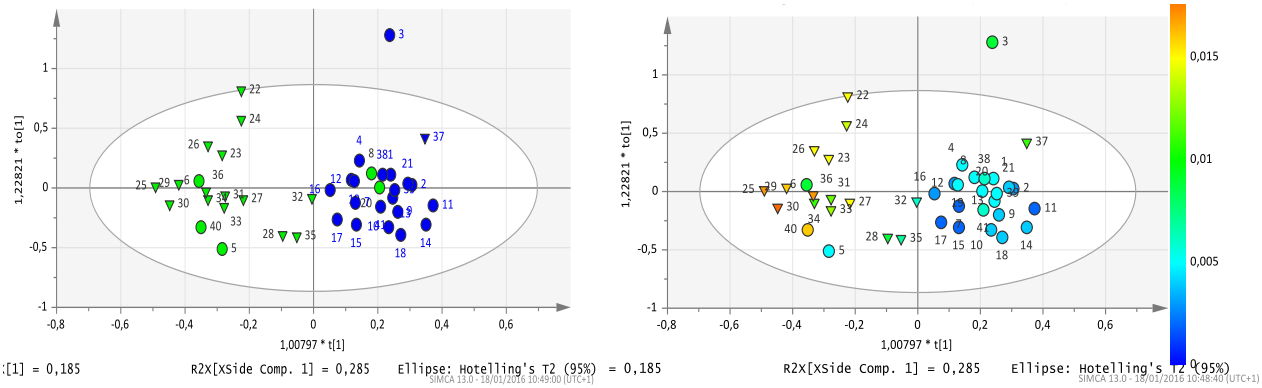


Fig. 22 Scatter plot obtained using OPLS-DA model. The samples were divided in two classes active (activity>50%) and not active (activity <50%) and the value of absorbance at 405 nm was used as y variable. The left part of the figure shows the separation between the active (green) and not active (blue), the black teas are represented by the triangle. The right part of the figure shows the same plot colored according to the values of absorbance at 405.

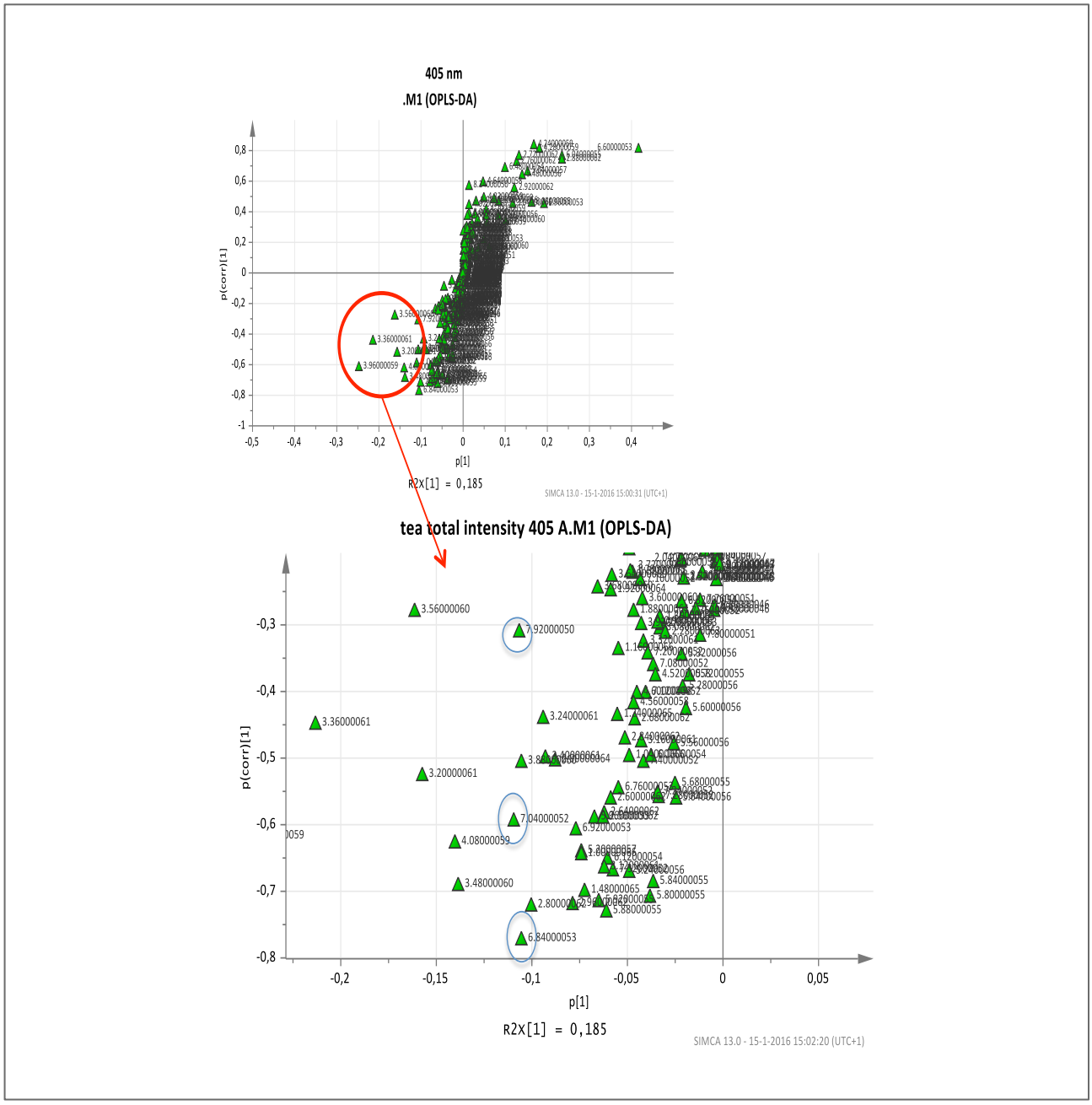


Fig. 21 S-plot of the OPLS-DA model, the signals important for the absorption at 405 and typical of the active teas are aromatic signals that can be linked to theaflavins and thearubigins skeleton.

The data obtained by the UV-Vis metabolomics study were also proved fractionating the most active tea (tea number 25). In fact, a C18 semipreparative column was performed on its raw extract (fig. 20) and the four obtained fractions were tested as collagenase inhibitors as well as analyzed by UV-Vis spectrophotometry. As expected the most active fractions (FR2-FR3) were found endowed with absorption at the waves lengths provided by the UV-Vis metabolomic method.

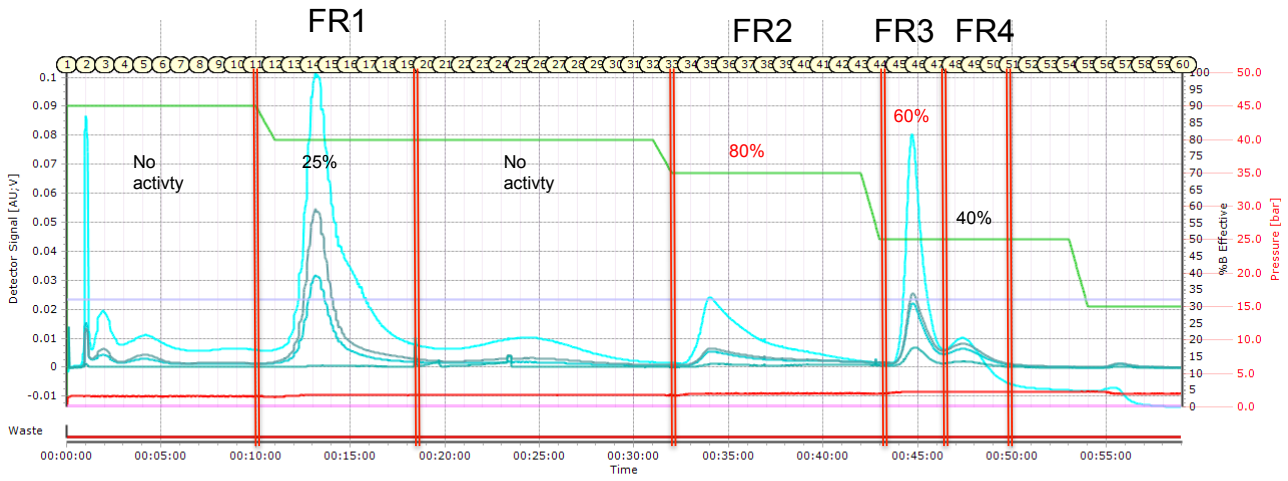


Fig. 23 C18 column performed on black tea 25, the last three fractions give absorption at the wavelengths indicate by the OPLS model and are also the most active ones (see percentage of activity indicated on the top in red).

Although the structures of the active compounds present in FR2 and FR3 are still under investigation, the developed UV-Vis based model was validated and it has been proved to be a useful, simple and low-cost technique to easily identify a tea with a strong collagenase inhibitory activity.

5. Conclusion

Medicinal plants as well as edible plants commonly used in the daily diet can be considered a valuable source for the maintenance of the extracellular matrix homeostasis, helping to prevent and treat many diseases and to modulate the ageing processes. In this work the activity of different medicinal plants was valued against collagenase, an enzyme belonging to the MMPs family, which deregulated activity has been found associated to several multifactorial diseases.

Metabolomics analysis combined with multivariate data treatment was used to investigate a large number of medicinal plants and establish some common chemical features conferring this biological activity. In particular, tannins were identified as an important class of compounds able to inhibit collagenase. A tannin-removal procedure was developed, allowing to prove this result and also to identify another class of secondary metabolites capable of strong collagenase inhibition, namely: glucuronide conjugated flavonoids as quercetin-3-O-glucuronide. In this context, *Alchemilla vulgaris* was found an important source of this compound.

Alchemilla vulgaris belongs to the Rosaceae family and is an herbaceous perennial plant, growing in temperate regions. It is topically used in the Mediterranean traditional medicine as anti-inflammatory and to facilitate wounds and burns healing.

According to our results, *Alchemilla vulgaris* was firstly selected as a medium activity plant against collagenase but after the tannin-removal procedure was identified as the plant less affected, in term of activity, by the tannins lost.

Therefore, this plant was better investigate and the relevant collagenase inhibitory activity found in *Alchemilla vulgaris* was attributed, through a bio-assay guided fractionation procedure, to the presence of quercetin-3-O-β-glucoronide, which showed highest activity than the control doxycycline and other common flavonoids. This result can support the traditional use of this plant and encourage further investigation on its pharmacological properties.

Tea plant (*Camellia sinensis*) was also studied for its collagenase inhibitory activity; in particular different varieties of tea were compared. The tested teas showed a wide range of collagenase inhibitory activity, in particular the majority of the black teas were found endowed with the highest percentage of activity. According to our results, different catechins are involved in the discrimination between the active and not active teas. This kind of secondary metabolites are highly variable among the different tea variety.

On the base of this supposition, theaflavin, a typical black tea catechin, was tested as collagenase inhibitor and it was proven to be particularly active when compared to the common green tea catechins. Thus, it was developed a UV-Visible metabolomics analysis able to correlate the UV-Vis

spectra of teas and the biological activity values by an OPLS model, which showed a positive correlation between high activity and increasing absorbance in the range between 350-440 nm. Absorptions at these wavelengths are generally considered typical of theflavins and thearubigins, characteristic of the black teas.

Another multivariate model (OPLS-DA), including the ^{1}H-NMR spectra of the teas, was performed. The samples were divided in two classes (active and not active) and NMR spectra were correlated to the absorbance values 405 nm. This model indicated a correlation between highest activity, absorbance at 405 nm and some ^{1}H-NMR signals that can be attributed to black teas catechins.

Moreover, the highest activity tea sample was further purified and only the active fractions showed UV-Vis spectra with absorbitions between 350-440 nm as predicted by the metabolomics analysis.

Even if the structure of the compounds present in these active fractions is still under investigation, in this work, was proved that black teas catechins are even more active than green tea catechins as collagenase inhibitors. Moreover, it was proposed a valuable and easily performed method to individuate a strongly active tea looking for collagenase inhibitory activity. In fact, the tea endowed with absorptions between 350-440 nm and especially at 405 nm can be considered promisingly for this activity. Considering that the absorbance measurement of an extract is a low-cost and simple procedure, the proposed method can be suitable, for instance, in order to provide information to select the best tea variety to develop an anti-wrinkles cosmetic or food supplement.

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