

Alma Mater Studiorum – Università di Bologna

DOTTORATO DI RICERCA IN

Scienze Chimiche

Ciclo XXIV

**Settore Concorsuale di afferenza: 03/D1-CHIMICA E TECNOLOGIE
FARMACEUTICHE TOSSICOLOGICHE E NUTRACEUTICO-
ALIMENTARI**

Settore Scientifico disciplinare: CHIM/08-Chimica Farmaceutica

**FROM FOLKE MEDICINE TO MEDICINAL CHEMISTRY: STUDY, USING
IN VITRO AND CELLULAR ASSAYS, OF RECEPTORS MECHANISMS
INVOLVED IN THE ACTIVITIES OF NATURAL COMPOUNDS**

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Esame finale anno 2012

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CHAPTER 1

Natural Products and Drug Discovery

For thousands of years, natural products have played an important role throughout the world in treating and preventing human diseases. Natural product medicines used in different countries and Medical Systems have come from various source materials including terrestrial plants, terrestrial microorganisms, marine organisms, and terrestrial vertebrates and invertebrates.

The value of natural products in this regard can be assessed using 3 criteria:

- the rate of introduction of new chemical entities of wide structural diversity, including serving as templates for semisynthetic and total synthetic modification
- the number of diseases treated or prevented by these substances
- their frequency of use in the treatment of disease.

An analysis of the origin of the drugs developed between 1981 and 2002 showed that natural products or natural-product derived drugs comprised 28% of all new chemical entities (NCEs) launched onto the market. [[Newman *et al.* 2003](#)]

Furthermore, 24% of these NCEs are synthetic or natural mimic compounds, based on the study of pharmacophores related to natural products. [[Newman *et al.* 2000](#)]

This combined percentage (52% of all NCEs) suggests that natural products are important sources for new drugs and are also good lead compounds suitable for further modification during drug development. The large proportion of natural products in drug discovery has stemmed from the diverse structures and the intricate carbon skeletons of natural products.

Since secondary metabolites from natural sources have been elaborated within living systems, they are often perceived as showing more “drug-likeness and biological friendliness than totally synthetic molecules” [[Koehn *et al.* 2005](#)]

making them good candidates for further drug development. [Balunas *et al.* 2005] [Drahl *et al.* 2005]

The investigation of the pharmacological activity of vegetal extracts represents the start point for the research of active molecules in the vegetal mixture and for the clinical investigation of the effectiveness of the whole extract which may administered as a food supplement.

Scrutiny of medical indications by source of compounds has demonstrated that natural products and related drugs are used to treat 87% of all categorized human diseases, including as antibacterial, anticancer, anticoagulant, antiparasitic, and immunosuppressant agents, among others. [Newman *et al.* 2003]

In the case of antibacterial agents, natural products have made significant contributions as either direct treatments or templates for synthetic modification. Of the 90 drugs of that type that became commercially available in the United States or were approved worldwide from 1982 to 2002, ~79% can be traced to a natural product origin. [Newman *et al.* 2003]

In the United States, 84 of a representative 150 prescription drugs are represented by natural products, including, predominantly, as anti-allergy/pulmonary/respiratory agents, analgesics, cardiovascular drugs, and for infectious diseases. [Newman *et al.* 2003]

Furthermore, natural products or related substances accounted for 40%, 24%, and 26%, respectively, of the top 35 worldwide ethical drug sales from 2000, 2001, and 2002. [Butler 2004]

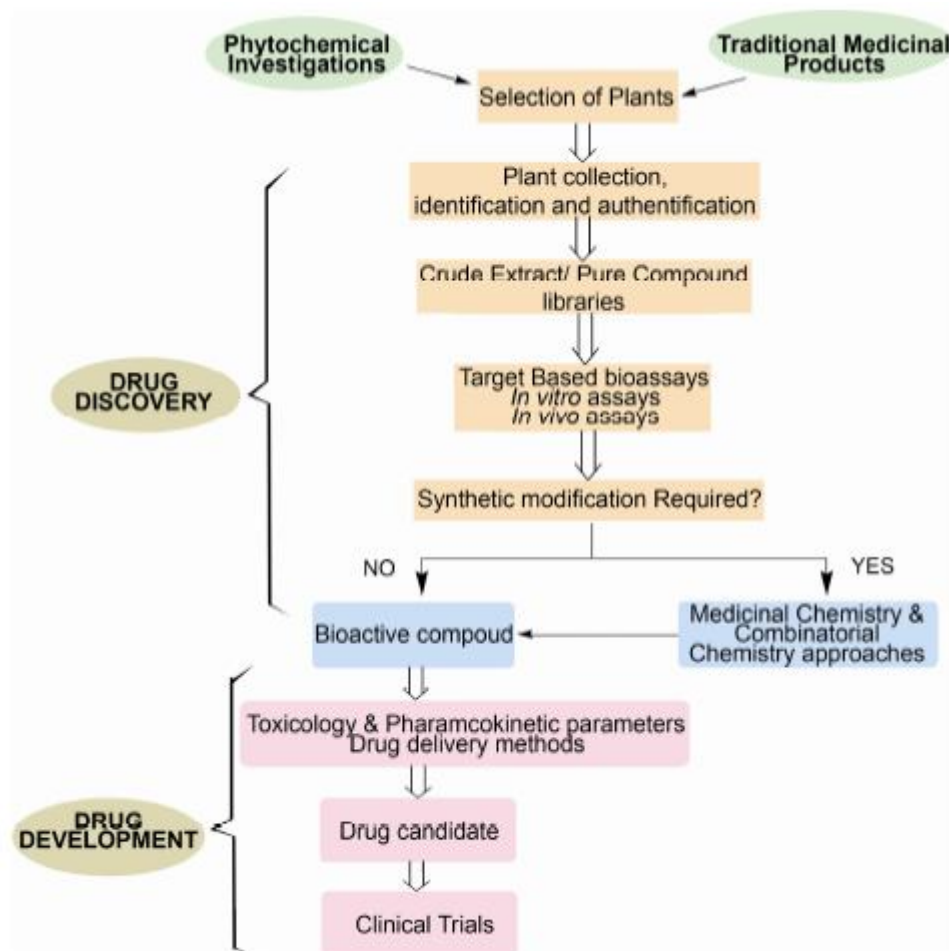


Fig. 1: Drug Discovery from vegetal extracts

The process leading to the discovery of new active compounds from vegetal extracts can involve

- the evaluation of the pharmacological activity of the whole extract, obtained in the same way it is done in Folk Medicine
- Fractionation of the extract
- Evaluation of the pharmacological activity of the different fractions and identification of the most active fraction
- Further Fractionation of the active fraction and identification of the active compound(s)
- Synthesis of analogs through modern medicinal chemistry-based molecular modification

The drug discovery process presupposes the knowledge of the Traditional Medicine in order to identify the vegetal extracts we can consider to give rise to the Research of new active compounds from natural extracts.

Traditional Chinese Medicine and Ayurvedic Medicine: different approaches

The two most prevalent forms of traditional medicine (TM) in Asia are the traditional Chinese medicine (TCM) and the traditional Indian medicine (represented by Ayurveda).

Over the years, TCM and Ayurveda have diffused all over the world. The two medical traditions represent a larger and larger part in the global market, presumably due to the rising interest not only among the consumers but also among the Medical Doctors. [Patwardhan *et al.* 2005]

The historical, cultural and social foundations of the Asian states are based on the three main philosophical traditions, represented by the Vedic philosophy (giving rise to Ayurveda), Taoism (giving rise to TCM) and Confucianism. Ayurveda and the Vedic philosophy represent the main phylosophies in the West Asian countries including India, Pakistan, Tibet, whereas TCM is the main philosophy in the East Asian countries including China, Korea, Japan, Vietnam. [Patwardhan *et al.* 2005]

Another Traditional Medicine, derived from TCM, is the Korean medicine (TKM). The Sasang constitutional medicine (SCM), first introduced by Jema Lee in 1894, belongs the TKM which shares the same principles of the TCM. [Song 2005]

Many different aspects in Ayurveda, TCM and SCM are common.

SCM, TCM and Ayurveda show the same basic holistic approach to healthcare which presupposes that the subject is considered as a whole entity. According to these medical traditions, pathological conditions are the results of single or combined disturbances/imbances on the physical, psychological, social and spiritual levels. Medical interventions therefore necessarily take into account the multifaceted and complex relationship between the spirit, mind and body,

and the aim of therapy is not the elimination of the isolated disease or symptom but the treatment of the body as a whole. [Zollman *et al.* 1999]

The diagnosis, in Traditional Medicine, is based on the subjective examination, consisting of observing, listening, inquiring and palpating, of the patients by the Medical Doctors and the Therapy is exerted through a range of therapeutic modalities such as herbal medication, acupunctural therapy and manual therapy.

In general, the herbal remedies used in this kind of Medicine, are a mixture of different vegetal extracts and the therapeutic effect is the result of the synergistic action of all the administered plant extracts which aim to restore the internal body imbalance. [Kim *et al.* 2009] [Khan *et al.* 2001]

The Approach belonging to the so called “holistic Medicine” involves the Examination of the patient who has to be treated as a whole, complex organism and the therapy can not include the same substances for the cure of a Disease, while it provides the substances able to cure the patient belonging to a prevalent constitution who develops a Disease. The Concept is to cure the Person, not the Disease. In other words, the same Disease may be treated with different herbal formulae or therapeutic methods depending on the characteristics of the patient.

Individuals are born with different traits and characteristics. SCM and Ayurveda emphasize the importance of variation in the constitutional makeup among individuals. These two medical traditions are based on the recognition and acceptance of the inherent constitutional differences between individuals, a concept that is central in SCM and Ayurvedic therapeutics. [Song 2005] [Sharma *et al.* 2007]

In contrast, the pathological presentation of the patient at the time of examination is the foremost consideration in TCM, whereas the other factors (such as the progression of disease, family history and congenital conditions) are taken into consideration but only in a secondary capacity. Although TCM, SCM and Ayurveda show the same qualities of holistic medicine in which they all treat an individual as a whole, they each start off from different viewpoints. TCM therapy begins with the evaluation and differentiation of syndrome (or the identification of disease patterns) [Tang *et al.* 2008], whereas the

constitutional typing and determination of the constitutional proclivity represent the first steps in SCM and Ayurveda therapy. Whereas the TCM therapy uses reducing and tonifying methods to redeem the external pathogenic factors such as blood stasis and qi deficiency, the therapeutic goal in SCM lies in the restoration and minimization of the imbalance in the quadrifocal organ scheme. In other words, although the therapeutic methods and materials may overlap, TCM and SCM use them for completely different reasons from completely different rationales. Ayurveda assigns an individual into one of the seven main constitutional types, or *prakriti*, based on the inherent imbalance of the three energy forces, or *dosha*, that are each called *Vatta*, *Pitta* and *Kapha*. SCM is rooted in the quaternity central to the Sasang philosophy and classifies the constitutional makeup of an individual into one of the four constitutional types namely, the Taeyang type (TY), the Soyang type (SY), the Taeum type (TE) and the Soeum type (SE). In SCM, the inherent proclivity in the constitutional imbalances exacerbates the weaknesses of the constitutional type, leading to specific patterns in susceptibility to particular pathologies. SCM therapy, therefore, is focused on minimizing these weaknesses in order to restore the internal balance. [Song 2005] [Sharma *et al.* 2007]

In SCM, the concepts of physiology and pathology are based on the quadrifocal scheme or quaternity which differs from the bifocal scheme or dichotomy of the Yin-Yang theory, representing the philosophical basis of TCM. The model explaining the internal organ structure in SCM is called “seesaw” model. In this system, the SE and SY types correspond to the spleen-kidney seesaw, where the spleen is responsible for the intake of food and the kidney for the discharge of waste products. A strong kidney system and a weak spleen system are typical characteristics of the SE type, whereas a strong spleen system and a weak kidney system belong typically to the SY type. The TE type has a strong liver system and a weak lung system, whereas the TY type is characterized by a strong lung system and a weak liver system [Song 2005] [Kim *et al.* 2009]

The concept of lung, liver, spleen and kidney in SCM was originally derived from the TCM theories but later evolved into a different physiopathological concept.

According to SCM, the weakness of each constitutional type corresponds to the preservative energy related to the most hypoactive viscera which represents the essential energy necessary to maintain homeostasis. The clearing Yin energy, the warming Yang energy, the dispersive energy and the accumulative energy are the requisite energies for the SE, SY, TE and TY types, respectively. The main therapeutic goals in SCM are the reinforcement and the preservation of the requisite energies.

The Ying Yang theory represents the base of the TCM pathology, even if the main theory is represented by five elemental phases theory. The five elements, which are wood, fire, earth, metal and water, exist in a mutual relationship between them. The restoration of the balance among these elements is the aim of the TCM. A five elements theory is also present in the Ayurvedic physiology and pathology [Tirtha 2005], even if this concept in Ayurveda differs from TCM. Infact, the five elements in the Ayurvedic theory are considered to have a sequentially fortifying relationship only, whereas the TCM elemental phases interact mutually in assisting and controlling relationships.

The therapeutic remedies used in Asian TM traditions are, in general, made with on botanical sources. The SCM and the TCM use, generally, the same herbal remedies, however the basic principles of usage and the underlying rationale are completely different. In SCM, the prime consideration concerns the identification of the constitutional type of the patient which is a critical step for the selection of the medicinal herbs and formulae for treatment. A particular medicinal herb is compatible with only one specific constitutional type and can therefore be used for that constitutional type only and be mixed with other herbs compatible with that constitutional type only. Use of a medicinal herb on an incompatible constitutional type can result in little effect or even induce adverse effects. For example, Radix Ginseng, an SE medicinal herb, and Radix Rehmanniae Glutinosae, an SY medicinal herb, should not be used in combination with each other. Also, a medicinal herb cannot be used across different constitutional types, but can be used for different symptomatology or diseases within that constitutional type [Kim *et al.* 2001 a] [Kim *et al.* 2001 b]

In contrast, TCM medicinal herbs are classified according to the therapeutic effects of the herb itself, namely, dispersive quality, Yin tonifying quality and so forth. Consequently, a particular medicinal herb can be applied to any patient afflicted with the same disease or pathology regardless of the individual's constitutional type. For instance, Radix Ginseng is sometimes used in combination with Radix Rehmanniae Glutinosae in some TCM formulae [Liu *et al.* 2005]. Ayurvedic and SCM therapeutics are based on constitutional approach, and the medicinal herbs are selected or excluded according to their compatibility or incompatibility to the constitutional makeup of a given individual. Ayurvedic medicinal herbs are distinguished by their effects on the three doshas, whereas SCM medicinal herbs are categorized according to their effects on the different constitutional types. For instance, Cortex Cinnamomi, a commonly used medicinal herb, is described in the Ayurvedic practice as being able to repress Vitta and Kapha while enhancing Pitta, whereas in SCM it is suggested to be compatible with the SE type and incompatible with the SY type. On a slightly different note, the actual specimen of medicinal herbs used in Ayurveda and SCM are likely to be different from each other due to the differences in the regional flora. [Kim *et al.* 2001 a] [Liu *et al.* 2005]

Traditional Medicine in the West

Theophrastus (370-285 B.C.), who was a philosopher from Athens and a Student of Aristotle, is considered the founder of the West botany. The Botanical Theophrastus' works, *Historia plantarum* and *Causae plantarum*, cover almost every part of the modern Botany, including morphology, physiology, taxonomy and pharmacognosy. These texts represent a significant part of the ancient knowledge in the field of botany. Ippocrate Kos, a physician of the ancient Greek who used methods of cure which have been used up to the Roman world and to the Middle Ages, was the first to classify, systematically, 300 plants species. One of the oldest botanical gardens is the garden of Alexandria of Egypt (from the fourth century B.C., under the Ptolemies). Another important botanical garden is the garden established in Athens, around 340 B.C., by the will of Aristotle with the aim of study and research about plants.

In the Roman times, very important works about pharmacognosy and pharmacotherapy are written. In these works, the drugs are no longer reported as a simple list or as an appendix to the disease (like in the writings of Hippocrates), but they are described by systematic and descriptive criteria which include their dosage, their method of administration, their adverse effects.

Since the first century A.C., in Rome, it was common practice to cultivate gardens with medicinal plants. Among the most important works of this period, we must remember the *De medicina* of Celsus (18 AD); the important work in 5 volumes of Dioscorides Pedanius Anazarbeo (I century AD), *De Materia Medica*, which represent all the medical knowledge at the time, including that relating to the medicinal properties of plants. This encyclopedia was considered a great authority throughout the Middle Ages, almost to the sixteenth century. For the first time, the plants are not reported in the alphabetical order, but according to their affinities. In these works, the descriptions are often influenced by philosophical, magical and astrological conceptions.

Another important physician of the Roman time is Claudius Galen (190-291) who cataloged the drugs according to the heat (or mood), allowing the choice of drug with this parameter for each disease (*Methodus medendi*). After the fall of the Roman Empire and the barbarian invasions, the scientific knowledge was preserved in the monasteries or developed by the Arabian world.

The modern phytotherapy origins in the Renaissance period with the birth of the first medical schools and universities (second medical school of Salerno. XI-XIII, University of Montpellier sec. XII). Paracelsus, the Medici, the Este, Leonardo da Vinci encouraged the research. During this period, there has been a shift away from empiricism of the alchemists in favor of a scientific testing with more sophisticated means of investigation. This allowed Carl Linnaeus (1707-1778 AD) to develop the systematic study of plants and to establish strict rules for the cultivation and harvesting of medicinal herbs. In the period from late 1700 to early 1800, in the field of Medicine many important discoveries occurred.

In the nineteenth century medical science has made an exponential progress, accompanied by surprising novelty in the field of chemistry: Wöhler 1800-1882, a student of Berzelius, obtained urea in the laboratory while he was trying to prepare the ammonium thiocyanate. After this first synthesis, Hermann Kolbe, a professor of chemistry at the University of Narburg (1818-1884), student of Wöhler, succeeds in the synthesis of acetic acid. In 1859, he discovered the chemical structure of salicylic acid and he was able to synthesize the molecule in the laboratory. The French chemist Marcellin Berthelot (1827-1907) could synthesize dozens of organic chemicals such as methyl alcohol, ethyl alcohol, methane, benzene and acetylene. From the early years of the '900, with the development of chemical technology and science, there has been a shift away from medicine herbal medicine and a growing attention to synthetic drugs, with the support of the first chemical-pharmaceutical companies that arose in those years. In the second half of the 900 medicine in Europe, called classic, is the only one to be in common use. However, in recent years, there has been a significant turnaround, with the return to herbal medicine and alternative medicine in general: there are now realities intimately related, traditional medicine and alternative medicine. Today we are able to separate the different active ingredients contained in plant extracts and to investigate which component is attributable to the pharmacological effect of the extract studied could, in this way to combine the ancient with the modern science, for the continuous search for new therapeutic molecules that the natural world offers us.

CHAPTER 2

Castanea Sativa Mill.



Fig. 2: *Castanea sativa* Mill.

Chestnut is a genus gathering eight or nine species of deciduous trees and shrubs, belonging to the Family of Fagaceae. The origins of this genus are represented by the temperate regions of the northern hemisphere. There are four main species referred as European chestnut (*Castanea sativa* Mill), Chinese chestnut (*Castanea mollissima*), Japanese chestnut (*Castanea crenata*), American chestnuts gathering *dentata* (American chestnut - Eastern states),

Castanea pumila (American- or Allegheny chinkapin, also known as "dwarf chestnut" - Eastern states), *Castanea alnifolia* (Southern states), *Castanea ashei* (Southern states), *Castanea floridana* (Southern states) and *Castanea paupispina* (Southern states). [[Artemas Ward 1911](#)] [[Mencarelli 2001](#)]



Fig. 3: *Castanea sativa* Mill Distribution

This work is focused on *Castanea sativa* Mill.

The sweet chestnut tree is a native of southern Europe, the Caucasus, Asia Minor and northern Africa. The young branches are reddish brown with light lenticels (pores in the bark), the leaves are elongated, feather-shaped and serrated. dark green on top and a lighter green underneath. The seeds grow in a green-brown cupule or outer shell with long spiky hairs which can be up to 5 centimetres long. Depending on the variety, one cupule may contain one to three seeds, which become brown when ripe, in October or November. The bark of the tree takes on a grey hue with age and is grooved and typically twisted. The chestnut wood is characterized by early formation of heartwood, and the sapwood is very thin. The heartwood is brown while the sapwood is light gray. A typical characteristic of the sweet chestnut is the large pores of the springwood that are clearly visible on the end-grain. They are also apparent as ridges in the long-section. The pores of the summerwood are much finer and barely visible. The longitudinal section is either clearly striped (radial cut) or

with a wavy grain (cross cut). A characteristic of chestnut wood is the lack of wood rays on the end-grain.

The sweet chestnut blooms in June or July, its blossom consisting of the male catkins, which are long, yellowish anthers, and the reddish females inflorescences.

The sweet chestnut, representing the most characteristic tree of southern Europe together with olive and fig, has always been of great economic importance especially in Italy, France and Switzerland.

It has been cultivated on a large scale for its nourishing as well for its wood.

The wood and the bark of sweet chestnut contain a lot of tannins, so in Europe it has been used for the colouring and tanning of hides.

The tannin content of a mature sweet chestnut wood (a tree at least thirty years old) in Southern Europe is 10 to 13 percent higher than that of the chestnut trees in the north. Italy has the largest commercial production of tannin.

The wood is very durable and serves, as the French say, literally *du berceau au cercueil*, from the cradle to the grave” (Coffin). It is used for poles (for instance, in the Kentish hop gardens), fence posts, railway sleepers, beams, garden furniture and, on account of being waterproof, for making wine barrels. In addition, it is used in order to produce castanets, mostly cut from chestnut wood, as their name indicates.

The Sweet Chestnut Wood Extract is, used in Folk Medicine, as an antidote against the bite of a rabid dog, as well as against dysentery, coughing and vomiting, and baldness. In a Swedish herbal the sweet chestnut is also mentioned as a remedy for whooping cough.

In folk medicine, the water extract of the bark is used in order to cure diarrhoea of different origin. In order to verify the existence of an activity toward gut motility of a Natural Extract of Chestnut Wood extract rich in ellagitannins, named ENC, its pharmacological activities have been tested in the experimental models described in the following paragraph.

In addition since ENC shows antimicrobial activities toward pathologic gastrointestinal agents, a potential antispasmodic activity may be useful to treat diarrhoea, which represents a world-wide health trouble, as described in the following paragraph.

CHAPTER 3

ENC (Natural Extract of Chestnut Wood)

Purified ENC (supplied by SilvaTeam, San Michele di Mondovì, Italy) is obtained by low pressure heating treatment. The water-soluble fraction is retained and subsequently dehydrated. The fine brown powder (92-95% dry matter) contains 77% of pure tannin on a dry matter basis. The chemical composition of the ENC batch used in the experiments was as follows: water, 2.9%; tannin, 77.8%; non-tannin, 17.7% (oligosaccharides, salts, vegetable resins, and gums coming from the hydrolysis process of chestnut wood); insoluble, 1.6%; crude fibers, 0.24%; ash, 1.7%. The tannin percentage was obtained by gravimetric analysis of vegetable tanning agents by using the filler Freiberg-Hide powder method. [Kuntzel 1954]

3.1 Chemical characterization of ENC

Before starting the evaluation of the biological activity of the extract, the analysis in order to identify the main organic compounds present in ENC has been exerted.

The total phenol (TP) content of tannin of chestnut extract was firstly determined by Folin-Ciocalteu method. [Singleton *et al.* 1965] This colorimetric test was used for a preliminary characterization of the extract, in fact, as each phenolic compound produces a different colour yield per unit mass in colorimetric assay, it is very disputable to choose a single phenol as reference standard for the total phenol calculation. [Mueller-Harvey 2001] The molar absorptivity of the standard chosen is peculiar and dependent on the number and the kind of chromophores present in the molecule, and on the solvent used for the detection. This peculiarity should be considered during the spectrophotometric quantization of total phenols, because it determines the intrinsic approximation of such analytical technique. [Pelillo *et al.* 2004]

Basing on literature data [De Vasconcelos *et al.* 2010] [Scalbert *et al.* 1989] [Vázquez *et al.* 2008] [Živković *et al.* 2010], gallic acid was selected as reference standard for a TP content by Folin-Ciocalteu spectrophotometric

method. TP content of the examined chestnut bark extract was 54.9 % of dry weight (g GAE/100 g of extract).

A quasi-quantitative analysis of the extract and was realized by HPLC. Tentative identification of tannins and phenolic compounds was made on the basis of retention time, molecular weight, spectroscopic properties and MS fragmentation characteristics (ESI negative mode), as described in Comandini *et al.* [Comandini *et al.* 2011] Table 1 reports the compounds characterized in chestnut bark extract, including ellagic acid, gallic acid and 4 ellagitannins (vescalin, castalin, vescalgin and castalgin).

Table 1 Amounts of separated tannins and phenolic compounds in chestnut bark extract, expressed as g EAE /100 g and as g GAE/100g.

Compound	g EAE/100 g^a	g GAE/100g^a
Vescalin	0.56 ± 0.02	1.18± 0.06
Castalin	0.69 ± 0.02	1.47± 0.06
Gallic acid	1.25 ± 0.04	3.68± 0.12
Vescalgin	2.31 ± 0.05	5.01± 0.11
Castalgin	2.26 ± 0.07	4.96± 0.08
Ellagic acid	1.70 ± 0.05	3.64± 0.10
Other compounds	1.92 ± 0.04	4.07± 0.04
Total	10.69 ± 0.28	24.01± 0.57

^a Values are means ± S.D. (n=3).

The three major components were vescalgin, castalgin and ellagic acid. Other minor compounds (quantified as Other compounds in Table 1), previously reported in chestnut bark [Lampire *et al.* 1998] [Canas *et al.* 1999] detected in trace levels are: 5-*O* galloylhamamelose, (3 ,5-dimethoxy-4 -hydroxyphenol)-1-*o*-β -D-(6'-*o*-galloyl)-glucoside isomer, *m*-digallic acid, kurigalin isomer and chestanin. The class “Other compounds” also included a not identified ellagitannin, which eluted between vescalgin and castalgin in the chromatographic trace, and represented 14.6% of the total concentration of the separated compounds.

The total concentration of ellagitannins and phenol compounds identified, expressed in ellagic acid, was about 10.69 g GAE/100 g.

Vescalgin and castalgin belongs to the class of ellagitannins and in particular to the Okuda's type II+ ellagitannins.

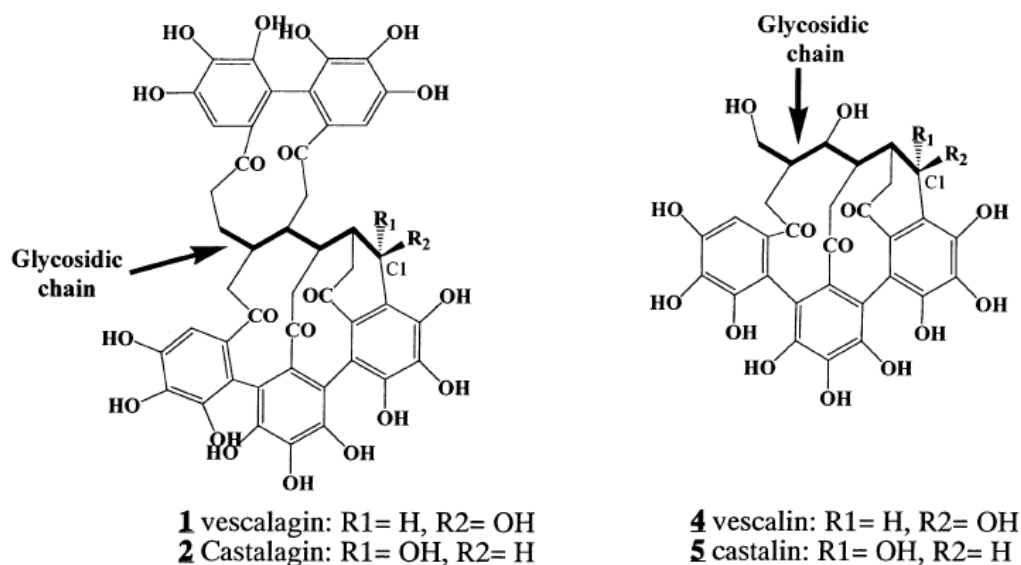


Fig. 4: Chemical structures of the 4 main ellagitannins present in ENC

3.2. General Structures of Ellagitannins.

Ellagitannins represent one of the major classes of polyphenolic natural products and derive from the secondary metabolism of dicotyledonous plant species of the Angiospermae. [Quideau 2004] [Quideau 2006] [Quideau *et al.* 2011] Their general chemical structures consist, basically, of a central sugar core, in general D-glucopyranose, to which are esterified gallic acid (1, i.e., 3,4,5-trihydroxybenzoic acid; fig.) units that are further connected together through C–C biaryl and C–O diaryl ether bonds as a result of intra- and intermolecular phenolic oxidative coupling processes. [Haslam and Cai 1994] [Quideau and Feldman 1996] [Khanbabaee and van Ree 2001] To date, thanks to the work of more than 50 years of investigations, from the seminal work of the German chemists Schmidt and Mayer to the outstanding contributions from the Japanese groups of Okuda, Yoshida, Nishioka and Kouno, about 1000 members of this subclass of so-called hydrolysable tannins (*vide infra*) have been isolated from different plant sources and completely characterised, thus determining the biggest group of known molecules belonging to the class of tannins. [Schmidt and Mayer 1956] [Okuda *et al.* 1995] [Okuda 2005] The number of about 1000 several molecular entities is very surprising when observing that they all plausibly are produced by a single precursor 3 [i.e., penta-O-galloyl- β -D-glucopyranose (β -PGG), see Scheme 1, Section 2], which is synthesized from two simple building blocks, represented by D-

glucopyranose and gallic acid. [Quideau 2004] A so evident structural diversity is the result of different various chemical reactions which, initially, involve oxidative (dehydrogenative) C–C coupling of galloyl groups on the glucopyranose core in either its 4C1- or its 1C4-conformation.

Further dehydrogenative transformations of galloyl and galloyl-derived groups are responsible for the induction of the hydration, decarboxylation, carbo- and oxocyclization, ring opening or ring contraction events, as well as of oligomerization processes via oxidative C–O coupling reactions. Hydrolytic cleavage of galloyl and galloyl-derived groups, glucopyranose ring opening (often followed by C-aryl glucosidation), additional galloylations, oligomerizing condensation reactions, as well as other condensation and conjugation events with other entities such as simple gallic acid derivatives, ascorbic acid, [Tanaka 2009] monosaccharides and flavanoids further expand the structural diversity and complexity of the ellagitannin family.

3.3 Different Groups of ellagitannins

3.3.1 Monomeric ellagitannins

The variety of ellagitannin structures is very large and its subdivision into different categories following a logically ordered manner has been carried out by different authors.

Haslam made a first subdivision of the two known subclasses of hydrolysable tannins (i.e., gallotannins and ellagitannins) into three groups: [Haslam 1982]

- group A, corresponding to the gallotannins with a core of β -penta-O-galloyl-D-glucopyranose (β -PGG, 3) which is linked to several other galloyl ester groups further linked in depside fashion, as shown in Scheme 1 by the typical hexagalloylglucose 4 (i.e., 3-O-digalloyl-1,2,4,6-tetra-Ogalloyl- β -D-glucopyranose).
- group B, characterized by the presence of two C–C-coupled galloyl ester groups at the 2,3- and/or 4,6-positions of a 4C1-glucopyranose core such as tellimagrandins I (5b) and II (5a). The 6,6'-dicarbonyl-2,2',3,3',4,4'-hexahydroxybiphenyl group, in general, represents the hexahydroxydiphenoyl (HHDP) unit, which is the structural characteristic defining hydrolysable tannins as ellagitannins.

Ellagitannins undergo hydrolytic reactions determining the release of HHDP units which are inevitably converted into the bislactone ellagic acid giving the name to these structures.

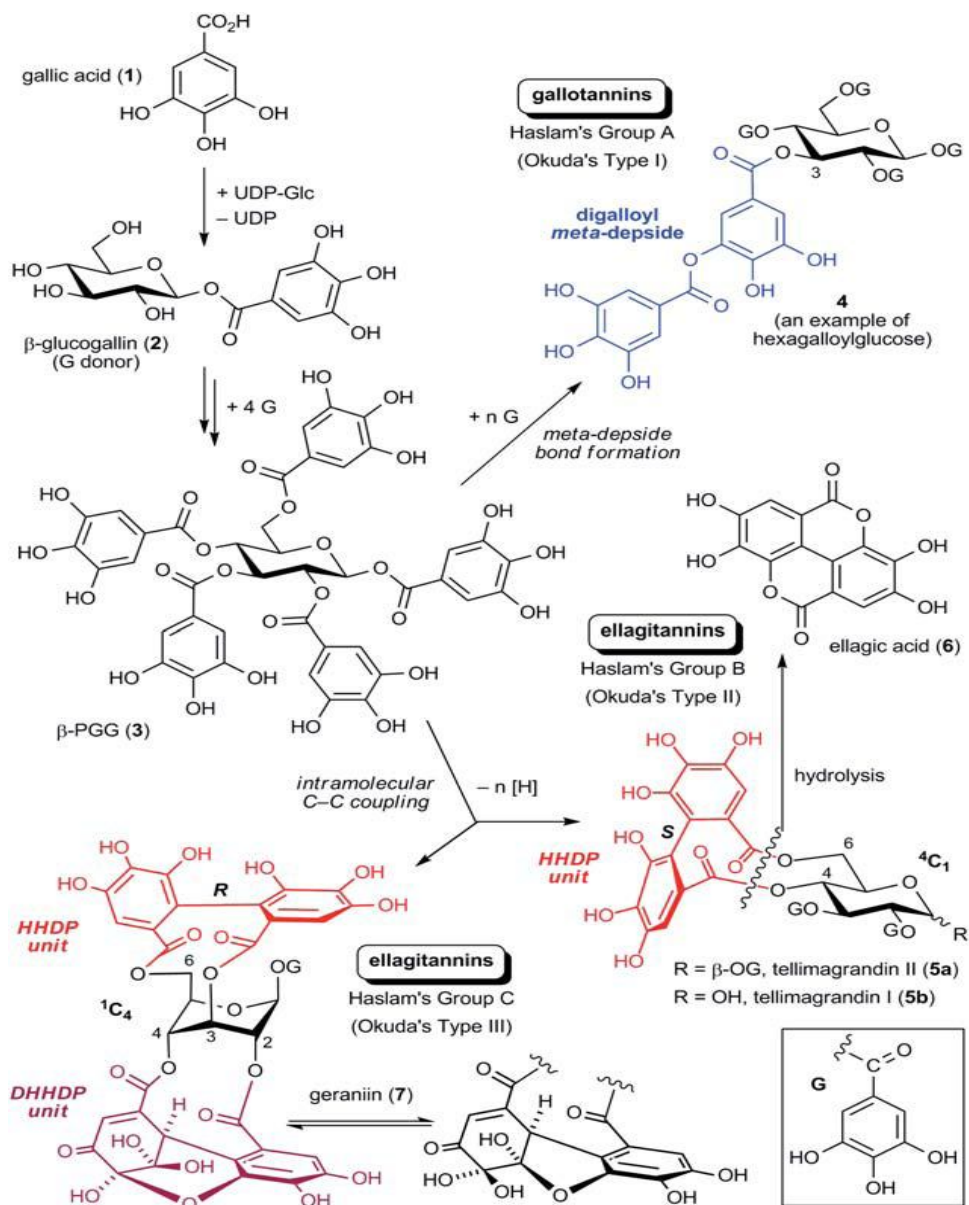


Fig. 5: Biosynthesis and classification of the different hydrolysable tannins (gallotannins and ellagitannins).

- Haslam's group C is represented by ellagitannins with HHDP units connected to the 1,6-, 2,4- and/or 3,6-positions of the D-glucopyranose ring in its least thermodynamically favored $1C_4$ -conformation, as exemplified by the structure of geraniin (7, Figure 5). [Haddock 1982]

These HHDP units show an axial chirality (i.e., atropisomerism).

In group B ellagitannins, these chiral biaryl units are found almost exclusively in the *S*-configuration, whereas ellagitannins of group C show both *R*- and *S*-configurations. [Quideau and Feldman 1996]

The HHDP bisester group undergoes a further oxidation leading to formation of the so-called hehydrohexahydroxydiphenoyl (DHHDP) unit, which isomerizes into an equilibrium mixture of hydrated five- and six-membered hemiacetalic rings in aqueous media. This additional oxidative metabolism regards, almost exclusively, the group C ellagitannins, so named ‘dehydroellagitannins’, such as geraniin (7, Fig. 5). there is a clear exception, represented by isoterchebin (8, Fig. 6), characterized by a DHHDP unit bridging the 4,6-positions of a D-glucopyranose ring in its 4C₁-conformation. [Okuda 1981]

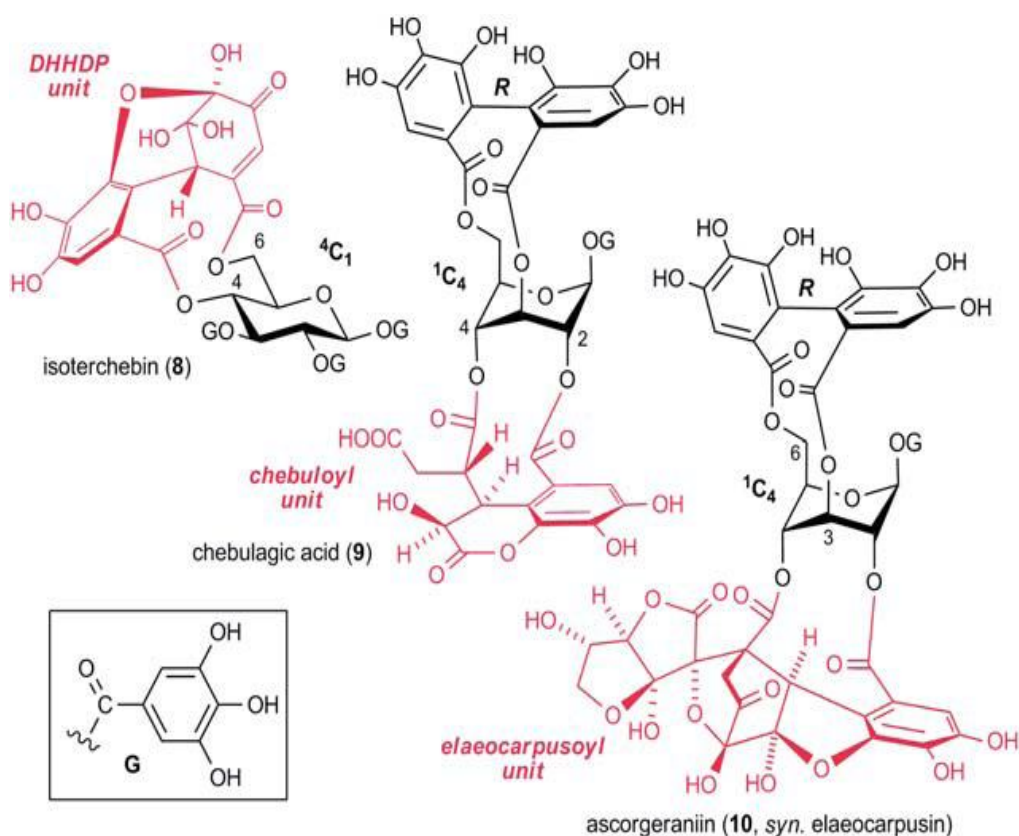


Fig. 6: Examples of ellagitannins featuring a DHHDP- or a DHHDPderived ester unit.

The DHHDP unit, consisting of a cyclohexenetrione C–C-bound to a pyrogallol motif, represents the site at which additional chemical reactivities are present, determining the origin of different other transformations producing various other types of monomeric ellagitannins. One example is represented by

chebulagic acid (9, Fig. 6) showing the DHHDP-derived chebuloyl unit esterified to the 2,4-positions of a 1C4-glucopyranose core, [Yoshida 1980] and ascorgeraniin (10, also known as elaeocarpusin), representing an example of an ellagitannin deriving from a condensation reaction between ascorbic acid and the DHHDP unit of geraniin (7). [Okuda 1986] [Tanaka 1986]

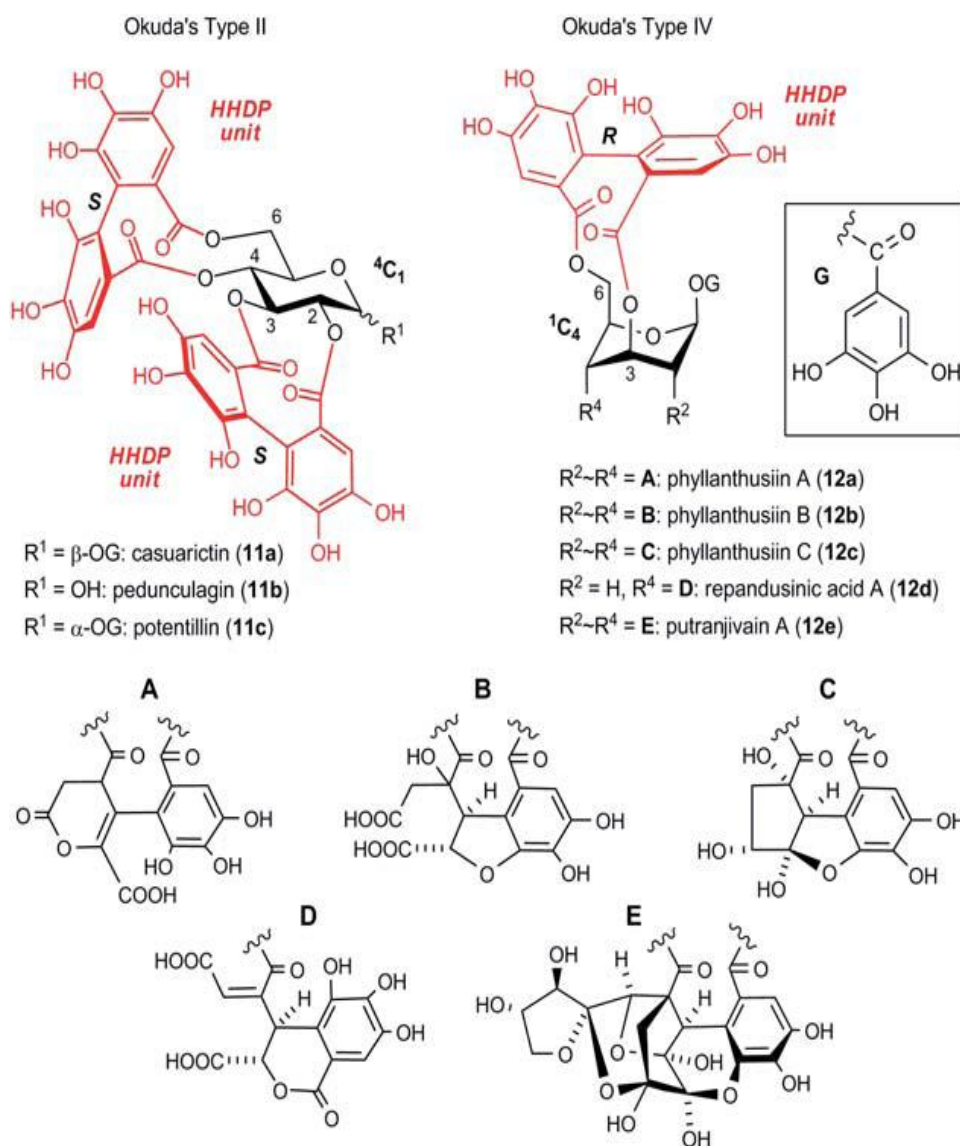


Fig. 7 Examples of Okuda's type II and type IV ellagitannins.

More recently, the old classification has been reviewed by Okuda and co-workers who proposed four main types of hydrolysable tannins basing on the oxidation level of their galloyl ester groups.[Okuda 2000] In this classification, which has been elaborated basing on a plausible progressive biogenetic

elaboration of hydrolysable tannins, first hypothesised by Schmidt and Mayer in 1956, gallotannins are defined as type I hydrolysable tannins. Type II includes the ellagitannins with a HHDP unit, involving, for example, the monomeric tellimagrandins II (5a) and I (5b) (Fig. 5), casuarictin (11a), pedunculagin (11b) and potentillin (11c) (Fig. 7), [Okuda 1982] [Yoshida 1983] and type III those with the DHHDP unit (i.e., dehydroellagitannins), such as geraniin (7, Fig 5).

As regards ellagitannins whose DHHDP unit undergoes additional transformations, such as the aforementioned chebulagic acid (9) and ascorgeraniin (10, Fig. 1), these structures form the type IV group. Two examples out of a large series of ester units derived from the parent DHHDP unit are represented by the chebuloyl and elaeocarpusoyl ester groups.¹³ Many other DHHDP-derived units exist.

These include (inter alia) phyllanthusiins A–C (12a–c), repandusinic acid A (12d), [Saijo 1989] [Yoshida 1992] and putranjivain A (12e), [Lin 1990] in which 2,4-DHHDP-derived unit is the result of the decarboxylation of the elaeocarpusoyl unit of ascorgeraniin (10). [Tanaka 2009] These ellagitannins are included into Okuda's type IV hydrolysable tannins group (Fig. 2).

In this classification many monomeric ellagitannins, whose structures deriving from chemical transformations other than those strictly mediated by oxidative processes, are excluded.

In this vein, some important structural modifications regard the opening of the D-glucopyranose core, the formation of C-aryl glucosidic bonds (vide infra) and condensation reactions then occurring at the glucose C-1 locus (see Section 7).

The steps involved in the biosynthesis of ellagitannins from their precursor, penta-O-galloyl- β -D-glucopyranose (β -PGG, 3, Fig 5), are just today starting to be comprehended, in particular thanks to the work exerted by Gross and co-workers. [Gross 1992] [Gross 1999] [Niemetz and Gross 2003a] [Niemetz and Gross 2003b]

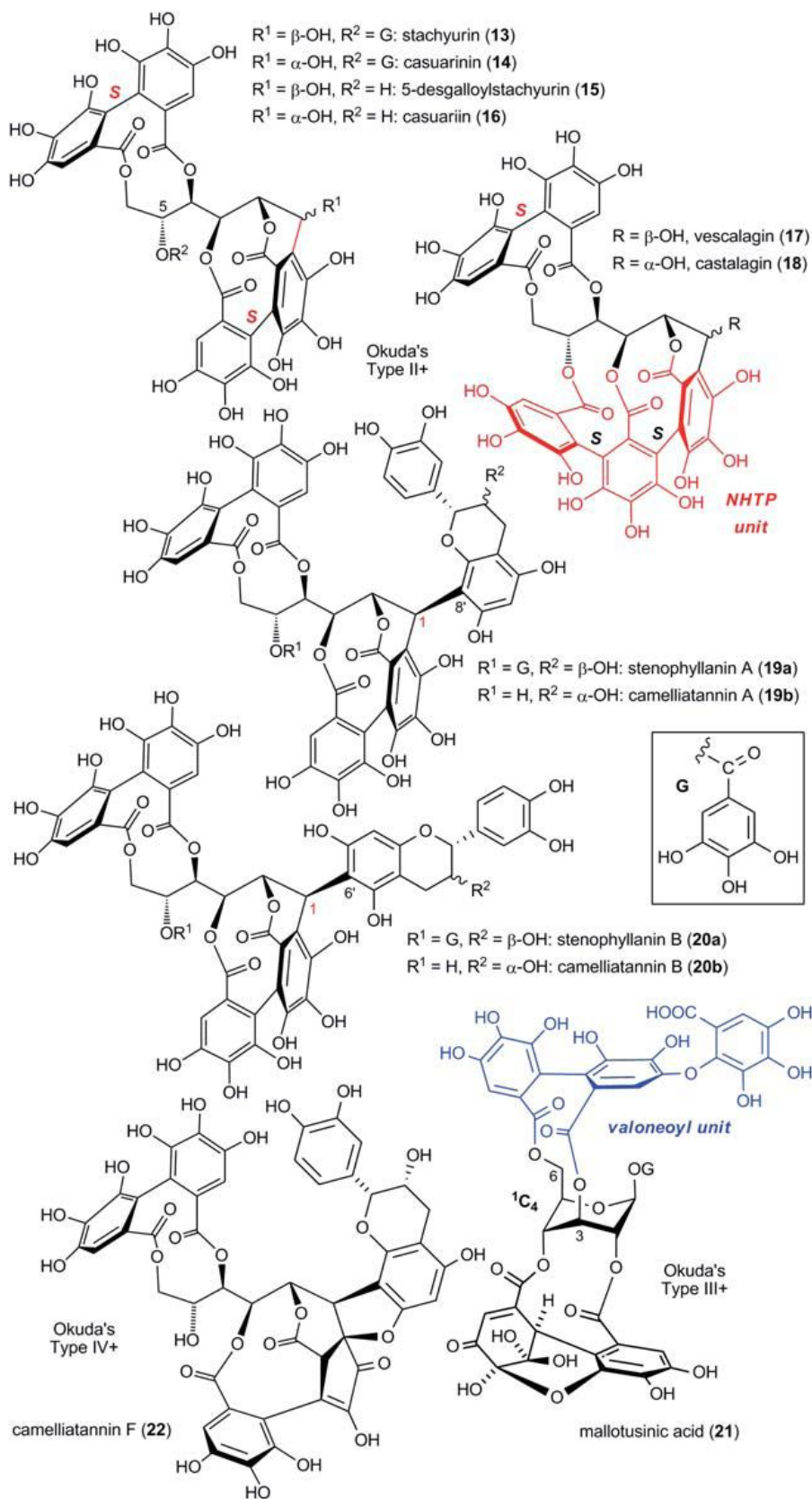


Fig. 8. Examples of Okuda's type II+, type III+ and type IV+ ellagitannins.

A β -pentagalloylglucopyranose-oxidizing enzyme favours the formation of the 4,6-HHDP-containing tellimagrandin II (5a) (Niemetz R., Schilling G. and Gross G. G., *Chem. Commun.*, 2001, 35–36) and a different laccase-type phenol oxidase catalyzes the dimerization of 5a into the m-DOG-type dimer, cornusiin E (26, Fig. 5), through the induction of the formation of a so-called valoneoyl-type diaryl ether bridge between the 2-galloyl group of one monomer and the 4,6-HHDP unit of the other monomer. [Niemetz and Gross 2003a] [Niemetz and Gross 2003b] Because these ellagitannins share the characteristic structural features with the primary types II–IV, these structures have been classified into types II+–IV+.22 Type II+ mainly includes HHDP-bearing C-glucosidic ellagitannins, such as stachyurin (13) and casuarinin (14) and their 5-O-desgalloylated variants 15 and 16, but also nonhydroxyterphenoyl (NHTP)-containing analogs such as vescalagin (17) and castalagin (18) (Fig. 8).

Flavanoid hybrids (i.e., flavano-ellagitannins, often globally referred to as complex tannins), including, among others, stenophyllanins A/B (19a/20a) and camelliatannins A/B (19b/20b), resulting from condensation reactions with the flavan-3-ol catechin or epicatechin at the C-1 center of their open-chain glucose core, belong to this type II+ subclass, too (Fig. 8).

Dehydroellagitannins showing moieties which are the result of a diaryl ether linkage with another phenolic or polyphenolic unit are included into the type III+ group.

Mallotusinic acid (21, Fig. 8), with a valoneoyl group (see Fig. 5 in Section 2.2), linked to the 3,6-positions of a 2,4-DHHDP-bearing 1C4-glucofuranose core, constitutes a representative example of this class. [Okuda 1978] [Okuda 1980]

Transformed dehydroellagitannins with moieties deriving from a C–C linkage with another phenolic or polyphenolic unit belong to the type IV+ group. One example of a structure belonging to this type resulting from the oxidative metabolism of the type II+ camelliatannin A (19b) is represented by the C-glucosidic epicatechin-containing complex tannin camelliatannin F (22, Fig. 8).

3.3.2 Oligomeric ellagitannins

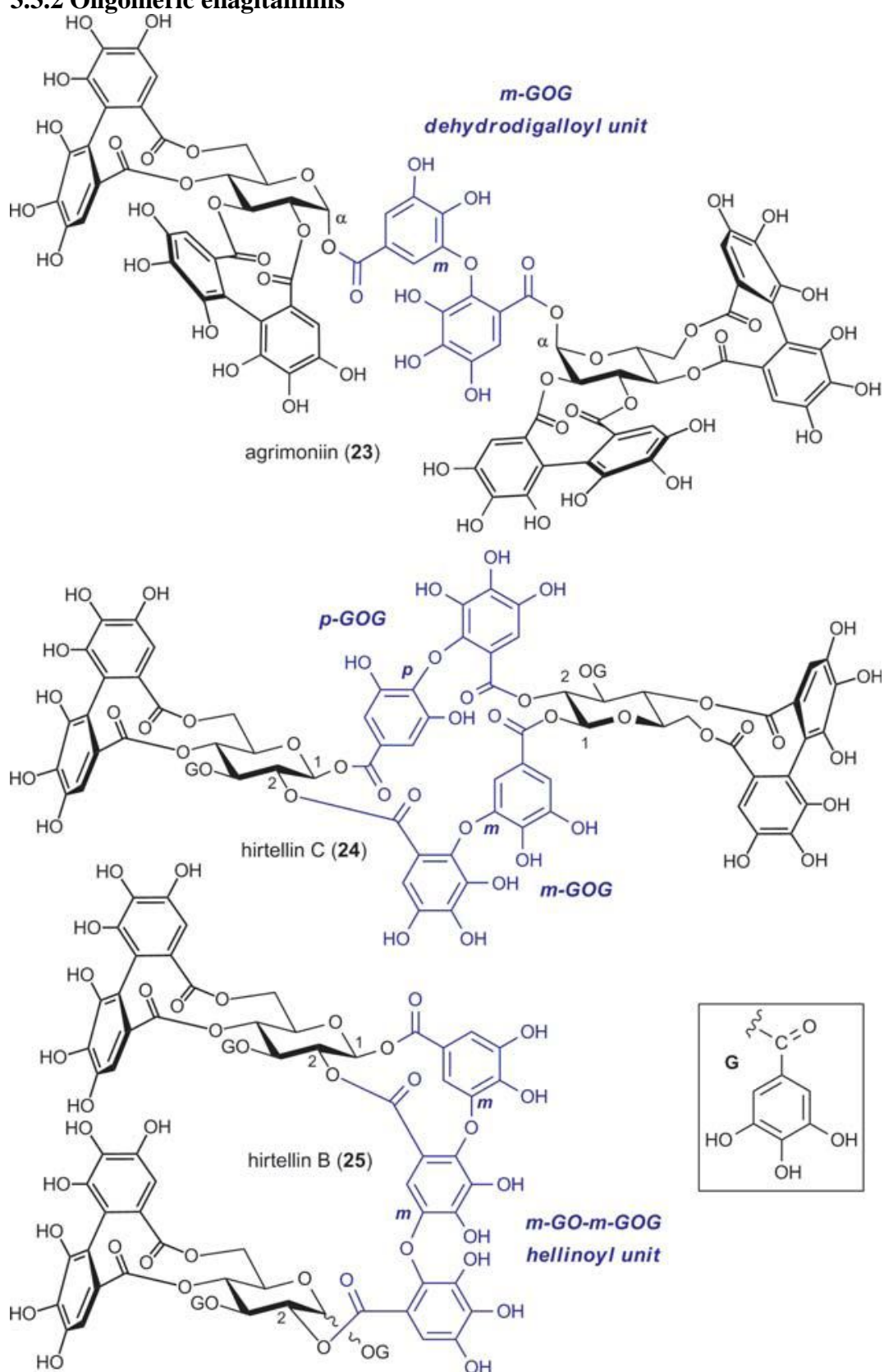


Fig. 9 Examples of Okuda's GOG- and GOGOG-type oligomeric ellagitannins.

Basically, types II and II+ oligomerize through various modes based on oxidative coupling reactions between free and C–C-coupled galloyl groups (i.e., HHDP units) on different glucopyranosic ellagitannins, as well as on reactions of condensation occurring at the C-1 center of C-glucosidic ellagitannins. In addition, oligomeric structures have been classified into five types basing on the nature of the inter-unit linkage between monomers: (i) GOG (and GOGOG), (ii) DOG, (iii) GOD, (iv) D(OG)₂ and (v) C-glucosidic type, for which G $\frac{1}{4}$ galloyl, O $\frac{1}{4}$ oxygen and D $\frac{1}{4}$ HHDP. [Okuda 1993]

In GOG- and GOGOG-type oligomers, the inter-unit linkages consist of two (or three) G units bound together through a diaryl ether bond, as exemplified by agrimoniin (23, Fig. 9), [Okuda 1982] which has a meta-GOG type linking unit (i.e., one of the oxygen atoms meta-positioned to the carboxyl group-bearing carbon of one G unit is C-linked to one of the unsubstituted ortho-positions of the other G unit). In addition, this kind of unit refers to as the dehydrodigalloyl (DHDG) unit. Sometimes, isodehydrodigalloyl units (i.e., the para-GOG type) are present in some oligomers as those isolated from plant species belonging to the family of Tamaricaceae, including *Reaumuria hirtella*, able to produce a dimer, hirtellin C (24), [Yoshida 1993] resulting from a double oxidative coupling of two molecules of tellimagrandin II (5a). This double mutual coupling is observed between the O-1-galloyl group of one monomer and the O-2-galloyl group of the other monomer and vice versa, but one C–O coupling produces the m-GOG type unit, whereas the other determines the formation of the more sterically encumbered p-GOG type unit. [Yoshida 1993]

In addition, the same plant species in Tamaricaceae are able to combine the same monomeric ellagitannin tellimagrandin II (5a) in different ways through the oxidative C–O coupling of galloyl groups, as exemplified by the structure of hirtellin B (25). [Yoshida 1991] The two O-2-galloyl groups are bound together through a m-GOG type unit, the oxygen-donating O-2-galloyl group being similarly linked to the O-1-galloyl group of the same monomeric unit. The resulting m-GO-m-GOG type unit is also referred to as the hellinoyl group. In addition, the DOG-type units are further classified into their meta and para variants. In these units, a HHDP unit is O–C-linked to a G unit. The m-

DOG type or valoneoyl unit is observed in many oligomeric ellagitannins, [Okuda 1993] including, among others, the aforementioned cornusiin E dimer (26, Fig. 5), and in some monomeric ellagitannins of type III+ of which mallotusinic acid represents an example (21, Fig. 3).

The dimer oenothain B (27a) [Hatano 1990] present in significant amounts in *Oenothera* and *Epilobium* species (Onagraceae) and in *Lythrum anceps* (Lythraceae), and its α -monogalloylated variant woodfordin C (27b), 39 isolated together with 27a from *Woodfordia fruticosa* (Lythraceae), represent significative examples of macrocyclic ellagitannin structures with two valoneoyl groups as macroring-forming inter-unit linkages (Fig. 5).

The m-DOG-type valoneoyl unit represents probably the most often encountered inter-unit linkage in ellagitannin oligomerization through oxidative coupling processes. In addition, it has been observed in some biogenetically intriguing dimers consisting of a glucopyranosic monomer m-DOG-linked to an open-chain C-glucosidic monomer. Two examples of this kind of dimer are represented by Reginins B (28a) and A (28b), which have the 4,6-HHDP unit of a pedunculagin (11b) monomer bound to the O-5-galloyl group of either stachyurin (13) or casuarinin (14) (Fig. 5). [Xu 1991] The para-DOG type unit, also referred to as the tergalloyl unit, is less common, probably due to its higher steric demand. One example representing this class of oligomers is the dimer of tellimagrandin I (5b), named eucalbanin C (29), which has been isolated from *Eucalyptus alba* (Myrtaceae).

In the GOD-type unit, which seems to be present only in its meta version, a HHDP unit of one monomer is C–O-linked to a G unit of another monomer. Furthermore, this kind of inter-unit linkage refers to as the sanguisorboyl unit and is observed in few oligomers, whose examples can be represented by the dimer sanguiin H-6 (30), isolated from *Sanguisorba officinalis* (Rosaceae). [Tanaka 1985]

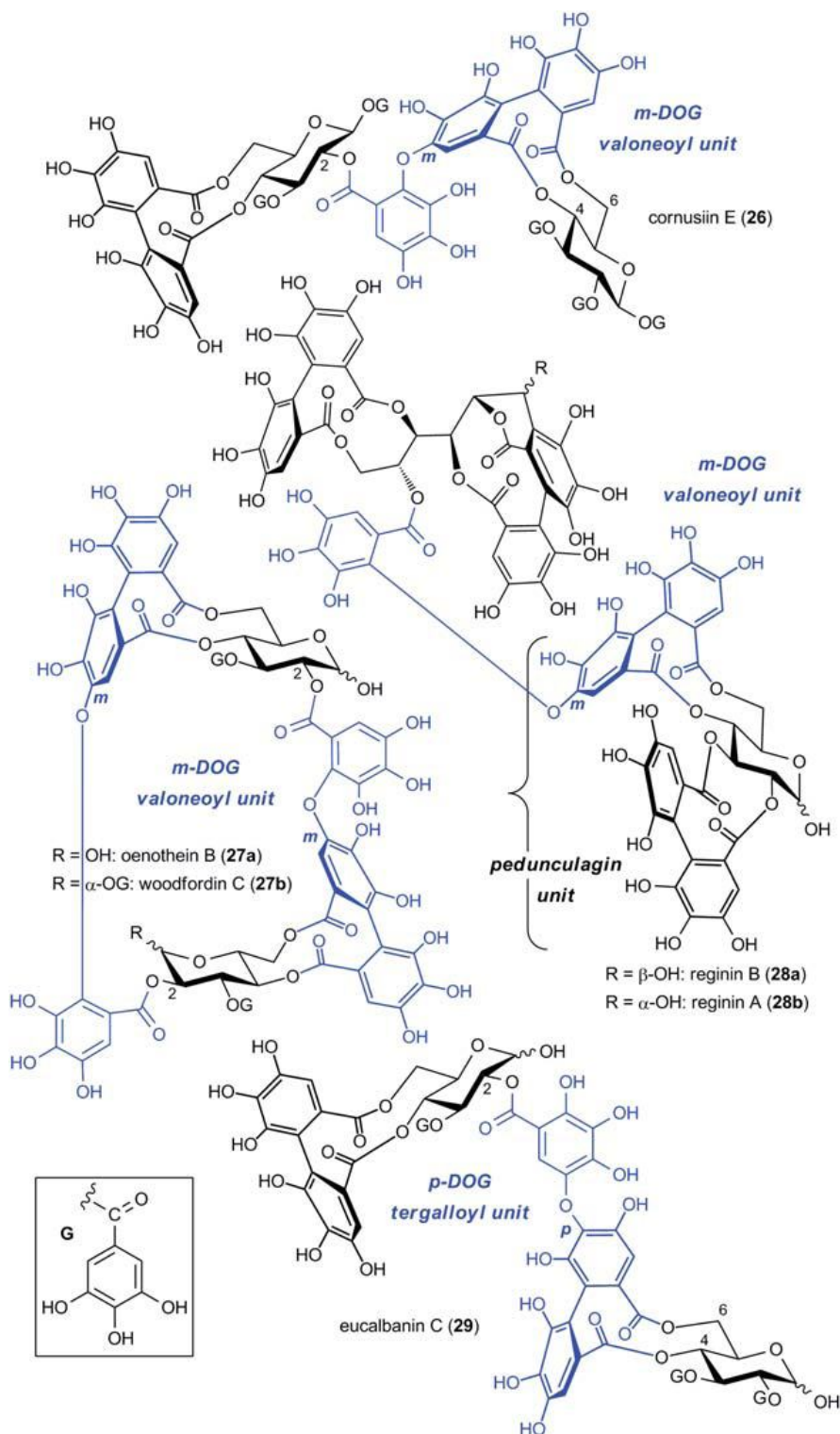


Fig. 10 Typical examples of Okuda's DOG-type dimeric ellagitannins

As ellagitannins and vegetal extracts rich in ellagitannins show many beneficial biological activities toward the cardiovascular system as described in the following paragraph, the effects of ENC have been tested for their ability to affect some cardiovascular functions and to exert some protective effects.

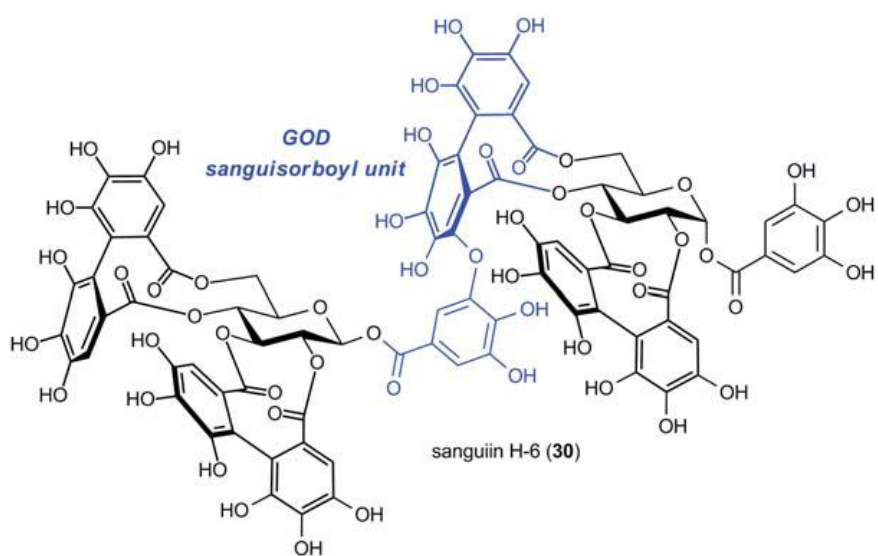


Fig. 11 A typical example of Okuda's GOD-type dimeric ellagitannin

CHAPTER 4

Biological effects of ellagitannins and vegetal extracts rich in ellagitannins toward cardiovascular system

Cardiovascular system

Ellagitannins are shown to determine many beneficial effects towards the cardiovascular system.

A positive association between walnuts and pomegranates consumption and cardiovascular health benefits has been observed. Both contain relevant amounts of phenolic antioxidants, and in particular ellagitannins (ETs) that have been shown responsible, at least partly, of these physiological properties. [Espín 2007a]

These phytochemicals possess many biological effects in vitro that have been connected to pharmacological (ET-rich medicinal plants) and nutritional (ET-rich foods) effects in vivo. These activities are mainly related to the field of prevention of cardiovascular diseases and cancer. The in vivo biological effects are, at least in part, due to the high free-radical scavenging activity observed in vitro assays. Many nutraceuticals, medicinal plant extracts and food products rich in hydrolysable tannins, and particularly in ETs, are currently marketed and proposed for their potential benefits on cardiovascular health.

Many plant species containing ETs have been used for the treatment of diseases, particularly in Asia [Okuda *et al.* 2009]. Among these plants, Agrimonia pilosa (agrimoniin), Camelia japonica (camelliatannin A), Cornus officinalis (cornussin A), Geranium thunbergii (geraniin), Geum japonicum (gemin-A), Liquidambar formosana (casuarictin), Mallotus japonicus (mallotusinic acid); Oenothera erythrosepala (oenothein B), Punica granatum (granatin B), Rosa rugosa (rugosin) and Terminalia chebula (chebulinic acid) are to be included. All the mentioned medicinal plants are clinically used for their antioxidant, anti-diarrheic, anti-microbial and immunomodulatory activities.

ETs are also present in significant amounts in different berries, such as strawberries, red and black raspberries [Zafrilla et al. 2001], blackberries, and nuts including walnuts [Fukuda, T et al. 2003], pistachio, cashew nut, chestnuts, oak acorns [Cantos et al. 2003] and pecans [Villarreal-Lozoya et al. 2007]. In addition, they are shown to present in large amounts in pomegranates [Gil et al. 2000], and muscadine grapes (Sandhu AK, et al., 2010)., and are important constituents of wood, particularly oak wood. [Glabasnia et al. 2006] EA, in addition, has also been found to be present in different types of honey. [Ferrerres et al. 1996]

Free EA and several glycosidic derivatives, including glucosides, rhamnosides, arabinosides and the corresponding acetyl esters, are present in these food products. [Zafrilla et al. 2001]

As oxidative stress is linked to atherosclerosis which represents the etiological base of cardiovascular diseases, [Kaneto et al. 2010] the antioxidant activity of fruit and plant extracts rich in EA, GA and (or) hydrolysable tannins has been considered, in part, responsible for the beneficial effects of Ets. [Serrano et al. 2009] [Basu et al. 2009]

Furthermore other beneficial biological activities of ellagitannins have been reported.

The anti-atherogenic, anti-thrombotic, anti-inflammatory and anti-angiogenic effects of fruits and plants extracts rich in EA, GA and (or) hydrolysable tannins (ETs and GTs) have been observed in several in vitro studies. Pomegranate juice and extracts, which contain large amounts of EA and Ets, have been shown to have multiple anti-atherogenic effects.

Paraoxonases (PONs) are lactonases inhibiting LDL-cholesterol peroxidation. The PON1 is located on High Density Lipoproteins and it is responsible for its antioxidant activity.

Pomegranate juice is able to prevent lipoproteins oxidation through the up-regulation of the expression and activity of PON1 and PON2 in hepatic cells [Khateeb et al. 2010] and in macrophages [Shiner et al. 2007a] and inducing the association of PON1 to HDL [Fuhrman et al. 2010]. In addition, several pomegranate extracts are able to determine a reduction of the levels of cholesterol in macrophages through the inhibition of the uptake of native and

oxidised LDL (ox-LDL) and the stimulation of high density lipoprotein (HDL) efflux [Aviram *et al.* 2008] and to protect monocytes and endothelial cells from peroxide and ox-LDL damage. [Sestili *et al.* 2007]

The endothelium possess anti-atherogenic and anti-thrombotic properties due to nitric oxide (NO), synthesized by endothelial nitric oxide synthase (eNOS), which regulates the vascular function: it inhibits platelets aggregation, induces vasorelaxation and represses the expression of inflammatory proteins and adhesion molecules such as the intercellular adhesion molecule (ICAM-1) and the vascular adhesion molecule (VCAM-1) both involved in the endothelial migration of leukocytes. [Thomas *et al.* 2003]

An adjunctive anti-atherogenic action of pomegranate is represented by its ability to induce the expression of eNOS in human artery endothelial cells and by its ability to inhibit activated platelets aggregation as well as to reduce the production of the circulating platelet activating agent thromboxane A₂ (TXA₂). [Mattiello *et al.* 2009]

The inhibition of LDL oxidation and the decrease of the levels of ICAM-1 and VCAM-1 in human endothelial cells has been reported also with other extracts rich in ellagitannins. [Papoutsi *et al.* 2008]

Other medicinal plants such as *Phyllanthus amarus* L (Euphorbiaceae) rich in ETs exert anti-inflammatory effects through the increase of the expression of inducible NOS (iNOS) and of different cytokines in macrophages. [Kolodziej, *et al.* 2005]

Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix and remodeling of the vascular wall. Induction of MMPs is associated to vascular smooth cells migration and atherogenic processes.

Medicinal plant extracts, such as *Phyllanthus urinaria*, containing large amounts of EA, are able to exert anti-angiogenic effects by decreasing the MMP12 activity in human endothelial cells. [Huang *et al.* 2009] Furthermore, the extracts rich in GA, grape or red wine extracts, have been shown to determine some anti-thrombotic effects through the inhibition of platelets aggregation and the activation of the platelets and endothelial cells adhesion molecule (PECAM-1) [De Lange *et al.* 2007].

Another important molecule involved in the vascular function is represented by the potent growth factor and inducer of angiogenesis, the vascular endothelium growth factor (VEGF). Angiogenesis is considered to represent an important factor in the atherosclerotic process and VEGF may have both detrimental and beneficial effects [Holm et al. 2009]. Red wine polyphenol extracts have been shown to be able to reduce the release of VEGF from human aortic smooth muscle cells [Oak et al. 2006]: this mechanism may represent a way through which these compounds exert beneficial effects against the formation of the atherosclerotic plaque. Furthermore, dealcoholized red wine is able to reduce hepatic intracellular levels of cholesterol as well as the secretion of apolipoprotein B100 (ApoB100) [Pal et al. 2003], a component of the LDL particles essential for the binding of LDL particles to the receptor for cellular uptake [Chan et al. 2006].

Other fruit extracts such as mulberry extract rich in GA are able to reduce the growth, migration and MMPs activity of rat thoracic smooth muscle cells [Chanet al. 2009] whereas the plant extract from *Rhus coriaria* rich in GTs determines endothelium-dependent vasorelaxation in isolated rabbit aortic rings [Beretta et al. 2009]. Overall, these results lead to suppose that either a component or components present in the tested extracts, presumably EA, GA or hydrolysable tannins, exert potential preventive effects towards the development of atherosclerotic lesions.

A total of thirteen studies about the responses of different vascular cell models exposed to EA and (or) punicalagin, which are the main pomegranate polyphenols [Zhang et al. 2009], suggest that these two compounds may contribute to determine the anti-atherogenic effects of pomegranate extracts or juice. In addition, EA has been shown to possess anti-inflammatory effects through the reduction of the levels of prostaglandin synthases [Karlsson et al. 2010] and the reduction of the expression levels of adhesion molecules including ICAM-1, VCAM-1 and E-selectin [Papoutsis et al. 2008] [Yu et al. 2007]. Furthermore, EA is able to exert anti-angiogenic effects through the reduction of the levels of the metalloproteinase MMP12 [Huang et al. 2009] and the inhibition of VEGF-induced endothelial and vascular smooth muscle cells migration [Labrecque et al. 2005]. EA and punicalagin have also been

shown to reduce or delay lipoproteins oxidation [Anderson et al. 2001] and to increase the expression of paraoxonases PON1 and PON2 [Fuhrman et al. 2010] [Khateeb et al. 2010]. These two compounds also modulate the metabolism of cholesterol and the uptake of native and ox-LDL in macrophages [Aviram et al. 2008]. In addition, punicalagin has been shown to induce NO production in bovine aortic endothelial cells [Chen et al. 2008] and to reduce IL-2 expression in lymphocytes [Lee et al. 2008].

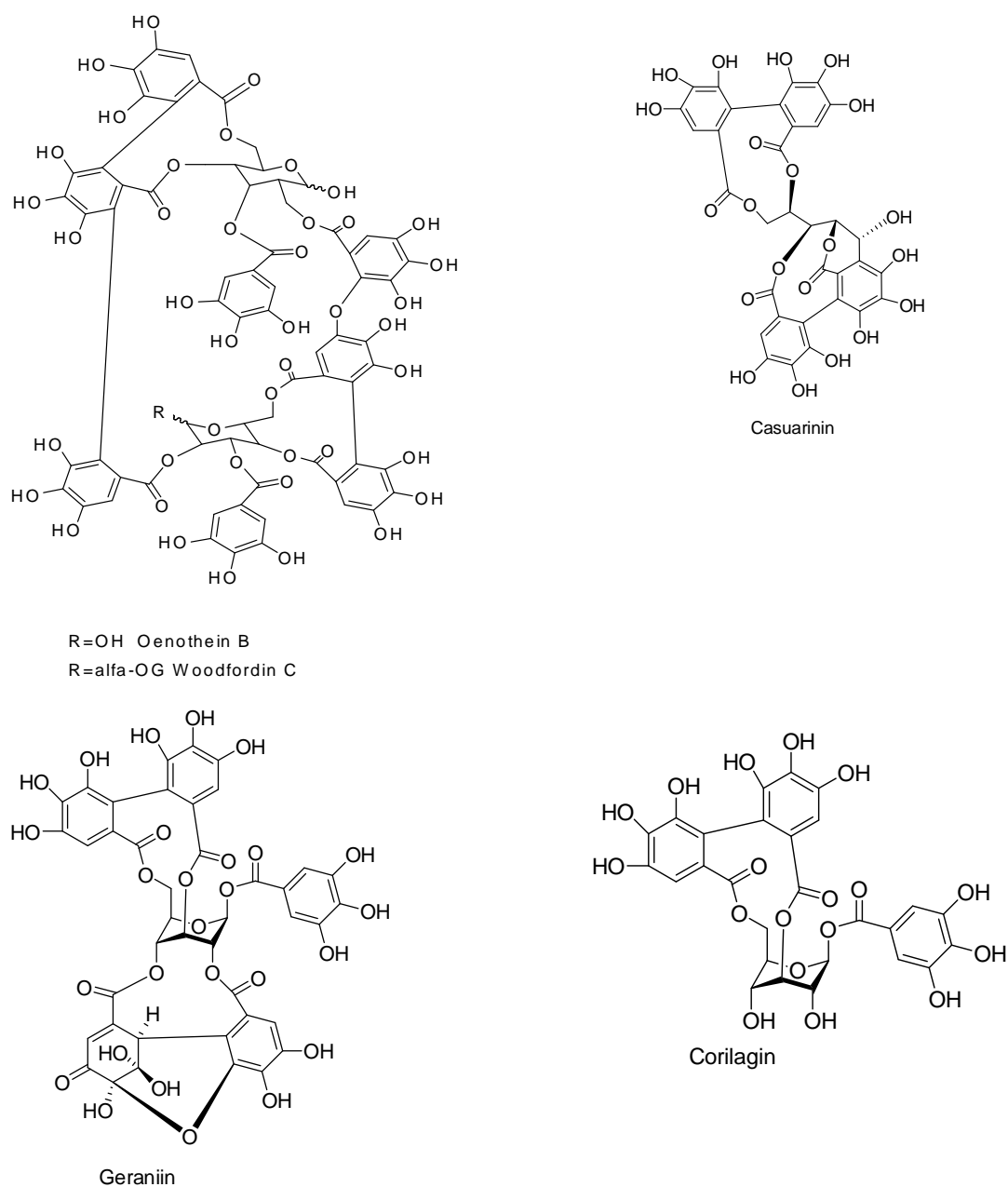
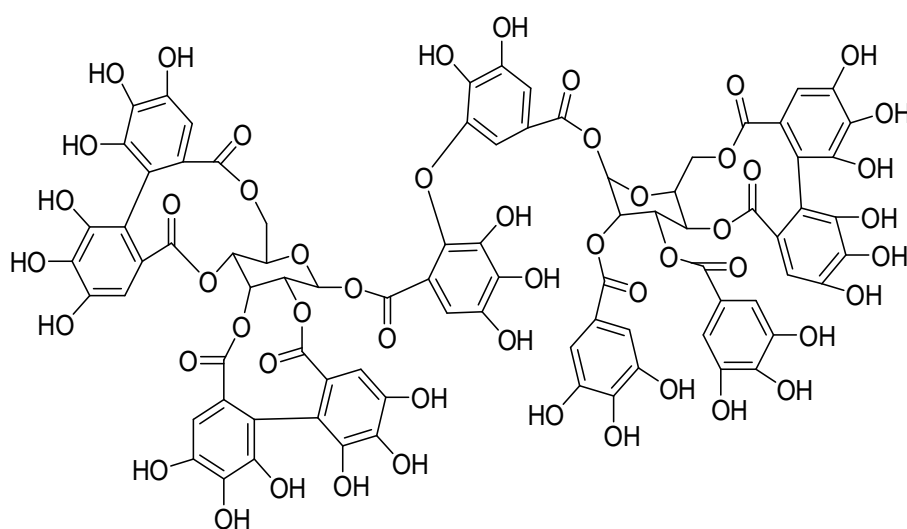


Fig. 12 A typical example of Okuda's GOD-type dimeric ellagitannin

In addition, different other ETs isolated from several plants used in traditional medicine have been shown to exert anti-inflammatory, anti-atherogenic and metabolic effects. For example, macrocyclic hydrolysable ETs such as oenotherin B, corilagin, cuphiin D, geraniin, woodfordin C, casuarinin or agrimoniin are able to determine immunomodulatory effects through the alteration of the levels of various cytokines and (or) the production of NO. [Schepetkin et al. 2009] [Zhao et al. 2008] [Kolodziej et al. 200] [Okabe et al. 2001] [Chen et al. 2000] (Pan et al. 2000) [Ishii et al. 1999] [Murayama et al. 1992]

Corilagin is able to reduce TNF- α levels, different interleukins such as and iNOS, whereas it enhances iNOS and cytokines in macrophages. Furthermore, other anti-atherogenic properties of corilagin include the inhibition of monocytes adhesion to endothelial cells and the proliferation of vascular muscle cells.

In addition, some ETs may affect lipid metabolism, thus, atherosclerosis development. For example, EA and some ETs present in the Chinese plant *Geum japonicum* (gemin-A (Fig 13) and -B, casuarinin, pedunculagin, etc.) have been shown to reduce the activity of fatty acid synthase (FASN) [Liu et al. 2009], an important lipogenic enzyme involved in the catalyze of the synthesis of long-chain saturated fatty acids [Menendez et al. 2009].



Gemin A

Fig. 13 Gemin A

ETs such as lagerstroemin, flosin B, stachyurin, etc. present in large amounts in Lagerstroemia speciosa (L.) Pers., used in Folk Medicine as anti-diabetic and weight loss herb, are able to modulate insulin-like glucose uptake in adipocytes and to inhibit adipocyte differentiation [Hattori et al. 2003].

In addition, GA, GTs and some derived gallic esters have been tested for their potential anti-inflammatory and anti-atherosclerotic effects, through in vitro vascular cell models.

Several studies have repeatedly shown that GA exhibits none or very weak activity on some of the tested models. For example, GA does not affect the stimulated release of VEGF from vascular smooth muscle cells [Oak et al. 2006] and does not alter the levels of eNOS expression [Wallerath et al. 2005] and NO production [Huisman et al. 2004] in endothelial cells. Although, GA is able to modulate the vasorelaxation properties of the endothelium of isolated rat aorta [Sanae et al. 2002] [Sanae et al. 2003] it does not relax the pre-contracted rat aortic rings (Andriambeloson, E. et al., 1998) As regards platelets functionality, GA has no effect towards ADP-induced platelets aggregation or PECAM-1 activation (de Lange, D.W. et al., 2007) however, it inhibits P-selectin-mediated adhesion between platelets and monocytes (Appeldoorn, C.C.M. et al., 2005) and it prevents the inhibitory effects of other polyphenols on induced platelets aggregation (Crescente, M. et al., 2009). It has been observed, in hepatic cells, that GA is able to slightly reduce the secretion of ApoB (Pal, S. et al., 2003) and, in macrophages to determine a small but significant induction of the tumor necrosis factor TNF- α . (Wang, J. et al., 2002a) In contrast, penta-O-galloyl-b-D-glucose, a gallotannin, seems to have better anti-inflammatory and anti-atherogenic activities than GA. The pentagalloyl glucose does not reduce iNOS expression and activity as well as NO production (Kim, M.-S. et al., 2009) (Chen, Y.-C. et al., 2000) (Pan, M.-H. et al., 2000). Important pentagalloyl glucose biological activities include the suppression of the expression of pro-inflammatory cytokines such as interleukins and TNF-a (Lee, S.H. et al., 2007) (Oh, G.S. et al., 2004), the inhibition of platelets aggregation (Jeon, W.K. et al., 2006), the relaxation of pre-contracted aortic rings and the reduction of the expression of VCAM-1, ICAM-1 or the monocyte chemoattractant protein-1 (MCP-1) in human

endothelial cells (Kang, D.G. et al., 2005). In addition, the pentagalloyl glucose is able to promote glucose transport in adipocytes and to inhibit adipocytes differentiation exerting potential beneficial effects in diabetes and metabolic syndrome. (Klein, G. et al., 2007) (Ren, Y. et al., 2006)

Many in vitro cell studies indicate that EA, GA and hydrolysable tannins possess potential antiatherogenic properties.

Some of these ETs physiological derivatives have now been identified: EA and its colonic metabolites, UroA and UroB, as well as their derived glucuronides, sulphates and methylated compounds are the molecules most likely to reach and enter the endothelium and vascular system. In addition, most published reports indicate that the circulating concentration of EA and urolithins metabolic derivatives is in the nM to low μ M range (Cerdá, B. et al., 2004) (Cerdá, B. et al., 2005a) (Espín, J.C. et al., 2007b). In relation to GA absorption and metabolism, both GA and its primary metabolite, 4-methyl GA (4-OMeGA), have been identified in the urine and plasma of human volunteers with plasma concentrations in the low μ M range (Loke, W.M. et al., 2009) (Mennen, L.I. et al., 2008). In rats, the plasma levels of GA and 4-OMeGA reached a C_{max} of approximately 1.8 and

0.4 μ M, respectively, after the consumption of grape seed extract (Ferruzzi, M.G. et al., 2009). Few information is disposable for the metabolic fate and bioavailability of other macrocyclic hydrolysable tannins which, probably, are not absorbed intact and do not reach the systemic blood stream and the vascular cells in its original form.

As regards the activity of the metabolites, few reports on the anti-inflammatory effects of some methyl EA derivatives and of 4-OMeGA have been published. In particular, 4-OMeGA has been demonstrated to reduce the expression of iNOS, IL-1 β and TNF- α in macrophages (Na, H-J. et al., 2006) as well as the expression of adhesion molecules ICAM-1 and VCAM-1 or the production of VEGF in endothelial cells (Lee, G. et al., 2006) (Jeon, K.S. et al., 2005). However, these studies were carried out using very high concentrations of the metabolite (from 2.5 to 100 μ M). In vivo studies about the cardiovascular effects of hydrolysable tannins have also been conducted.

Ellagic acid has been shown to augment the partially activated thromboplastin time and to decrease the platelets number, fibrinogen, kininogen and prekallilrein plasma levels in addition to inducing a hypotensive effect.

(Majid, S. et al., 1991), reported that the administration of EA in drinking water to mice for 8 weeks, determines an antioxidant effect with augmented activity of GSH and GR in liver and lungs and reduced levels of MDA. In another experiment carried out in rabbits, 1% EA, given together with atherogenic diet for eight weeks, has been shown to determine a reduction of atherosclerotic lesion, oxidative DNA damage and apoptosis in the aorta (Yu, Y.M. et al., 2005). In addition, the administration of coencapsulated EA in nanoparticles with coenzyme Q10 to rats fed with a high-fat diet has been observed to determine an improvement in the endothelial function and a reduction of total cholesterol and triglycerides in plasma (Ratnam, D.V. et al. 2009)

Many studies regarding EA have been carried out with ETs or EA-containing foodstuff. In general, these studies have been exerted using pomegranate or derived products such as pomegranate extracts or juice. These studies lead to suppose that the potential cardioprotective effect of ETs and/or EA is not linked to a single effect but EA seems to affect several parameters involved in cardiovascular health. The principle effect observed is the reduction of oxidative stress in plasma and tissues, including the aortic tissue. Several studies indicate that EA induces a reduction in plasma and macrophage lipid peroxidation levels (Aviram, M. et al., 2000) (Aviram, M. et al., 2008) (Kaplan, M. et al., 2001) (Rosenblat, M. et al., 2006b), and an effect on nitric oxide metabolism augmenting the activity and the expression of eNOS and levels of NO (de Nigris et al., 2005, 2007a,b). In addition, the antioxidant activity reducing the oxidative stress associated with atherosclerosis is coherent with the observed decreased levels of 8-oxo-dG in aorta and urine (Yu et al., 2005; Fukuda et al., 2004), decreased plasma isoprostane levels and modulation of redox sensitive transcription factors like ELK-1, p-JUN and p-CREB (de Nigris, F. et al., 2005) (De Nigris, F. et al., 2007b). Another parameter modulated by ETs is the effect on the lipid profile. The intake of diverse pomegranate-derived extracts or juice seems to modify the blood lipids

profile regardless of the animal model used (hypercholesterolemic diet, streptozotocin treated, Zucker diabetic fat rats, ApoE deficient mice).

A general reduction of triglycerides, total cholesterol, LDL, VLDL, and non esterified free fatty acids plasma levels has been reported (Li, Y. et al., 2005a) (Lei, F. et al., 2007) (Aviram, M. et al 2008) (Bagri, P. et al., 2009) (Ratnam, D.V. et al., 2009) (Huang, T.H. et al., 2005a) as well as a modulation of genes involved in lipid metabolism such as PPAR- α , FATP, CPT-1, ACO and AMPKa2 (Huang, T.H. et al., 2005a) (Shimoda, H. et al., 2009). In addition, EA and ETs consumption seems to affect parameters related to lipoproteins such as their susceptibility to oxidation. In this line, studies carried out in ApoE deficient mice have shown a reduction of the LDL oxidation and a decreased ox-LDL uptake by macrophages (Aviram, M. et al., 2000) (Aviram, M. et al., 2008) (Kaplan, M. et al., 2001) (Rosenblat, M. et al., 2006b). Furthermore, the effect of different products derived from pomegranate on the increased activity and expression of paraoxonase enzymes (PON1 and PON2) that are increased in plasma and macrophages, respectively, following consumption of pomegranate products have been reported (Kaplan, M. et al., 2001) (Aviram, M. et al., 2008)

In addition, EA and pomegranate exert hypotensive and anti-diabetic effects. An extract of Terminalia arjuna, administered i.v. to rats, determines a reduction of blood pressure and heart rate (Takahashi, S. et al. 1997).

The administration of 100–300 mg/kg/day for 4 weeks of pomegranate juice extract to diabetic rats treated with angiotensin II decreased mean arterial blood pressure and the biochemical changes induced by diabetes and angiotensin II (Mohan, M. et al., 2009). The administration of pomegranate flower extract augments oral glucose tolerance and reduces the fasting glucose plasma levels (Huang, T.H. et al., 2005a) (Bagri, P. et al., 2009) (Hontecillas, R. et al, 2009) showing some anti-diabetic effects for these compounds. The probable mechanisms determining these anti-diabetic effects involve an augment of PPAR- γ expression in cardiac, skeletal muscle and adipose tissue (Huang, T.H. et al., 2005b) (Hontecillas, R. et al, 2009) Some mentioned studies have been exerted using pomegranate-derived extracts that not only contain polyphenols but also fibre, sugars, organic acids and other compounds that may contribute

to the observed effects. However, even if some compounds not belonging to the class of hydrolysable tannins such as the triterpenoid 3 β -hydroxy-olea-12-en-28-oic acid have been reported to possess beneficial effects towards the cardiovascular system, these compounds show a very low bioavailability. (Huang, T.H. et al., 2005a) Clinical data about ET-containing foodstuffs consumption and cardiovascular diseases have been reported. Most of these foodstuffs include pomegranate and walnuts.

Both pomegranates and walnuts (Banel, D.K. et al., 2009) have been observed to exert some cardioprotective effects. Despite both contain large amounts of ETs, in the case of pomegranate the beneficial effects are believed to be due to the fraction of ETs with antioxidant effects, whereas as regards the beneficial effects of walnuts, these are considered to be induced mostly by their lipid fraction (Ros, E. et al., 2006). However, other constituents such as different polyphenols, phytosterols, tocopherols, L-arginine and magnesium could contribute to the cardioprotective effects of walnuts (Casas-Agustench, P. et al., 2010) (López-Uriarte, P. et al., 2010)

Aviram and Dornfeld (2001) reported the cardiovascular benefits of pomegranate juice in a placebo-controlled and no crossover study which has been carried out in only 13 healthy volunteers. In this study the augment of (20%) of serum PON1 (an HDL-associated esterase that can protect against lipid peroxidation) together with the ex-vivo reduction susceptibility of LDL oxidation have been observed. No changes in serum lipid profile have occurred. Even if the authors assessed that the active compounds are the 'antioxidant flavonoids' of the juice, this is probably untrue because flavonoids represent the minor constituents in pomegranates compared to the non-flavonoid polyphenols ETs, so perhaps, authors mixed up the terms flavonoid and polyphenol.

In addition to the mentioned study, fifteen further human intervention studies with pomegranate have been carried out. Most studies have been done in order to justify the cardiovascular health benefits observed on the base of the impressive in vitro antioxidant activity of pomegranate (Gil, M.I. et al., 2000). However the EA and ET fraction, once ingested, undergoes an extensive metabolism by the gut microbiota to produce principally urolithins A and B

with negligible antioxidant activity (Cerdá, B. et al., 2004). The activity of punicalagin, incubated in vitro with macrophages, has been tested in order to explain the in vivo effects such as the increase of PON2 (Shiner, M. et al., 2007a). However, considering the fact that punicalagin concentration used in the mentioned study will never be reached in the bloodstream, other mechanisms should have to be involved.

Furthermore, several studies about pomegranate and cardiovascular system (many of them with the same co-authors), in addition to the improvement of serum lipid profile and serum antioxidant activity, reported other beneficial effects including the reduction of systolic blood pressure (Aviram et al., 2001, 2004) and decrease of carotida intima-thickness (Aviram et al., 2004) (Davidson, M.H. et al., 2009).

Other additional mechanisms which may contribute to the cardiovascular protection of pomegranate juice have been related to its potential estrogenic-related effects (Sturgeon, S.R. et al., 2010). These effects occur through the inhibition of cyclooxygenase, 17 β -hydroxysteroid dehydrogenase and aromatase activities observed in vitro and in animal models due to the tentative action of constituents such as punicalic acid, EA, and anthocyanins.

Larrosa and colleagues (2006b) (Larrosa, M. et al., 2006b) showed dose-dependent estrogenic and anti-estrogenic activities of urolithin A and B in vitro, using molecular and cellular models.

In the study of Seeram and colleagues (Seeram, N.P. et al., 2006a) involving in postmenopausal women (n = 11), a significant increase in serum estrone levels has been observed, however, but this had no any significant estrogenic-related effects.

The beneficial effects of walnuts consumption on cardiovascular disease have been widely reported.

A recent review of 25 intervention trials reported that nut consumption improves blood lipid levels in a dose-dependent manner. (Sabaté, J. et al., 2010)

Interestingly, different types of nuts (such as almonds, which do not contain ETs) show a similar activity profile regarding the influence on blood lipid

levels which could limit the possible specific role of ETs in the mediation of these effects.

As regards this field, walnuts or canola oil consumption, showing similar fatty acid composition, have been shown to exert similar LDL-cholesterol lowering effects (Chisholm, et al. 2005).

However, recent reports claim for cardiovascular benefits beyond blood lipid lowering (Ros, E., 2009). In addition, walnuts consumption has been associated to an increase of plasma total antioxidant capacity (FRAP and ORAC assays) and the decrease of plasma lipid peroxidation (TBARs and MDA) and these effects are believed to be due to plasma phenolic content. (Torabian, S. et al. 2009)

Different studies to assess the properties of walnuts to impart 'functional properties' in meat products, i.e. improvement of antioxidant (Canales, A. et al., 2007) or thrombogenic (Canales, A. et al., 2009) status have been conducted and a possible contribution of ETs-derived metabolites in the determination of these effects can not be excluded, even if a combined synergistic effect of different walnut constituents has been supposed for the beneficial effects.

In general, the number of human intervention studies dealing with cardiovascular protection and ET-containing foodstuffs is small.

A recent study about the effect of pomegranate juice on patients with a moderate risk for cardiovascular disease involved 289 participants with a follow-up for 18 months. (Davidson, M.H. et al., 2009)

As regards walnuts, more intervention studies are disposable. Sabaté and colleagues (2010) have recently reviewed 25 nut consumption trials among 583 participants, which make an average sample size of 22 participants per trial. In different studies, referring to blackberries, strawberries, muscadine grape and *E. officinalis* extract, the sample size ranged from 6 to 30 people.

The lack of crossover studies represents the weak point about the human intervention studies carried out with pomegranate. In fact, only one crossover study with pomegranate ETs with the principle aim to evaluate the effect of ET consumption on strength recovery after eccentric exercise has been carried out (Trombold, J.R. et al., 2010).

The scientific evidence supporting cardioprotective effects upon walnuts consumption is stronger than that related to pomegranate consumption taking into account the number of intervention studies, sample size and number of crossover studies which confer relevant statistical power to the results.

Bioavailability and metabolism issues represent critical points to identify the probable compounds involved in the cardiovascular-related effects observed. In the pomegranate studies, the tentative bioactive compounds are ETs. According to previous reports, the main detected metabolite in bloodstream (at micromolar level) is urolithin A glucuronide (Cerdá et al., 2004) (Seeram et al., 2006b) (Espín, J.C. et al., 2007b) (Tomas-Barberan, F.A. et al., 2009) leading to suppose that this compound must be somehow involved in the effects observed, and this action is not necessarily related to a traditional free-radical scavenging ability but probably to its interference with the signalling cascades such as those involved in atherothrombosis (monocyte adhesion to endothelium, cytokine production, regulation of transcription factors, etc. (González-Sarrías, A. et al., 2009) (González-Sarrías, A. et al., 2010b) (Larrosa et al., 2010). If urolithins (in particular UroA glucuronide) are involved in the cardioprotective effects of ET-containing foodstuffs, the role of gut microbiota in the biological effects of ET-containing foodstuffs has to be considered, as the ability of each individual to produce the gut microbiota-derived metabolites urolithins would be critically related to the biological effects. In other words, the activity deriving from the intake of pomegranate or walnuts could be different depending on the gut microbiota. In fact, people can be divided into high, low and very low urolithin-producers (Cerdá et al., 2005, 2006) (González-Sarrías, A., et al., 2010a).

This could be a reason explaining the diverse results obtained in some studies carried out with both pomegranates and walnuts. Therefore, human intervention studies with ETs should include a sample size of population enough ($n > 60$) to obtain statistically significant results depending on the capacity of the individuals to produce urolithins.

Oxidative stress has been reported to exert a relevant role in many cardiovascular diseases, such as atherosclerosis, hypertension, myocardial infarction, etc. (Levonen, A.L., et al., 2008). For this reason, the 'antioxidant

activity' (measured with many different techniques and models) of a compound has been often related to the potential cardioprotective effects of such compound.

CHAPTER 5

Cardiovascular effects of ENC

5.1 Heart

Since ellagitannins and vegetal extracts rich in ellagitannins have many beneficial effects toward the cardiovascular system, the effects of ENC toward the cardiovascular system of ENC have been tested.

Sweet chestnut bark extract was tested for its cardiovascular profile in guinea pig left atrium and left papillary muscle driven at 1 Hz and in spontaneously beating right atrium to evaluate its negative inotropic and/or chronotropic effects, respectively. The data, relative to about 10 minutes of incubation with the extract, are reported in Table 1. It should be noted that the extract (1 mg/ml) produced a positive inotropic effect ($+ 218 \pm 17 \%$) in left atrium even though with a concomitant reduction in heart rate ($- 59 \pm 3.6 \%$). A single dose (1 mg/ml) of ENC reduces the heart rate in right atrium and simultaneously increases the contraction on left atrium. Chestnut extract (1 mg/ml) revealed a positive inotropic effect are on also seen on the left papillary muscle stimulated at 1 Hz where we evaluate effect on the ventricular contraction (inotropy). All the reported effects are reversible after 30 minutes of washing.

Table 1. Activity of Sweet Chestnut bark extract in Guinea pig heart preparations.

Comp	Tissue		
	Left Atrium	Right Atrium	Papillary Muscle
	Positive Inotropic Activity ^a	Negative Chronotropic Activity ^b	Positive Inotropic Activity ^c
Ext 1mg/ml	218 ± 17	59 ± 3.6	42 ± 2.1

^a Increase in developed tension on isolated guinea-pig left atrium, expressed as percent changes from of the controls ($n = 5-6$). The left atria were driven at 1 Hz. ^b Decrease in atrial rate on in guinea-pig spontaneously beating isolated right atrium, expressed as percent changes from the control ($n = 7-8$). Pretreatment heart rate ranged from 165 to 190 beats/min. ^c Increase in developed tension on isolated guinea-pig left papillary muscle, expressed as percent changes from the control ($n = 5-6$). The left papillary muscle were driven at 1 Hz. Data represent mean ± S.E.M.. All data refer to 10 minutes of incubation.

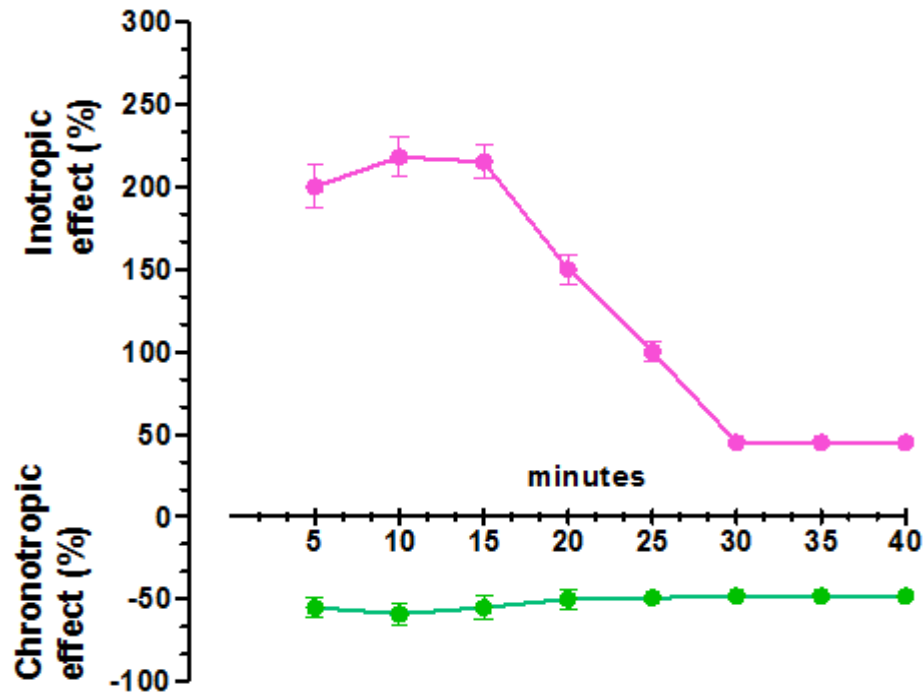


Fig 14: Time course of extract (1 mg/mL) on positive inotropic effect in guinea pig left atria driven at 1 Hz (magenta) and on negative chronotropic effect in spontaneously beating right atria (green), respectively. Values are means \pm SEM (n = 5–6). Where error bars are not shown these are covered by the point itself.

To evaluate the time course of simultaneous positive inotropic and negative chronotropic negative effects on left and right atria respectively, we measured the effect of single dose (1 mg/mL) every 5 minutes to for 30 minutes. The data are collected in table 3 and shown in Figure 14.

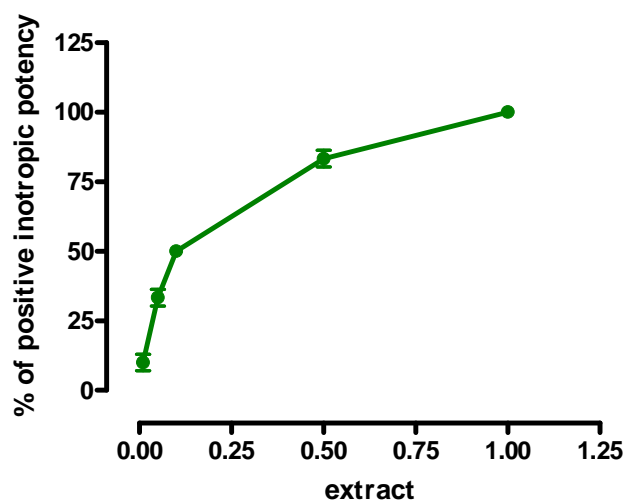


Fig. 15. Potency of extract on positive inotropic effect in guinea pig left papillary muscle driven at 1 Hz (magenta). Values are means \pm SEM (n = 4–7). Where error bars are not shown these are covered by the point itself.

The intrinsic positive inotropic activity is reduced by about 50 % after 30 minutes. In fact, calculating the inotropic and chronotropic potency by cumulative curve after 30 minutes of incubation for each concentration, the intrinsic activity does not exceed 50% with the exception of positive inotropy on papillary muscle (Figure 15). Data are collected in Table 3.

Table 2. Cardiac Activity and potency of ENC

	Positive Inotropy					Negative Chronotropy	
	Left Atria		Left Papillary Muscle			Right Atria	
	Activity ^a	Potency ^b	Activity ^a	Potency ^b		Activity ^a	Potency ^b
Ext	45 ± 0.7		52 ± 1.9	0.09	0.07-0.13	48 ± 2.3	

^a Increase in developed tension on isolated guinea-pig left atrium at 1 mg/ml, expressed as percent changes from the control ($n = 5-6$). Data represent mean ± S.E.M.. The left atria were driven at 1 Hz. 1 mg/ml gave the maximum effect for extract. ^b The 50% of effect concentration (EC50) was expressed as mg/mL concentration and was calculated from concentration-response curves (Probit analysis by Litchfield and Wilcoxon [Tallarida 1987] with $n = 6-7$). ^c Increase in developed tension on isolated guinea-pig left papillary muscle at 1 mg/ml, expressed as percent changes from the control ($n = 5-6$). The left papillary muscle were driven at 1 Hz. 1 mg/ml gave the maximum effect for extract. Data represent mean ± S.E.M.. ^d Decrease in atrial rate on guinea-pig spontaneously beating isolated right atrium at 1 mg/mL, expressed as percent changes from the control ($n = 7-8$). Data represent mean ± S.E.M.. Pretreatment heart rate ranged from 165 to 190 beats/min. 1 mg/ml gave the maximum effect for extract.

These very interesting results led us to exclude some mechanisms responsible for the effects seen for extract.

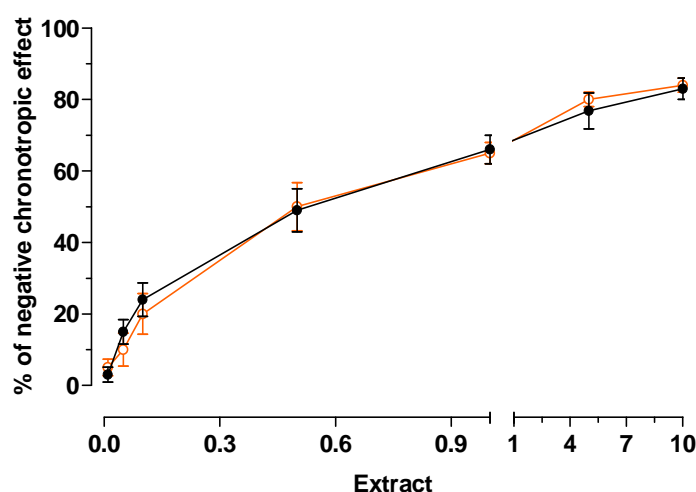


Fig 16. Cumulative concentration-response curves for extract in absence (●) and in presence of atropine (1 μM) (○) in guinea-pig spontaneously beating right atria. Each point is the mean ± SEM of four-six experiments. Where error bars are not shown these are covered by the point itself.

To clarify the mechanisms involved in the observed negative chronotropic effect and in order to elucidate the implication of cholinergic system, the

spontaneous right atrium was treated with extract in presence and in absence of 1 μ M atropine to test an implication of cholinergic system in chronotropic effect.

The figure 16 shows the cumulative negative chronotropic effect of chestnut extract in presence of atropine. As shown in Figure 16 the presence of atropine does not modify the effect of the extract on heart rate. The right atrium preparations were exposed simultaneously to extract (1 mg/ml) with atropine (1 μ M) (Figure 17). As shown in Table 3, the pA_2 of atropine does not change in the presence of the extract. The extract does not change even the pA_2 inotropic effect on spontaneous activity of the right atrium: perhaps indicating that the negative chronotropic effect is not mediated by cholinergic receptor modulation.

Table 3. Antagonist affinities at guinea pig right atria, expressed as pA_2 Values

Comp		Right Atrium ^a	
		Inotropy	Chronotropy
Atropine	pA_2^b	9.45 \pm 0.04	9.21 \pm 0.03
Atropine + ENC	pA_2^b	9.41 \pm 0.03	9.19 \pm 0.02

^a The agonist was carbachol. ^b Each pA_2 values was obtained for three different concentrations and were calculated from Schild plots, [Arunlakshana 1959] constrained to slope -1.0 [Tallarida 1987]. Results are presented as mean \pm S.E..

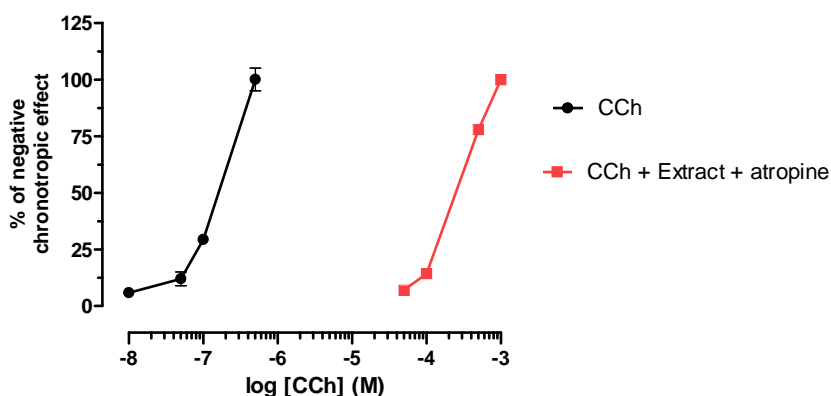


Fig. 17. Effect of extract on carbachol-induced negative chronotropic activity of isolated guinea pig spontaneously beating right atrium. Cumulative dose-response curves were obtained before and after simultaneously exposure to extract (1 mg/mL) and atropine (1 μ M) for 30 min. Each point is the mean \pm SEM ($n = 5-6$). Where error bars are not shown these are covered by the point itself.

For the positive inotropic effect, showed by the extract on guinea pig left atrium driven at 1 Hz, has been verified the involvement of the adrenergic

receptor. The positive inotropic effects of chestnut extract (1 mg/ml) in left atrium, also persist in the presence of propranolol (1 μ M), (Figure 18).

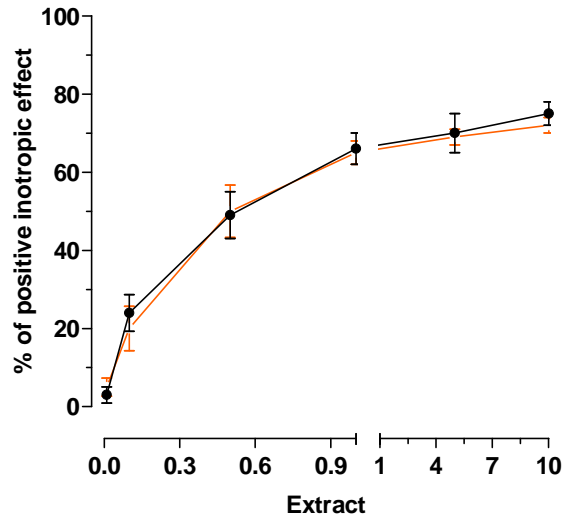


Fig. 18. Cumulative concentration-response curves for **extract** in absence (—●—) and in presence of propranolol (1 μ M) (—□—) in guinea-pig left atria driven at 1 Hz. Each point is the mean \pm SEM of four-six experiments. Where error bars are not shown these are covered by the point itself.

5.2 Guinea pig aortic strips

Chestnut extract was tested on K^+ (80 mM) or Na (1 μ M) depolarizing guinea pig aortic strips to assess their vasorelaxant activity. Chestnut extract (1 mg/ml) spasmolytic activity was less than 50% against potassium chloride and norepinephrine. The figure 6 shows the effects of extract against the contraction induced by KCl and NA. As shown in figure 19, the effects are almost completely reversible.

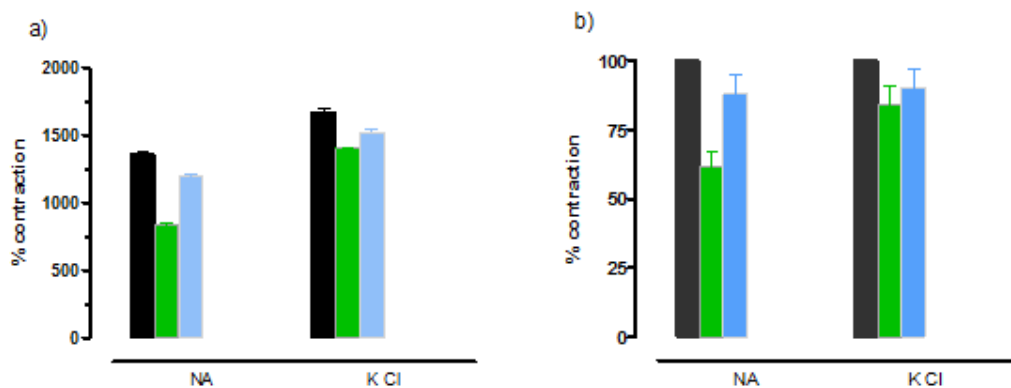


Fig 19. Spasmolytic activity of extract against contraction induced by NA (1 μ M) and potassium chloride (80 mM). a) The effect was expressed in milligrams of contraction. b) The effect was expressed in percentage of the control. (black) effect of agonist. (green) effect of agonist in presence of chestnut

extract (1 mg/ml). (blu) effect of agonist after washout of extract. Each point is the mean \pm SEM of five-six experiments. Where error bars are not shown these are covered by the point itself.

For extract was also evaluated the antispasmodic activity against KCl. The curve of contraction to KCl in the presence of extract (1 mg/ml) is non significantly different from the control.

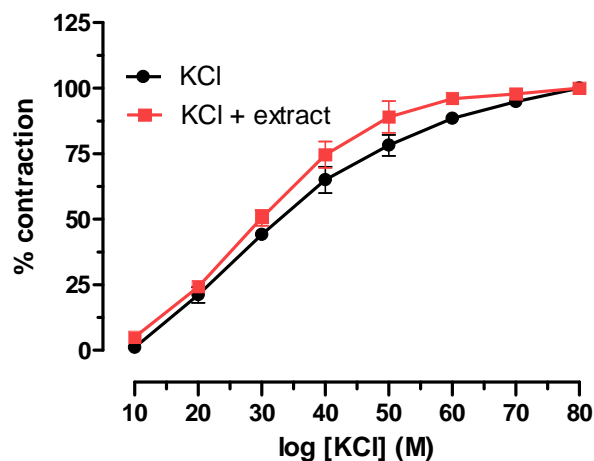


Figure 20. Effect of ENC on potassium chloride-induced contraction in isolated guinea pig aortic strips. Cumulative dose-response curves were obtained before and after exposure to ENC (1 mg/ml) for 30 min. Each point is the mean \pm SEM ($n = 5-6$). Where error bars are not shown these are covered by the point itself.

During incubation with ENC (1 mg/ml) for 30 minutes we observed a interesting contraction that stabilizes within 30 minutes that we have calculated the potency [$EC_{50} = 0.18$ mg/ml (c.l. 0.14–0.19)]. The maximum effect is reached at 1 mg/ml. This contraction is inhibited by 44.7 ± 3.8 % by nicardipine (1 μ M).

5.3 Antioxidant and cytoprotective effects of Sweet Chestnut bark extract in cultured rat cardiomyocytes

Sweet Chestnut bark extract is particularly rich in tannins (77% p/p), therefore we have investigated the ability of the extract to protect cultured cardiomyocytes from oxidative stress.

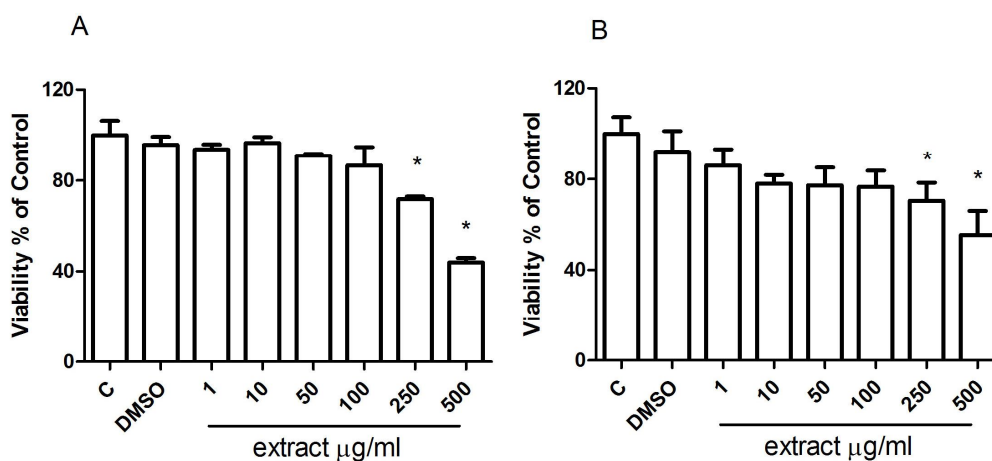


Fig. 21 Cell Viability of cultured cardiomyocytes treated with chestnut extract. Rat cardiomyocytes were treated with chestnut extract, solubilized in DMSO, as described in Materials and methods, data are reported as means \pm S.D.. (A) Cell viability was analysed by the MTT test as reported in *Materials and Methods*, (B) Cell viability was analysed by flow cytometry. Cells were double labelled with Annexin V-PE 7 AAD, and analyzed by a Guava EasyCyte flow cytometer. Statistical analysis was performed by one-way analysis of variance (ANOVA) followed by Dunnett's test., * $p < 0.05$ with respects to controls.

Figure 21 shows, by MTT (A) and flow cytometry analysis (B), that chestnut extract did not exert any toxic effect on cultured primary cardiomyocytes on a wide range of concentrations, (1-100 $\mu\text{g/ml}$). A significant decrease in ROS production, as detected by DCFH-DA assay, was observed in chestnut extract-treated cardiomyocytes following exposure to H_2O_2 . The ability of the extract to reduce ROS production was already detected at 1 $\mu\text{g/ml}$ concentration.

Vehicle controls containing equivalent volumes of DMSO (0.2% v/v) did not show any significant difference in comparison to cells exposed to H_2O_2 . ROS levels were significantly reduced in extract-treated cells after 24 h in a dose dependent fashion. Incubation of cardiomyocytes with 100 μM H_2O_2 for 30 min caused a significant decrease in cell viability (Figure 21), as detected by MTT reduction assay. Treatment of cardiac cells with chestnut extract at the concentration of 50-100 $\mu\text{g/ml}$ for 24h prior to H_2O_2 exposure partially protected against oxidative damage, as shown by the significant increase in cell viability with respect to H_2O_2 -treated cells.

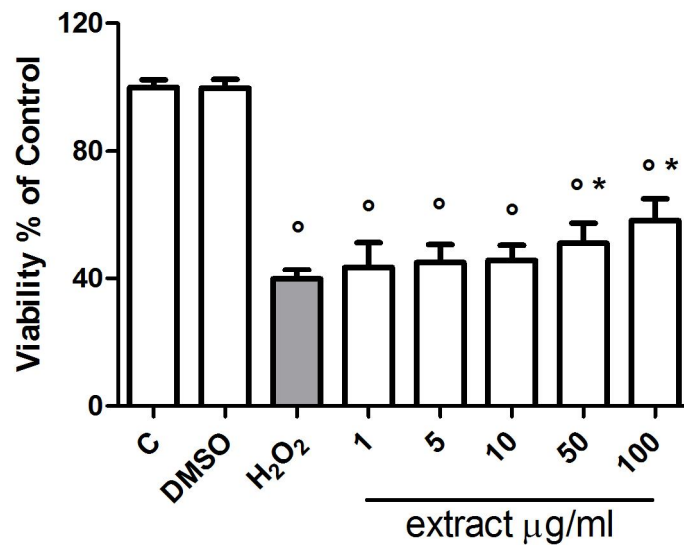


Fig. 22 *Effect of Sweet Chestnut bark extract treatment on cell viability in cardiomyocytes exposed to H_2O_2 .* Cardiomyocytes were treated with chestnut extract (1-100 $\mu\text{g/ml}$) for 24h before the addition of 100 μM H_2O_2 , and cellular damage was assessed by the MTT assay and reported as percent cell viability in comparison to control cells. Each bar represents the mean \pm S.D.. of four independent experiments. Data were analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test, * $p < 0.05$ with respect to H_2O_2 -treated cells, ° $p < 0.05$ with respect to control cells.

5.4 Discussion

Many studies have described the relation between cardiac dysfunction and potential initiating factors such as smoking, excessive alcohol consumption, diet, obesity, stress and so on. In several cases, the effect of one this factors alone or in combination with other initiating factors, as atherosclerosis and hypertension, represents a first step in the development of myocardial ischemia that plays a central role in the onset and development of different cardiac dysfunctions such as angina, infarction, arrhythmias or heart failure. [Soufi M, et al., 2006] [Woo KS, et al., 1999]

Different approaches are commonly applied to reduce cardiovascular risks. Beside the early identification of risk factors, lifestyles and nutrition habits play a fundamental role in preventing or counteracting cardiovascular diseases. Epidemiological studies have indicated the existence of an inverse correlation between the intake of fruits and vegetables, rich in antioxidant phytochemicals, and the risk of developing cardiovascular diseases [Genkinger JM, et al., 2004] [Kris-Etherton PM, et al., 2002]. Many phytochemicals have been shown to exert antioxidant effect and to counteract many oxidative stress related diseases, like cardiovascular diseases [Mizrahi A, et al., 2009]

The high potential of *Castanea Sativa* Mill. was firstly discovered by the Romans not only for fruits but also for leaves, flowers and bark. The bark and wood of chestnut trees are the prime sources of tannins; different *Castanea* tissues are rich in both simple phenolics and more complex tannins but the chestnut wood contains much higher levels of phenolics than the chestnut fruits [De Vasconcelos MCBM, et al., 2010]. The extract used in this study has been demonstrated to contain more than 10% (w/w) of phenolic compounds, of which tannins as Vescalgin and Castalgin are the more representative. It has previously been demonstrated that many ellagitannins, including castalagin and vescalagin, have potent antitumor, antioxidant, antimicrobial and antimalarial properties [Cerdà B, et al., 2004] [Seeram NP, et al., 2005] [Reddy NM, et al., 2007].

Tannins, including proanthocyanidins, exert many biological effects [Haslam E., 1996]. Tannins exert a double action towards the cardiovascular system: a direct action on heart and blood vessels by modulation of cardiovascular parameters and an indirect action through their antioxidant activity. In fact they are able to inhibit lipid peroxidation and lipoxygenases in vitro, and to scavenge radicals such as hydroxyl, superoxide, and peroxy [Gyamfi MA, et al., 2002]. While antioxidant effects of condensed tannins have been reported by several studies, [Guo Q, et al., 1999] there is little information on the antioxidant activity of water soluble tannins [Ito H., 2011] [Yoshida T, et al., 2010]. Hydrolysable tannins, for their high degree of hydroxylated aromatic functions, show high antioxidant activity [Koleckar V, et al., 2008].

In many countries Pycnogenol® a preparation based on *Pinus maritima* bark extract, a pine from the south of France, is used as a cardioprotective food supplement [Packer L, et al., 1999]. The main bioactive compounds of this product are oligomeric proanthocyanidins and phenolic monomers.

No data about the cardioprotective effects of chestnut bark extract are reported in the literature, therefore in order to validate this hypothesis we have characterized the extract, and measured its antioxidant and cytoprotective activities in cultured rat cardiomyocytes. Moreover we have investigated its inotropic and chronotropic effects in guinea pig cardiac preparations and its activity in guinea pig aorta strips.

Extract induced transient negative chronotropic effect in isolated spontaneously beating right atria and simultaneously positive inotropic effect in left atria driven at 1 Hz. On papillary muscle the positive inotropic effect is persistent. This effect is particularly interesting, in fact it is known that a persistent positive inotropic effect is useful for ventricular support of heart function and for preventing stagnation of blood into the ventricles.

Surprisingly cardiac cholinergic receptors are not involved in the negative chronotropic effect, in fact the preincubation with atropine does not affect the negative inotropic effect. Since previous studies have shown that natural extract of chestnut is able to bind gut cholinergic receptors in a non-competitive reversible manner [Budriesi R, et al., 2010], it is possible to hypothesize that the extract is able to discriminate between the major cholinergic receptor subtypes. In particular, in heart tissue, the bradycardic seems not to be mediated by direct interaction with this system.

The positive inotropic effects are not related to adrenergic receptors because the effect of the extract persists even in the presence of propranolol. These results demonstrate that both chronotropic and inotropic effect of the extract are not due to the main receptor mechanisms involved in heart function regulation.

As regards the vascular smooth muscle, natural extract of chestnut did not significantly change the contraction induced by potassium (80 mM) or that induced by noradrenaline (1 μ M). In a previous paper [Budriesi R, et al., 2010] we demonstrated that bark chestnut extract exerts antispasmodic activity towards the contraction induced by potassium chloride in different gut segments. It is well known that the ileum contraction induced by potassium chloride involves the activation of L-type calcium channels. [Bolton TB. 1979] It is not surprising that the extract does not produce any effects on large vessels, as there are many drugs such as calcium channel entry blockers that acts selectively in potassium induced ileum contraction without having effects on aortic strips. [Budriesi R, et al, 2009]

Extract did not exhibit antispasmodic effect on the potassium induced contraction although, during the incubation period, it showed a weak intrinsic

contractile activity. This weak contraction induced by extract is inhibited by nicardipine.

These preliminary findings do not allow us to make statements about the mechanism by which the extract induces its direct cardiovascular effects. certainly the homeostasis of calcium is involved in its cardiovascular protective effects.

Extracts from *Castanea Sativa* leaves have been shown to exert an antioxidant effect in different “in vitro” model systems and to be useful in the prevention of photoaging and oxidative stress mediated skin diseases [Almeida IF, et al., 2008] [Calliste CA, et al., 2005].

Recently, Frantic et al. [Frankič T, et al., 2011] have investigated the effect of sweet chestnut wood extract in pigs treated with high doses of n-3 PUFAs to induce oxidative stress; the authors demonstrated that the extract treated pigs showed a decreased level of many biomarkers of oxidative stress as urine MDA and isoprostanes, and lymphocytes DNA damage, suggesting the use of chesnut wood extract in animal nutrition to prevent oxidative stress.

Even though previous studies have demonstrated the ability of chesnut (*Castanea crenata*) inner shell extract to protect HepG2 cells from t-BHP induced oxidative stress [Noh JR, et al., 2010], to our knowledge no data are available to elucidate the effects of castanea sativa bark extracts on cardiac cells.

In this study the extract did not shows any toxic effect in cultured cardiomyocytes in a wide range of concentrations (1 to 100 µg/ml) and resulted in a significant protection against H₂O₂ -induced cytotoxicity and in a marked decrease in intracellular ROS production.

CHAPTER 6

Diarrhoea, a world-wide health trouble

Gastro-intestinal water-borne infections represent among the most emerging and re-emerging infectious diseases throughout the world. These infections heat mainly the stomach and the gastro-intestinal tract. They are mostly endemic with a worldwide distribution and they have a heterogeneous aetiology. Most water-borne diseases that are caused by organism ranging from microscopic viruses (rotarine) of less than 18mm in diameter to parasites of 10cm in length culminate into diarrhoea and determine about 5 million reported deaths per year.

There are four main features, in diarrhoea, which reflect the basic underlying pathology and altered physiology:

- acute watery diarrhoea
- acute bloody diarrhoea,
- persistent diarrhoea
- diarrhoea with severe malnutrition of which 50% of worldwide cases of the condition present with watery diarrhoea.

Approximately 35% are persistent diarrhoea and 15% dysentery-diarrhoea with blood stains

Diarrhoea has been recognized as one of the most important health problems afflicting mankind, particularly those populations in socio-economically backward, and developing, third-world countries. (Gutierrez, R.M.P. et al, 2008) (Venkatesan, N. et al, 2005)

Dehydration represents the principle threat, though diarrhoea also reduces the absorption of nutrients, determining poor growth in children, low resistance to infections, and potentially long-term gut disorders.

Annually, at least 1,500 million episodes of diarrhoea affect children under the age of five years and 4 million children deaths are estimated to be caused by diarrhoea. (WHO. <http://www.who.int/aboutwho/en/preventing/preventing.htm>)

Kung'u *et al*, (Kung'W. N *et al*, 2002) reported that 37% of all cases of diarrhoea in the world occur in sub-Saharan Africa.

Morbidity and mortality due to acute diarrhea is significant even in the United States. (Cohen, ML.,1988) (Ho, MS, *et al*, 1988) The Foodborne Disease Active Surveillance Network (FoodNet) conducted a population-based telephone survey of 12,075 persons in the United States from 1998 to 1999 to assess diarrheal illness. Six percent reported an acute diarrheal illness at some point during the four weeks preceding the interview (annualized rate, 0.72 episodes per person-year). Rates of illness were highest among children younger than five years (1.1 episodes per person-year) and were lowest in persons aged ≥ 65 years (0.32 episodes per person-year). (Imhoff, B. *et al*, , 2004)

In addition, Sandler and colleagues observed that the most prevalent diseases were non-foodborne gastroenteritis (135 million cases per year) and foodborne illness (76 million cases per year). (Sandler, RS *et al*, 2002)

A study from England that included 9776 adults reported an incidence of infectious diarrhea of 19.4, 3.3, and 0.15 cases per 100 person years in a community cohort, those presenting to general practitioners, and cases reaching the national surveillance system, respectively. (Wheeler, JG *et al.*, 1999)

A retrospective, cross-sectional telephone survey of 3500 Canadian residents from February 2001 to February 2002 reported an incidence of acute gastrointestinal illness of 1.3 episodes per person-year. (Majowicz, SE *et al*, 2004)

The incidence of gastroenteritis was 45 per 100 person years in a prospective cohort study in the Netherlands involving 2206 people from the general population. (De Wit, MA, *et al*, 2000)

A report from the Centers of Disease Control and Prevention (CDC) found that foodborne diseases account for approximately 76 million illnesses, 325,000 hospitalizations, and 5000 deaths each year in the United States based upon surveillance data from multiple sources. While acute diarrhea occurs in most cases of foodborne illness, there are other causes of acute diarrhea such as inflammatory bowel diseases, (Mead, PS, *et al.*, 1999) including, among others, Crohn's disease (CD) and ulcerative colitis (UC).

In order to overcome the menace of diarrhoea in developing countries, especially the discomfort and inconvenience of frequent bowel movements, the World Health Organization (WHO) has introduced a programme for diarrhoeal control which involves the use of traditional herbal medicines. Several medicinal plants have been reported to be useful in the treatment, management and/or control of diarrhoea. (Abdullahi, et al, 2001) (Aniagu, S.O., et al, 2005) (Agunu, A., et al., 2005)

ENC and gastro-intestinal tract

6.1 Ileum and proximal colon

In order to explain the pharmacological role of ENC, rich in hydrolyzable tannins, in the modulation of intestinal motility, its effects have been evaluated. Isolated guinea pig ileum and proximal colon segments were used to evaluate the ability of the extract to inhibit contractions evoked by agonists such as carbachol (CCh), serotonin (5-hydroxytryptamine [5-HT]), BaCl₂, KCl and histamine. (Budriesi et al, 2010)

The biological activity of ENC against CCh-induced contraction was studied in the isolated guinea pig ileum using papaverine as the standard reference.

6.1.1 Guinea Pig Ileum

As shown in Figure 23a, ENC reduced the maximum response to CCh in a concentration-dependent manner and behaved as a non competitive antagonist. The maximum response to carbachol was reduced in a concentration-dependent manner by the concentration of extract. Papaverine acts in a similar way, but its potency was greater.

In guinea pig ileum, the maximum effect of ENC was reached within a 30-minute incubation at a concentration of 1 mg/mL (Fig. 23b). The dose-response curve obtained with CCh after a 45-minute incubation (ENC 1 mg/mL) did not differ from the curve obtained after a 30-minute incubation ($P < -05$).

In order to verify if the effects of ENC are reversible, we studied the concentration-response curves to CCh after exposure to 1 mg/mL ENC at different washout times. As displayed in Figure 1c, the response to CCh was completely recovered after 60 minutes of tissue washout.

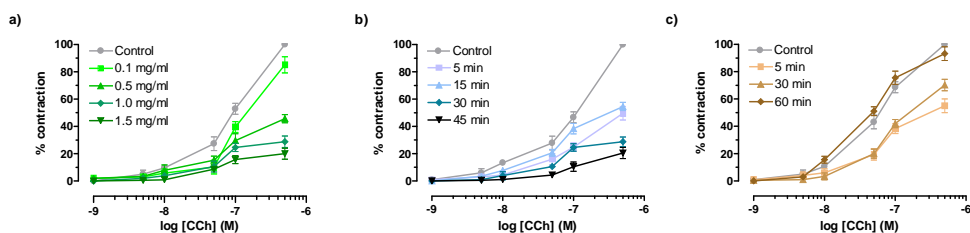


Figure 23. (a) Effect of ENC on carbachol (CCh)-induced contraction in isolated guinea pig ileum. Cumulative concentration-response curves were obtained before and after exposure to ENC for 30 minutes. Data are mean \pm SEM values ($n = 5-6$). (b) Time course of ENC effect on CCh induced contraction in isolated guinea pig ileum (100%). Cumulative concentration-response curves were obtained before and after exposure to ENC (1 mg/mL) for 5, 15, 30, and 45 minutes. Data are mean \pm SEM values ($n = 4-7$). (c) Time course of effect of ENC (1 mg/mL) on CCh induced contraction in isolated guinea pig ileum. Cumulative concentration-response curves were obtained before and after exposure to ENC (1 mg/mL) and following washing for 5, 30, and 60 minutes. Data are mean \pm SEM values ($n = 3-5$). Where error bars are not shown these are covered by the point itself

The antispasmodic activity of ENC was better investigated against a variety of different spasmogenic agents in the guinea pig ileum. ENC reduced the histamine-induced spasms by a non competitive mechanism (Fig. 24a), and the inhibition was completely reversed after 60 minutes of tissue wash out.

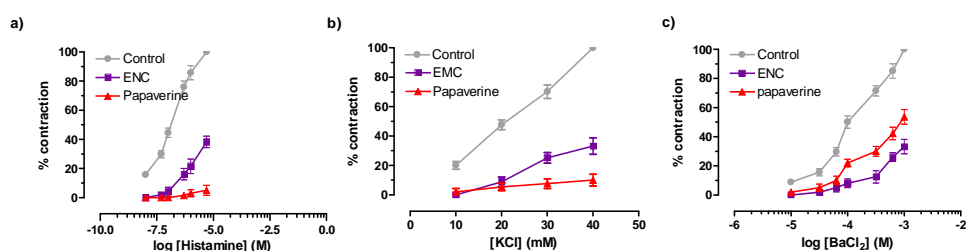


Figure 24. (a) Effect of ENC on histamine-induced contraction of isolated guinea pig ileum. Cumulative dose-response curves were obtained before and after exposure to ENC (1 mg/mL) and papaverine (0.01 mg/mL) for 30 minutes. Data are mean \pm SEM values ($n = 5-6$). (b) Effect of ENC on KCl-induced contraction in isolated guinea pig ileum. Cumulative dose-response curves were obtained before and after exposure to ENC (1 mg/mL) and papaverine (0.01 mg/mL) for 30 minutes. Data are mean \pm SEM values ($n = 5-6$). Where error bars are not shown these are covered by the point itself. (c) Effect of ENC on BaCl₂-induced contraction in isolated guinea pig ileum. Cumulative dose-response curves were obtained before and after exposure to ENC (1 mg/mL) and papaverine (0.01 mg/mL) for 30 minutes. Data are mean \pm SEM values ($n = 5-6$). Where error bars are not shown these are covered by the point itself.

In Figure 24a, the effect of ENC (1 mg/mL) on spastic contractions induced by histamine is reported in comparison with that of papaverine (0.01 mg/mL). Both papaverine and ENC showed the same activity profile. The contraction induced by KCl (Fig. 24b) was diminished by pretreatment with ENC as well as by pretreatment with papaverine (Fig. 24b). The effect was completely reversible after 60 minutes of tissue washing. In contrast, ENC (1 mg/mL) did not significantly affect the contraction induced by KCl, 80 mM.

Spasmodic contractions elicited by BaCl₂ were reduced by ENC in a concentration-dependent manner (Fig. 24c). The effect was similar to that induced by papaverine and was completely reversible after tissue washout (60 minutes).

6.1.2. Guinea Pig Proximal Colon

In the proximal colon model, the tissues were stimulated by CCh (muscarinic receptors) or by 5-HT (serotonergic receptors).

In the first series of experiments the inhibition of CCh-induced motility was investigated following the protocol used for the guinea pig ileum. Concentration-response curves to CCh were measured in the presence or absence of ENC (Fig. 25a), with papaverine being used as the standard reference. ENC showed a lower potency in the guinea pig proximal colon relative to that elicited in the guinea pig ileum (Table 4). Moreover, ENC antagonized the carbachol response in a non competitive manner, like papaverine, under the same experimental conditions.

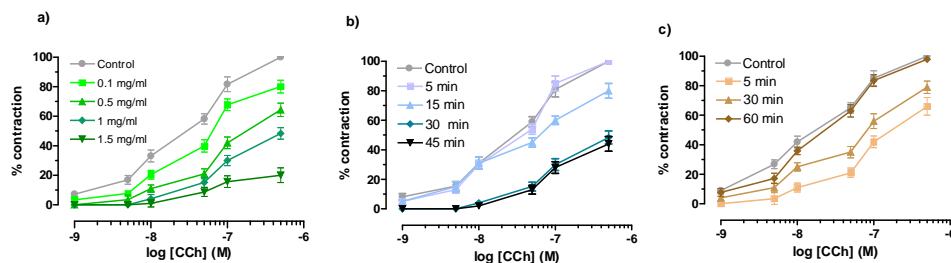


Figure 25: (a) Effect of ENC on CCh-induced contraction in isolated guinea pig proximal colon. Cumulative concentration-response curves were obtained before and after exposure to 0.5, 1.0, and 1.5 mg/mL ENC for 30 minutes. Data are mean \pm SEM values ($n = 5-6$). (b) Time course of ENC effect on CCh-induced contraction in isolated guinea pig colon (100%). Cumulative concentration-response curves were obtained before and after exposure to 1 mg/mL tannins for 5, 15, 30, and 45 minutes. Data are mean \pm SEM values ($n = 4-7$). (c) Effect of ENC (1 mg/ mL) on CCh induced contraction in isolated guinea pig proximal colon. Cumulative dose-response curves were obtained before and after exposure to ENC (1 mg/mL) and following washing for 5, 30, and 60 minutes. Data are mean \pm SEM values ($n = 3-5$). Where error bars are not shown these are covered by the point itself.

As observed in the ileum, the maximum effect was reached after 30 minutes of incubation (results were not significantly different from those obtained after 45 minutes of incubation [$P < .05$]) (Fig. 25b). The non competitive antagonism was completely reversed by 60 minutes of tissue washout (Fig. 25c).

Table 4. Antagonist affinities, expressed as IC₅₀ Values, in the different guinea pig gut smooth muscle segments.

Guinea pig Gut Smooth Muscle Segments	ENC		Papaverine	
	IC ₅₀ ^a	95% conf lim	IC ₅₀ ^a	95% conf lim
Ileum	0.44	0.35–0.55	0.0035	0.091–0.0089
Proximal colon	1.50	1.22–1.84	0.25	0.15–0.58

^a IC₅₀ was expressed as mg/ml conc. and calculated from concentration-response curves (Probit analysis by Litchfield and Wilcoxon with *n* = 6–7) (Tallarida, 1987).

Then, 5-HT agonist activity in guinea pig proximal colon was also investigated (Fig. 26). As shown in Figure 5a, 5-HT produced a double response in the proximal colon: the former was a fast and strong contraction, inhibited by atropine, probably due to acetylcholine liberation from the intramural parasympathetic ganglion cells; the latter was a relaxation due a direct stimulation of 5-HT receptors of smooth muscle cells.

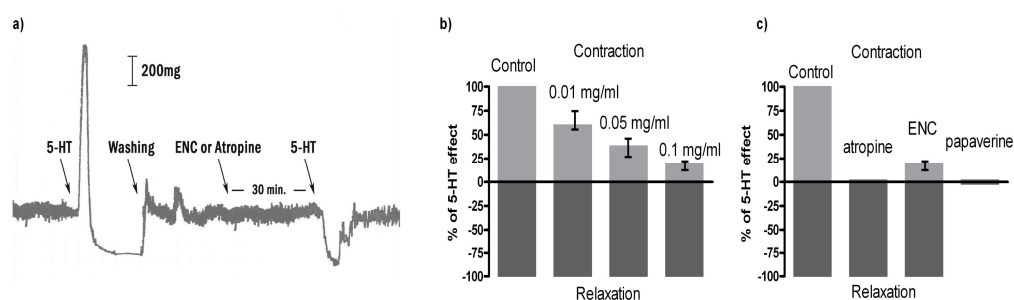


Figure 26: (a) Typical data chart recorded in guinea pig proximal colon stimulated by 5-HT (50 μM) alone and after treatment with ENC (1 mg/ml) or atropine (1 μM) in guinea pig proximal colon. Arrows indicate the treatments. (b) Effect of 5-HT (50 μM) on contraction or relaxation in guinea pig proximal colon before, and after exposure to 0.01, 0.05 and 0.1 mg/ml of ENC for 30 minutes. All data are the mean ± SEM (*n* = 3-5). (c) Effect on 5-HT-induced contraction or relaxation (50 μM) in guinea pig proximal colon obtained before (control) and after exposure to ENC (1 mg/ml), atropine (1 μM) and papaverine (0.1 mg/ml) for 30 min. Results are expressed as the mean ± SEM (*n* = 4-7). Where error bars are not shown these are covered by the point itself.

ENC ability to inhibit proximal colon movements elicited by 5-HT was evaluated taking papaverine as the reference. Contraction elicited by 5-HT in proximal colon segments was inhibited by ENC in a dose-dependent manner, whereas the relaxation induced by 5-HT was not affected (Fig. 26a,b). The effects of ENC in proximal colon appeared similar to those induced by atropine (1 μM) (Fig. 26c), whereas papaverine completely blocked both contraction and relaxation elicited by 5-HT. The spontaneous contractions and the basal tone of guinea pig ileum and proximal colon were not affected by ENC incubation.

In our experimental models, the purified ENC is able to relax guinea pig ileum and proximal colon smooth muscle spasms induced by several mechanisms.

The effect of the extract on contraction induced by 5-HT is very particular as 5-HT in proximal colon elicits two contrasting actions: it induces a strong contraction mediated by release of acetylcholine from the intramural parasympathetic ganglion cells, followed by a relaxing action due to a direct stimulation of 5-HT receptors of smooth muscle cells. In proximal colon segments ENC prevents the contraction induced by 5-HT, whereas it does not affect the relaxing action mediated by 5-HT. This observation points out the selectivity of ENC, which can inhibit the cholinergic activity but does not interfere directly with serotonergic receptor activation. This molecular mechanism could be helpful both in reducing colon contractile activity and in promoting the relaxing activity of 5-HT.

The observed data indicate that ENC could be helpful in controlling diarrhea through its antispasmodic effects on ileum and colon segments of the intestine, and this action is the result of the synergic effect of ENC. In fact, ENC exerts an antibacterial activity against many food-borne pathogen bacteria like *Staphylococcus aureus* and *Vibrio* spp.. Furthermore, some compounds, belonging to the class of hydrolyzable tannins found in ENC, have been shown to exert a strong antiviral activity.

6.2 Discussion

Experiments performed in guinea pig ileum showed that ENC, like papaverine, used as the reference standard, inhibited the maximum response to CCh in a noncompetitive manner. Furthermore, this blockade was reversed after tissue washing. The restoration of basal tone of ileum tissue, by removing ENC with several washings, suggests this intestinal segment was not damaged as its spontaneous motility was maintained. ENC displayed an IC_{50} value (0.44 mg/mL) lower than that of papaverine (0.0035 mg/mL). The difference in potency between ENC and papaverine could be partially explained by the fact that a phytocomplex is made up of various components containing a pool of substances, whereas papaverine is a well characterized chemical compound.

ENC produced its antispasmodic activity not only by reduction of CCh-induced contractions, but also by reduction of those due to histamine, KCl, and BaCl₂. Histamine contracts the guinea pig ileum by interacting with histamine receptor subtypes.[Atta AH, et al., 2005] ENC antagonizes contractions evoked by histamine in a noncompetitive reversible manner with a lower potency respect than that calculated against CCh (IC₅₀ = 0.73 mg/mL and 0.44 mg/mL, respectively). The spasms induced by BaCl₂, an agent able to release bound calcium with ganglion-stimulating properties, were inhibited by the extract. This could be due to the reduction of smooth muscle responsiveness by interfering with Ca²⁺ availability and bound Ca²⁺-releasing mechanisms. ENC acts as a non competitive reversible antagonist, and its potency is equal to that measured in antagonize the effect of CCh.

KCl depolarizes the ileum strips, resulting in spastic contractions by activation of voltage-dependent Ca²⁺ channels. (Bolton TB, 1979)

ENC reduces KCl-induced contractions by a noncompetitive reversible mechanism showing a potency threefold lower than that elicited against CCh or BaCl₂. This antispasmodic activity might involve the inhibition of voltage-dependent Ca²⁺ channels. Under the same experimental conditions papaverine (0.01 mg/mL) induces a full spasmodic activity block. However, ENC (1 mg/mL) does not possess a spasmolytic action and does not affect the contractile response induced by KCl (80 mM). Concerning the guinea pig proximal colon, inhibition induced by ENC in CCh contraction is qualitatively similar to that induced in guinea pig ileum, but, like papaverine, ENC is less potent in this intestinal segment. One of the most interesting findings was the results obtained with ENC in guinea pig proximal colon preparation stimulated with 5-HT. Contractions induced by the stimulation of neuronal 5-HT receptors in proximal colon are mediated by the cholinergic System, whereas the stimulation of the intestinal smooth muscle 5-HT receptors causes relaxation. The data reported in Table 3 show that ENC, like atropine, inhibits contraction induced by 5-HT (50 μM), whereas ENC does not affect the following relaxation.

It could be concluded that ENC strongly interferes with the cholinergic System with poor or insignificant serotonergic activity. Moreover, ENC seems to

modulate the bound Ca^{2+} -releasing mechanisms and to a minor extent the voltage-gated calcium channels.

In the experimental models I used, the purified ENC has been shown able to relax guinea pig ileum and proximal colon smooth muscle spasms induced by several mechanisms.

The effect of the extract on contraction induced by 5-HT is very particular as 5-HT in proximal colon elicits two contrasting actions: it induces a strong contraction mediated by release of acetylcholine from the intramural parasympathetic ganglion cells, followed by a relaxing action due to a direct stimulation of 5-HT receptors of smooth muscle cells. In proximal colon segments ENC prevents the contraction induced by 5-HT, whereas it does not affect the relaxing action mediated by 5-HT. This observation points out the selectivity of ENC, which can inhibit the cholinergic activity but does not interfere directly with serotonergic receptor activation. This molecular mechanism could be helpful both in reducing colon contractile activity and in promoting the relaxing activity of 5-HT. In conclusion, ENC could be helpful in controlling diarrhea through its antispasmodic effects on ileum and colon segments of the intestine, and this action is the result of the synergic effect between the antispasmodic effect and the antimicrobial effect of ENC. ([Jamroz D, et al., 2009](#)) ([Frankič T, et al., 2011](#))

CHAPTER 7

Biological activity of ENC toward other gastro-intestinal tracts and toward biliary tract

7.1 Stomach and Juneum

The effect of the ENC in other parts of the gastro-intestinal tract has been investigated. In particular, its effects toward the CCh-mediated contraction has been investigated.

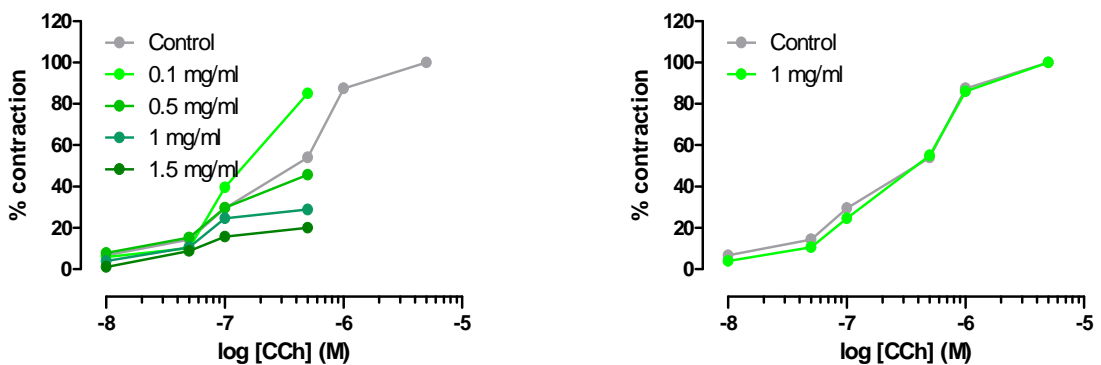


Figure 27: Effect of ENC on carbachol (CCh)-induced contraction in isolated guinea pig jejunum. Cumulative concentration-response curves were obtained before and after exposure to ENC for 30 minutes. Data are mean \pm SEM values (n = 5-6). (b) Carbachol (CCh)-induced contraction after 30 minutes washout. Data are mean \pm SEM values (n = 3-5). Where error bars are not shown these are covered by the point itself

ENC is able to reduce the maximum contraction induced by carbachol. The observed antagonism is non-competitive and it occurs in a concentration-dependent manner. In addition, it is reversible.

The effects toward the CCh-mediated contraction has been investigated also in the duodenum.

Also in this case, a non-competitive, reversible and concentration-dependent antagonism towards muscarinic receptors has been observed.

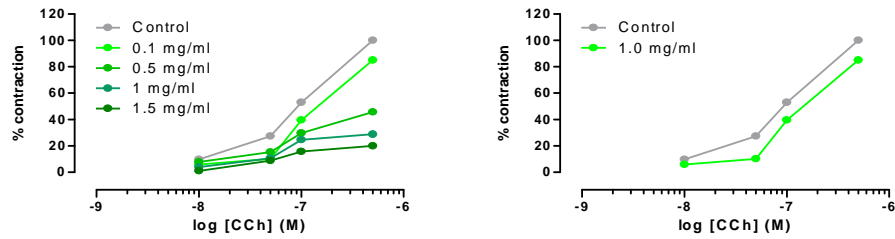


Figure 28: Effect of ENC on carbachol (CCh)-induced contraction in isolated guinea pig s. Cumulative concentration-response curves were obtained before and after exposure to ENC for 30 minutes. Data are mean \pm SEM values (n = 5-6). (b) Carbachol (CCh)-induced contraction after 30 minutes washout . Data are mean \pm SEM values (n = 3-5). Where error bars are not shown these are covered by the point itself.

The data show that natural extract of chestnut wood exerts spasmolytic effects in stomach, ileum, duodenum and proximal colon, by a mechanism perhaps involving unspecific cellular pathways. These findings, taken together with the antiviral, and antibacterial activities against many food-born pathogen bacteria like *Staphylococcus aureus* and *Vibrio* spp., (Buzzini P. et al, 2008), and antispasmodic properties of tannins, suggest that tannins may be relevant to treat diarrhea.

7.2 Biliary tracts

The usual inhibitors of gut peristaltic contraction, such as loperamide, in addition to reducing the ileal and colonic motor function, inhibit the gallbladder motility, probably through an indirect cholinergic mechanism. (Hopman WP et al, 1990)

A decrease of the gallbladder motility determines a reduction of the bile flow; this effect, in patients suffering from illnesses predisposing to gallbladder motility alterations, increases the risk of cholelithiasis. (Thimister PW et al, 1997)

7.3 Gallstones: an increasing health trouble

Gallstone disease represents one of the most frequent and expensive digestive diseases in developed countries, as its prevalence in adults ranges from 10% to 15% (Portincasa P. et al 2006) (Wang DQH et al, 2004) (Everhart JE, et al, 1999) (Sandler RS, et al, 2002). Many patients with gallstones remain “silent”; about a third of patients develop the symptoms and/or the complications. In the United States, medical expenses for the treatment of gallstones exceeded \$6 billion in the year 2000. Furthermore, the prevalence of gallstones seems to be

increasing and about one million new cases are discovered each year ([Liver Disease Subcommittee of the Digestive Disease Interagency Coordinating Committee. Action Plan for Liver Disease Research. Bethesda: NIH; 2004. p. 145-50](#)).

Approximately, 75% of the gallstones in the United States and westernized countries, including Italy, are cholesterol gallstones ([Diehl AK., 1991](#)) ([Attili AF. et al, 1995](#)) ([Attili AF, et al., 1997](#)). The remaining gallstones, represented by pigment stones which have less than 30% cholesterol by weight, can be subclassed into two groups: black pigment stones (about 20% of all gallstones, found in the gallbladder and/or bile duct, containing mainly insoluble bilirubin pigment polymer mixed with calcium phosphate and carbonate, and cholesterol) and brown pigment stones (about 5% of all gallstones, found mainly in bile ducts, containing calcium bilirubinate, calcium palmitate, and stearate and cholesterol)([Sherlock, S. et al., 2002](#)).

Cholesterol gallstones are associated with well known risk factors, such as obesity, type 2 diabetes, dyslipidaemia, and hyperinsulinaemia ([Portincasa P, et al., 2006](#)) ([Grundy SM., 2005](#)) ([Grundy SM, et al., 2005](#)) ([Eckel RH, et al., 2005](#)) ([Tsai CJ, et al., 2004](#)). Furthermore, fibrates, such as gemfibrozil, bezafibrate, fenofibrate, clofibrate, clinically used as hypolipidemic agents, are shown to augment significantly the risk of gallbladder stones formation by increasing the lithogenicity of bile. ([Caroli-Bosc FX, et al., 2001](#)) ([Leiss O, et al., 1986](#)) ([Liang CC, et al., 2011](#))

Cholesterol cholelithiasis is prevalent in populations used to consume a “Western” diet (i.e. enriched in saturated fatty acids, cholesterol, and rapidly absorbed refined carbohydrates), rather than a more “prudent” diet (i.e. enriched in monopolyunsaturated fats, fruit, vegetables and low in refined carbohydrates) associated with physical activity ([Tsai CJ, et al., 2004](#)) ([Tsai CJ. et al., 2004](#)) ([Tsai CJ, et al., 2006](#)) ([Tsai CJ, et al., 2004](#)) ([Tsai CJ, et al., 2005](#)) ([Leitzmann MF, et al., N Engl J Med 1999;341\(11\):777–84](#)) ([Tsai CJ, et al., Ann Intern Med 2004;141\(7\): 514–22](#)) ([Leitzmann MF. et al., 1998](#)).

Thus, the prevalence of cholesterol gallstone disease is significantly higher in North and South American as well as European populations than that in Asian and African populations ([Diehl AK., 1991](#)). In China, the prevalence of

cholesterol gallstones appears to increase with the “westernization” of the traditional Chinese diet (Zhu X, et al., 1995) (Huang YC, et al., 1984) (Sun H, et al., 2009). Even in Japan, the adoption of Western-type dietary habits has resulted in a marked increase of the prevalence of cholesterol cholelithiasis over the past 40 years (Nakayama F, et al., 1970) (Nagase M, et al., Am 1978). The complex pathogenesis of cholesterol gallstones depends on the concurrent existence of hepatic hypersecretion of cholesterol into bile leading to bile supersaturation with cholesterol, accelerated nucleation/crystallization of cholesterol in gallbladder bile, impaired gallbladder motility leading to gallbladder stasis, and increased cholesterol availability from the small intestine, as well as *LITH* genes and genetic factors (Wang HH, et al., 2008) (Portincasa P, et al., 2008).

A complex genetic basis plays a key role in determining individual predisposition to develop cholesterol gallstones in response to environmental factors (Wittenburg H, et al., 2007) (Wang DQH, et al., 2005) (Lammert F, et al., 2005)

7.4 ENC effects towards gallbladder

ENC has shown significant effects on promoting gallbladder contraction. In addition, it is able to relax Oddi sphincter. These effects may be the basis of treating acute pancreas adenitis. In this experiment, we found that ENC significantly increases the resting tension and contractile frequency of isolated guinea pig gallbladder strips. Neither atropine(10-8 M), nor SR27897 (10-8 M) reduces the ENC-mediated gallbladder contraction, suggesting that neither muscarinic nor CCK-1 receptors are involved in the observed activity. The latter action is inhibited by nicardipine, leading to suppose the involvement of calcium channels in the observed activity.

The gallbladder contraction and the relaxing effect toward Oddi’s Sphincter occur also in guinea pigs fed a lithogenic diet, suggesting that ENC may be useful also in subjects at high risk of developing gallstones-such as patients affected by metabolic syndrome- who already have pathological alterations of gallbladder which may reduce its physiological contractility.

Furthermore, the prokinetic effect of ENC toward gallbladder has been tested in human gallbladder obtained from patients undergoing laparoscopic cholecystectomy for acute cholecystitis secondary to gallstone disease. The results indicate that ENC shows a contractile activity also in human gallbladder. In particular it has been shown that the contractile effect of ENC is more potent in women than in men, even if more samples are needed to complete this study.

Since there are no drugs able to contract the gallbladder, the identification of a novel compound able to determine gallbladder contraction is very interesting.

Also oral contraceptives are shown to favor gallstone formation. (Khan MK, et al., 2007)

In these particular conditions, a substance which stimulates the contraction of gallbladder may be useful for the prevention of gallstones formation thus it may avoid the cholecystectomy.

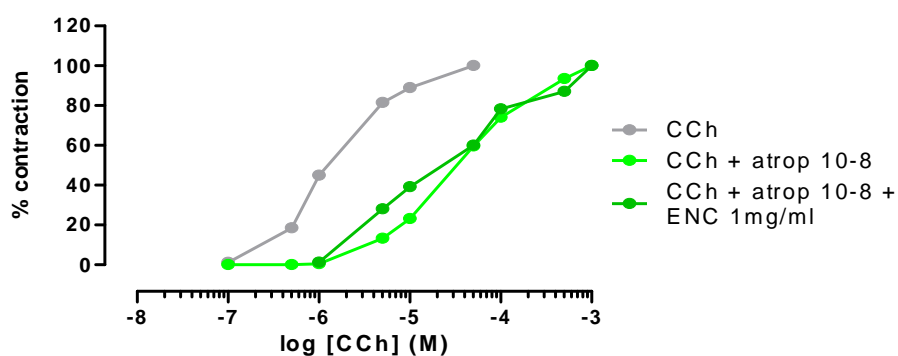


Figure 29 Effect of atropine on carbacol-induced contraction of isolated guinea gallbladder. Cumulative dose-response curves were obtained before () and after exposure to atropine 10-8 M alone and in presence of *Castanea Sativa* Mill. extract (1.0 mg/ml) for 30 min. Each point is the mean \pm SEM ($n = 5-6$). Where error bars are not shown these are covered by the point itself.

Carbachol determines a contraction of gallbladder smooth muscle, as shown in fig. 29. The competitive antagonism of atropine is not affected by the presence of ENC, at the concentration of 1 mg/mL. The Atropine pA2 is not significantly affected by ENC.

As ENC is able to affect the calcium flow through the membrane, it has been conducted a series of experiments where gallbladder has been depolarized through KCl, 80 mM. The contraction induced by KCl, 80 mM, is not altered by the presence of ENC. Nicardipine, a calcium antagonist, reduces the contraction mediated by carbachol, in a concentration-dependent manner.

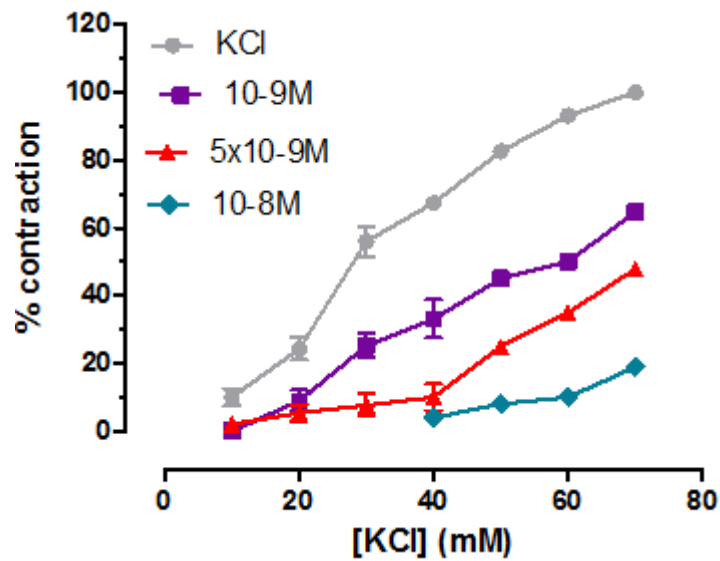


Figure 30. Antispasmodic activity. Effect of Nicardipine on KCl-induced contraction on isolated guinea gallbladder. Cumulative concentration–response curves were obtained before and after exposure nicardipine for 30 min. Data are mean \pm SEM values ($n = 5-6$). Where error bars are not shown these are covered by the point itself.

The effect of nicardipine on ENC-induced contraction has been evaluated.

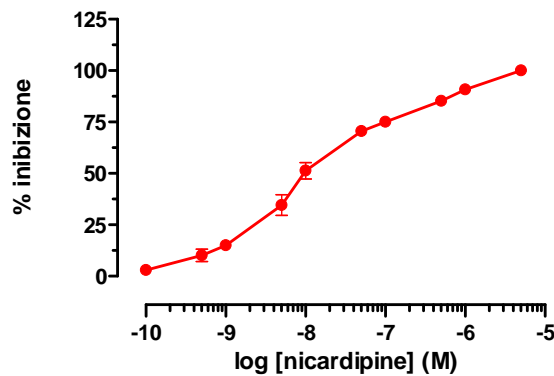


Fig. 31. *Spasmoditic activity.* Effect of Nicardipine on KCl-induced contraction on isolated guinea gallbladder. Cumulative concentration–response curve was obtained after exposure to 80 mM of KCl for 60 min (tempo necessario a rendere la contrazione costante). Data are mean \pm SEM values ($n = 5-6$). Where error bars are not shown these are covered by the point itself.

The contraction induced by ENC at the concentration of 1 mg/mL has been compared with the contraction induced by different agonists such as carbachol (cholinergic agonist), KCl (Calcium opener), A71623 (CCK1 agonist).

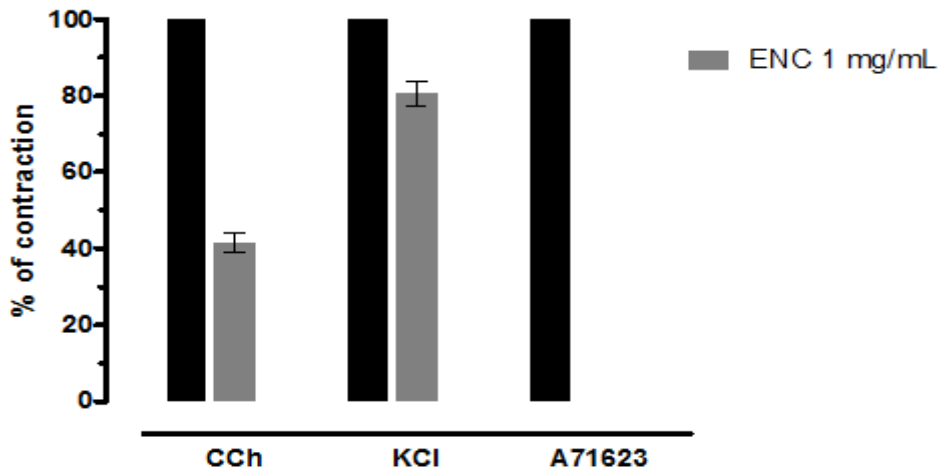


Fig. 32. Effect of ENC induced contraction on isolated guinea gallbladder compared with those induced by CCh, KCl, and A71623. Data are mean \pm SEM values ($n = 5-6$). Where error bars are not shown these are covered by the point itself

As shown in table 5, we have calculated the potency of the contractile action versus gallbladder of ENC.

Table 5:

Comp	Gallbladder Activity ^a	EC ₅₀ of Activity	
	Activity (M \pm SEM)	EC ₅₀ ^b (μ M)	Activity (M \pm SEM)
ENC	88 \pm 1.6	0.032	0.018–0.054

^a Increase in developed tension on isolated guinea-pig gallbladder at 10⁻⁴ M, expressed as percent changes from the control ($n = 5-6$). ^b Calculated from concentration-response curves (Probit analysis by Litchfield and Wilcoxon [Tallarida 1987] with $n = 6-7$).

In the figure 20, the concentration-dependent curves of gallbladder contraction induced by ENC, carbachol and KCl.

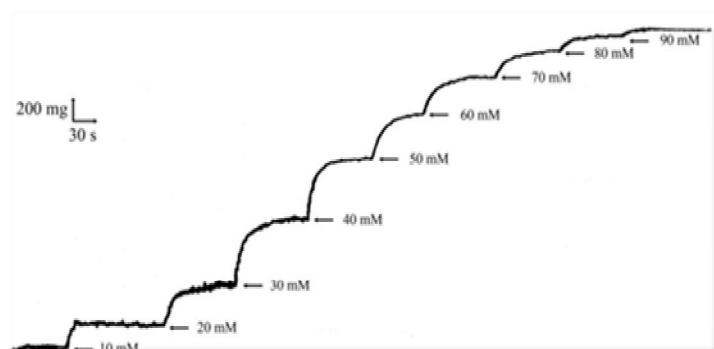


Figure 33: Typical tracing of the contractile response to ENC. Each point is the mean \pm SEM ($n = 5-6$).

In order to understand the possible mechanisms involved in ENC-induced gallbladder contraction, we have made some experiments, where we have

injected the antagonists, such as atropine (muscarinic antagonist) or SR27897 (CCK1 receptor antagonist) into the organ solution before injecting the ENC (1 mg/mL). Since neither atropine (10^{-8} M) nor SR27897 (10^{-8} M) affect the gallbladder contraction, neither muscarinic receptors nor CCK1-receptors are involved in the ENC-mediated gallbladder contraction.

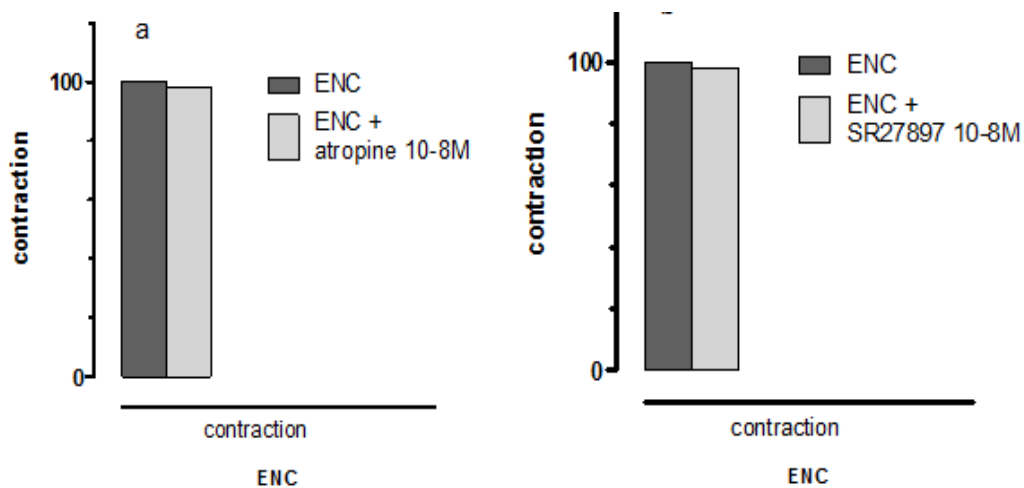


Fig. 34. a) Effect of atropine on ENC (1.0 mg/ml)-induced contraction of isolated guinea gallbladder. Curves were obtained before and after exposure to atropine 10^{-8} M for 30 min. b) Effect of SR 27897 on ENC (1.0 mg/ml)-induced contraction of isolated guinea gallbladder. Curves were obtained before and after exposure to SR 27897 10^{-8} M for 30 min. Each point is the mean \pm SEM ($n = 5-6$). Where error bars are not shown these are covered by the point itself. * $P < 0.05$ versus controls

Since this contraction is inhibited by nicardipine, we suppose the contractile mechanism involves the calcium channels.

Since the cholecystokinetic activity is clinically useful in those clinical conditions which predispose to gallstones formation, favouring the bile flow, we have evaluated the activity of ENC towards Oddi's sphincter smooth muscle.

7.5 Effects of ENC toward Oddi's sphincter smooth muscle

Sphincter of Oddi dysfunction (SOD) refers to an abnormality of Sphincter of Oddi (SO) contractility. It is a benign, noncalculous obstruction to flow of bile or pancreatic juice through the pancreaticobiliary junction, i.e., the sphincter of Oddi. SOD may be manifested clinically by pancreaticobiliary pain, pancreatitis, or deranged liver function tests. It is actually made up of two entities. SO dyskinesia refers to a primary motor abnormality of the SO which may result in a hypotonic sphincter but more commonly, a hypertonic sphincter. In contrast, SO stenosis refers to a structural alteration of the

sphincter, probably from an inflammatory process with subsequent fibrosis. Because it is often impossible to distinguish patients with SO dyskinesia from those with SO stenosis, the term SOD refers to both groups of patients. Also papillary stenosis, ampullary stenosis, biliary dyskinesia, and post-cholecystectomy syndrome refers to SOD, even if the gallbladder can be intact. SOD occurs most after cholecystectomy, but it can occur also when gallbladder is present in situ. (Stuart Sherman, et al., 2001) The integrity of the relaxation function of the sphincter of Oddi is a prerequisite for normal delivery of bile into the duodenum. Sphincter of Oddi relaxation is mainly executed by non-adrenergic, non-cholinergic NANC nerves that are essentially nitrenergic in several species including guinea pigs and rabbits. (Szilvassy Z, et al., 1998) (Szilvassy Z, et al., 1996) The therapeutic approach in patients with SOD is intended to reduce the resistance of the SO to the flow of bile or pancreatic juice. (Cheon YK, et al., 2009)

In figure 22, it is shown the Oddi's sphincter smooth muscle contraction in response to carbachol and to KCl. As shown in the same figure, ENC determines a relaxation.

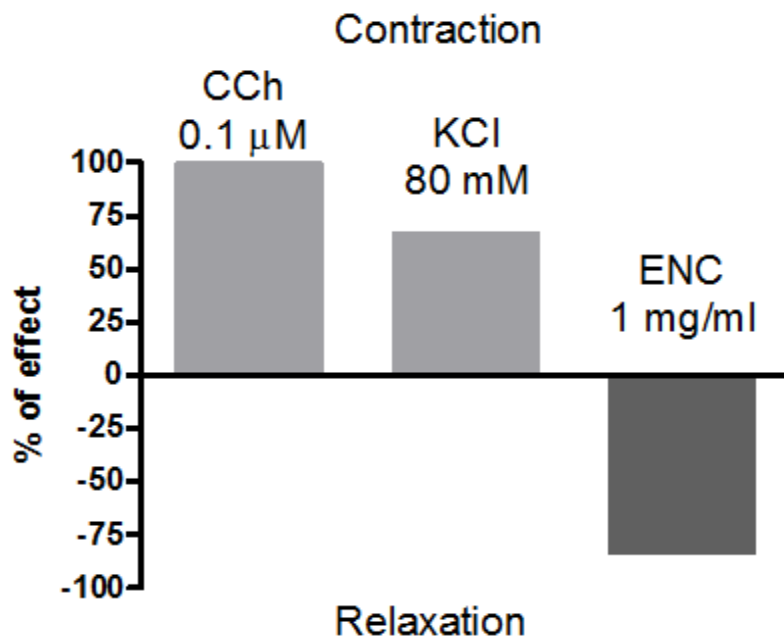


Figure 35. Effect after exposure for 30 minutes of CCh (0.1 μM), KCl (80 mM) and to ENC (1 mg/mL) on contraction or relaxation in guinea pig Oddi sphincter. Data are mean ± SEM values (*n* = 4–7). Where error bars are not shown these are covered by the point itself

In order to investigate the effects of ENC towards gallbladder and Oddi's sphincter smooth muscle in pathological conditions.

7.6 Guinea Pigs fed a lithogenic diet

The administration of a lithogenic diet in guinea pigs, for a 28 days period, determines gallstones formation. In this condition gallbladder smooth muscle is altered. (Chen Q, et al., 1999) (Portincasa P, et al., 2004)

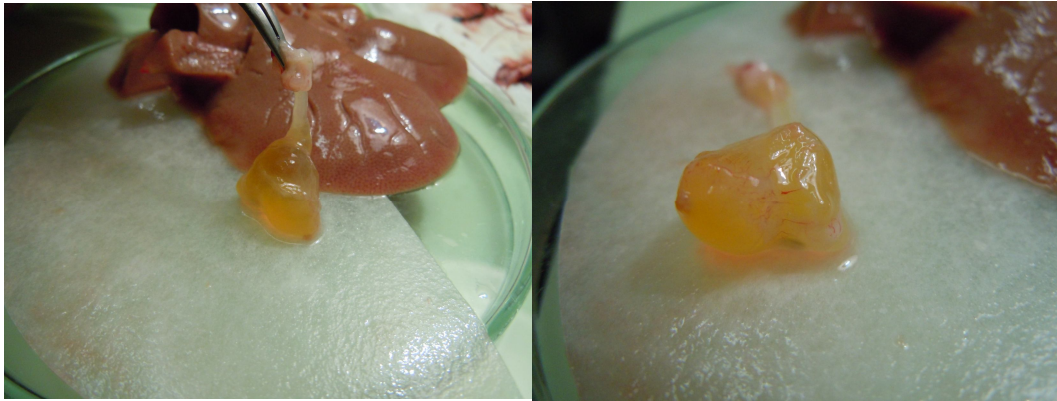


Fig 36: Gallbladder and liver of guinea pigs fed a normal diet and a lithogenic diet.

The gallbladder from these guinea pigs has been tested with ENC for its ability to determine contraction also in pathological conditions.

The obtained results indicate that ENC is able to contract the pathological gallbladder with a similar potency to the contraction of a non pathological gallbladder.

The same experiment has been exerted with Oddi's Sphincter. Also in this case the biological activity of ENC is maintained.

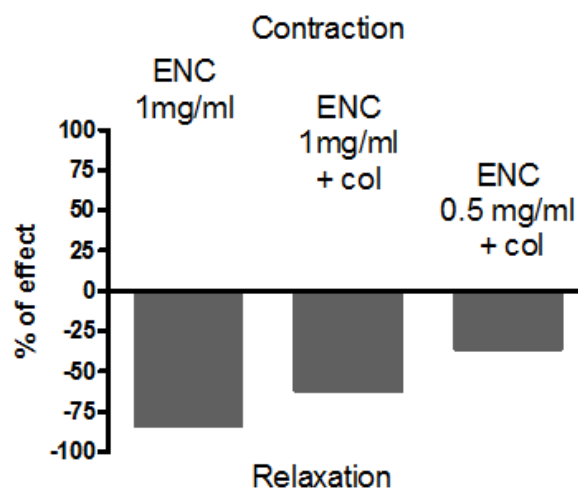


Figure 37. Effect of ENC on isolated guinea oddi sphincter. Cumulative dose–response curve was obtained to ENC Data are mean \pm SEM values ($n = 5-6$).

Since gallbladder contraction represents a very important activity in order to prevent gallstones formation and there are no drugs able to exert this effect, we decided to investigate the effects of ENC towards human gallbladder contraction, using strips of gallbladder, taken from patients with with gallstones, surgically removed by laparoscopic cholecystectomies.

7.7 Effects of ENC towards human gallbladder

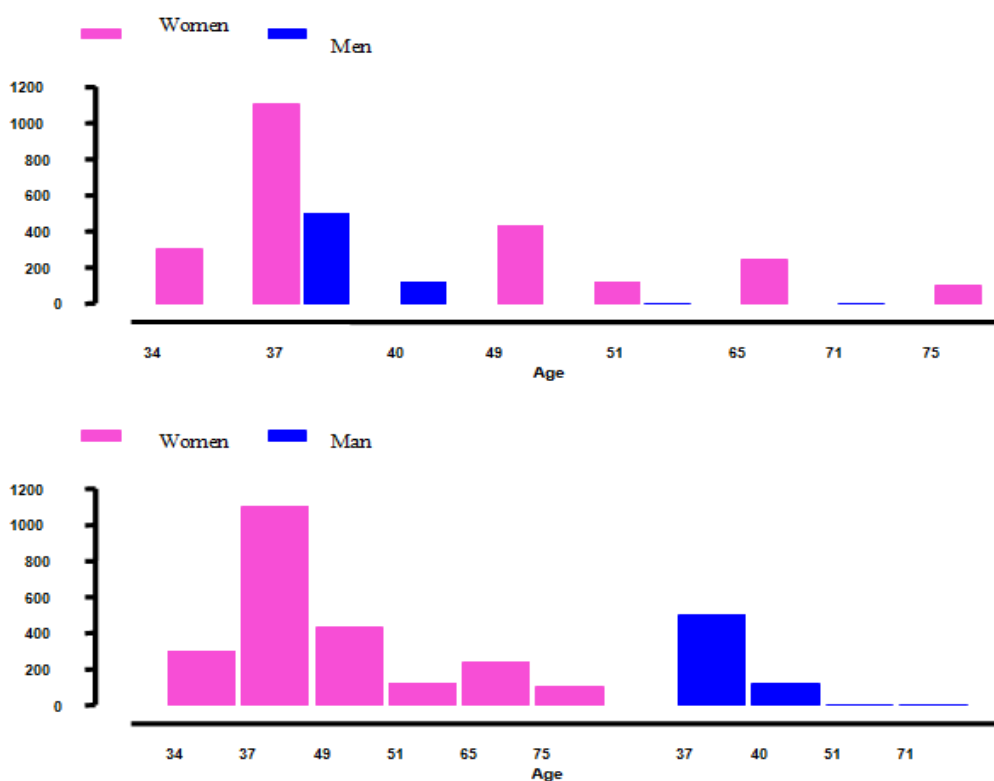


Figure 38: Cholecystocynetic activity of ENC towards human gallbladder contraction taken from woman (pink) and from men (blue)

ENC is able to contract also human gallbladder and that this action is more accentuated in woman than in men, and it is more evident in young patient than in older patients.

As this action can be clinically useful, the process towards the identification of the active compounds has been started.

CHAPTER 8

ENC Fractionation

8.1 Single Fractions Activity towards gallbladder contraction in guinea pigs

Fractionation through solvents with an increasing polarity

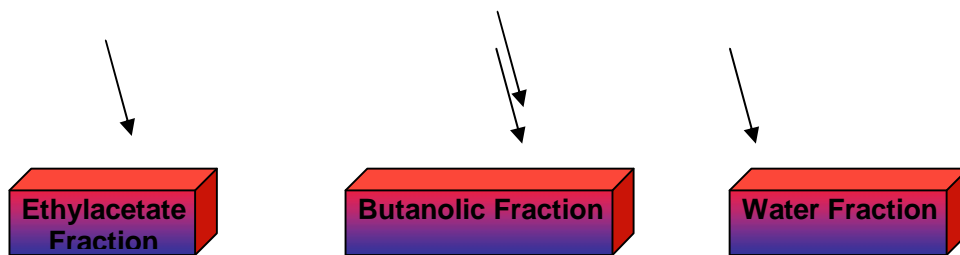


Figure 39: Fractionation of ENC through solvents with an increasing polarity

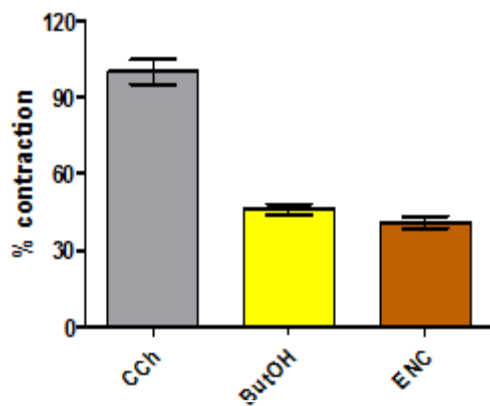


Figure 40: Contraction of gallbladder induced by butanolic fraction (0,1 mg/mL). compared with the contraction induced by CCh (10-8M) and ENC (0,1 mg/mL). Data are mean \pm SEM values (n = 4-7). Where error bars are not shown these are covered by the point itself

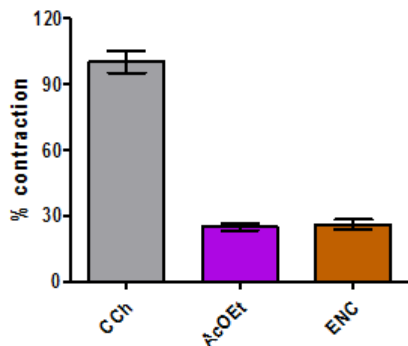


Figure 41: Contraction of gallbladder induced by EthylAcetate fraction (0,1 mg/mL). compared with the contraction induced by CCh (10-8M) and ENC (0,1 mg/mL). Data are mean \pm SEM values (n = 4-7). Where error bars are not shown these are covered by the point itself

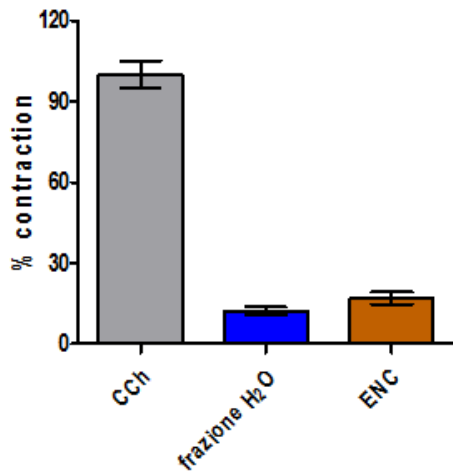


Figure 42: Contraction of gallbladder induced by Water fraction (0,1 mg/mL). compared with the contraction induced by CCh (10⁻⁸M) and ENC (0,1 mg/mL). Data are mean ± SEM values (n = 4–7). Where error bars are not shown these are covered by the point itself

All these fractions have been tested for their ability to contract gallbladder smooth muscle. For each fraction, it has been reported the comparison with carbachol and ENC. None of these fractions showed a higher potency than ENC.

It has been exerted a fractionation through flash chromatography.

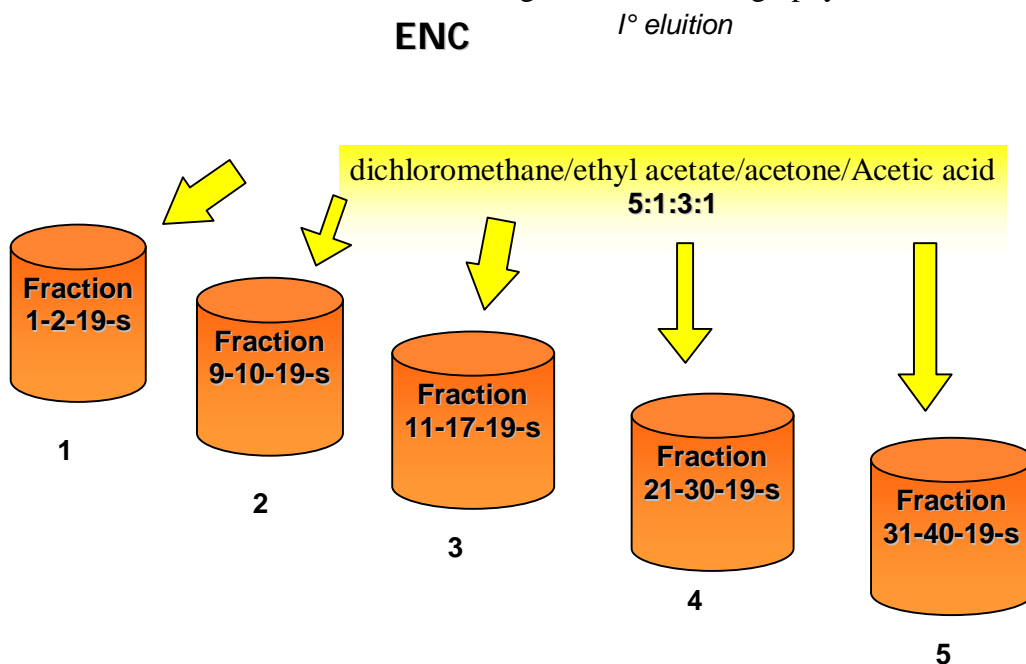


Figure 43: Fractionation of ENC through Flash Chromatography

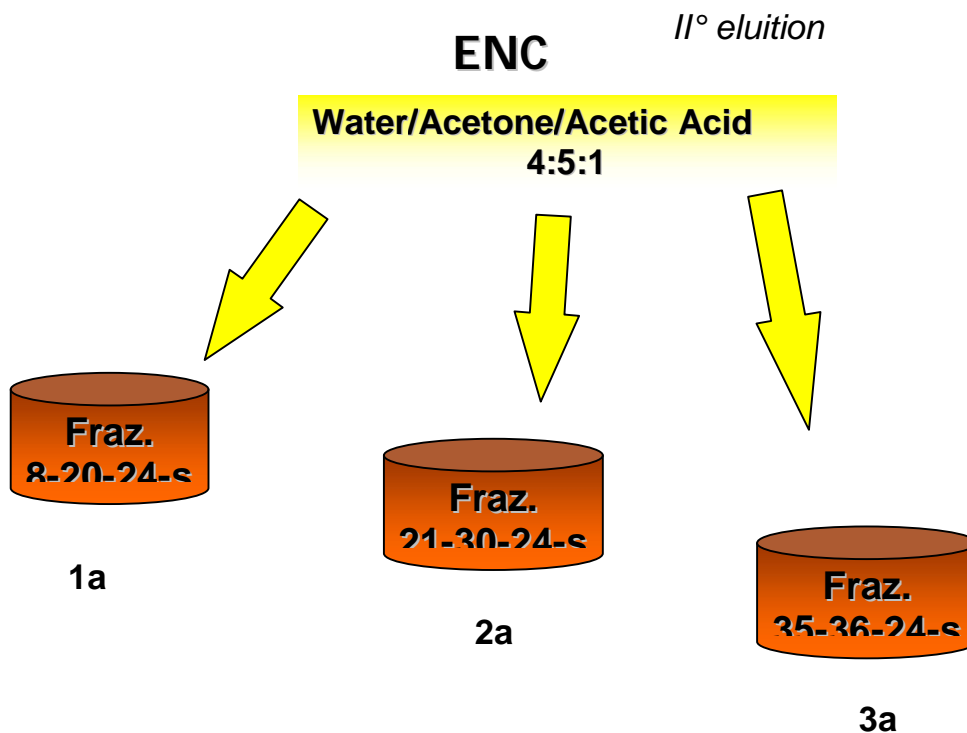


Figure 44: Further fractionation of ENC through Flash Chromatography

A preliminary analysis of some fractions has been carried out, through the mass spectrometry analysis, in order to observe the possible presence of some ellagitannins, comparing it with the already published mass spectra of ellagitannins from chestnut wood.

8.2 Effects of ENC fractions towards gallbladder smooth muscle motility

The single fractions, have been tested for their ability to contract gallbladder.

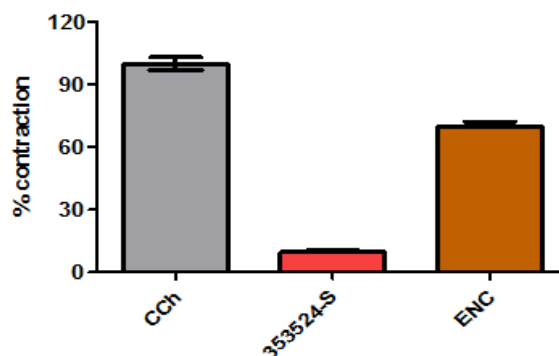


Figure 45: Contraction of gallbladder induced by 35-36-24-s fraction (0,1 mg/mL). compared with the contraction induced by CCh (10-8M) and ENC (0,1 mg/mL). Data are mean \pm SEM values (n = 4-7). Where error bars are not shown these are covered by the point itself

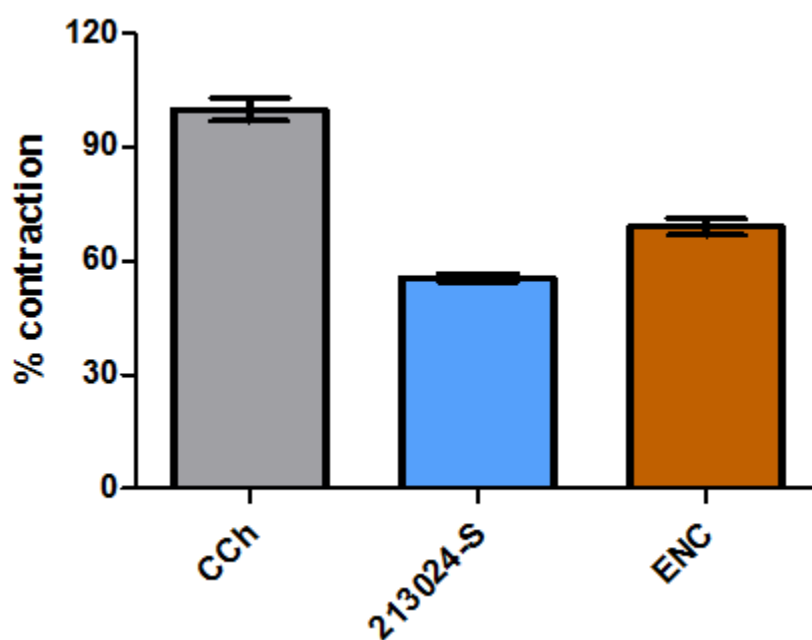


Figure 46: Contraction of gallbladder induced by 21-30-24-s fraction (0,1 mg/mL). compared with the contraction induced by CCh (10-8M) and ENC (0,1 mg/mL). Data are mean \pm SEM values (n = 4-7). Where error bars are not shown these are covered by the point itself.

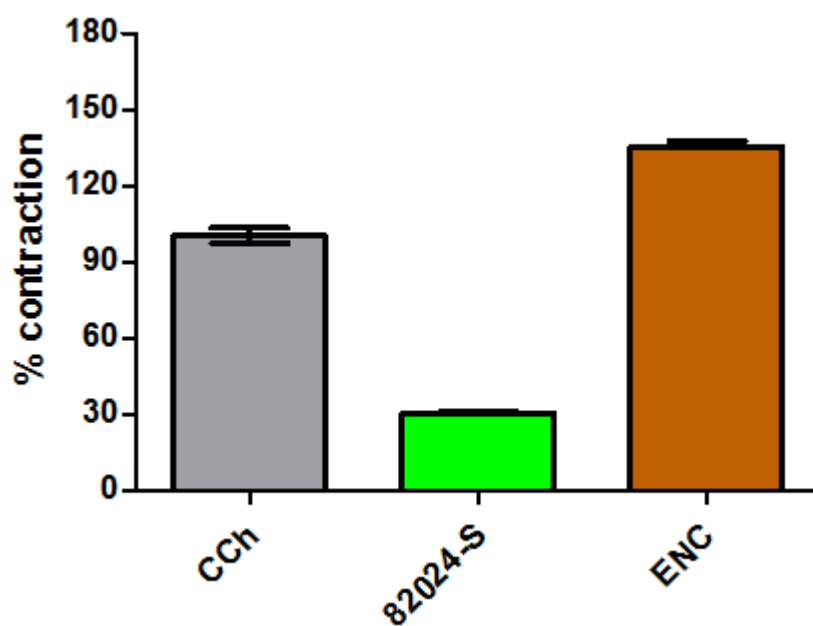


Figure 31: Contraction of gallbladder induced by 8-20-24-s fraction (0,1 mg/mL). compared with the contraction induced by CCh (10-8M) and ENC (0,1 mg/mL). Data are mean \pm SEM values (n = 4-7). Where error bars are not shown these are covered by the point itself

These findings lead us to suppose that the active fraction does not contain ellagitannins.

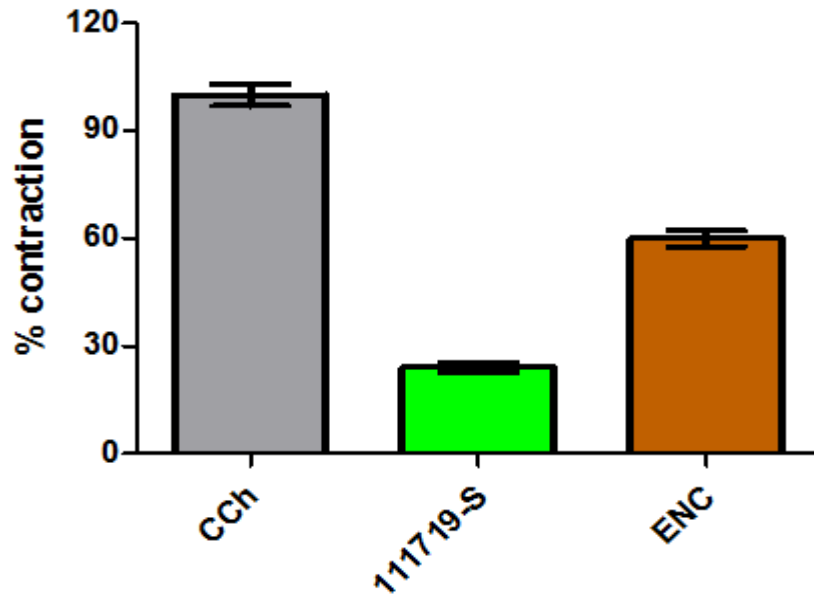


Figure 47: Contraction of gallbladder induced by 11-17-19-s fraction (0,1 mg/mL). compared with the contraction induced by CCh (10-8M) and ENC (0,1 mg/mL). Data are mean \pm SEM values ($n = 4-7$). Where error bars are not shown these are covered by the point itself

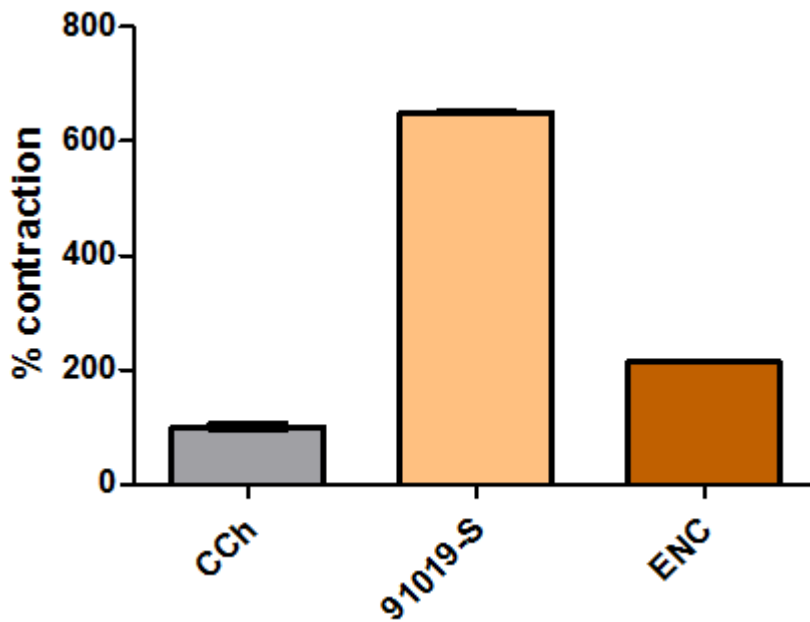


Figure 48: Contraction of gallbladder induced by 9-10-19-s fraction (0,1 mg/mL). compared with the contraction induced by CCh (10-8M) and ENC (0,1 mg/mL). Data are mean \pm SEM values ($n = 4-7$). Where error bars are not shown these are covered by the point itself

8.3 Mass Spectra analysis

Mass spectra analysis have been exerted in order to compare the mass spectra of the fractions with the mass spectra of ellagitannins to observe the possible

presence of structures with masses analogue to those of ellagitannins present in several chesnut wood extracts.

The results indicate the presence of some structures with the same masses of vescalagina, castalagina, vescalin, castalin, ellagic acid.

Further procedures are required in order to further separate the fractions and to exert several chemical analysis in order to identify the chemical structures.

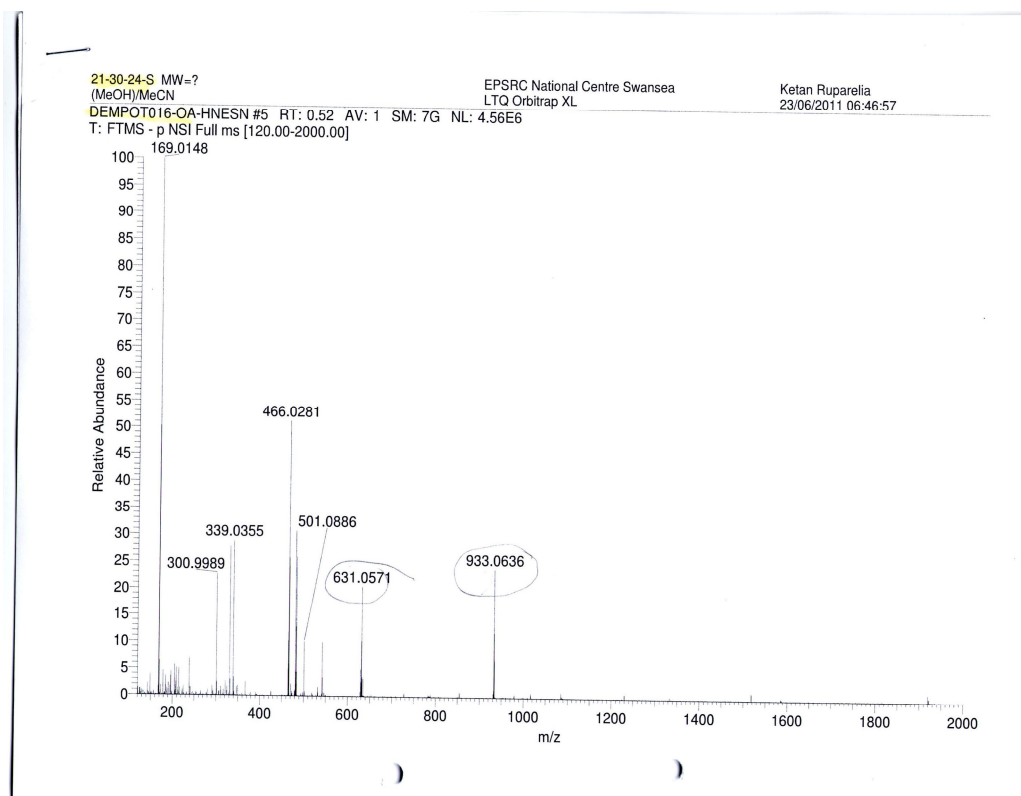


Fig. 49 Mass Spectra 21-30-24s

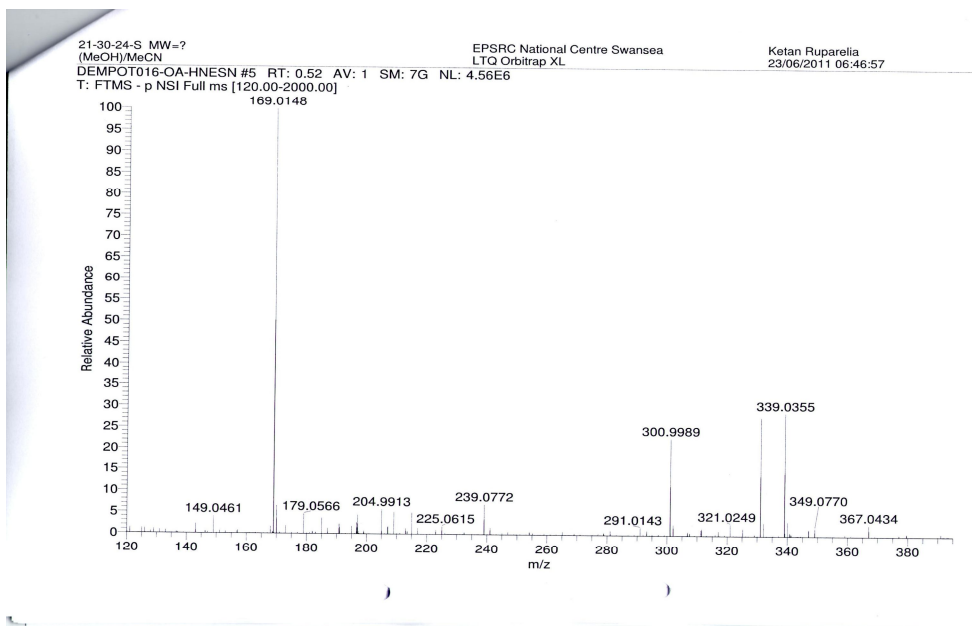


Fig. 50 Mass Spectra 21-30-24s

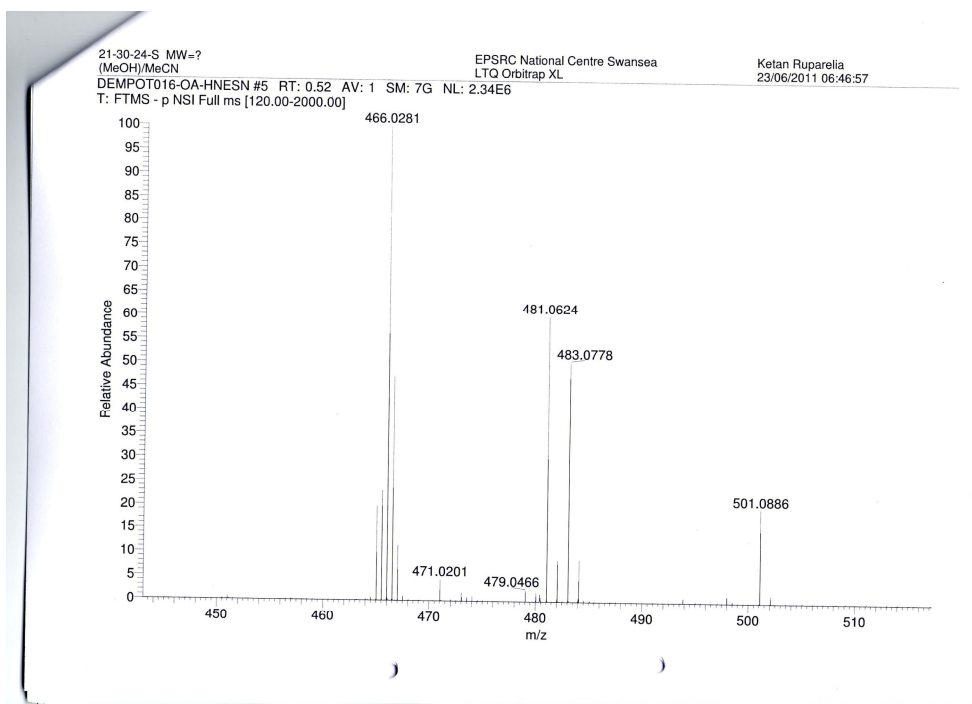


Fig. 51 Mass Spectra 21-30-24s

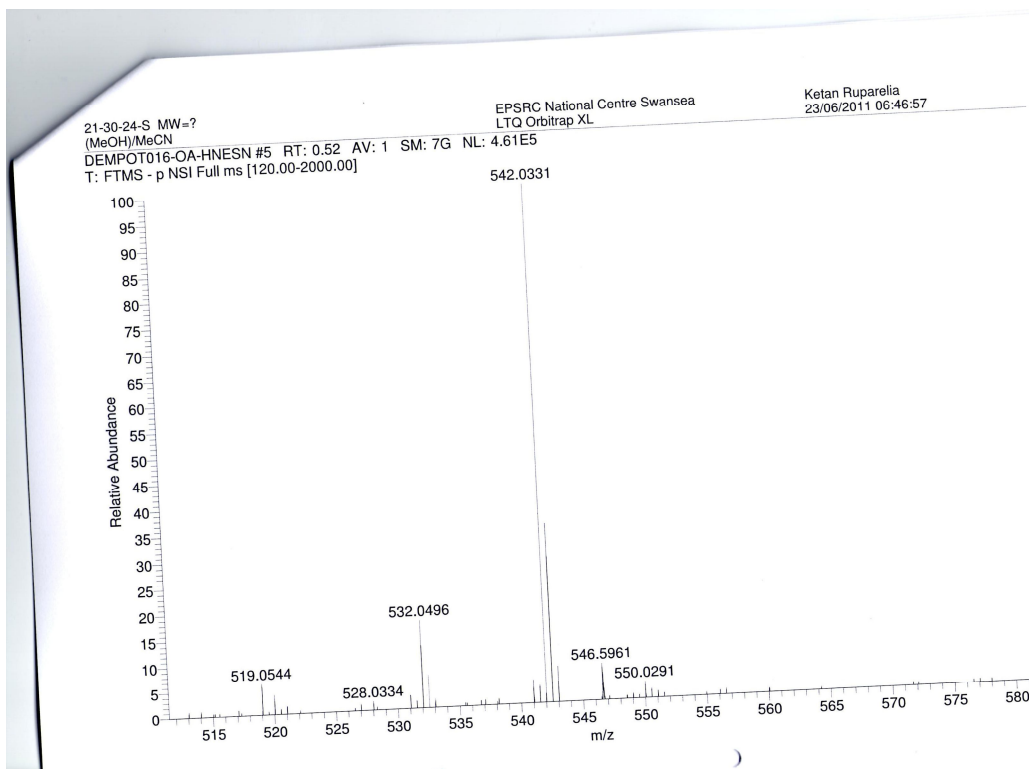


Fig. 52 Mass Spectra 21-30-24s

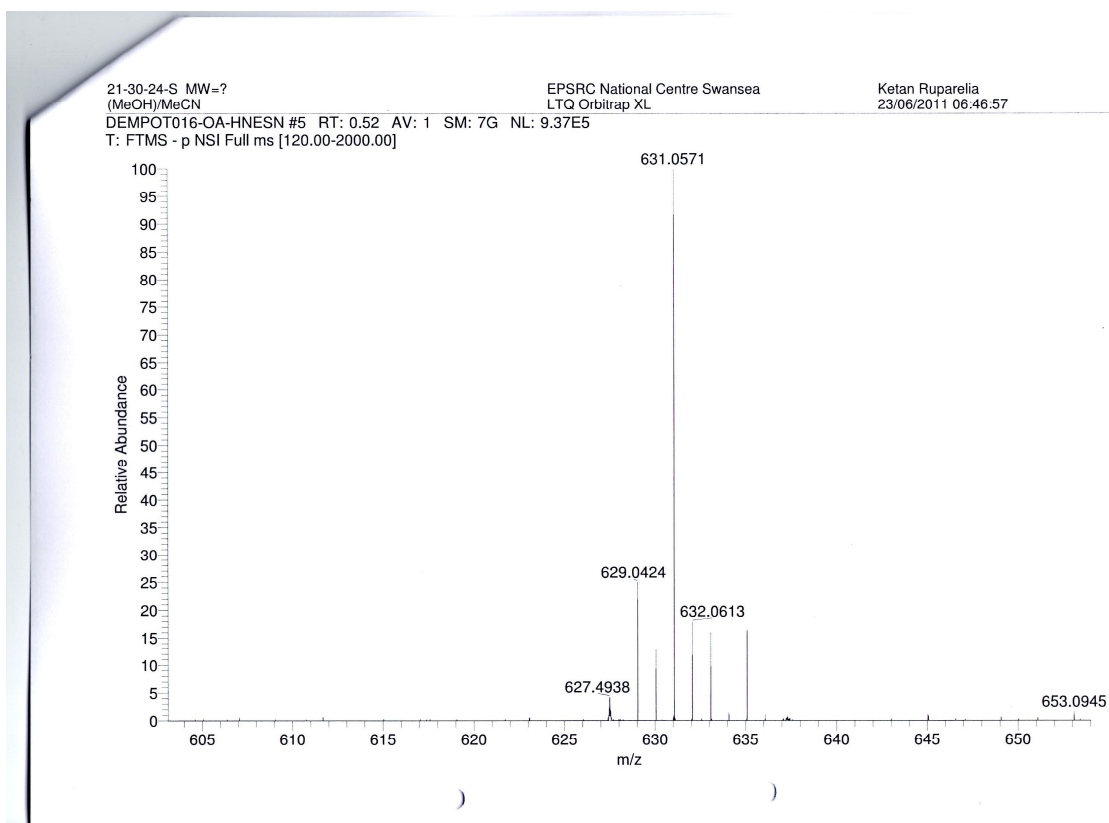


Fig. 53 Mass Spectra 21-30-24s

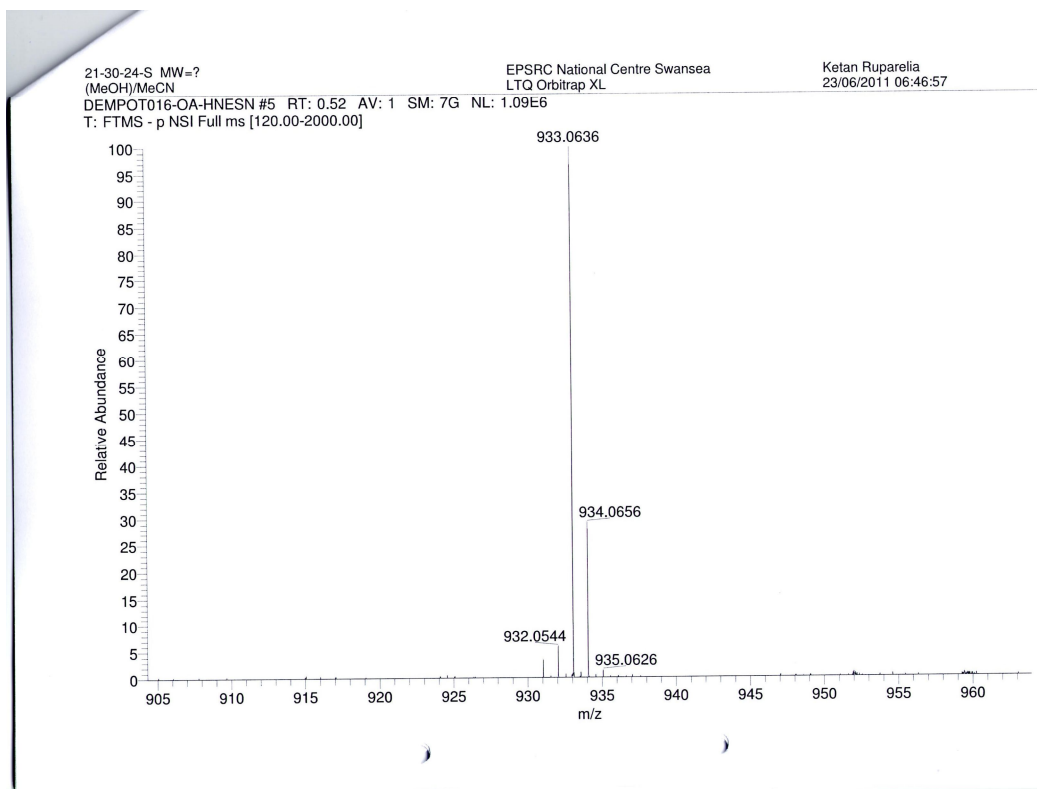


Fig. 54 Mass Spectra 21-30-24s

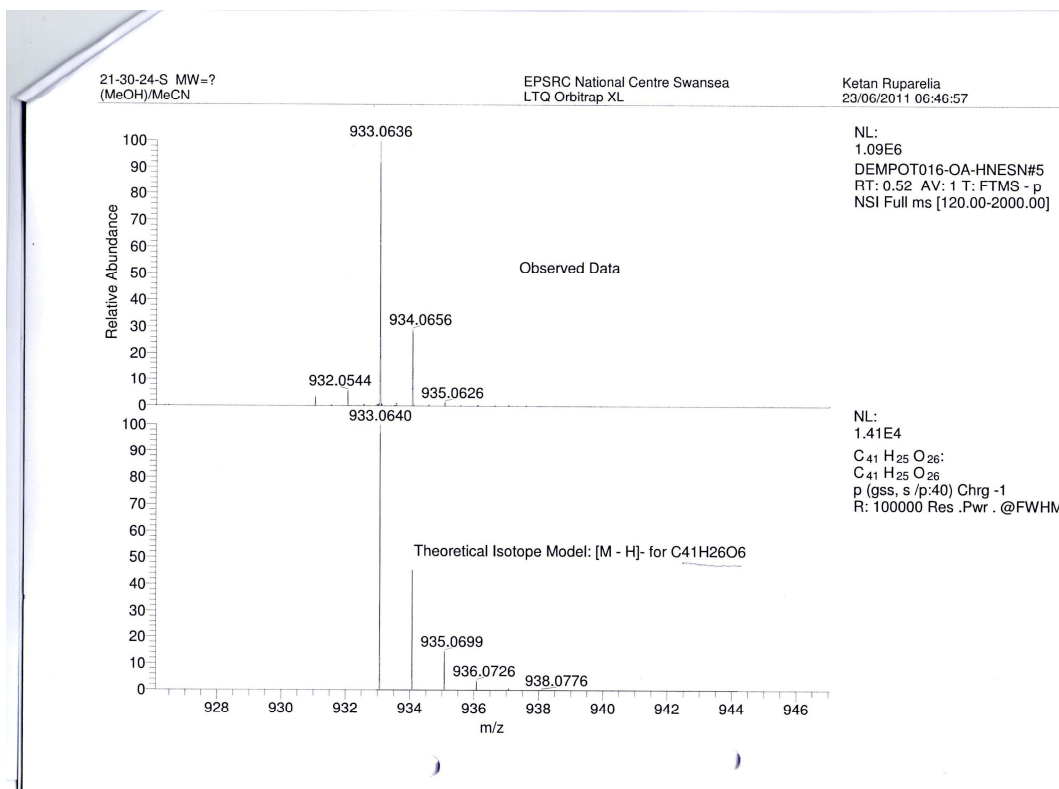


Fig. 55 Mass Spectra 21-30-24s

Conclusions

ENC exerts many different biological activities which may render this extract a potential food supplement able to contribute to prevent some cardiovascular diseases and to contribute to the improvement of the health state in subjects suffering from cardiovascular diseases.

It can be interesting to identify the active molecule(s) able to exert the negative chronotropic and positive inotropic effects.

As regards the gastro-intestinal system, the ENC is able to reduce the peristalsis in different tracts, such as stomach, jejunum, ileum, colon, suggesting its potential role as adjuvant in the treatment of diarrhoea. The cholecystokinetic action occurs in healthy guinea pigs, in guinea pigs fed a lithogenic diet and in human gallbladders taken from patients suffering from cholelithiasis. The latter action is very interesting and together with the Oddi's sphincter relaxing effect may be useful for the prevention of cholelithiasis.

As there are no drugs able to contract gallbladder smooth muscle, the process leading to the identification of the active compound(s) has been started and we concluded and the active fraction has been identified.

As in this fraction the preliminary analysis through mass spectra suggest that it does not contain ellagitannins, we hypothesized that ellagitannins do not represent the main active compounds.

Since vescalagin, castalagin, vescalin, castalin have been found to be present in ENC, we have compared the mass spectra of the ENC fractions with those of these ellagitannins published by M. Sanz and colleagues ([Sanz M. et al., 2010](#))

The fact that in 21-30-24s the peaks of vescalagin, castalagin, vescalin and castalin are present lead to suppose that these molecules may be present in this fraction.

Further fractionations and molecular analysis are needed to confirm this hypothesis.

Material and Methods

ENC (supplied by SilvaTeam, San Michele di Mondovì, Italy) obtained by low pressure heating treatment. The water-soluble fraction is retained and subsequently dehydrated. The fine brown powder (92-95% dry matter) contains 77% of pure tannin on a dry matter basis. The chemical composition of the ENC batch used in the experiments was as follows: water, 2.9%; tannin, 77.8%; non-tannin, 17.7% (oligosaccharides, salts, vegetable resins, and gums coming from the hydrolysis process of chestnut wood); insoluble, 1.6%; crude fibers, 0.24%; ash, 1.7%. The tannin percentage was obtained by gravimetric analysis of vegetable tanning agents by using the filler Freiberg-Hide powder method.

Guinea pigs of either sex (200-400 g) obtained from Charles River (Calco, Como, Italy) were used. The animals were housed according to the ECC Council Directive regarding the protection of animals used for experimental and other scientific purposes. All procedures followed the guidelines of the Animal Care and Use Committee of the University of Bologna (Bologna, Italy). The animals were sacrificed by cervical dislocation, and the organ (ileum and proximal colon) required was set up rapidly under a suitable resting tension in a 15-mL organ bath containing appropriate physiological salt solution consistently warmed (see below) and buffered to pH 7.4 by saturation with 95% O₂/5% CO₂ gas.

Guinea pig ileum

The terminal portion of the ileum (3-4 cm near the ileocecal junction) was cleaned, and segments 2-3 cm long of ileum were set up under 1 g of tension at 37°C in organ baths containing Tyrode's solution of the following composition: 118mM NaCl, 4.75 mM KCl, 2.54 mM CaCl₂, 1.20mM MgSO₄ • 7H₂O, 1.19 mM KH₂PO₄ • 2H₂O, 25 mM NaHCO₂, and 11 mM glucose. When BaCl₂ was used as the agonist, MgSO₄-7H₂O was replaced by MgCl₂-6H₂O. The two segments obtained (2-3 cm) were set up under 1 g of tension in the longitudinal direction along the intestinal wall. Tissues were allowed to equilibrate for at least 30 minutes, during which time the bathing solution was changed every 10

minutes. Concentration-response curves were constructed by cumulative addition of the agonist (CCh, histamine, KC1, and BaCl₂). The concentration of agonist in the organ bath was added only after the response to the previous addition had attained a maximal level and remained steady. Contractions were recorded by means of a displacement transducer (model FT 03, Grass Instruments, Quincy, MA, USA) using Power Lab software (ADInstruments Pty. Ltd., Castle Hill, NSW, Australia). In any cases, parallel experiments in which tissues did not receive any antagonist were run in order to check for any variation in sensitivity. Concentration-response curves to agonist were obtained at 30-minute intervals, with the first one being discarded and the second one being taken as the control. Following incubation with the antagonist (ENC and papaverine), a new concentration-response curve to agonist was obtained.

Guinea pig proximal colon

Starting approximately 1 cm distal from the cecocolonic junction, two segments of about 1 cm of the guinea pig proximal colon were cut. The proximal colon was cleaned by rinsing it with De Jalon solution of the following composition: 155 mM NaCl, 5.6 mM KC1, 0.5 mM CaCl₂, 6.0 mM NaHCO₃, and 2.8 mM glucose. Then the mesenteric tissue was removed. The two segments were suspended in organ baths containing gassed, warm de Jalon solution under a load of 1 g maintained at 37°C. Tension changes in longitudinal muscle length were recorded. Tissues were allowed to equilibrate for at least 30 minutes, during which time the bathing solution was changed every 10 minutes.

Concentration-response curves to agonist (CCh) were recorded isotonicly and obtained at 30-minute intervals, with the first one being discarded and the second one being taken as the control. Following incubation with the antagonists (ENC and papaverine), a new concentration-response curve to agonist was obtained. Some experiments were performed using 5-HT as the agonist. Noncumulative dose-response curves to 5-HT were obtained also in the presence of 1 μ M atropine. Longitudinal muscle contractions or relaxations were recorded isotonicly by the mean of the Grass Instruments FT 03 force displacement transducer using Power Lab software. In all cases, parallel

experiments in which tissues did not receive any antagonist were run in order to check for any variation in sensitivity.

Determination of dissociation constants

In functional experiments, antagonism activity against different agonists of ENC was estimated by determining the concentration of the non competitive antagonist that inhibited 50% (IC₅₀) of the maximum response to the agonist. Three different antagonist concentrations were used, and each concentration was tested at least four times. A pharmacological computer program was used to analyze data. A *P* value of < .05 was considered significant. All the figures were created by using GraphPad (La Jolla, CA, USA) software.

Guinea pig gallbladder. Cholinergic (muscarinic receptor) activity. The gallbladder was removed, opened and washed several times in Krebs solution to remove bile. Two strips of each gallbladder approximately 0.5 cm wide X 1.5 cm long were mounted in organ baths containing Krebs solution (15 ml) of the following composition (mM): NaCl, 118; KCl, 4.7; CaCl₂, 2.5; MgSO₄·7H₂O, 1.2; KH₂PO₄·2H₂O, 1.2; NaHCO₂ 24.9; glucose 11.1; maintained at 37 °C and gassed with 95% O₂ and 5% CO₂. A resting pre-load of 0.5 g was applied to each muscle strip which was then allowed to equilibrate for 1 h. During which time the Krebs solution was changed every 20 min. Concentration-response curves were constructed by cumulative addition of the agonist (carbachol or ENC). [Van Rossum 1963] The concentration of agonist in the organ bath was added only after the response to the previous addition had attained a maximal level and remains steady. Contractions were recorded using isometric transducers (FT. 03, Grass Instruments, Quincy, MA) using Power Lab software (AD Instruments Pty Ltd, Castle Hill, Australia).

For evaluation of antagonistic activity, following incubation with the antagonist (atropine or ENC) for 30 min, a new dose-response curve to agonist was obtained. In all cases, parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity.

Guinea pig gallbladder. L-type calcium channel modulator activity. The gallbladder was removed, opened washed and put into Krebs-Henselait

solution (15 ml) of the following composition (mM): NaCl, 122; KCl, 5.4; CaCl₂, 2.5; MgSO₄·7H₂O, 1.2; NaH₂PO₄·H₂O, 1.2; NaHCO₃ 25; glucose 10; maintained at 37 °C and gassed with 95% O₂ and 5% CO₂ before cut into longitudinal strips 4 mm wide 7 mm long. The isometric tension was recorded by a force transducers (FT. 03, Grass Instruments, Quincy, MA) using Power Lab software (AD Instruments Pty Ltd, Castle Hill, Australia).

Antispasmodic activity: After 1 hr of equilibration at resting tension of 1 g, which was reported to be optimal for measurement of changes in the tension of gallbladder strips for guinea pig [Moumami 1991], the strips were contracted with KCl. A cumulative dose response-curve was constructed and taken as control. Following incubation with the antagonist (nitrendipine or ENC) for 30 min, a new cumulative dose response-curve was obtained.

Spasmolytic activity: The strips were secured at one end to plexiglass hooks and connected via the surgical thread to a force displacement transducer (FT 0.3, Grass Instruments Corporation) for monitoring changes in isometric contraction and were subjected to a resting force of 1 g and washed every 20 min with fresh Krebs-Henselait solution for 1 h. After the equilibration period, guinea-pig aortic strips were contracted by washing in PSS containing 80 mM KCl (equimolar substitution of K⁺ for Na⁺). When the contraction reached a plateau (about 45 min) various concentrations of the compounds (nitrendipine or ENC) were added cumulatively to the bath allowing for any relaxation to obtain an equilibrated level of force. Addition of the drug vehicle had no appreciable effect on K⁺-induced contraction (DMSO for all compounds). All data are presented as mean ± S.E.M.. The IC₅₀ were calculated from log concentration-response curves. [Tallarida RJ, et al., 1987]

Experiment in calcium free solution: The guinea pig gallbladder was used to assess the activity of ENC on calcium free solution. Aortic strips were isolated and cleaned as previously described and placed in organ bath containing the Krebs-Henselait solution maintained at 37°C. Tissue were equilibrated for 1 h under an optimal tension of 1 g. After incubation with ENC for 30 min, addition of Ca²⁺ (2.5 mM) induced an increase in the contraction.

Guinea pig gallbladder. Cholecystokin (CCK1) activity. The gallbladder was removed, opened and washed and put into Krebs-Henselait solution (15 ml) of

the following composition (mM): NaCl, 122; KCl, 5.4; CaCl₂, 2.5; MgSO₄·7H₂O, 1.2; NaH₂PO₄·H₂O, 1.2; NaHCO₂ 25; glucose 10; maintained at 37 °C and gassed with 95% O₂ and 5% CO₂ before cut into longitudinal strips 4 mm wide 7 mm long. The tissue were allowed to equilibrate under a resting tension of 0.5g for 1 h, during which time they were washed repeatedly. Isometric contractions were recorded using a force displacement transducer (FT 0.3, Grass Instruments Corporation) connected to a multichannel data acquisition system (Power Lab® software AD-Instruments Pty Ltd, Castle Hill, Australia). Cumulative concentration-response curves for CCK1 agonist (A71623) were obtained according to Van Rossum [Van Rossum 1963] in the absence and in presence of fixed concentration of antagonist (SR27897 or ENC) for 30 min before the agonist. Each tissue was exposed to one concentration of antagonist only. In all cases, parallel experiments in which tissues did not receive any antagonist were run in order to check any variation in sensitivity.

Oddi sphincter. Cholinergic (muscarinic receptor) activity. The distal bile duct, from 1 cm above its junction with the pancreatic duct, through to its junction with the duodenum, including the sphinter of Oddi and 1 cm of contiguous duodenum. The isolated Oddi's sphincter was immediately placed organ bath (15 ml) in oxygenated (95% O₂ and 5% CO₂) Krebs solution of the following composition (mM): NaCl, 132.5; KCl, 4.69; CaCl₂, 2.12; MgSO₂·7H₂O, 0.6; NaH₂PO₄·H₂O, 1.3; NaHCO₂ 16.39; glucose 7.66; maintained at 37 °C. The tissue was allowed to equilibrate for 60 min with Krebs solution replaced at 15 min intervals. After equilibration the contractile response induced by charbacol was made with an isometric transducer (FT 0.3, Grass Instruments Corporation) connected to a multichannel data acquisition system (Power Lab® software AD-Instruments Pty Ltd, Castle Hill, Australia). After incubation with ENC for 30 min, a new cumulative dose-response curve to charbacol was made.

Guinea-Pig Atrial Preparations and treatments.

Guinea-pigs (males and females, 300–400 g) obtained from Charles River (Calco, Como, Italy) were housed in a controlled environment with a 12:12-h light-dark cycle at 22°C and provided with chow diet and water ad libitum.

Guinea-pigs were sacrificed by cervical dislocation. After thoracotomy the heart was immediately removed and washed by perfusion through the aorta with oxygenated Tyrode solution containing (mM): NaCl 136.9; KCl 5.4; CaCl₂ 2.5; MgCl₂ 1.0; NaH₂PO₄·xH₂O 0.4; NaHCO₃ 11.9; and glucose 5.5. The physiological salt solution (PSS) was buffered at pH 7.4 by saturation with 95% O₂ – 5% CO₂ gas, and the temperature was maintained at 35 °C. The following isolated guinea-pig heart preparations were used: spontaneously beating right atria and left atria driven at 1 Hz were used. For each preparation, the entire left and right atria were dissected from the ventricles, cleaned of excess tissue, hung vertically in a 15 mL organ bath containing PSS continuously bubbled with 95% O₂ – 5% CO₂ at 35 °C, pH 7.4. The contractile activity was recorded isometrically by means of force transducer (FT 0.3, Grass Instruments Corporation, Quincy, MA, USA) using Power Lab® software (AD-Instruments Pty Ltd, Castle Hill, Australia). The left atria were stimulated by rectangular pulses of 0.6–0.8 ms duration and about 50% threshold voltage through two platinum contact electrodes in the lower holding clamp (Grass S88 Stimulator). The right atria were in spontaneous activity. After the tissues were beating for several min, a length-tension curve was determined, and the muscle length was maintained at the value which elicited 90% of maximum contractile force observed at the optimal length. A stabilization period of 45–60 min was allowed before the atria were challenged by various agents. During the equilibration period, the bathing solution was changed every 15 min and the threshold voltage was ascertained for the left atria. Atrial muscle preparations were used to examine the inotropic and chronotropic activity of the Sweet Chestnut extract (0.01-10 mg/mL), dissolved in PSS. During the generation of cumulative concentration-response curves, the next higher concentration of extract was added only after the preparation reached a steady state. Some experiments were performed with a single extract

concentration (1 mg/mL). All data are reported as means \pm SEM. The EC₅₀ values were calculated from concentration-response curves. [Tallarida 1987]

2.6.1 Muscarinic activity. Was determined on guinea-pig spontaneously beating right atria. Tissues were suspended in PSS (see above) at 35 °C, pH 7.4 and bubbled with 95% O₂ – 5% CO₂. Chronotropic activity was recorded isometrically. Tissues were stabilized for about 60 min, with changes in bathing solution every 15 min. Cumulative log concentration–response curves to the agonist Carbachol (CCh) (0.01–1 μ M) was constructed. Following incubation with the antagonist atropine (1 μ M) or by simultaneous administration of atropine (1 μ M) with extract (1 mg/ml) a new concentration–response curve to CCh was obtained. Parallel experiments in the absence of antagonist were run. Following incubation with the antagonist atropine (1 μ M) in the absence or presence of Sweet Chestnut extract (1mg/ml), a new concentration–response curve to CCh was obtained. Parallel experiments in the absence of antagonist were run. One set of experiments was carried out using a single concentration of extract (1 mg/ml): in particular negative chronotropic activity was induced by a single dose of extract. Following incubation with atropine (1 μ M) for 30 min, a new effect with Sweet Chestnut extract (1 mg/ml) was done.

Adrenergic activity Was determined on guinea pig left atria driven at 1 Hz. Tissues were suspended in PSS (see above) at 35 °C, pH 7.4 bubbled with 95% O₂ – 5% CO₂. Following a equilibration period (45 min) of during which the PSS was changed every 15 min, a contraction to Sweet Chestnut extract (1 mg/ml) was performed. Following incubation with propranolol (10⁻⁶ M) for 30 min, a new contraction to extract (1 mg/ml) was obtained. Simultaneously reproducibility of the contraction obtained by first to the second trials in the absence of propranolol (10⁻⁶ M) was confirmed.

Guinea-Pig Left Papillary Muscle preparation.

The left ventricular papillary muscles were rapidly isolated from the heart and suspended in an organ bath (15 mL) containing modified Ringer solution of the following composition (mM): NaCl 135; KCl 5; CaCl₂ 2; MgCl₂ 1; NaHCO₃ 15; and glucose 5.5; bubbled with 95% O₂-5% CO₂, pH 7.4 at 35 °C in an organ bath. The papillary muscles were driven through a pair of platinum

electrodes (field stimulation) by square pulses (1Hz, 5-7ms, 50% threshold voltage). The developed tension was recorded isometrically. The preparation was equilibrated for at least 60 min before the start of experiments. The papillary muscle preparations were used to examine the inotropic activity of the extract (0.01-10 mg/mL), dissolved in PSS. During the generation of cumulative concentration-response curves, the next higher concentration of extract was added only after the preparation reached a steady state. Some experiments were performed with a single extract concentration (1 mg/mL). All data are reported as means \pm SEM. The EC₅₀ was calculated from log concentration-response curves. [Tallarida 1987]

2.8 Guinea-Pig Aortic Strips preparation.

The thoracic aorta was removed and placed in Tyrode solution containing (mM): NaCl, 118; KCl 4.75; CaCl₂ 2.54; MgSO₄ 1.20; KH₂PO₄ 1.19; NaHCO₃ 25; and glucose 11; equilibrated with 95% O₂-5% CO₂ at pH 7.4. The vessel was cleaned of extraneous connective tissue. Two helicoidal strips (10 mm x 1 mm) were cut from aorta beginning from the end proximal to the heart. Vascular strips were then tied with surgical thread (6-0) and suspended in a jacketed tissue bath (15 mL) containing aerated PSS at 35 °C in a jacketed tissue bath. Aortic strips were secured at one end to plexiglass hooks and connected via the surgical thread to a force displacement transducer (FT 0.3, Grass Instruments Corporation) for monitoring changes in isometric contraction. Aortic strips were subjected to a resting force of 1 g and washed every 20 min with fresh PSS for 60 min. After the equilibration period, guinea-pig aortic strips were contracted by washing in PSS containing 80 mM KCl (equimolar substitution of K⁺ for Na⁺) or 1 μ M Noradrenaline (NA). When the contraction reached a plateau (about 45 min or 15 min respectively) different concentrations of the extract (0.01- 10 mg/mL) were added cumulatively to the bath allowing for any relaxation to obtain an equilibrated level of force. Some experiments were performed with a single extract concentration (1 mg/mL). All data are reported as means \pm S.E.M.. The IC₅₀ were calculated from log concentration-response curves. [Tallarida]

Antioxidant and Cytoprotective Activities.

Cell Culture and Treatments. Neonatal cardiac myocytes were isolated as previously reported. [Hrelia 2002] The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institutes of Health (NIH Publication 85-23, revised 1996) and approved by the Ethics Committee of our institution. Briefly, cells, obtained from the ventricles of 2-4-day-old rats, were grown until complete confluence. Cells were treated with different concentration of extract (1-500 $\mu\text{g/ml}$) for 24 h, and control cells were treated with equivalent concentrations of DMSO alone.

2.4.2 Determination of cell viability. Cardiomyocyte viability of control and treated cells was measured using the MTT assay as previously reported. [Angeloni 2008] For the flow cytometry analysis the cells were double labelled with Annexin V conjugated to Phycoerythrin (Annexin V-PE) and 7-Amino-actinomycin D (7 AAD), and immediately analyzed on a Guava EasyCyte flow cytometer (Guava Technologies, Hayward, CA) in accordance with the manufacturer's instructions as reported in [Angeloni C, et al., 2011]. The percentage of viable cells was reported with respect to the total number of cells.

Detection of Intracellular Reactive Oxygen Species. The formation of ROS was evaluated using a fluorescent probe, DCFH-DA, as previously reported [Angeloni C, et al., 2007] Briefly, controls and treated cells were washed with PBS and then incubated with 5 μM DCFH-DA in PBS for 30 min. After DCFH-DA removal, the cells were incubated with 100 μM H_2O_2 for 30 min. Cell fluorescence from each well was measured using a microplate spectrofluorometer (λ excitation = 485 nm and λ emission = 535 nm). Intracellular antioxidant activity was expressed as the percentage of inhibition of intracellular ROS produced by H_2O_2 exposure.

Determination of cytoprotective effect. Cytoprotection against H_2O_2 iduced cell damage was assessed using the MTT assay as previously reported. [Angeloni C, et al., 2008]. Control and treated cells were exposed to 100 μM H_2O_2 in PBS for 30 min after which cells were changed to a fresh culture medium. After 24 h, MTT was added to the medium at the final concentration of 0.5 mg/mL and incubated for 1 h at 37 °C. DMSO was added to dissolve the formazan crystals and the absorbance was measured at 595 nm using a

microplate reader VICTOR3 V™ Multilabel Counter. Data were expressed as percentage of viable cells with respect to controls times.

Statistical analysis

Data obtained from rat neonatal cardiomyocytes cell culture are presented as means \pm S.D. and have been analyzed by one-way analysis of variance (ANOVA) followed by Dunnett's test, and P value less than 0.05 has been considered significant.

Data on atria, papillary muscle and aortic strips were analyzed by the Student's t-test and presented as means \pm S.E.M. [Tallarida]. and P value less than 0.05 has been considered significant. The potency of drugs defined as EC50 was calculated from log concentration-response curves (Probit analysis using Litchfield and Wilcoxon [Tallarida] or from concentration-response curves or GraphPad Prism® [Motulsky H, et al., 2003a] [Motulsky H.J., Prism 5 Statistics Guide, GraphPad Software Inc., San Diego CA, 2007, www.graphpad.com]. Antagonist activity was estimated by determining the concentration of the non competitive antagonist that inhibited 50% of the maximum response to the agonist. Three different antagonist concentrations were used and each concentration was tested at least four.

Spectrophotometric determination of total phenol content

The total phenol (TP) content of tannin extract was determined by adapting the method used by Pirisini et al. [Pirisini et al., 2000] After the extraction with methanol and a suitable dilution of the sample, TP content was determined by using the Folin-Ciocalteu reagent and measuring the absorbance at 750 nm (Shimadzu Spectrophotometer UV-VIS 1204, Kyoto, Japan). TP content was calculated using gallic acid for the construction of the calibration curve ($r^2 = 0.9967$), expressing the results as g of gallic acid/100 g of dry extract, designated as gallic acid equivalent (GAE)/100 g.

HPLC-DAD-MS analysis

The dry extract was dissolved in methanol and analysed in HPLC-DAD-MS, adapting the method described by Comandini et al. 2011. [Comandini et al., 2011] Tannins and other phenolic compounds were quantified as g of ellagic acid/100 g of dry extract and indicated as ellagic acid equivalent (EAE)/100 g.

ENC fractionation

4 grams of ENC were dissolved in methanol, coated on silica gel, and applied on top of a 40 × 4.1 cm silica gel column.

Column chromatography was performed on silica gel 60A (particle size 35-70 μ) and the chromatography was eluted with the following eluent dichloromethane/ethyl acetate/acetone/Acetic acid (5:1:3:1).

We collected 20 ml fractions according to thin layer chromatography (TLC) profiles.

5 different fractions have been identified and named:

1. 1-2-19-s
2. 9-10-19-s
3. 11-17-19s
4. 21-30-19s
5. 31-40-19s

These fractions were evaporated under vacuum and stored in the freezer. TLC separations were performed on precoated silica gel 60 F254 plates. Visualisation of the separated bands was carried out under UV light (365 nm).

The column was further eluted with the following eluent water/ acetone/Acetic acid (4:5:1), collecting 20 ml fractions according to thin layer chromatography (TLC) profiles. 4 fractions have been obtained and named:

1. 1-7-24s
2. 11-20-24s
3. 21-30-24s
4. 35-36-24s

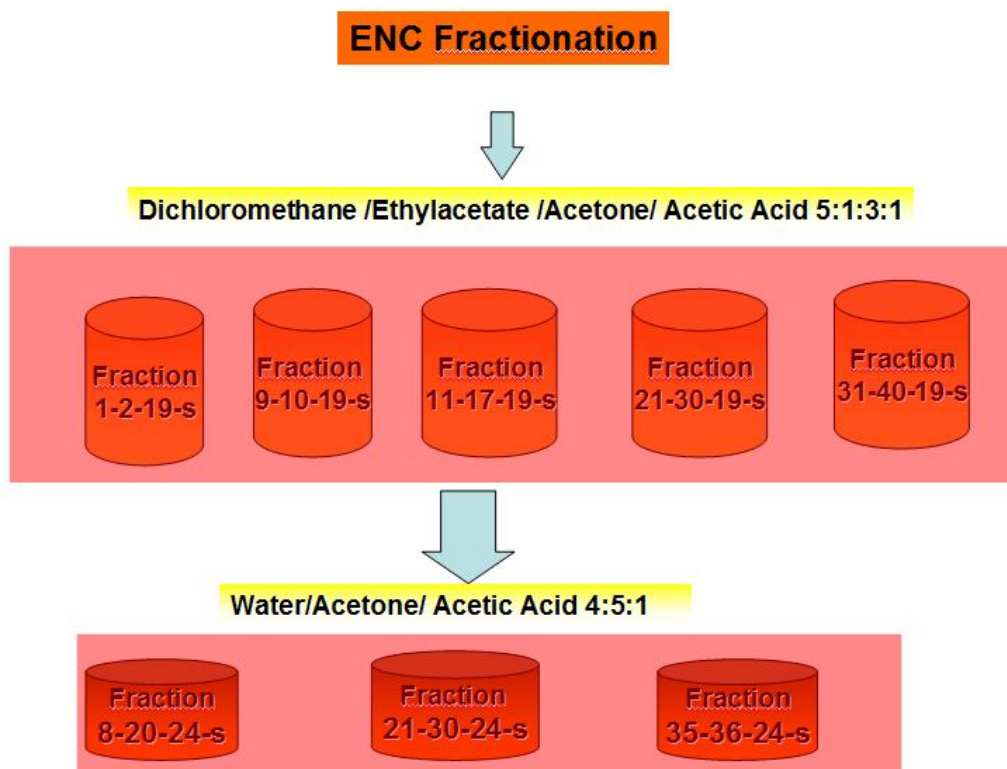


Figure 56: Fractionation of ENC through Flash Chromatography

A method able to isolate ellagitannins has been applied as described by Ignacio García-Estévez and colleagues. (Ignacio García-Estévez, M. et al., 2010)

The powder of ENC has been first applied onto a Waters C-18 Sep-Pak® (500 mg) cartridge (Millipore Corp., Milford, MA, USA), previously activated with methanol and equilibrated with 2.5% acetic acid in water. The first fraction (fraction a) was collected from the moment of the application of the sample onto the C-18 cartridge to the end of the loading step and during the elution with 5mL of 2.5% acetic acid in water. The second fraction (fraction b) was eluted with 5mL of ethyl acetate and the third (fraction c), with 5mL of methanol. Fractions a and b were evaporated under reduced pressure and re-dissolved in 2.5% acetic acid to a final volume of 2 mL.

Fraction c was also evaporated in order to remove methanol and was re-dissolved in 2.5% acetic acid to a final volume of 5 mL.

Fraction a was subsequently submitted to another fractionation in a hand-packed Sephadex LH-20 minicolumn (10mm×30mm) previously activated with methanol and equilibrated with ultrapure water. In this second fractionation, three different eluents were employed obtaining four eluates as

follows: the first eluate (fraction 1) was obtained with 2mL of ultrapure water, the second (fraction 2), with 2mL of 100% ethanol (96% vol.), the third (fraction 3) with 1mL of 100% methanol and the last (fraction 4), with 5mL of 100% methanol. All these eluates were evaporated under reduced pressure and re-dissolved in 2.5% acetic acid to a final volume of 2 mL.

The fraction 4 contains ellagitannins and this fraction is used in order to have a standard to compare for the TLC analysis.

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