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The effect of tannins from *Castanea sativa* Mill. on inflammation induced by *Helicobacter pylori* in gastric epithelial cells

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INTRODUCTION 6

THE CULTURE BEHIND SWEET CHESTNUT AGRICULTURE 6
PHARMACOGNOSY OF SWEET CHESTNUT 7
TANNINS FOR EPITHELIAL DEFENSE 10
GASTRITIS 12
TANNINS FOR *HELICOBACTER PYLORI*-RELATED GASTRITIS 18

AIMS OF THE STUDY 20

MATERIALS AND METHODS 22

MATERIALS 22
PLANT MATERIAL AND EXTRACTION 23
CELL VIABILITY TEST 24
CELL CULTURE AND IL-8 MEASUREMENT 24
NF-κB DRIVEN TRANSCRIPTION AND IL-8 PROMOTER ACTIVITY 25
HELICOBACTER PYLORI CULTURE AND INFECTION OF CELLS 26
BACTERIAL ADHESION TO CELLS 26
MINIMUM INHIBITORY CONCENTRATION (MIC) ASSESSMENT 27
GENE EXPRESSION 28
TOTAL PHENOL CONTENT 29
VANILLIN INDEX 29
UPLC MS/MS METHOD FOR MULTIPLE CLASSES OF PHENOLICS 30
IN VITRO SIMULATED GASTRIC DIGESTION 31
EVALUATION OF THERMAL STABILITY 31
STATISTICAL ANALYSIS 32

RESULTS AND DISCUSSION 33

PART 1: STUDY ON CHESTNUT FROM *CASTANEA SATIVA* MILL. 33
EFFECT OF CHESTNUT EXTRACTS ON IL-8 RELEASE INDUCED BY TNF 33

BIOLOGICAL ACTIVITY AND CHARACTERIZATION OF CHESTNUT PARTS 35

EFFECT OF CHESTNUT EXTRACTS ON IL-8 RELEASE INDUCED BY *H. PYLORI* INFECTION 39

A MECHANISM FOR THE INHIBITION OF *H. PYLORI*- OR TNF-INDUCED IL-8: THE EFFECT OF CHESTNUT EXTRACTS ON THE NF-κB PATHWAY 40

EFFECT OF EXTRINSIC VARIABLES ON THE BIOLOGICAL ACTIVITY OF CHESTNUT EXTRACTS 42

APPLICATION OF THE EVIDENCES ON CHESTNUT BIOACTIVITY TO CHESTNUT FLOUR PRODUCTION: THE BIOLOGICAL EFFECT OF CHESTNUT BY-PRODUCTS 43

PART 2: STUDY ON LEAVES FROM *CASTANEA SATIVA* MILL. 46

EFFECT OF LEAF EXTRACTS ON IL-8 RELEASE INDUCED BY TNF 46

CHARACTERIZATION OF LEAF EXTRACT 47

IMPLEMENTATION OF THE MODEL TO STUDY THE IMPACT OF NATURAL COMPOUNDS ON THE INTERACTION AMONG *H. PYLORI* AND GASTRIC EPITHELIUM 49

EFFECT OF LEAF EXTRACTS ON IL-8 RELEASE INDUCED BY TNF OR *H. PYLORI* 53

A MECHANISM FOR THE INHIBITION OF *H. PYLORI*- OR TNF-INDUCED IL-8: THE EFFECT OF LEAVES EXTRACTS ON THE NF-κB PATHWAY 55

EFFECT OF SIMULATED DIGESTION ON THE BIOLOGICAL ACTIVITY OF LEAF EXTRACTS 56

BIOLOGICAL ACTIVITY OF ELLAGITANNINS FROM LEAF EXTRACTS 57

ANTIBACTERIAL AND ANTI-ADHESIVE EFFECT OF LEAF EXTRACTS 61

CONCLUSIONS 66

BIBLIOGRAFIA 69

ABSTRACT (ENGLISH)

The eradication therapy against *Helicobacter pylori* (*H. pylori*) accounts for the reduction of IL-8 levels in gastric mucosa and the related risk of gastric cancer and ulcer onset. However, the current therapeutic approach has being challenged by antibiotic resistance. The discovery of natural compounds from edible plants with anti-inflammatory and anti-bacterial activities may contribute to the control of the disease and improve the safety profile of eradication. Plants producing tannins, including ellagitannins, have already shown pre-clinical gastroprotective evidences, but pharmacological validations are still required. Accordingly, *Castanea sativa* Mill. (sweet chestnut) is a well-known source of tannins, traditionally used for gastro-intestinal and respiratory tract diseases. On the base of the traditional indication, extracts from sweet chestnut are currently allowed as botanical ingredients by several European countries; however, their phytochemical composition and biological activity are poorly investigated, thus limiting the substantiation of health claims.

The aim of the phD project was to characterize hydroalcoholic extracts from fruit and leaves of sweet chestnut, with a particular attention to tannin composition. In parallel, the extracts were evaluated as potential candidates against gastritis and *H. pylori* infection through a multidisciplinary approach, including in vitro pharmacological and microbiological experiments. In the view of nutraceutical application, the work took into account the importance of the botanical origin of the extracts and evaluated the impact of gastric digestion on the stability of natural compounds.

The results obtained on the fruit (chestnut) have been published by the research group (Sangiovanni and Piazza et al. 2018): in brief, following a bio-guided approach, hydroalcoholic extracts from chestnut of five different varieties were screened for their ability to impair TNFα-induced IL-8 release in human gastric epithelial cells (AGS). Two varieties (Venegon and Verdesa) emerged for their promising inhibitory activity (IC_{50}s < 10 μg/mL) and were further characterized for their polyphenolic content. HPLC-MS analysis revealed the presence of condensed tannins (oligomeric proanthocyanidins) in extracts from the outer part of chestnut (pericarp and episperm), which

correlated with IL-8 impairment (IC_{50}s < 0.5 μg/mL); on the contrary, the edible part (endosperm) was devoid of proanthocyanidins and ineffective. Of note, typical hydrolysable tannins from the bark of sweet chestnut, namely gallotannins and ellagitannins, were absent in chestnut.

In line with the literature regarding tannins, extracts maintained their inhibitory activity after the simulation of gastric digestion. Moreover, the bioactivity was modestly affected by the application of brief thermal treatments (till 100 °C for 30 min), thus suggesting the possible reuse of chestnut shell for the enrichment of a bioactive chestnut flour. Consequently, an episperm-fortified flour was prepared respecting the original composition of chestnut, then extracted and assayed again for IL-8 impairment (IC_{50} = 16.35 μg/mL), thus demonstrating the potential application of chestnut by-products in nutraceuticals.

Following a similar approach, hydroalcoholic extracts from leaf belonging to the most promising varieties (Venegon and Verdesa) were investigated. Contrarily to the fruit, the ellagitannins castalagin and vescalagin were revealed in leaf (1-2%), thus prompting further studies on the anti-*H. pylori* effect.

In particular, the antibacterial and anti-inflammatory activity was evaluated in a co-culture model of gastric epithelial cells infected with the bacteria (tumoral AGS in comparison with non-tumoral GES-1). Leaf extracts inhibited IL-8 release in both the infected cells with IC_{50}s lower than 30 μg/mL and impaired the bacterial growth at 100 μg/mL. After simulated gastric digestion, these bioactivities were moderately impaired (2 folds); in parallel, a slight decrease in total phenols content was observed, thus suggesting their possible stability at gastric level after oral consumption. Ellagitannins castalagin and vescalagin exhibited the same inhibitory effects (IC_{50} on IL-8 < 16 μM; MIC = 25 μM), but the inhibitory concentrations were unable to completely explain the effect of the whole extract; therefore, the synergy with other polyphenols could play a role.

The experimental work sustained for the first time the potential anti-gastritis application of by-products from sweet chestnut, such as chestnut shell and leaf, elucidating the role of tannins for the biological activity.

ABSTRACT (ITALIAN)

La terapia di eradicazione contro Helicobacter pylori (H. pylori) comporta la riduzione dei livelli di IL-8 nella mucosa gastrica e il controllo del rischio di cancro e ulcera. L'approccio terapeutico corrente è minacciato dall'antibiotico resistenza. Pertanto, la scoperta di composti vegetali di origine dietetica con effetto anti-infiammatorio e anti-batterico potrebbe contribuire al controllo della gastrite e migliorare il profilo di sicurezza della terapia. Le piante contenenti tannini, inclusi gli ellagitannini, hanno già dimostrato effetti gastro-protettivi in studi preclinici, ma richiedono ulteriore validazione farmacologica. Castanea sativa Mill. (castagno) rappresenta una fonte di tannini utilizzata tradizionalmente contro disturbi gastro-intestinali e respiratori.

Gli estratti di C. sativa Mill. sono ammessi come ingredienti nutraceutici in diversi paesi europei sulla base del loro impiego tradizionale; d'altra parte, la loro composizione fitochimica e la loro attività biologica sono scarsamente studiate. Ciò comporta un limite per la documentazione di indicazioni salutistiche (health claims).

L'obiettivo del seguente progetto di dottorato è stato di caratterizzare estratti idroalcolici ottenuti da frutti e foglie di C. sativa Mill., con un'attenzione particolare alla valutazione della composizione in tannini. In parallelo, gli estratti sono stati studiati come potenziali candidati contro la gastrite ed H. pylori, attraverso un approccio multidisciplinare che ha compreso esperimenti in vitro di tipo farmacologico e microbiologico. Nell'ottica di una possibile applicazione nutraceutica, il lavoro ha considerato l'importanza dell'origine botanica degli estratti e ha valutato l'impatto della digestione gastrica sulla stabilità dei composti bioattivi.

I risultati conseguiti sugli estratti del frutto (la castagna) sono stati pubblicati dal gruppo di ricerca (Sangiovanni and Piazza et al. 2018): in breve, seguendo un approccio bio-guidato, gli estratti idroalcolici di frutti da 5 varietà di castagno sono stati utilizzati nel modello di rilascio di IL-8 indotta da TNFα in cellule epiteliali gastriche (AGS). Due delle varietà (Venegon e Verdesa) sono emerse per la loro attività inibitoria promettente (IC_{50} < 10 μg/mL) e di conseguenza sono state caratterizzate per il loro contenuto polifenolico. L'analisi HPLC-MS ha rilevato la presenza di

tannini condensati (proantocianidine oligomeriche) negli estratti ottenuti dalle componenti esterne della castagna (pericarpo ed episperma), i quali hanno correlato con l'inibizione di IL-8 (IC_{50}s < 0.5 µg/mL); al contrario, la parte edibile del frutto (l'endosperma) è risultato privo di proantocianidine e attività biologica. I tannini idrolizzabili tipici della corteccia di castagno, ovvero gallotannini ed ellagitannini, sono risultati assenti nel frutto.

In accordo con la letteratura riguardante i tannini, gli estratti hanno mantenuto la loro attività inibitoria in seguito alla simulazione in vitro della digestione gastrica. Inoltre, l'attività biologica è stata modestamente intaccata da brevi trattamenti termici (fino a 100°C per 30 min), suggerendo la possibilità di recuperare le parti esterne per l'arricchimento di una farina di castagne bioattiva. Di conseguenza, una farina arricchita di episperma è stata preparata rispettando la composizione originale della castagna, quindi è stata estratta e valutata per l'inibizione di IL-8 (IC_{50} = 16.35 µg/mL), rivelando una potenziale applicazione nutraceutica.

Seguendo un approccio simile sono stati studiati gli estratti idroalcolici delle foglie appartenenti alle varietà più promettenti (Venegon e Verdesa). Diversamente dal frutto, nella foglia sono stati riconosciuti gli ellagitannini castalagina e vescalagina (1-2%), i quali hanno stimolato l'approfondimento di un potenziale effetto contro H. pylori. In particolare, sono stati valutati l'effetto anti-batterico e anti-infiammatorio in un modello di co-coltura di cellule epiteliali gastriche infettate con il batterio (cellule tumorali AGS a confronto con cellule non-tumorali GES-1). Le foglie hanno inibito il rilascio di IL-8 con IC50 inferiori a 30 µg/mL in entrambi i modelli cellulari e hanno inibito la crescita batterica a 100 µg/mL. La simulazione della digestione gastrica ha moderatamente inficiato gli effetti biologici (2 volte) e il contenuto in fenoli totali, suggerendo la loro probabile stabilità a livello gastrico in seguito ad assunzione orale. Per gli ellagitannini castalagina e vescalagina è stato dimostrato lo stesso effetto inibitorio (IC_{50} di IL-8 < 16 µM; MIC = 25 µM), ma a concentrazioni che non spiegano completamente l'effetto dell'estratto originale; di conseguenza, la sinergia con altri polifenoli potrebbe giocare un ruolo.

Il lavoro sperimentale ha posto le prime basi per il recupero a scopo nutraceutico di scarti agricoli della coltura del castagno, quali le parti esterne del frutto e le foglie, sottolineando il ruolo dei tannini per la loro attività biologica.

Introduction

The culture behind sweet chestnut agriculture

The interaction among European population and sweet chestnut (*Castanea sativa* Mill.) started thousands of years ago. According to Krebs et al. (2004) the most likely natural range of chestnut tree was delimited by six macro-regions in Europe and Middle East, which probably allowed chestnut populations to survive during the main glacial events: the tree was naturally present around the southern coast of the Black Sea, in several areas of Italy (among Tyrrhenian cost and Apennines ridge, Cuneo-region and Colli Euganei), in Balkan peninsula and northern Syria. From palynological studies, the first trace of spreading due to human activity appeared back to around 2100 B. C. in the Anatolian peninsula, northeastern Greece and southeastern Bulgaria (Zeist and Bottema 1991), but the most precise and direct indication of cultivation is the ancient Greek and Latin literature. Together with grapevine and olive, Greek colonies of *Magna Grecia* mediated the contact of sweet chestnut with the Latin world (Dion 1977; Forni 1990). From the literary works of Theophrastus (H.P. III.10.1), Athenaeus (Ath. Deipn. ii 54 b) and Pliny (N.H. XV.92) emerged that the fruit was considered hard to digest and sensorially poor by Greeks and Latins as well. Notably, dietary and medicinal uses coexisted, since Nicander (Nic. Alex. 268–271) and Dioscorides (Dios. Med. I.145) reported the use of chestnut for wound healing and astringency, respectively. Actually, except for Pliny, the Latin literature referring to dietary use of chestnut is relatively rare. According to the detailed revision of Conedera et al. (2004) chestnut was probably unfavored by Roman aristocracy; on the contrary, it was a symbol of rural and modest life. The driving force for chestnut diffusion through Romans into Europe was, presumably, wood exploitation, but the marginal use by rural population for self-sufficiency slowly contributed to select better quality of fruits. More probably, a

major interest in systematical cultivation for food production developed after the Roman period and was associated with the socio-economic structures of medieval times.

However, the value of chestnut for self-sufficiency is still in the memory of inhabitant from mountains in Italy, who used chestnut flour to produce “the bread” during hard times. Nevertheless, chestnut cultivation was, and still remain today, a resource for landscape and soil stability. The relationship among farmers and sweet chestnut is strong and representative of a territory identity, especially in Italy: despite chestnut production is far from remunerative, producers had great efforts to obtain 16 quality denominations (DOP and IGP) on the total of 26 in Europe. Interestingly, the sector is mainly composed by old producers, who rarely transform the product or differentiate their activity, thus suffering for the loss of added economical value (Castellotti and Doria 2016).

To (re)discover a new purpose for chestnut cultivation, like the production of functional ingredients for health, may contribute to preserve the great cultural baggage beyond sweet chestnut agriculture.

Pharmacognosy of sweet chestnut

The medicinal use of *Castanea sativa* Mill. (**Fig. 1**) paralleled the exploitation for fruit and wood production from ancient times. Pliny described chestnut for the regulation of gastro-intestinal transit or against hemoptysis (N.H. XXIII.150); Nicander (Nic. Alex. 268–271) mentioned the kernel from fruit for lips and esophageal ulcers, while Dioscorides (Dios. Med. I.145) recommended the episperm for astringency. The latter is also proper to the bark, whose decoction was traditionally used against dysentery and for leather tanning (Senatore 2004). On the contrary, leaves were used for respiratory irritation and persistent cough, sometime associated with eucalyptus (*Eucalyptus globulus* Labill.), thyme (*Thymus vulgaris* L.) and roundleaf sundew (*Drosera rotundifolia* L.) (Agradi 2005; Campanini 2012).

Fig. 1. *Botanical picture of Castanea sativa Mill.*

Everyone experimented astringency like a common property of plants, such as grapevine (*Vitis vinifera* L.), nuts (*Juglans regia* L.) or pomegranate (*Punica granatum* L.), which is related to the interaction of tannins with epithelial proteins on the mouth or the skin. Indeed, as suggested by ancient texts, tannins are widely distributed in sweet chestnut, but their chemical identity was only recently investigated, thanks to scientific advance. The bark contains high amount of ellagitannins, named castalagin and vescalagin (Comandini 2014, Ricci 2016), which have never been deeply investigated in the other tissues. However, the level of tannins-related phenolic compounds can be high in chestnut peel and low in the edible part (Ribeiro 2007, Barreira 2008, Vekiari 2008, Živković 2008). According to the main interest for food consumption, the edible part of chestnut have been better characterized for nutrients than non-nutrients compounds, as precisely reported in nutritional tables from CREA (www.crea.gov.it; italian *Council for agricultural research and analysis of agroeconomic*): crude kernel contains high amount of starch (41.7%), proteins (6%) and fiber (13.8%); the main micronutrients are iron (1.9 mg/100 g), potassium (738 mg/100 g) and phosphorus (131 mg/100 g).

In analogy with the bark, leaves may contain an important amount of tannins (6-8%), flavonoids (>1%) and a small fraction of triterpenes (Wagner and Bladt 1983). If flavonoids have been widely identified as quercetin and kaempferol glycoside (Wagner and Bladt 1983, Cerulli 2018, Silva 2020, Almeida 2010), tannins were not comprehensively investigated. Indeed, leaves as well as burs and flowers may contain gallotannins (like chestanin and trigalloyl-glucose) and other unknown hydrolysable tannins (Cerulli 2018, Quave 2015, Caleja 2019, Esposito 2019).

On the contrary, the knowledge on the efficacy of chestnut preparation for human health is certainly lower than the one on nutritional and phytochemical composition. Despite the long traditional use for digestive and respiratory disorders, the biological activity of sweet chestnut has been scarcely investigated by modern studies. Nevertheless, according to the "*BELgium FRance ITaly*" project on botanical ingredients for food supplements started in 2011, extract from sweet chestnut are already present in the European market. The Italian guidelines integrated the positive list of botanicals, attributing several claims on the base of the traditional use: sweet chestnut leaves are allowed for the physiological effect on bronchial secretion fluidity, regularity of intestinal transit and antioxidant defense (D.M. 10 Agosto 2018, Allegato 1). Moreover, bark, fruit and buds are admitted without a specific indication.

The scientific evidence for health claims of sweet chestnut is actually limited to *in vitro* and few *in vivo* studies, concerning the antioxidant, antibacterial and spasmolytic properties. Regarding the first, Braga et al. (2014) have already noted that many authors reported the presence of antioxidant compounds in chestnut by-products like burs, chestnut peel and leaves. The attention to the recovery of chestnut by-products for their health value is still evident from very recent publications in high quality journals (Esposito 2019; Silva 2020). The antioxidant and scavenging effect against lipid peroxidation, ROS production or ferric ions oxidation was established for bark, fruit peel and leaf extracts containing polyphenols (Živković 2008, Barreira 2008, Chiarini 2013, Vazquez 2008, Almeida 2010, Calliste 2005). However, only polar extracts from leaf were further investigated in a specific biological context of oxidative stress by Almeida et al. (2015) and Cerulli et al. (2018), who

discovered an NRF-2-independent photoprotective effect in keratinocytes. Interestingly for topical application, the allergenic potential for skin exposure have been previously excluded by patch test in 20 healthy volunteers (Almeida 2008).

Few authors carried out experiment against MRS bacteria using sweet chestnut honey and chestnut by-products (peel from fruit, burs and leaves). Ethanol extract from the latter inhibited *S. aureus* and *S. epidermidis*, with MIC ranging from 10 to 50 mg/mL, but with lower or negligible effects on gram-negative bacteria. The inner peel (episperm) represented an exception for the wider spectrum, inhibiting also *Enterococcus* spp., *K. pneumoniae* and *P. aeruginosa* at similar concentrations (Silva et al. 2020). Conversely, the apolar fraction from leaves, rich in triterpenes (oleanane and ursene derivatives), was unable to inhibit *S. aureus* growth, but impaired quorum sensing (<25 µg/mL) and *in vivo* abscess development (50 µg, topically) (Quave 2015). Secondly, the honey exerted anti-*S. aureus* effect through membrane alteration and bacteriostatic activity, although less representative of the original polyphenolic composition (Kucuk 2007, Kolayli 2016, Combarros-Fuertes 2020). Of note, methanol extract from honey, containing polyphenols (0,2%), inhibited also the growth of fungi (*Candida* spp.) and *H. pylori* (Kucuk 2007).

Finally, keeping in mind the traditional use against dysentery, several authors investigated the spasmolytic effect on small intestine, gall bladder (Budriesi 2010, Micucci 2014) and cardiomyocytes (Comandini 2013), suggesting a mechanism of action dependent from calcium flux and independent from CCK or cholinergic signals.

Tannins for epithelial defense

Most of the pathological contexts in which tannin-containing plants have been traditionally used have a common feature: the epithelial inflammation provoked by wounds or infections. Complications of wounds are frequent today, but even more before the discovery of hygiene measures and antibiotics.

Castanea sativa Mill. is not an exception, looking at the traditional preparations against dysentery or ulcers of the first digestive tract. Other drugs containing tannins have been deeply investigated to treat respiratory infections, cutaneous wounds, gastro-intestinal ulcers and intestinal disorders, reaching *in vivo* and, sometimes, clinical evidences. Of note, the presence of gallotannins, ellagitannins (hydrolysable tannins) or proanthocyanidins (condensed tannins) may result equally of interest for epithelial defense.

For condensed tannins, a famous example is the procyanidins-rich fraction from *Pinus pinaster* Ait., responsible for the efficacy of Picnogenol® in many clinical trials including the adiuvant treatment of venous ulcers (Toledo 2017). Another example of procyanidins-rich extract is EPs 7630®, the proprietary root extract from *Pelargonium sidoides* DC currently used to treat respiratory infections, inducing mucosal fluidity and immune response (Patiroglu 2012). Again, grape seed extract proanthocyanidins demonstrated wound healing properties *in vivo* (Khanna 2002, Hemmati 2011) and in several clinical trials (Hemmati 2015, Izadpanah 2019).

Regarding hydrolysable tannins, a well-known source of gallotannins is *Hamamelis virginiana* L., exhibiting clinical efficacy against hemorrhoids (Knoch H.G. 1991) and tradition application for dermatological and microvascular disturbs; similarly, gallotannins-rich proprietary extract from oak (*Quercus rubur* L.), named QRB-7™, finds clinical application for wound healing and hemorrhoids (Jacobs 2008). Examples of ellagitannins-rich plants are less clinically investigated, but our group has recently reviewed the evidence on wound healing properties of berries (Piazza 2020), among which pomegranate (*Punica granatum* L.) was considered for *in vivo* studies and a promising case-report has been published as well (Fleck 2016).

Despite one of the most reported traditional uses of tannins-rich plants is for peptic ulcer, its pharmacological validation is actually not intense, according to the number of publications per year on Pubmed. Despite many extracts and purified tannins showed anti-ulcer efficacy *in vivo* (De Jesus et al. 2012), only rare cases reached clinical investigation against gastritis, such as cranberry (Gotteland et al. 2008, Zhang et al. 2005). Citing other examples from red fruits, the presence of

ellagitannins and ellagic acid is mandatory for *in vivo* efficacy of *Punica granatum* L. against gastric ulcer and gut inflammation, as reviewed by Colombo et al. (2013). Accordingly, extracts from *Rubus* spp. enriched in ellagitannins have been successfully studied against ethanol-induced gastric injury (Sangiovanni 2013).

A last important example, cited in ESCOP monographs, is *Filipendula ulmaria* L. (ESCOP, 2003): the major constituents, similarly to *Castanea sativa* Mill., are quercetin glycosides (spireoside) and ellagitannins (tellimagrandin II), both responsible for gastroprotection *in vivo* (Manayi 2013). Remembering ellagitannins recorded in *Castanea* spp., castalagin and vescalagin display a specific C-glycosidic structure and are common to some others genus like *Lythrum* and *Quercus*. Of note, castalagin (50 mg/Kg) resulted the most active constituent for ulcer prevention *in vivo* present in *Quercus* spp. leaves (Khennouf 2003) and exerted anti-inflammatory effect *in vitro* on neutrophils (Granica 2015).

Gastritis

One of the most common epithelial inflammation is gastritis, namely the histologically documented inflammation of gastric mucosa. The correlation between histologic or endoscopic findings of gastritis and symptoms like abdominal pain or dyspepsia is poor, therefore, there is no typical clinical manifestation of gastritis. The etiologic factors leading to gastritis are heterogeneous and classified on the base of the clinical history, histologic features, anatomic distribution or pathogenic mechanisms (**Fig. 2**).

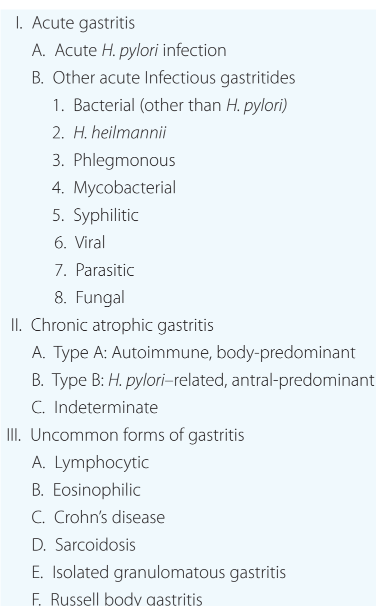

I. Acute gastritis
A. Acute *H. pylori* infection
B. Other acute Infectious gastritides
1. Bacterial (other than *H. pylori*)
2. *H. heilmannii*
3. Phlegmonous
4. Mycobacterial
5. Syphilitic
6. Viral
7. Parasitic
8. Fungal
II. Chronic atrophic gastritis
A. Type A: Autoimmune, body-predominant
B. Type B: *H. pylori*–related, antral-predominant
C. Indeterminate
III. Uncommon forms of gastritis
A. Lymphocytic
B. Eosinophilic
C. Crohn's disease
D. Sarcoidosis
E. Isolated granulomatous gastritis
F. Russell body gastritis

Fig. 2. *Classification of gastritis (Harrison's Principles of Internal Medicine).*

The most common cause of the disease is *Helicobacter pylori* (*H. pylori*) infection, recognized from the 80's as a risk factor for the onset of peptic ulcer

disease (PUD), gastric cancer and B-cell or MALT (*mucosal associated lymphoid tissue*) lymphoma (**Fig. 3**).

The precise mechanisms by which the infection causes the aforementioned clinical outcomes is unknown, but the eradication has been established as efficient preventive measure.

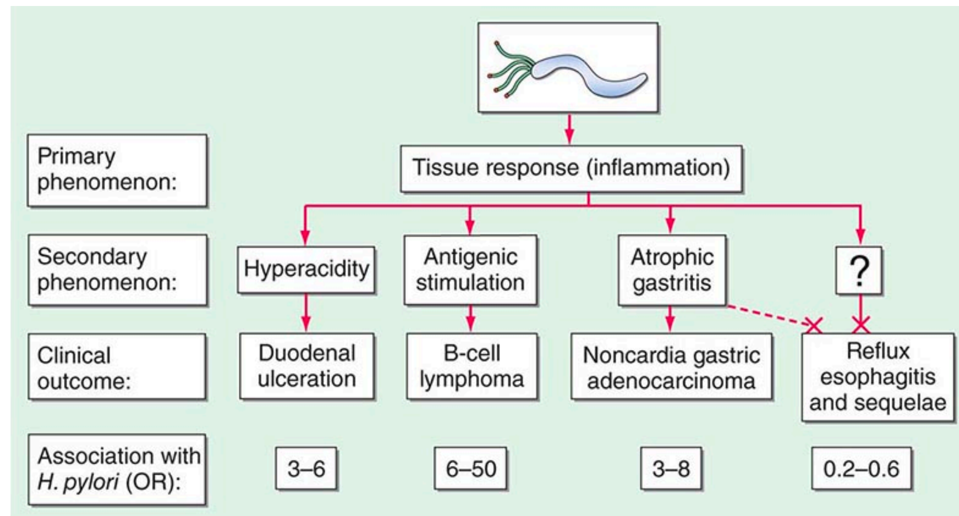


Fig. 3*, Association among H. pylori infection and upper gastrointestinal diseases. Essentially all persons colonized with H. pylori develop a host response, which is generally termed chronic gastritis. H. pylori colonization increases the lifetime risk of peptic ulcer disease, gastric cancer distal from cardia, and B-cell non-Hodgkin's gastric lymphoma (odds ratios [ORs] for all, >3). In contrast, a growing body of evidence indicates that H. pylori colonization (especially with cagA^{+} strains) protects against adenocarcinoma of the esophagus (and the occurrence of gastric cardia) and premalignant lesions such as Barrett's esophagus (OR, <1). Although the incidences of peptic ulcer disease (cases not due to nonsteroidal anti-inflammatory drugs) and noncardia gastric cancer are declining in developed countries, the incidence of adenocarcinoma of the esophagus is increasing. (Adapted from Blaser MJ 1999).*

Since *H. pylori* colonizes only the human stomach, the demonstration of the causal relationship with gastritis has been challenging and represented a great step in the scientific history.

The first isolation of the bacteria from active gastritis patients was obtained by Warren and Marshall in 1983: following the discovery, Marshall supported the infective theory of gastritis by self-administration of *H. pylori* virulent strains, thus recording for the first time the symptoms of an acute infection in human. Symptoms included bloating and vomiting, while endoscopic exam revealed polymorphonuclear infiltration and epithelial damage. However, not all infected people, representing almost one on third in the developed country and till 80% in rural societies, develop gastritis symptoms (Suerbaum 2002, Marshall 2005). In predisposed patients the infection may evolve in the most frequent form of chronic gastritis (Type B or *H. pylori*-related) in which lymphocytes and plasma cells infiltration play a pivotal role in inflammatory processes.

The interplay between bacteria and host is still enigmatic and may explain different fates of chronic infection (**Fig. 4**). The expression of virulence factors, such as urease, proteins from bacterial secretion system (*type 4 secretion system*, T4SS), toxins like *citotoxin associated gene A* (CagA) and *vacuolating cytotoxin* (VacA) and adhesins, as well as genetic factors, ethnicity or the age of infected people may account for the severity of gastritis. For example, CagA positive *H. pylori* are associated with PUD, but gastric ulcer and duodenal ulcer have different pathogenesis on the base of infection background: antral predominant gastritis may result in hypergastrinemia and hyperchloridria, leading to duodenal ulcer (Blaser and Atherton 2004), while diffuse infections (corpus or pan-gastritis) are associated with gastric ulcer and hypochloridria caused by gastric atrophy. Gastric atrophy is then recognized as crucial step for intestinal metaplasia and

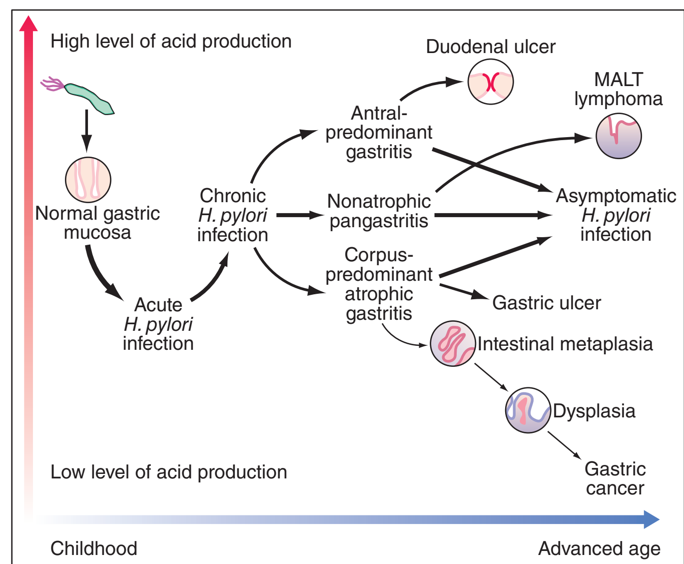


Fig. 4. *Natural history of H. pylori infection (Suerbaum 2002)*

gastric cancer outbreak (1-3% of infected people), following a still unknown pathogenic process (Taylor 1995, Blaser and Atherton 2009). Intriguingly, patients with antral-gastritis and duodenal ulcer seem to be partially protected from the development of gastric cancer (Hansson 1996).

The common features of *H. pylori*-related gastritis at histological level are inflammation and oxidative stress orchestrated by leukocytes, infiltrating the mucosa following chemokines attraction (Naito 2002). A crucial inflammatory signal is IL-8, which is mostly released by gastric epithelia and which level positively correlates with *H. pylori* infection and gastritis severity (Ando 1996, Crabtree 1993; Crabtree 1994, Uemura 1997). IL-6 and TNFα are other mediators involved in the inflammatory process (Basso 2010). Notably, IL-8 is implied in the specific recruitment of neutrophils, while IL-6 has been implied in the attraction of mononuclear cells in the gastric site (Ando 1998). TNFα and *H. pylori* are able to induce IL-8 release in gastric cells trough a partially different signaling converging in the NF-κB activation (Sharma 1998, Foryst-Ludwig 2000). In clinical experiments, activated NF-κB co-localizes with IL-8 in *H. pylori*-infected mucosa of patients with gastritis (Isomoto 2000). Accordingly, the eradication therapy leads to early decrease of IL-8 levels and neutrophils infiltration, but IL-8 increases again if gastric biopsies are reinfected *ex vivo*, thus confirming the causal relationship (Ando 1998).

The virulence factors produced by *H. pylori* are responsible for its survival and colonization, but also for the inflammatory process and the consequent mucosal damage (**Fig. 5**). The genomic region *cagPAI* encodes for the T4SS and the toxin CagA: the first is responsible for cell-host adhesion and injection of toxins including CagA. Proteins from the T4SS and other injected bacterial structures may cover a key role for the induction of pro-inflammatory cytokines (Wiedemann *et al*., 2009). They are also consistently associated with the activation of NF-κB via NOD1 signaling, a typical innate pathway (Viala 2004, Gorrel 2013). CagA may contribute to NF-κB activation but its role as a necessary factor was not established (Brandt 2005; Shweitzer 2010).

The biological role of CagA is not fully elucidated, however, the toxin is able to inhibit apoptosis *in vitro* and *in vivo* (Mimuro *et al*., 2007; Ohnishi *et al*, 2008); once in the host cell it may undergo to

phosphorylation, thus exerting mitogenic effects and cytoskeletal changes through the interaction with E-cadherin (Mimuro *et al*., 2002; Churin *et al.*, 2003). Notably, the infection of *cagA* positive *H. pylori* correlates with IL-8 levels and with the severity of the inflammation, thus representing a risk for the onset of adenocarcinoma (WHO 1994, Crabtree 1995, Jones 2010).

VacA is another important toxin encoded by *vacA* gene, involved in the pathogenesis of peptic ulcer and gastric cancer, exhibiting several antagonistic effects in respect to CagA. It induces vacuolization, lysosomes impairment, apoptosis, immunomodulation and produces damages at gastric parietal cells, thus suggesting an involvement in atrophy development (Wang 2008). Controversially, VacA has been reported to induce the activation of NF-κB along with immunosuppression in T cells (Takeshima 2009).

LPS, together with CagA and VacA, interferes with innate and specific immunity, thus counteracting the bacterial clearance. This evasion strategy is also responsible for the persistence of the infection and its chronic course (Chmiela *et al.*, 2014). The variable regions of LPS have been implied in the bacterial diversity and survival in the stomach (Moran *et al.*, 2002; Skoglund *et al.*, 2009). Another fundamental protein for *H. pylori* persistence is the enzyme urease: it exploits urea to obtain ammonia in order to increase the pH of gastric microenvironment; of note, mutant strains lacking urease are not able to successfully colonize the human gastric mucosa (Eaton *et al.*, 1991).

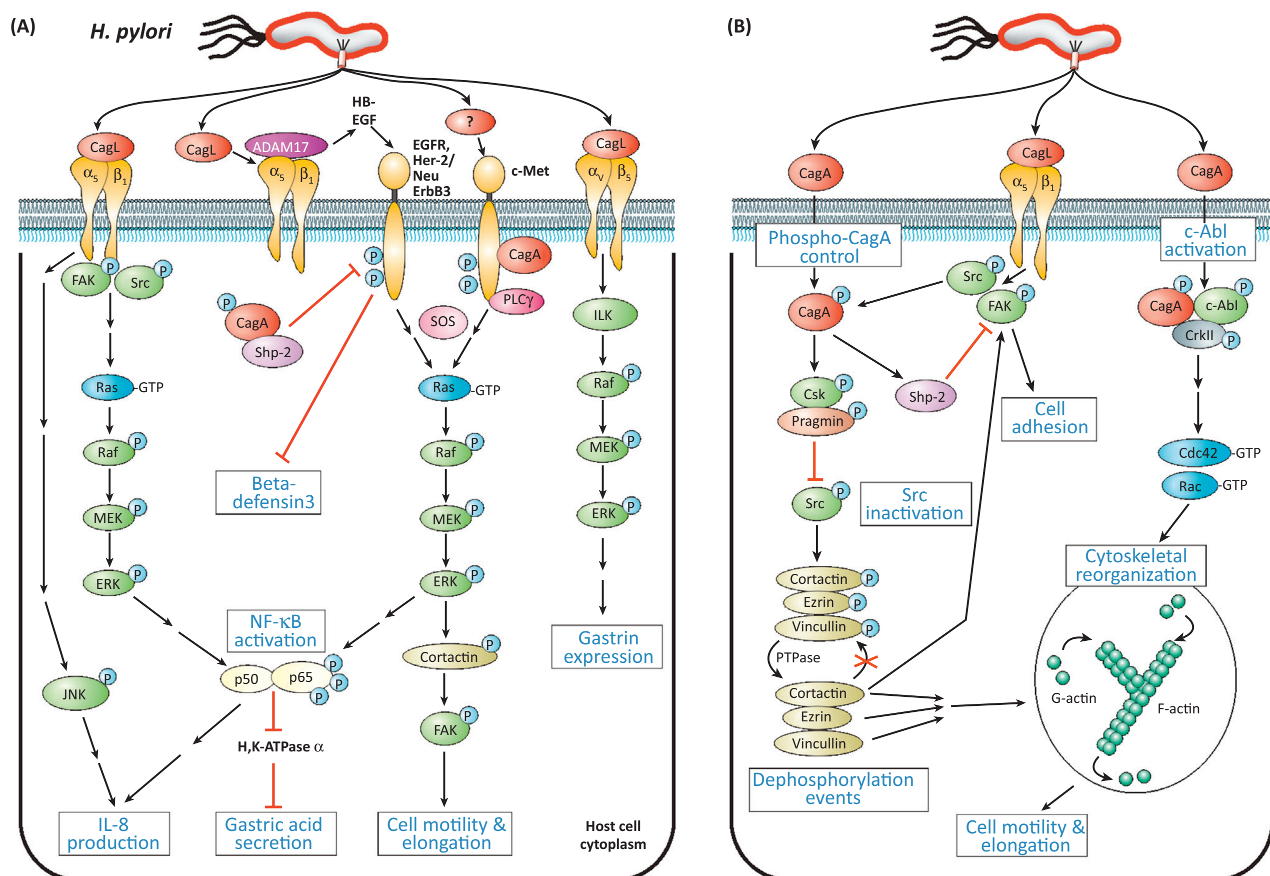


Fig. 5. *Type IV Secretion System (T4SS)-Dependent Interactions of H. pylori with Host Cell Surface Receptors and Downstream Signaling Events. (A) The T4SS targets various integrin receptors and receptor tyrosine kinases. In particular, CagL can bind to integrin/5β1, which triggers IL-8 production via several kinases (Src, Raf, MEK, ERK, and JNK) and transcription factor NF-kB. Upon delivery, CagA targets the c-Met receptor and promotes PLC-dependent mitogenic responses. CagL dissociates metalloprotease ADAM17 from integrin /5β1 and activates EGFR-dependent, NF-kB-mediated suppression of H, K ATPase and gastric acid secretion. MAPKs induce the serine phosphorylation of cortactin, which controls FAK phosphorylation and cell elongation. EGFR/MAPK signaling triggers the expression of b-defensin-3. In addition, a novel CagL/integrin β5/ILK signaling complex was characterized and found to be crucial for Hp-induced gastrin expression. (B) Induction of intracellular nonreceptor tyrosine kinase signaling by phosphorylated CagA. Phospho-CagA modulates various downstream signaling events such as binding of phosphatase SHP-2, inhibiting FAK activity. This process counteracts integrin- and cortactin-mediated FAK activation controlling cell adhesion. Phospho-CagA interacts with Csk, which inhibits Src activity. Inactivation of Src affects various actin-binding proteins, for example, cortactin, ezrin, and vinculin, followed by actin-cytoskeletal rearrangements and cell elongation. The latter signaling is enhanced by a trimeric complex formed by phospho-CagA, Abl, and the adapter protein CrkII, which stimulates the small Rho GTPases Cdc42 and Rac1. ERK, extracellular-regulated kinase; FAK, focal adhesion kinase; JNK, c-Jun N-terminal kinase; HB-EGF, Heparin-binding epidermal growth factor; ILK, integrin-linked kinase; MEK, mitogen-activated ERK kinase; P, phosphate; SHP-2, SH2 domain-containing protein tyrosine phosphatase-2. (Adapted from Neumann et al. 2017, Trends in microbiology, 25:4, 316)*

Tannins for *Helicobacter pylori*-related gastritis

The discovery of *H. pylori* infection by Warren and Marshall is relatively recent, especially if we consider the following efforts to establish and accept its causal association with peptic ulcer, leading to the Nobel Prize in 2005 only. The discovery completely changed the therapeutic approach to gastritis, whose treatment was relegated to mild diet and anti-acids. In agreement with the scientific and cultural environment of the time, the antibiotic therapy became rapidly the first line to prevent ulcer and gastric cancer: citing the amazing book of M. D. Martin J. Blaser, *Missing Microbes*, the scientific community considered "the only good *H. pylori* as a dead *H. pylori".*

Accordingly, if tannins have always been used against peptic ulcer, their direct anti-*H. pylori* effect has been assessed just in the last 20 years. Moreover, the research on new anti-gastritis compounds is generally affected by the difficulty of simulating the very complex relationship among the bacteria and its human host (Marchetti 1995; Hirayama 1999). To date, the hypothesis of potential benefit of tannins for gastritis would mostly derive from traditional efficacy, gastric ulcer models (ethanol or FANS injury) or microbiological studies involving *H. pylori*.

Regarding the latter, either condensed or hydrolysable tannins may exert anti-*H. pylori* activity, in analogy with previously cited evidence on ulcers and wounds healing. However, according to Funatogawa et al. (2004), ellagitannins are more potent than B- and C-type proanthocyanidins, with MICs of 6,25 to 50 µg/ml against four *H. pylori* strains. Other studies involving condensed tannins reported the effect of procyanidins from *Peumus boldus* Mol. and *Pelargonium sidoides* DC against the bacteria: the first inhibited *H. pylori* growth (MIC=16 µg GAE/ml), urease activity and adhesion to gastric cells (Pastene 2014); the second exhibited anti-adhesive effect *in situ* (0,1-10 mg/mL) (Wittschier 2007). The idea that *H. pylori* adhesion is the major target for condensed tannins was sustained by other results obtained with Picnogenol® and cranberry juice (Burger 2002, Rohdewald and Beil 2008).

The gastroprotective action of tannins should not limit to direct anti-*H. pylori* activity, since their polyphenolic structures may also exhibit anti-inflammatory and antioxidant effects.

Several *in vivo* and *in vitro* studies attributed anti-inflammatory and antioxidant mechanisms to tannins from edible fruits at gastric level. Regarding hydrolysable tannins, *Rubus* spp. ellagitannins prevented ethanol-induced ulcer inhibiting oxidative stress and IL-8 levels in mice, but also in inflamed gastric human cells acting on the NF-κB pathway (Sangiovanni et al. 2013); in analogy, *Fragaria* spp. ellagitannins and procyanidin B1 impaired IL-8 release *in vitro* (Fumagalli 2016). Again, pomegranate extracts and ellagic acid counteracted FANS-induced ulcer and glutathione depletion *in vivo* (Lai et al. 2009).

Other authors investigated condensed tannins bioactivity, reporting inhibition of neutrophils infiltration, enhancement of antioxidant defense, increase of PGE_2 levels and pH regulation as the main contributors for prevention of peptic ulcer *in vivo* by proanthocyanidins (Iwasaki 2004, Kim 2013, Cires 2017). Notably, structural units of tannins, namely gallic acid, ellagic acid and catechin may exert anti-ulcer effect *in vivo* per se (Paturi 2014, Pal 2010, Baserra 2011). However, only few examples of studies underline the protective role of tannins from wine and green tea in gastritis using a mice model of *H. pylori* or VacA infection (Ruggiero 2007); thus, studies on the role of tannins about *in act* infection *in vitro* as well as *in vivo* are poor, thus limiting the speculation on the possible efficacy in humans.

Aims of the study

Despite the traditional use of sweet chestnut (*Castanea sativa* Mill.) for respiratory and gastro-intestinal diseases, these inflammatory contexts have never been included in pharmacological studies, even though *in vitro* literature attributed some pertinent biological effect to sweet chestnut, like antioxidant and antibacterial effects.

A strong global debate on antibiotics overuse raised in the last years, leading to important policies to limit the dose and the frequency of antibiotic courses for humans and animals, like the World Antibiotic Awareness Week by WHO. Like for other infective pathologies, the treatment of *H. pylori*-related gastritis is afflicted by increasing antibiotic resistance (Bjorkholm 2001) and may need substitutive or supportive approaches. Moreover, the ongoing explanations on the relationship among *H. pylori* and human host brought to consider the concept of *amphibiosis*, namely occurring when a microorganism may be pathogenic or not depending on factors like environment and host (Atherton and Blaser 2009). Notably, this concept stimulates to understand how to control the detrimental outcomes of *H. pylori* infection, like ulcer and gastric cancer, in favour to the positive outcomes, like protection from asthma and esophagitis (de Martel 2005, Chen Y. 2007). As a consequence, the current eradication strategy is being debated and vaccines or probiotics and complementary approaches are being experimented (Malfertheiner 2018, Gotteland 2008).

This work investigates, for the first time, the potential anti-gastritis properties of sweet chestnut with a bio-guided approach. The plant is a well-known source of tannins, which composition was clearly elucidated in bark only (Ricci 2016, Comandini 2014). However, tannins may occur in chestnuts as well as in by-product of cultivation, like burs, peel and leaves (Cerulli 2018, Quave 2015, Caleja 2019, Esposito 2019) also in association with other polyphenols of interest for human health, like flavonoids (Wagner and Bladt 1983).

Since tannins are gaining attention for their gastroprotective effects, the first aim was to evaluate *in vitro* anti-inflammatory activity of extracts from chestnuts and leaves by a model of gastric epithelial cells infected by *H. pylori* or challenged with pro-inflammatory cytokines. The possible influence of the variety of origin was also considered, since 5 different varieties from Northern Italy (Campo dei Fiori, Varese) were selected for the study. Moreover, the assays were conducted before and after simulated gastric digestion of the extracts to validate the biological stability in the gastric environment. In parallel, extracts were deeply characterized, with particular attention to the polyphenolic composition.

Materials and methods

Materials

Dulbecco's Modified Eagle's Medium/F12 (DMEM)/F12 (1:1), RPMI 1640 medium, penicillin, streptomycin, L-glutamine and trypsin-EDTA were from Gibco (Life Technologies Italia, Monza, Italy). Fetal bovine serum (FBS), and disposable materials for cell culture were purchased by Euroclone (Euro- clone S.p.A., Pero-Milan, Italy). Gastric epithelial cells from human adenocarcinoma (AGS, CRL-1739) were purchased from LGC Standard S.r.l. (Milano, Italy); while SV-40 immortalized GES-1 from human gastric epithelium were kindly donated by Dr. Dawit Kidane-Mulat, The University of Texas at Austin.

Mueller Hinton Broth, Brucella Broth and glycerol from BD (BD, Franklin Lakes, USA), agar from Sigma-Aldrich and defibrinated sheep blood from LifeTechnologies were used to cultivate and store *Helicobacter pylori*, *cag*+ strain 26695 from ATCC (ATCC 700392™, Virginia, USA).

The reagent 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), FITC and curcumin were from Sigma Aldrich (Milan, Italy). Ellagitannins castalagin and vescalagin were from Phytolab GmbH & Co. KG (Vestenbergsgreuth, Germany). All reagents used for the biological assays were HPLC grade. Human TNFα and Human IL-8 Elisa Development Kit were from Peprotech Inc. (London, UK). All chromatographic solvents were HPLC grade or LC–MS grade for MS experiments. Acetonitrile, methanol, ethanol, formic acid, hydrochloric acid, vanillin, and iron sulfate were from Sigma Aldrich (Milan, Italy).

Plant material and extraction

Chestnuts and leaves from five varieties (Venegon, Paié, Russirö, Verdesa and Piliscé) of *Castanea* sativa Mill. (**Fig. 6**) were collected by the farmer consortium in the regional area of Campo dei Fiori (Varese, Italy).

Fig. 6. *Pictures of chestnuts and leaves from different varieties of Castanea sativa Mill. Leaves were collected from Venegon and Verdesa varieties only.*

Fresh fruits were maintained under vacuum at 4°C, while leaves were dried in layer at r.t. before extraction. To obtain the extracts, 2.5 g of milled fruits were extracted twice with 50mL of water (aqueous extract) or ethanol/water 50:50 (hydroalcoholic extract) for 4 and 16 h, respectively, at room temperature under dark conditions. The mixture was filtered through Supervelox filter paper in order to remove plant debris; the extracts obtained were frozen with dry ice and alcohol and placed at −80 °C overnight, then lyophilized and maintained at −20 °C. An equal amount (2.5 g) of three parts of the fruits, endosperm (kernel) and the outer parts episperm (which directly covers the kernel) and pericarp (the woody part) (**Fig. 7**), were separated and extracted with hydroalcoholic solvent following the previously described procedure.

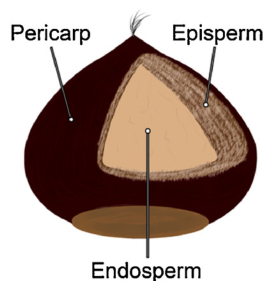


Fig. 7. *Image reproducing the organization of tissues from chestnut fruit. Endosperm (kernel), episperm and pericarp can be easily recognized and separated for extraction.*

Commercially available flour, produced from the endosperm of dried chestnut (variety Venégon), and relative industrial by-product, constituted by episperm and pericarp, were extracted by hydroalcoholic solvent. Leaves extracts were obtained by hydroalcoholic extraction as well.

The yield (w/w) of each extraction was calculated as percentage of the dried extract weight in respect to the weight of the starting material.

Before proceeding with the biological evaluation, the extracts were dissolved in sterilized distilled water and DMSO (water:DMSO 75:25, 30 mg/mL for fruits, water:DMSO 50:50, 25 mg/mL for leaves), then stored in aliquots at −20 °C. Pure ellagitannins were dissolved in water:DMSO 50:50 at 5 μM.

Cell viability test

The integrity of the cell morphology before and after treatment was assessed by light microscope inspection. Cell viability was measured, after 6 h treatment, by the 3,4,5-dimethylthiazol- 2-yl-2-5-diphenyltetrazolium bromide (MTT) method. This method evaluates the activity of a mitochondrial enzyme, which is an index of cell viability. The toxicity of extracts or pure compounds at the concentration tested was excluded.

Cell culture and IL-8 measurement

AGS or GES-1 cells were grown at 37 °C in DMEM F12 or RPMI, respectively, supplemented with 100 units penicillin per mL, 100 mg streptomycin per mL, 2 mM L-gluta- mine, and 10% heat-inactivated FBS (Euroclone S.p.A, Pero, Italy), under a humidified atmosphere containing 5% CO_2. Cells were grown in 24-well plates for 48 h (30'000 cells per well) before treatment. For the experiments, growth medium is replaced by FBS free media. The IL-8 secretion, induced by TNFα at 10 ng/mL or *H. pylori* 26695, was tested after 6h treatment in the presence of undigested or digested extracts (0.1–100 µg/mL). Curcumin (10 µM) or EGCG (20 µM) were used as the reference inhibitors. IL-8 was quantified using a Human Interleukin-8 ELISA Development Kit as described below. Briefly, Corning 96 well EIA/RIA plates from Sigma- Aldrich (Milan, Italy) were coated with the antibody provided in the ELISA Kit (Peprotech Inc., London, UK) overnight at 4°C. After blocking the reaction, 200 µl of samples in duplicate were transferred into wells at room temperature for 1 h. The amount of IL-8 in the samples was detected by spectroscopy (signal read: 450 nm, 0.1 s; Viktor, PerkinElmer) by the use of biotinylated and streptavidin–HRP conjugate antibodies, evaluating the 3,3′,5,5′-tetramethylbenzidine (TMB) substrate reaction. Quantification of IL-8 was done using an optimized calibration curve from IL-8 standard supplied with the ELISA Kit (50–1000.0 pg/mL).

NF-κB driven transcription and IL-8 promoter activity

To evaluate the NF-κB driven transcription and IL-8 promoter, AGS cells were plated in 24-well plates (30,000 cells per well). After 48 h, cells were transiently transfected by the calcium-phosphate method with the reporter plasmid NF-κB-LUC (50 ng/well) and IL-8-LUC (100 ng/mL). The first contains three responsive elements for NF-κB, while the second for NF-κB, AP-1 and C/EBP, all of them controlling the luciferase gene. The plasmid NF-κB-LUC was a gift of Dr. N. Marx (Department of Internal Medicine-Cardiology, University of Ulm, Ulm, Germany), while IL-8-LUC were kindly

provided by Dr. T. Shimohata (Department of Preventive Environment and Nutrition, University of Tokushima Graduate School, Japan). After 16 h, the cells were treated with the stimulus (TNFα 10 ng/mL or *H. pylori*) and the extract for 6 h. Curcumin (10 μM) or EGCG (20 μM) were used as reference inhibitors. At the end of this time, cells were harvested, and the luciferase assay was performed using the Britelite™ Plus reagent (PerkinElmer Inc., Walthman, MA, USA) according to the manufacturer's instructions.

***Helicobacter pylori* culture and infection of cells**

Helicobacter pylori 26695 (*H. pylori* 26695) is a genome sequenced strain from ATCC. The bacteria were stored in glycerol solution (50% sheep blood, 20% glycerol, 30% Brucella Broth) at -80°C before each use. With the collaboration of the laboratory of microbiology (Prof. A. Polissi, University of Milan), the strain was checked for virulence phenotype and antibiotic resistance as follows:

Strain	**Clarithromicin/ metronidazole sensitivity**	**Levofloxacin/ amoxicillin sensitivity**	**CagA**	**VacA (m1/2, s1/2 alleles)**
ATCC® 26695	Yes	Yes	Positive	s1m1

A suspension of bacteria (50-100 μL) was seeded in agar-blood Petri dishes containing 1% agar, 2.2% Mueller Hinton Broth and 5% sheep blood. After 72h in microaerophilic environment (5% O_2, 10% CO_2 and 85% N_2 at 37°C, 100% humidity) the bacteria were collected and suspended in PBS 1X to be counted by optical density at 600 nm (O. D of 5 corresponds to 2 x 108 bacteria/mL). AGS or GES-1 cells were then infected using a ratio of bacteria:cells of 50:1 for all biological assays.

Bacterial adhesion to cells

The inhibition of bacterial adhesion was evaluated by a cytofluorimetric method adapted from Niehues and Hensel (2009). Taking into account an infection rate of 50 bacteria for each AGS or GES-1 cell, an excess of bacterial suspension in PBS 1X was labeled by FITC probe solution (1% in DMSO). In particular, 2 µL of FITC were added for 10^8 bacteria and incubated for 45 min at 37°C. In parallel, cells were pre-treated in 24 well plate for 1h with plant extracts (10-200 µg/mL) and the reference inhibitor procyanidin A1 (500 µM) before infection. The FITC-*H. pylori* suspension was centrifuged (3150 g, 5 min) and washed twice (PBS 1X) to remove the excess of probe, then resuspended in PBS 1X and used for the infection of cells with a new medium. After 1h (37°C) cells were washed twice with PBS 1X, collected by trypsin/EDTA and centrifuged (3200 g for 5 min). The pellet was fixed by formaldehyde (2% in PBS) and incubated in ice bath for 10 min. Finally, cells were centrifuged, washed and resuspended in 0.5% BSA (PBS/EDTA 2 mM) for the analysis with NovoCyte flow cytometer (ACEA Biosciences, San Diego, CA, USA) and NovoExpress software (ACEA Biosciences).

Minimum Inhibitory Concentration (MIC) assessment

For MIC assessment, broth dilution method was performed. 100 µL/well of sample extract were prepared by serial dilution in Brucella Broth with 5% FBS, were added to 96 well plate. Then, *H. pylori* suspension was diluted to O.D. 0.1 in Brucella Broth with 5% FBS and 100 µL were added to each well of the plate, bringing the extract to the desired concentration to test antibacterial effect. The plate is finally incubated at 37°C under microaerophilic condition. After 72h optical density at 600 nm is read. Tetracycline was used as positive control (MIC=0.125 µg/mL).

Gene expression

AGS and GES-1 cells were grown in 24-well plates (30,000 cells per well) for 48 h; then, the cells were treated with the proinflammatory stimulus (TNFα, 10 ng/ml) or *H. pylori*. After 6h treatment, the medium was removed and cells were lysed through the addition of the Qiazol lysis buffer (QIAGEN GmbH, Germany) according to the manufacturer's instructions. Total RNA was further isolated from the cell lysates using the miRNeasy Mini Kit (QIAGEN). Total RNA was eluted in nuclease-free water and stored at −80°C.

The concentration of the isolated RNA was evaluated by spectrophotometry (NanoDrop ND-1000, ThermoFisher Scientific). The purity of the samples was estimated by measuring the ratio between the absorbance of the samples at 260 nm and 280 or 230. cDNA was synthetized from 400 ng of total RNA, after elimination of any residual genomic DNA, using the RT2 First Strand kit (QIAGEN).

The analysis of gene expression was performed using a multigenic (PCR) array, related to human genes involved in the inflammatory process (RT2 Profiler™ PCR array: PAHS-011ZE Human Inflammatory Cytokines and Receptors, QIAGEN Sciences, USA). In this array, each well contained the primers for a specific target gene (84 different target genes in total), or housekeeping gene for data normalization (five different housekeeping genes). Moreover, the array included several controls: one control for genomic DNA contamination, three controls for the repeatability of the reverse transcription reaction, and three controls for the repeatability of the PCR reaction.

The obtained cDNA was mixed with the SYBR Green Master Mix RT2 reagent (QIAGEN Sciences, USA) and loaded into the 384-well array. The real-time PCR was performed using the CFX384™ Real-Time PCR Detection System (coupled to C1000™ Thermal Cycler; Bio-Rad Laboratories Srl, Segrate, Italy). The threshold cycle value for each gene (Ct) was automatically provided by the management software CFX Manager™ (Bio-Rad), depending on the

amplification curves. The baseline and the threshold values were set manually as recommended by the PCR array manual. The analysis of the data was performed using the web portal SABiosciences (QIAGEN Sciences, USA). The C_t cut-off was set to 35. Data were normalized on the basis of housekeeping genes: hypoxanthine phosphoribosyltransferase 1, beta-2-microglobulin, ribosomal protein P0, beta-actin, and glyceraldehyde-3-phosphate dehydrogenase. In each experiment, the housekeeping genes with a variability higher than ±1 threshold cycle among the different experimental conditions were excluded from the analysis.

Total phenol content

Total polyphenol content was determined according to Folin–Ciocalteu's method, as reported by Singleton and Rossi. Freeze-dried samples (50 mg) were solubilized in 1 mL of a 50:50 water:methanol solution. Aliquots of 300 μL from different samples were mixed in test tubes with 1.5 mL of Folin–Ciocalteu's reagent diluted 10 times, and 1.2 mL of 7.5% (w/v) sodium carbonate. After 30 min, the absorbance was measured at 765 nm in a UV–vis spectrophotometer. The polyphenol content in samples was calculated using a standard curve of Gallic acid. Results were expressed as equivalents of Gallic acid in mg/g.

Vanillin index

The catechins and proanthocyanidins reactive to vanillin were analyzed according to the optimized and controlled vanillin-HCl method of Broadhurst and Jones, following the conditions described by Di Stefano et al. 0.5 mL (Di Stefano et al. 1989) of the initial extract and 0.5 mL of MeOH were collected in a 50 mL flask, shielded from light with aluminum foil, 6 mL of vanillin (4% in methanol) were added in the flask and 3 mL of HCl. To subtract the natural interference, 0.5 mL of the extract was prepared under the same conditions using 6 mL of pure methanol instead of vanillin solution.

The absorbance was measured at 500 nm in a 10 mm cell, against a blank reaction. Concentrations were calculated as (+)-catechin (mg/g).

UPLC MS/MS method for multiple classes of phenolics

Phenolic compounds from chestnut were determined in collaboration with Edmund Mach foundation (Prof. F. Mattivi), with solid expertise in tannins characterization. Briefly, an aliquot of the extract was filtered 0.22 µm in a HPLC vial. Chromatographic analysis was performed using a Waters Acquity UPLC system (Milford, USA) with a Waters Acquity HSS T3 column (100 mm x 2.1 mm; 1.8 µm). The flow was 0.4 mL/min and the gradient profile was 5% B for the initial condition; from 0 to 3 min linear gradient to 20% B; from 3 to 4.3 min, isocratic 20% B; from 4.3 to 9 linear gradient to 45% B; from 9 to 11 min, linear gradient to 100% B; from 11 to 13 min wash at 100% B and then from 13.01 to 15 min back to the initial conditions (B: acetonitrile containing 0.1% formic acid; A: water containing 0.1% formic acid). Mass spectrometry detection was performed on a Waters Xevo triple-quadrupole mass spectrometer detector (Milford, USA) with an electrospray (ESI) source. A total of 24 polyphenols were identified among the samples and proper calibration curves were obtained for each individual compound for precise quantification.

Similarly, a qualitative analysis of phenolic compounds from leaves was performed in collaboration with NaTuRa laboratory (Prof. L. Pieters), according to well-established methods (Bijttebier et al. 2016). Briefly, chromatographic analysis was performed using a Waters Acquity UPLC system (Milford, USA) with Waters RP18 UHPLC BEH SHIELD column. The mobile phase solvents consisted of water + 0.1% formic acid (A) and acetonitrile + 0.1% formic acid (B), and the gradient was set as follows (min/A%): 0.0/99, 9.91/74, 18.51/35, 18.76/0, 20.76/0, 20.88/ 99, 23.00/99. Mass spectrometry detection was performed on a Waters Xevo qTOF mass spectrometer detector (Milford, USA) with an electrospray (ESI^-) source.

Ellagitannins castalagin and vescalagin were also detected and further quantified in the laboratory of mass spectrometry at the University of Milan: HPLC-MS analysis was performed using Exion LCTM AC System (AB Sciex, Foster City, CA, USA) with column PHENOMENEX, Synergi Hydro-RP 80, coupled to Triple QuadTM 3500 system (AB Sciex, Foster City, CA, USA) with ESI^- source. The mobile phase solvents consisted of water + 0.1% formic acid (A) and methanol (B) set as follows (min/A%): 0.0/95%, 5.0/0, 8.0/0; 8.10/95%, 15.0/95%.

In vitro simulated gastric digestion

According to a well-established protocol, the gastric digestion was simulated using an in vitro approach previously described (Sangiovanni et al. 2015). Briefly, the extracts (100 mg) were incubated for 5 min at 37 °C with 6 mL saliva juice, then 12 mL gastric juice were added to the suspension and the sample was incubated for 2 h at 37 °C. At the end of the incubation, the digested sample was centrifuged for 5 min at 3000 g and the super- natant frozen and lyophilized. All the samples were then stored at −20 °C until use for biological assays.

Evaluation of thermal stability

Heating treatment was performed directly on 10 mg of dried ex- tracts, placed in pyrex vials, using a stove (Tecnovetro s.r.l., Monza, Italy) at different temperatures. The extracts were placed in the stove once the selected temperature was reached. The temperature was additionally controlled by a second thermometer during the incubation time. The heating treatment at 50 °C reflected the temperature reached by the fruit during the industrial drying process, while 100 °C was selected as boiling temperature, the same used for boiled chestnut preparation. After heating, 1–2 milligrams of the extract were weighted and solved in a mix of water and DMSO (3:1). The highest heating time (6 h)

at 100 °C led to carbonized insoluble particles, thus the extracts were centrifuged after the addition of the solvent to remove insoluble residues.

Statistical analysis

All data are expressed as mean ± s.d.; data were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni as post-hoc test. Statistical analyses were done using GraphPad Prism 5.0 software (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant. IC_{50} was calculated using GraphPad Prism 5.00 software.

Results and discussion

Part 1: study on chestnut from *Castanea sativa* Mill.

Effect of chestnut extracts on IL-8 release induced by TNFα

The present study followed a bio-guided approach, namely evaluating a potential target of inhibition to outline biological and then chemical differences among *Castanea sativa* Mill. Extracts; yields of extraction are summarized in **Tab 1.**

Tab. 1. Yields of extraction of chestnuts from *C. sativa* Mill.

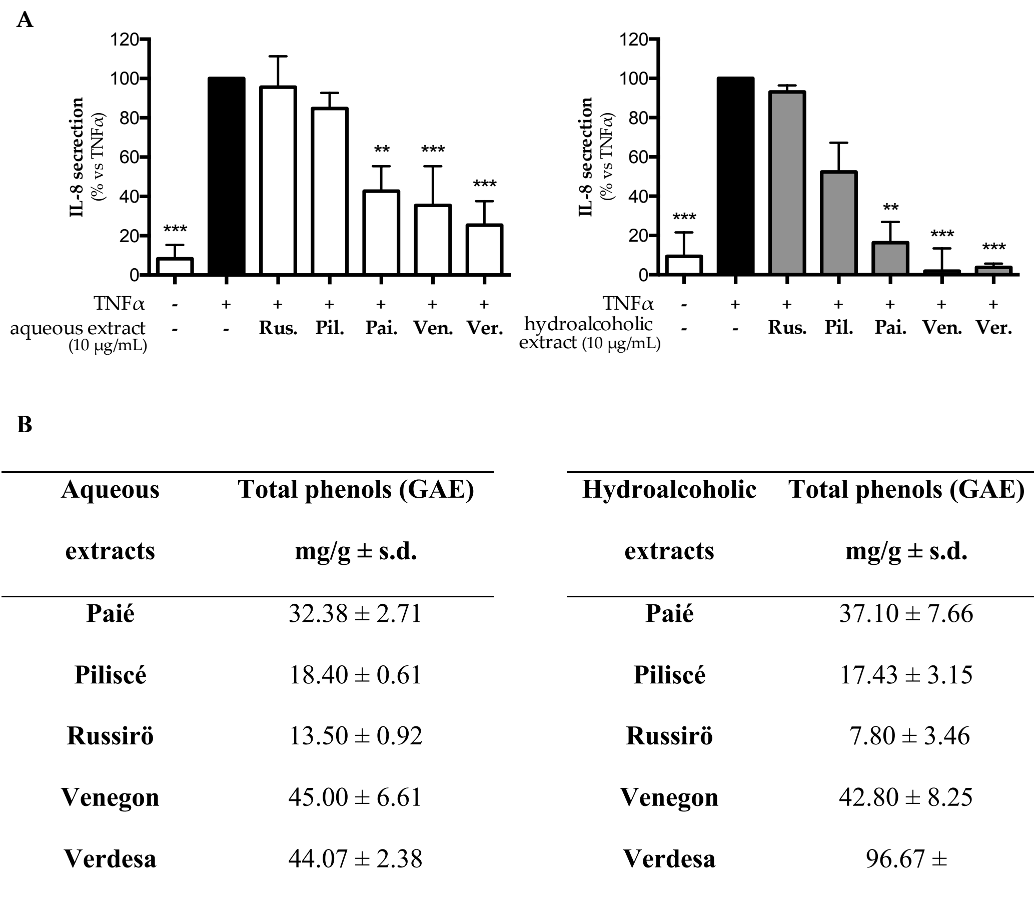
***C. sativa* Mill. Yields (% w/w)**	**Solvent of extraction**	**var. Venegon**	**var. Verdesa**	**var. Paié**	**var. Russirö**	**var. Piliscé**
Whole fruit	Water/ethanol	12.60	8.4	8.00	11.96	12.97
	water	11.40	16.3	10.3	11.10	12.19
Pericarp	Water/ethanol	6.00	3.92	3.3	3.48	3.48
Episperm	Water/ethanol	21.00	27.9	16	0.64	5.16
Endosperm	Water/ethanol	14.50	16.50	9.6	6.52	15.28

The chosen biomarker of gastric inflammation was IL-8, which is released by gastric epithelial cells after *H. pylori* infection as well as TNFα induction (Yasumoto 1992, Crabtree 1994).

For the first step, aqueous and hydroalcoholic extracts from whole chestnut were tested in AGS cells at the same concentration of 10 μg/mL against TNFα-induced IL-8 release. The variety of origin represented a main factor for the efficacy of the extracts, since Venegon, Verdesa and Paiè, but not Piliscè and Russirö inhibited IL-8 release (**Fig. 8A**). In parallel, total phenol content ranged from 1

to 10% of the extracts and correlated with the inhibitory effect (**Fig. 8B**), thus suggesting the importance of polyphenols for the biological activity of chestnut.

A



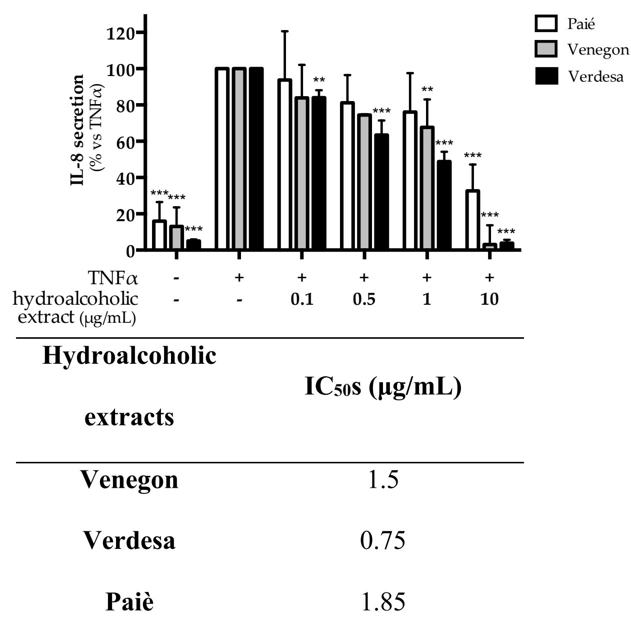
B

Aqueous extracts	Total phenols (GAE) mg/g ± s.d.
Paié	32.38 ± 2.71
Pilisсé	18.40 ± 0.61
Russirö	13.50 ± 0.92
Venegon	45.00 ± 6.61
Verdesa	44.07 ± 2.38

Hydroalcoholic extracts	Total phenols (GAE) mg/g ± s.d.
Paié	37.10 ± 7.66
Pilisсé	17.43 ± 3.15
Russirö	7.80 ± 3.46
Venegon	42.80 ± 8.25
Verdesa	96.67 ±

Fig. 8 (A) *Effect of aqueous and hydroalcoholic extracts (10 µg/mL) from chestnuts of 5 varieties on IL-8 release induced by TNFα in AGS cells.* ***(B)*** *Amount of total phenols measured for each extract (Folin–Ciocalteu's method), expressed as mg of gallic acid equivalents (GAE)/g extract from chestnuts*

The hydroalcoholic extracts from the most active varieties (Venegon, Verdesa, Paiè) were further investigated in concentration-response experiments ranging from 0.1 to 10 μg/mL. They impaired IL-8 release in a concentration-dependent manner with similar IC_{50s} ranging from 0.75 to 1.85 μg/mL (**Fig. 9)**, thus confirming the relationship between total phenol content and inhibitory effect.



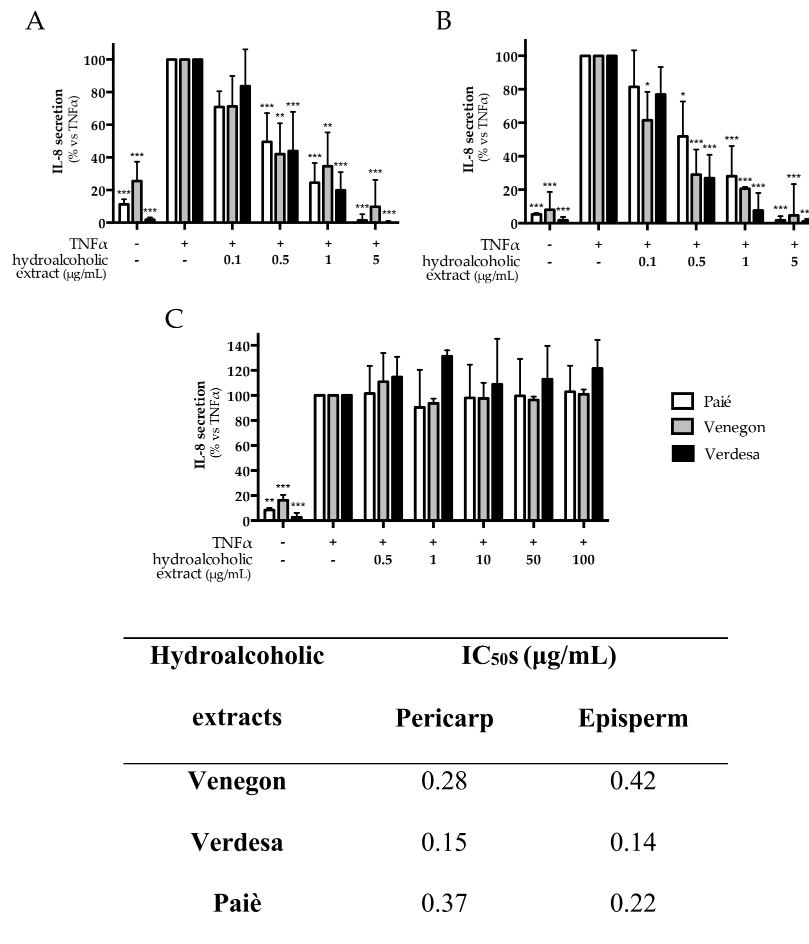
Hydroalcoholic extracts	IC_{50}s (μg/mL)
Venegon	1.5
Verdesa	0.75
Paiè	1.85

Fig. 9. *Concentration-dependent effect of hydroalcoholic extracts from chestnuts on IL-8 release induced by TNFα in AGS cells.*

Biological activity and characterization of chestnut parts

Several authors showed differences in terms of phenolic content and antioxidant activity among the edible part of chestnut (endosperm) and the outer peel (episperm and pericarp) (Ribeiro 2007; Barreira 2008; Vekiari 2008; Zivkovic 2008): in order to understand the contribution of each part of the fruit for the observed biological activity, endosperm, episperm and pericarp were extracted and assayed separately. Chestnut peel components exerted an inhibitory effect on TNFα-induced IL-8 release with a concentration-dependent fashion, thus reflecting in lower IC_{50s} (**Fig. 10A, 10B**) in respect to the whole fruit extract. On the contrary, the edible endosperm displayed no biological

activity at all (**Fig. 10C**), even though the literature suggested at least a minor presence of phenolic compounds in it.



Hydroalcoholic extracts	**IC_{50}s (µg/mL)**	
	Pericarp	**Episperm**
Venegon	0.28	0.42
Verdesa	0.15	0.14
Paiè	0.37	0.22

Fig. 10. *Concentration-dependent effect of hydroalcoholic extracts from chestnuts pericarp (**A**), episperm (**B**) and endosperm (**C**) on IL-8 release induced by TNFα in AGS cells.*

These findings prompted to investigate in detail the nature and distribution of polyphenols in chestnut. As reported in **Tab**. **2**, the outer parts of the fruit were rich in high molecular weight proanthocyanidins, which correlated with the most active varieties of origin. They strongly concentrated in the episperm extract, in particular, ranging from 65 to 92% in the active fruits, while they were undetectable in the endosperm.

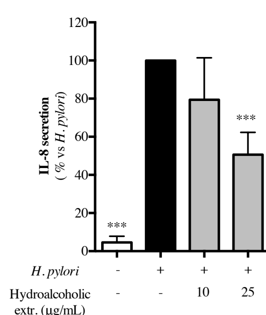
The anti-inflammatory activity was unrelated to the mean degree of polymerization (mDP); as an example, extracts from both pericarp and episperm of Venegon fruits displayed IC_{50} of about 0.15 μg/mL whereas the mDP accounted to 8.2 and 2.9, respectively. Notably, other minor constituents may participate to IL-8 inhibition, namely catechin and gallocatechin (till 0.6%), ellagic acid (from 0.5 to 2.3% in pericarp), isorhamnetin and quercetin glycosides (till 0.17%) and gallic acid (from 0.05 to 0.3% in the episperm). The high content in condensed tannins in inedible parts of the fruit was a peculiar finding, since the rest of the plant is characterized by hydrolysable tannins.

Tab. 2. Phytochemical characterization of hydroalcoholic extracts from endosperm, episperm and pericarp of chestnuts from 5 different varieties.

		LOQ	Paié			Piliscé			Russirö			Venégon			Verdésa		
		mg	End.	Epi.	Per.	End.	Epi.	Per.	End.	Epi.	Per.	End.	Epi.	Per.	End.	Epi.	Per.
Gallic acid	mg/g	0,01	n.d.	3.42	1.98	0.03	1.38	0.89	0.01	1.04	0.74	n.d.	2.65	1.74	0.09	0.49	0.82
Ellagic acid	mg/g	0,10	1.17	1.66	18.19	0.79	4.24	20.97	0.24	4.27	23.61	0.53	3.73	15.54	0.52	1.03	4.97
Vanillin reaction (+) catechin	mg/g	0,1	6.4	387.5	90.4	9.3	114.7	45.9	3.5	7.9	15.8	5.5	303.8	50.6	0.1	676	281.9
Proanthocyanidin B.S. (HMWP)	mg/g	2,0	n.d.	687.3	145.1	n.d.	235.7	76.6	n.d.	4.2	25.6	n.d.	651.4	150.2	n.d.	921	378.9
Flavanol monomers	µg/g		0	1.7	0.9	0	0.6	0.3	0	0.1	0.1	0	1.6	0.6	0	1.3	2.6
Procyanidins dimers	µg/g		0	0.4	0.2	0	0	0.3	0	0	0	0	0.5	0.6	0	0.2	3.5
Procyanidins oligomers	µg/g		0.7	11.1	211.1	0.7	53.1	7.5	0.1	0.7	2.2	0.7	13.4	188.6	0.1	352.4	112.8
mDP	%		1	2.3	11.8	1.3	9.1	3.3	1	1.7	1.8	1.3	2.9	8.2	1.5	13.2	5
p-hydroxybenzoic acid	µg/g	0,50	14.39	38.13	85.8	11.98	29.52	37.76	13.13	69.38	60.82	33.77	30.61	62.71	0.8	3.97	12.22
Vanillic acid	µg/g	0,10	37.95	25.39	60.54	4.55	21.11	22.19	2.57	7.54	11.35	21.57	16.29	45.27	0.92	0.83	3.98
Caffeic acid	µg/g	0,02	1.08	2.48	6.47	0.05	0.18	0.07	n.d.	n.d.	n.d.	0.76	9.56	1.83	0.58	0.02	n.d.
Ferulic acid	µg/g	0,01	25.08	11.76	2.59	1.56	2.07	0.27	0.01	0.25	0.35	21.52	47.47	11.14	8.43	1.85	1.58
Sinapic acid	µg/g	2,00	61.86	127.46	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	56.9	143.2	n.d.	53.43	n.d.	n.d.
t-coutaric acid	µg/g	0,05	41.36	37.42	3.65	5.19	n.d.	n.d.	5.5	1.41	n.d.	47.51	26.78	37.79	8.05	6.65	41.6
t-resveratrol	µg/g	2,00	n.d.	89.37	46.02	n.d.	16.6	12.84	n.d.	n.d.	5.03	n.d.	38.17	30.54	n.d.	36.94	48.5
t-piceide	µg/g	1,00	5.77	25.8	20.82	n.d.	8.02	4.86	n.d.	2.33	3.02	7.38	21.05	16.96	n.d.	3.03	8.87
Phlorizin	µg/g	0,10	n.d.	17.91	34.69	n.d.	1.23	7.78	n.d.	0.12	0.55	n.d.	16.09	53.13	0.52	22.48	13.88
Luteolin	µg/g	0,20	n.d.	1.34	11	n.d.	0.28	5.79	n.d.	n.d.	2.26	0.28	0.4	7.44	n.d.	0.35	9.31
Naringenin	µg/g	0,20	5.75	74.68	47.06	n.d.	3.29	5.19	n.d.	n.d.	0.55	5.14	39.98	54.54	1.09	14.09	20.62
Catechin	µg/g	5,00	n.d.	1533.04	2231.36	n.d.	170.38	213.67	n.d.	11.73	154.43	22.95	1974.71	1648.55	n.d.	286.76	1942
Gallocatechin	µg/g	100,00	n.d.	1642.02	613.7	n.d.	547.02	356.55	141.87	216.17	n.d.	n.d.	1228.7	571.09	n.d.	611.34	486.2
Procyanidin B1	µg/g	20,00	n.d.	249.47	1056.22	n.d.	n.d.	104.56	n.d.	n.d.	59.62	n.d.	507.95	941.71	n.d.	62.12	827.09
Procyanidin B3 (as B1)	µg/g	20,00	n.d.	287.11	336.46	n.d.	n.d.	81.1	n.d.	n.d.	52.88	n.d.	1193.32	319.1	n.d.	69.82	378.94
Taxifolin	µg/g	0,50	n.d.	18.44	18.08	n.d.	n.d.	2.1	n.d.	n.d.	1.02	n.d.	19.67	17.71	n.d.	1.18	13.17
Quercetin-3-Rha	µg/g	0,20	n.d.	0.75	3.02	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.86	7.48	n.d.	n.d.	4.5
Kaempferol-3-Glc	µg/g	0,20	n.d.	n.d.	2.97	n.d.	n.d.	1.03	n.d.	n.d.	n.d.	n.d.	n.d.	1.4	0.43	n.d.	0.47
Isorhamnetin-3-Glc	µg/g	0,10	n.d.	3.94	102.86	n.d.	n.d.	8.48	n.d.	n.d.	0.97	n.d.	1.94	135.92	n.d.	0.2	47.24
Isorhamnetin-3-rutinoside	µg/g	0,20	n.d.	0.97	5.82	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.37	4.48	n.d.	n.d.	0.83
Quercetin-3-glucuronide	µg/g	0,20	15.03	0.69	41.21	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.63	0.28	5.58	n.d.	n.d.	0.63
Quercetin-3-Glc + quercetin-3-Gal	µg/g	0,10	5.25	1.47	23.77	n.d.	0.14	0.53	n.d.	n.d.	0.69	0.43	0.75	10.55	0.29	0.2	4.42

Effect of chestnut extracts on IL-8 release induced by *H. pylori* infection

The promising results shown above encouraged to verify the anti-inflammatory effect against in act *H. pylori* infection. Thus, one of the most active variety (Venegon) was selected to counteract IL-8 release in AGS infected with *H. pylori*. The hydroalcoholic extract from whole chestnut of Venegon variety was inactive till the concentration of 50 µg/mL (data not shown), thus suggesting a weaker activity in this experimental setting. Then, basing on the previous data, an hydroalcoholic extract from chestnut peel (episperm + pericarp) was prepared in order to concentrate the bioactive



components of the fruit. As expected, the inhibitory activity was revealed at higher concentrations in respect to TNFα induction, obtaining a 50% of inhibition at 25 µg/mL (**Fig. 11**).

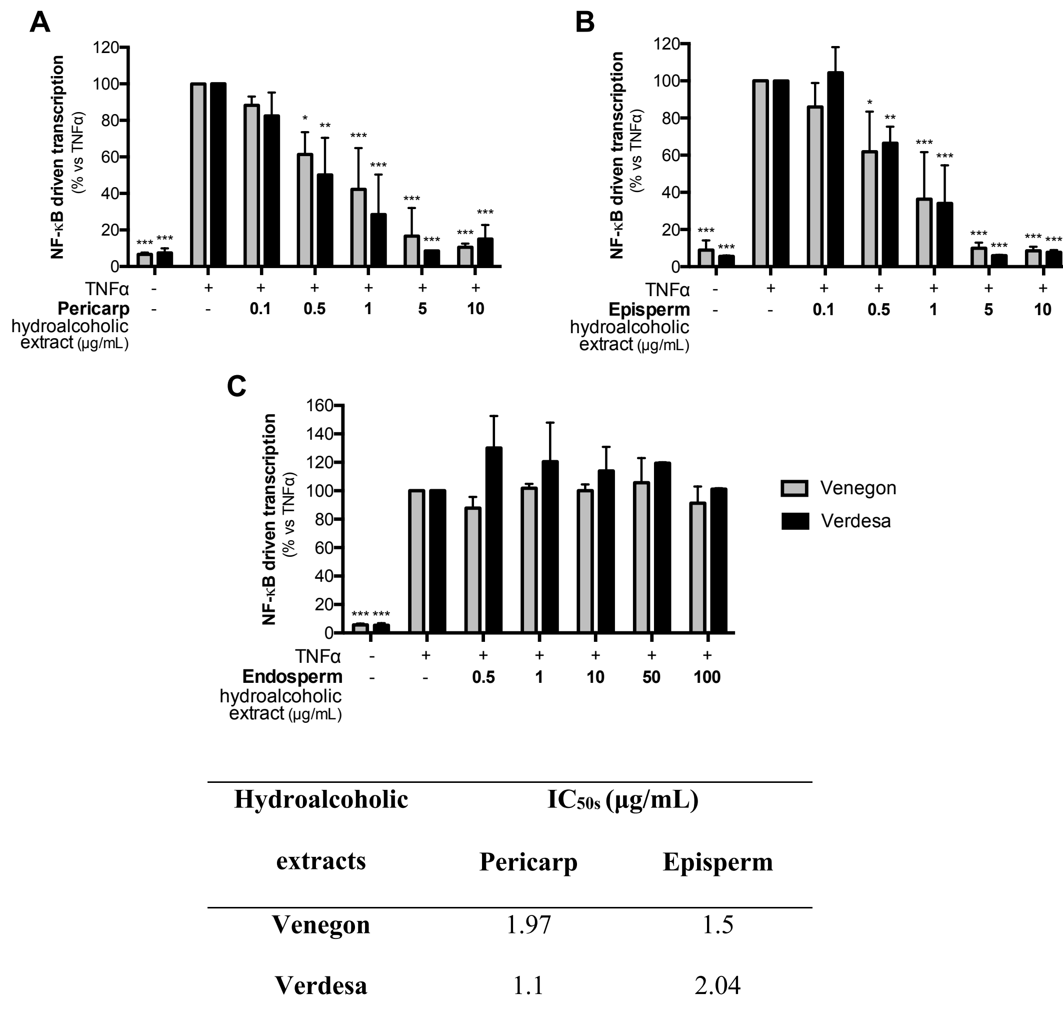
Fig. 11. *Effect of hydroalcoholic extracts from chestnuts peel (pericarp+episperm) on IL-8 release induced by H. pylori in AGS cells.*

Despite TNFα plays a role in the milieu of cytokines amplifying *H. pylori*-related inflammation, Foryst-Ludwig et al. (2000) outlined commons and differences regarding the molecular signaling triggered by the two insults: the observed differences have been mainly identified in the upstream signals and may explain, at least in part, the loss of potency against *H. pylori*-induced IL-8.

However, following experiments were conducted to get more insights on the potential interest of chestnut-derived extracts to counteract the inflammatory cues of gastritis.

A mechanism for the inhibition of *H. pylori*- or TNFα-induced IL-8: the effect of chestnut extracts on the NF-κB pathway

At this point of the experimental work, we speculated about how chestnut extracts may potentially reduce IL-8 levels during either *H. pylori*- and TNFα-mediated inflammation. In fact, NF-κB represents an important crosstalk in the downstream of bacterial and cytokine receptor enhancing IL-8 release (Yasumoto 1992, Crabtree 1994, Foryst-Ludwig 2000). In clinical experiments, nuclear NF-κB co-localizes with IL-8 increase in *H. pilori*-infected mucosa of patients with gastritis (Isomoto 2000). As a consequence, the work focused on the investigation of the NF-κB pathway as a crucial target. Extracts from chestnut peel (episperm and pericarp) from Venegon and Verdesa were able to interfere with the NF-κB driven transcription after TNFα induction in a concentration-dependent manner (**Fig. 12**).



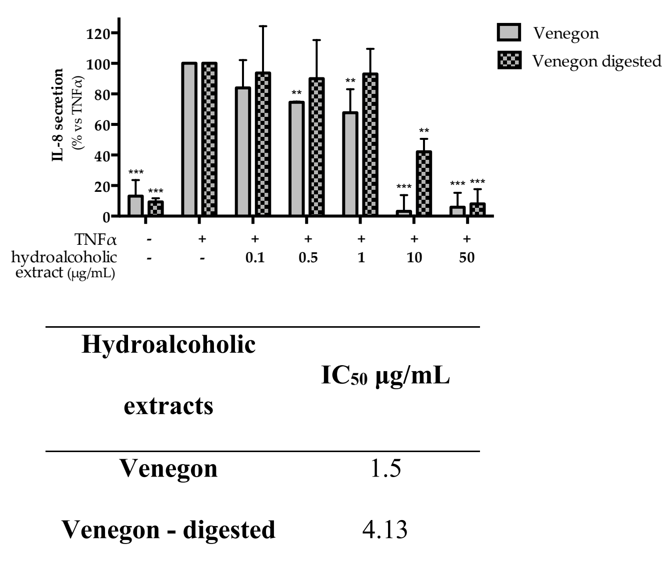
Hydroalcoholic extracts	IC_{50s} (µg/mL)	
	Pericarp	**Episperm**
Venegon	1.97	1.5
Verdesa	1.1	2.04

Fig. 12. *Concentration-dependent effect of hydroalcoholic extracts from chestnuts pericarp (**A**), episperm (**B**) and endosperm (**C**) on the NF-κB driven transcription induced by TNFα in AGS cells.*

IC_{50s} on the NF-κB activity ranged from 1.1 to 2.0 µg/mL, thus suggesting that NF-κB is at least partially involved in IL-8 inhibition.

Effect of extrinsic variables on the biological activity of chestnut extracts

A fundamental aspect to corroborate the potential use of the chestnut against gastric inflammation is the biological stability in the gastric environment. In fact, acid and enzymatic hydrolysis may affect polyphenols and their biological activity in the stomach. Consequently, extract from whole chestnut (Venegon) underwent to simulated gastric digestion and IL-8 inhibition was slightly altered as evident from IC_{50s} comparisons (**Fig. 13)**.



Hydroalcoholic extracts	IC_{50} µg/mL
Venegon	1.5
Venegon - digested	4.13

Fig. 13. *Concentration-dependent effect of hydroalcoholic extracts from whole chestnut on IL-8 release induced by TNFα in AGS cells before and after simulated gastric digestion.*

Accordingly, oral consumption and food processing of chestnut-derived extracts may require heating. Typical processes involved in chestnut consumption or chestnut flour production are drying (50°C), boiling (100°C) and baking (200°C). Therefore, chestnut extract from the varieties Venegon and Verdesa were heated for increasing times (till 6h) and the effect on IL-8 release was assayed again.

Mild temperature was not able to alter the biological activity, while high temperature (100°C) reduced the activity in a time-dependent manner, as shown in (**Fig. 14A, 14B**).

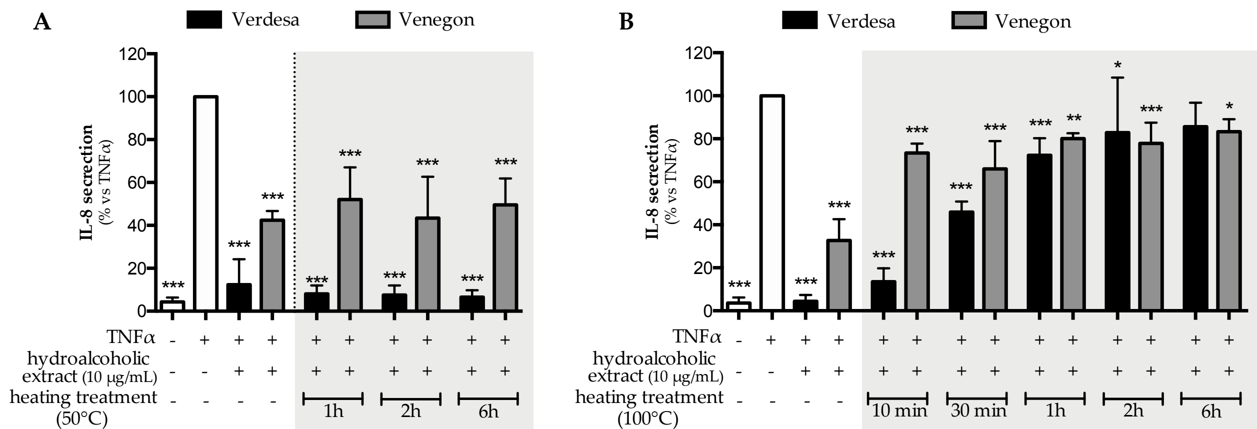


Fig. 14. *Heating treatment of dried hydroalcoholic extracts from chestnuts varieties: (**A**) the extracts inhibit IL-8 secretion in TNFα-treated AGS cells after heating at 50 °C for 6 h. (**B**) time and temperature dependent loss of IL-8 inhibition in TNFα-treated AGS cells during heating at 100 °C.*

A relevant inhibitory effect was maintained till 30 min at 100°C, while it almost completely disappeared after 2h, thus suggesting that short boiling processes may allow residual activity.

Application of the evidences on chestnut bioactivity to chestnut flour production: the biological effect of chestnut by-products

Commercially available chestnut flour is produced starting by the separation and cleaning of the endosperm from the fruit. The endosperm rich in starch is then dried and milled, while the peel is discarded, thus, it actually represents a by-product of food industry. The resulting flour is appreciated for the mild bitter flavor, which characterize salted and sweet food or beer. Notably, chestnut flour is naturally free of gluten, thus resulting useful to formulate substitutive flours for celiac people.

Our discovery on tannins distribution in chestnut prompted to suppose the potential formulation of a functional flour enriched with the episperm, namely the less woody part of peel containing bioactive compounds. In order to translate our idea to the real processes, an extract was directly prepared from

by-product, consisting of the not edible parts (pericarp and episperm) and resulting from mechanical peeling: in line with previous results (**Fig. 10**), data shown in **Fig. 15A** demonstrated the inhibitory activity on IL-8 with IC_{50} of 0.2 μg/mL. Of note, again, in vitro gastric digestion did not affect the biological activity of the extract, as shown in (**Fig. 15B**). As expected, the extract prepared from industrial flour, mainly constituted of endosperm, showed absence of activity (**Fig. 15C**), again confirming previous evidence. These results indicate that the mechanical peeling procedure does not alter the potential biological activity of the not edible chestnut parts.

In view of improving the nutraceutical properties of chestnut flour, we prepared a hydroalcoholic extract from a mixture of flour and episperm, maintaining the ratio found in the fruits (22:3, for endosperm and episperm, respectively). The extract prepared from flour enriched with episperm (final concentration 12%, w:w) showed inhibitory activity with an IC_{50} of 16.35 μg/mL, as shown in **Fig. 15D**. Consequently, we demonstrated that chestnut flour is devoid of any anti-inflammatory activity while industrial by-product retains the ability to inhibit IL-8 secretion. These findings provide experimental evidence in support of the potential use of chestnut peel for the preparation of nutraceuticals and functional foods, such as “fortified” flour. However, the development of a chestnut peel-based functional food may require an organoleptic test and the set of the best cooking process.

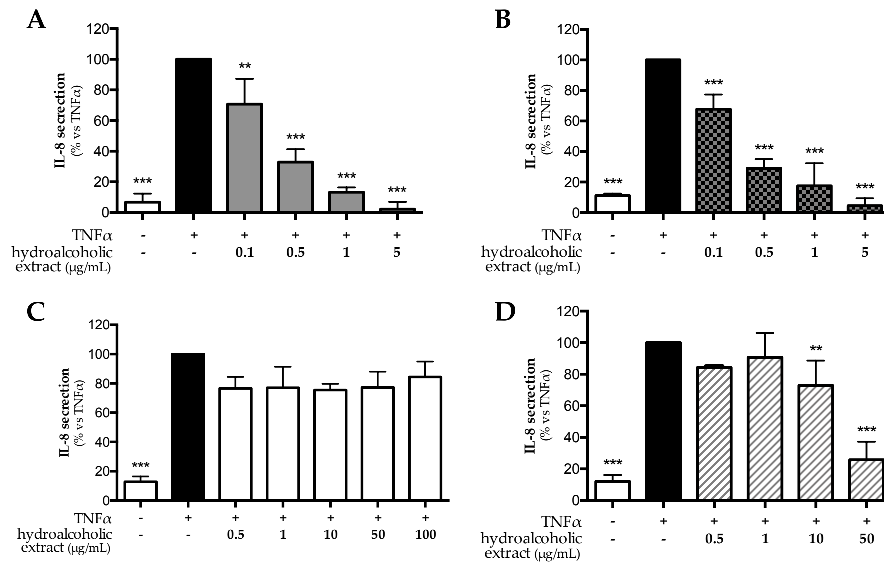


Fig. 15. *Concentration-dependent effect of hydroalcoholic extracts from chestnut peel (episperm + pericarp) on IL-8 release induced by TNFα in AGS cells before (**A**) and after simulated gastric digestion (**B**). Concentration-dependent effect of hydroalcoholic extracts from chestnut flour (milled endosperm) before (**C**) and after enrichment with episperm (**D**).*

Part 2: study on leaves from *Castanea sativa* Mill.

Effect of leaf extracts on IL-8 release induced by TNFα

Leaves and bark from sweet chestnut are well-known sources of tannins, which nature have been deeply investigated in bark only. Bark contains high amount of ellagitannins, mainly represented by castalagin and vescalagin. Of note, the exploitation for wood production requires disruptive processes for the plants, while the collection of leaves should be more sustainable and may integrate with chestnut harvesting procedures.

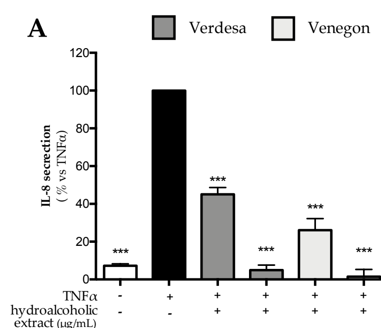
For these reasons the second part of this work focused on the evaluation of the anti-inflammatory effect of leaves extract at gastric level, in parallel with the characterization of tannin content.

Since hydroalcoholic solvent emerged as preferable to the aqueous one for chestnut extraction, it was selected to prepare also leaf extracts. For the purpose, varieties emerging from the previous work, Verdesa and Venegon, were selected for the extraction (**Tab. 3**) and tested against TNFα-induced IL-8 release in AGS cells.

Tab. 3. Yields of extraction of leaf from *Castanea sativa* Mill.

C. sativa **Mill. Yields (% w/w)**	**Solvent of extraction**	**var. Venegon**	**var. Verdesa**
Leaf	Water/ethanol	16.24	21.88

Hydroalcoholic extracts from leaf (10-25 μg/mL) exhibited inhibitory activity on IL-8 release (**Fig. 16A**), which correlated with the presence of high levels of total phenols, namely 289.72 and 265.60 mg/g of extract (corresponding to 29% and 27% w/w) for Verdesa and Venegon, respectively (**Fig. 16B**).



B

Hydroalchoolic extracts	Total phenols (GAE) mg/g ± s.d.
Verdesa	289.72 ± 8.20
Venegon	265.60 ± 18.28

Fig. 16. *(A) Effect of hydroalcoholic extracts from leaf of 2 different varieties of sweet chestnut on IL-8 release induced by TNFα in AGS cells. (**B**) Amount of total phenols measured for each leaf extract (Folin–Ciocalteu's method), expressed as mg of gallic acid equivalents (GAE)/g extract.*

Characterization of leaf extract

The preliminary results motivated a detailed characterization of polyphenols from leaves extracts. UPLC-MS analysis conducted in two different laboratories (NaTuRa lab., University of Antwerp, and Pharmacognosy lab., University of Milan) revealed the presence of ellagitannins discovered in the bark, namely the epimers castalagin and vescalagin, but never reported in leaf before.

Moreover, leaf extract analysis showed other relevant peaks belonging to ellagitannins and gallotannins, whose tentative identification was conducted by manual comparison with natural compounds databases and with the literature about *Castanea* spp. phytochemistry. Among them, the presence of the gallotannin chestanin and the related hydrolysis derivative chesnatin was considered highly probable, since it was already reported in other organs from *Castanea* spp. (Ozawa 1977; Esposito 2009). The structures of the mentioned tannins are depicted in **Fig. 17**.

As a consequence, the amount of castalagin and vescalagin was measured using the available analytical standards and ranged from 1.6 to 2% (sum of the two molecules) in Venegon and Verdesa leaves, respectively.

Regarding other classes of polyphenols, Wagner and Bladt (1983) have already published a TLC-based characterization of chestnut leaf polar extract in their manual: they showed the presence of common phenolic acids and flavonol-glycosides, confirmed in our extracts as well by the same TLC method (data not shown) and further identified by MS analysis: quinic acid, kaempferol glycosides (e. g. astragalin, kaempferol dicoumaroyl hexoside), quercetin glycosides (e. g. hyperoside) and quercetin glucuronides (e. g. miquelianin) were identified. The **Tab. 4** resumes the list of the principal mentioned compounds with molecular weight and relative major MS^2 fragments.

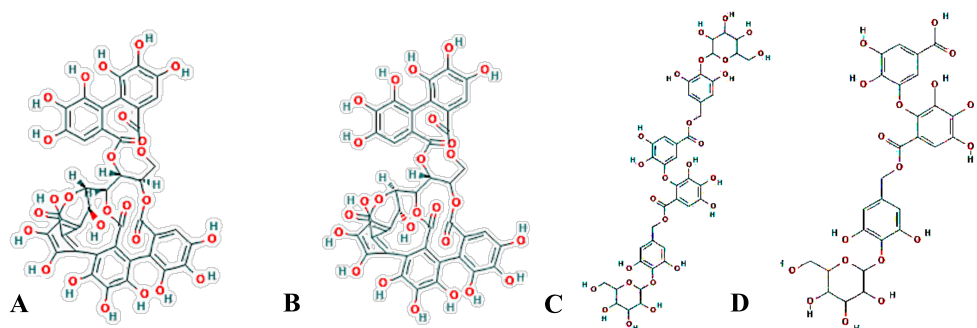


Fig. 17. *Chemical structure of tannins identified in sweet chestnut leaf extracts: (**A**) castalagin (**B**) vescalagin (**C**) chestanin (**D**) chesnatin.*

Tab. 4. List of the main compounds revealed in leaf extracts by qualitative UPLC-qTOF analysis

Tentative identification	rt (min)	M.W.	MS ion $[M-H]^-$	MS/MS ions
Vescalagin/Castalagin	3.68	934	933.0599	467.0 $[M-H]^{2-}$/300.9 [Ellagic ac.-H]$^-$
Chestanin	10.03	938	937.1873	467.0 $[M-H-C_{20}H_{16}O_{13}]^-$/637.1 $[M-H-C_{13}H_{16}O_8]^-$
Miquelianin (Quercetin-3-O-glucuronide)	12.03	478	477.0688	301.0 [Querc.-H]$^-$
Hyperoside (Quercetin-3-O-galattoside)	11.60	464	463.0881	301.0 [Querc.-H]$^-$
Astragalin (Kaempferol-3-O-glucoside)	12.70	448	447.0922	285 [Kaempf.-H]$^-$
kaempferol dicoumaroyl hexoside	16.74	740	739.1870	593.1 [M-H-coumartoyl]$^-$/285.0 [Kaempf.-H]$^-$/453.1 [M-H-Kaempf.]$^-$
Quinic acid	0.71	192	191.0568	-

Implementation of the model to study the impact of natural compounds on the interaction among *H. pylori* and gastric epithelium

Despite the traditional use of tannin-containing plants against ulcers, ellagitannins have been less investigated than proanthocyanidins against *H. pylori*-induced gastric inflammation. In particular, their biological activity may include antibacterial and anti-inflammatory effect which still require pharmacological validation. Our discovery about their presence in sweet chestnut leaves motivated the implementation of the previous *in vitro* model to deeply investigate their role in the interaction among *H. pylori* and gastric epithelium.

With this purpose, the response to *H. pylori* infection in AGS cells was compared with GES-1 cells. The immortalized, non-pathological, GES-1 cell line is a relatively recent *in vitro* model in comparison to the well-established tumoral AGS cell line, which was originated in 1979 according

to ATCC cell bank. Despite the phenotype of GES-1 should represent an advantage to mimic the physiological condition of gastric epithelium, the literature shows few papers, published during the last 10 years, in which GES-1 are applied for inflammatory studies.

As a first step in the characterization of classical inflammatory cues linked to *H. pylori* infection, AGS and GES-1 were compared for TNFα (10 ng/mL) challenge. The PCR array (**Fig. 18A**) revealed that, in basal conditions, both cell lines express very low levels of inflammatory factors, while, in general, the response to TNFα is rather similar. Several NF-κB dependent genes are induced in either AGS and GES-1: examples are CCL20, TNF, LTB, CXCL1-5, CXCL8 (IL-8), CXCL10, and CXCL11. Several exceptions are represented by a subset of NF-κB target genes expressed by GES-1 only, such as IL-6, MMP-9, CCL2 (MCP-1) and IL1B, thus suggesting that NF-κB activation is necessary but not enough to induce gene expression in AGS.

Accordingly, TNF super family (TNFSF) is generally more over-expressed in GES-1 than AGS, even with a downregulation of TNFSF13B in AGS. Again, CXCR2, namely the receptor for CXCL8 and other pro-inflammatory and proliferative CXC chemokines, is over-expressed in AGS and down-regulated in GES-1: these differences may not surprise considering the tumoral nature of AGS.

As a consequence, AGS may lack factors with a key role in sustaining inflammatory processes in response to TNFα, in favor to a proliferative phenotype.

The ELISA assayed on cell culture media confirmed that the chemokine IL-8 is similarly released by AGS and GES-1 (**Fig. 18B**), while IL-6 and MMP-9 are released by GES-1 only (data not shown).

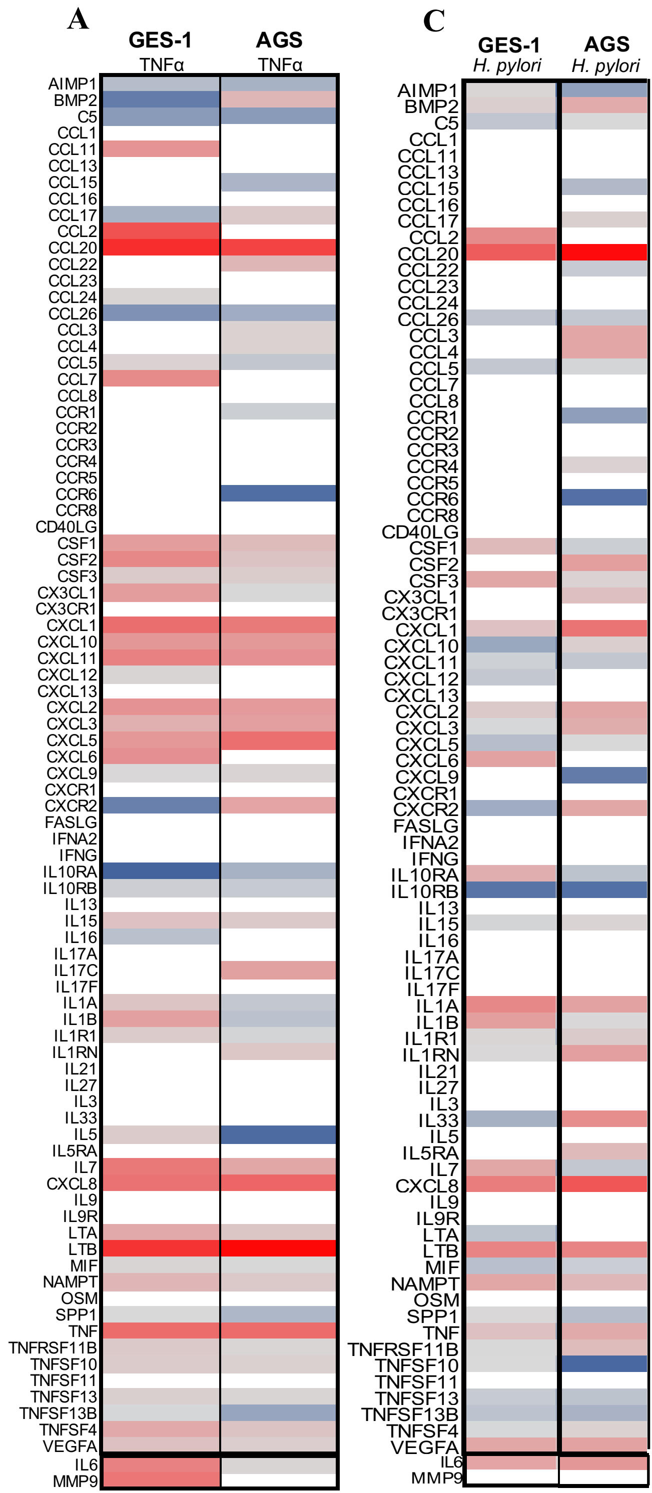
In analogy, AGS and GES-1 were compared for the response to *H. pylori*. The bacteria challenged a similar qualitative response in AGS or GES-1 cells (**Fig. 18C**): the main over-expressed genes were, again, several NF-κB dependent genes like CCL20, CXCL8, IL1A, LTB and TNF for both cell lines. The gene CXCL8 and the antimicrobial gene CCL20 are the most over-expressed, exhibiting a relatively higher expression in AGS than GES-1 cells. However, in this case, several genes were induced in AGS but not GES-1: for example, CCL3, CCL4, CSF2, CX3CL1 and, again, CXCR2;

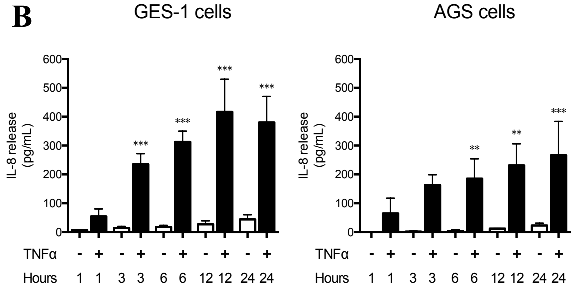
while, in agreement with TNF-α induction, some other genes are induced in GES-1 but not AGS: for example, CCL2 and IL1B.

In agreement with PCR data, CXCL8 was also detected in culture media of the two cells line after 6h of infection (**Fig. 18D**), IL-6 was released by GES-1 only, while MMP-9 was not released (data not shown).

Despite TNFα caused a relatively wider and stronger over-expression than *H. pylori*, CCL20 and CXCL8 are among the most over-expressed genes in both contexts of induction, thus remarking the involvement of common activation pathways, mainly related to NF-κB.

Taken together, the results allowed to compare the inflammatory profile of the two cell lines during *H. pylori* infection or TNFα induction. For first, they confirmed an important overlap for the two inflammatory profiles; secondly, they suggested the lack of fundamental inflammatory factors, e. g. transcription factors, in AGS in respect to GES-1. As a consequence, the data prompted a parallel assessment of the bioactivity of sweet chestnut leaf extracts on GES-1 and AGS, starting by the crucial mediators IL-8 and NF-κB. However, this methodological setting supported the hypothesis that GES-1 cells may represent a more affordable model than AGS for pharmacological studies on *H. pylori*-epithelium interaction.





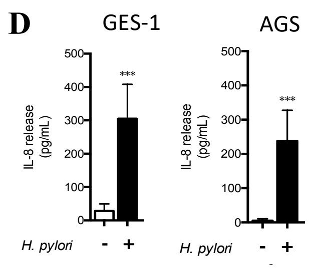
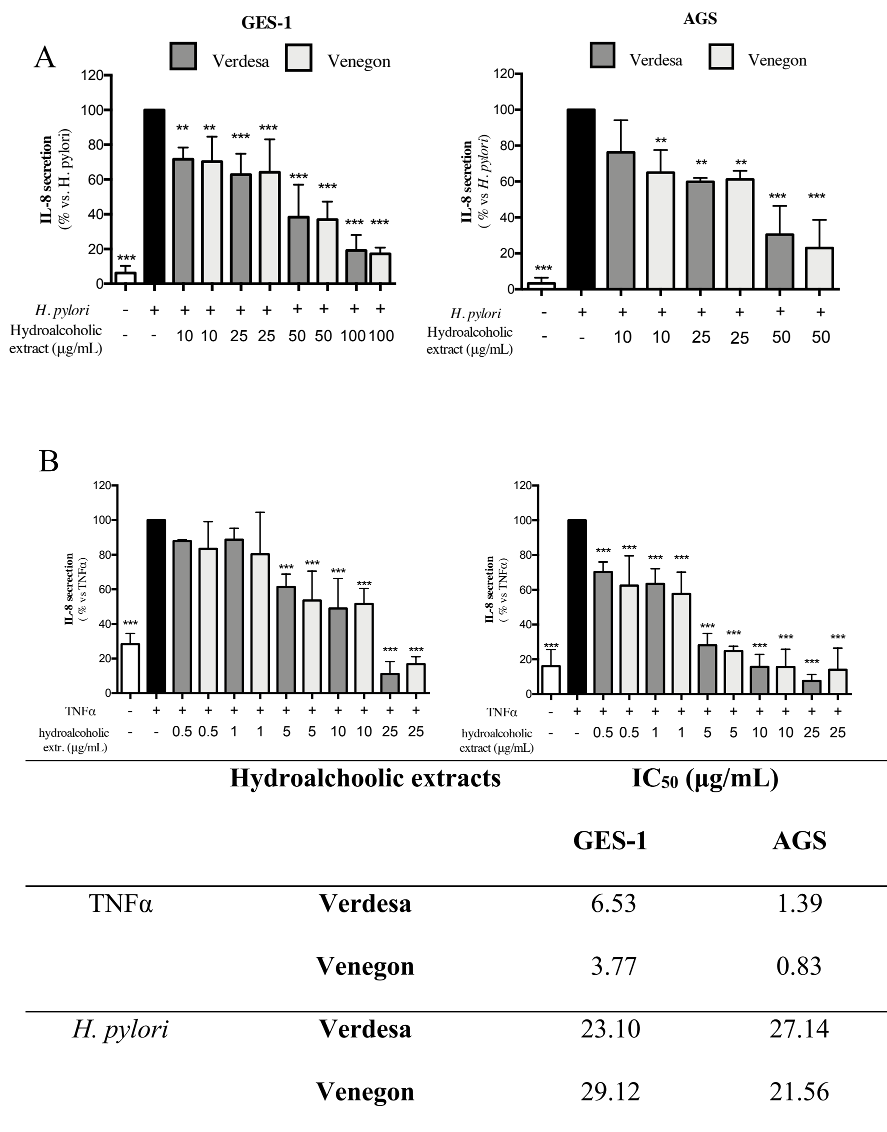


Fig. 18. *Comparison of GES-1 and AGS response regarding gene expression and IL-8 release after TNFα (**A, B**) or H. pylori (**C, D**) induction.*

Effect of leaf extracts on IL-8 release induced by TNFα or *H. pylori*

According with the major role of IL-8 in gastric inflammation and its over-expression in both AGS and GES-1 cells, the hydroalcoholic extracts from leaves (0.5 to 50 μg/mL) were investigated in IL-8 release experiments. They exhibited similar inhibitory activity on IL-8 release induced by *H. pylori* (**Fig**. **19A**) or TNFα (**Fig**. **19B**) in AGS and GES-1. Observing the data for each inflammatory trigger, the biological effect of leaves against TNF-α is more pronounced than against *H. pylori* in both AGS and GES-1. Regarding the specificity of each cell line, the effect of leaves against TNF-α is stronger in AGS than GES-1. On the contrary, the IC_{50}s of leaves extracts are similar in both AGS and GES-1 infected with *H. pylori* (22-29 μg/mL), thus remarking the involvement of a target with crucial role in both cells line. Consequently, the role of NF-κB in the inhibitory mechanism of IL-8 release was investigated, selecting GES-1 cells only.



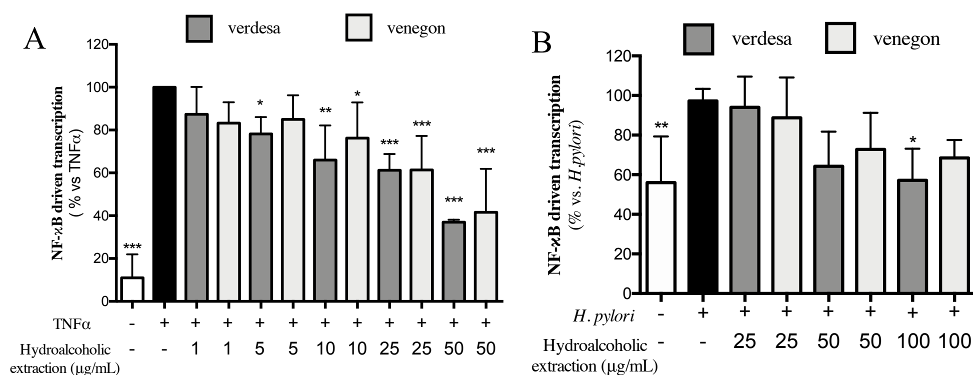
	Hydroalchoolic extracts	IC50 (µg/mL)	
		GES-1	AGS
TNFα	**Verdesa**	6.53	1.39
	Venegon	3.77	0.83
H. pylori	**Verdesa**	23.10	27.14
	Venegon	29.12	21.56

Fig. 19. *Effect of hydroalcoholic extracts from leaf of 2 different varieties of sweet chestnut on IL-8 release induced by H. pylori **(A)** or TNFα **(B)** in AGS and GES-1 cells.*

A mechanism for the inhibition of *H. pylori*- or TNFα-induced IL-8: the effect of leaves extracts on the NF-κB pathway

Extracts from Venegon and Verdesa leaf were able to interfere with the NF-κB driven transcription after TNFα (**Fig. 20A**) or *H. pylori* (**Fig. 20B**) induction in GES-1 cells, in a concentration-dependent manner: IC_{50}s were similar for the two leaf extracts from different varieties.

Under TNFα stimulation, the ratio of NF-κB IC_{50}s with the ones on IL-8 release was almost 10 folds, thus suggesting that NF-κB is at least partially involved in the inhibitory mechanism. Differently, IC_{50s} were similar under *H. pylori* infection, thus remarking the relevance of targeting NF-κB in this specific context.

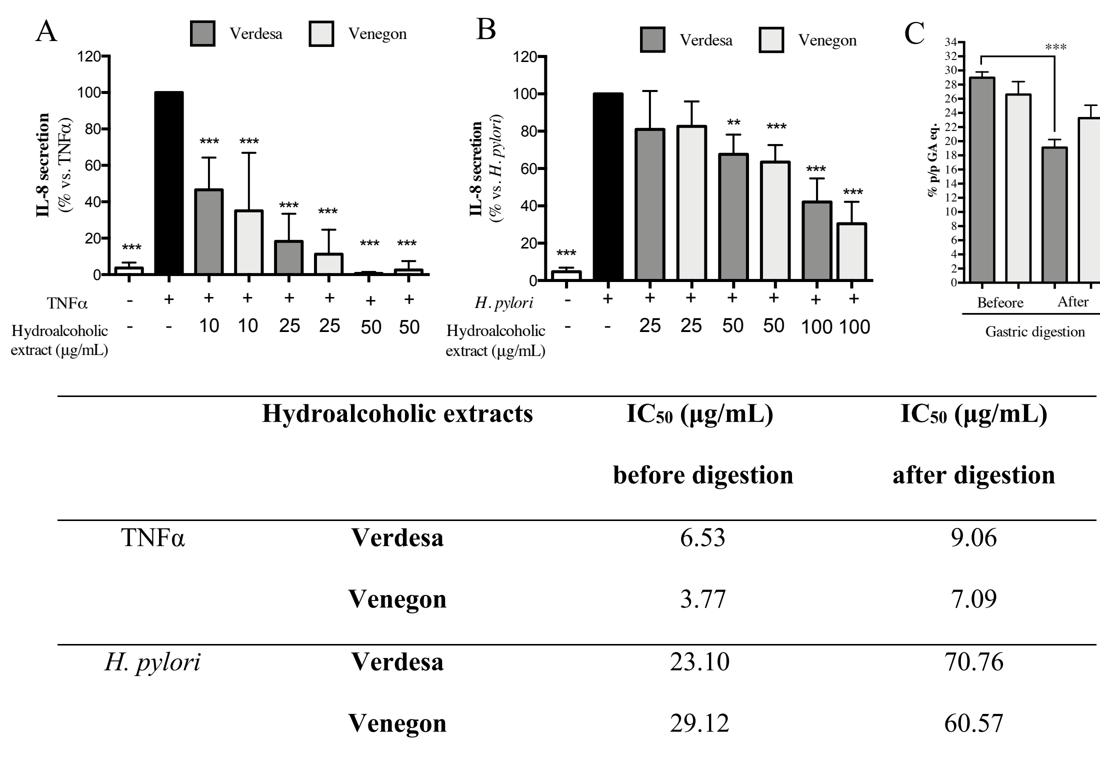


	Hydroalcoholic extracts	IC_{50} (µg/mL)
TNFα	**Verdesa**	25.10
	Venegon	31.46
H. pylori	**Verdesa**	43.48
	Venegon	36.79

Fig. 20. *Effect of hydroalcoholic extracts from leaf of 2 different varieties of sweet chestnut on the NF-κB driven transcription induced by H. pylori (**A**) or TNFα (**B**) in GES-1 cells*

Effect of simulated digestion on the biological activity of leaf extracts

In analogy with the previous work on chestnut (Part 1), the biological stability of leaf extract was evaluated by IL-8 measurement after the gastric simulated digestion. Despite the substantial maintenance of the inhibitory activity, IC_{50s} on IL-8 release displayed a slight increase after the digestive process, either under TNFα (**Fig. 21A**) or *H. pylori* (**Fig. 21B**) stimulation of GES-1 cells. These data paralleled with a moderate reduction in total phenols content (**Fig. 21C**), thus suggesting at least a partial stability of polyphenols responsible for the effect.



	Hydroalcoholic extracts	IC_{50} (µg/mL) before digestion	IC_{50} (µg/mL) after digestion
TNFα	**Verdesa**	6.53	9.06
	Venegon	3.77	7.09
H. pylori	**Verdesa**	23.10	70.76
	Venegon	29.12	60.57

Fig. 21. *Effect of hydroalcoholic extracts from leaf of 2 different varieties of sweet chestnut after gastric simulated digestion on IL-8 release induced by H. pylori (**A**) or TNFα (**B**) in GES-1 cells. IC_{50}s are shown in comparison with undigested extracts. (**C**) Amount of total phenols measured for each leaf extract (Folin–Ciocalteu's method) before and after simulated digestion, expressed as % w/w of gallic acid equivalents (GAE)/ extract.*

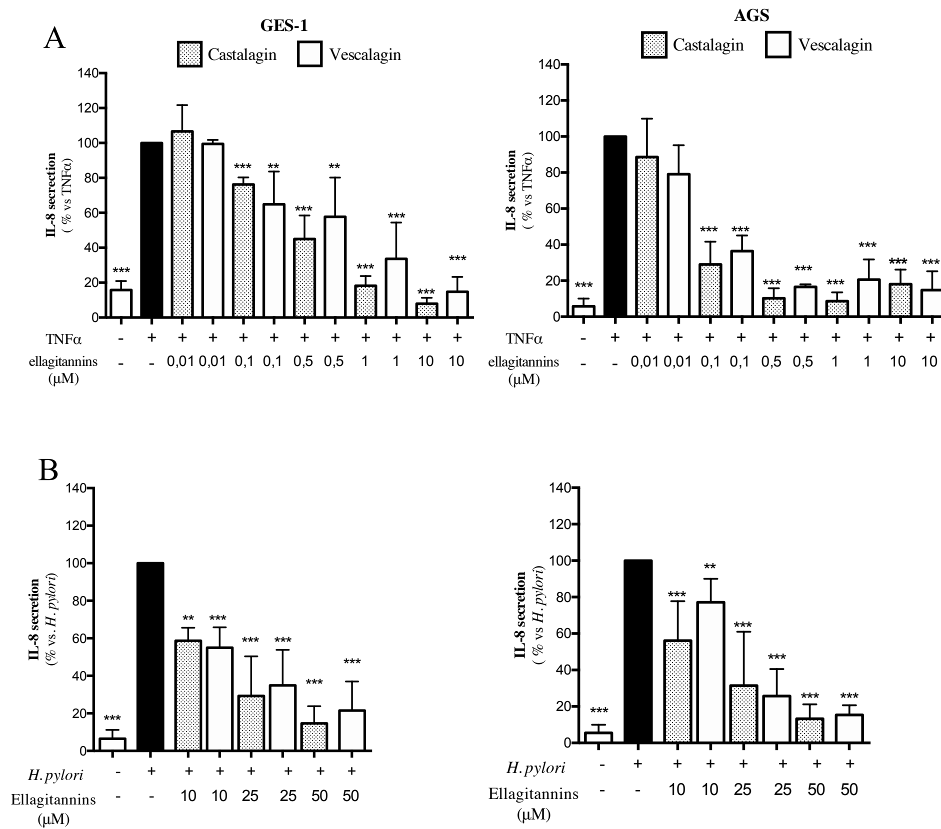
Since ellagitannins should be generally stable in the gastric environment and our analysis detected a relevant amount of them in leaf extracts, the following work aimed to discover the role of isolated ellagitannins castalagin and vescalagin for the bioactivity of the total extract.

Biological activity of ellagitannins from leaf extracts

According to our analytical data, the structure and the quantity of polyphenols in chestnut or leaf extracts was completely different. The very high level of proanthocyanidins concentrated in chestnut peel extract allowed to attribute them most of the biological activity to this class of polyphenols. Conversely, extracts from leaf contained hydrolysable tannins, including ellagitannins. Of note, ellagitannins are less investigated than proanthocyanidins for the bioactivity at gastric level: consequently, we pointed out castalagin and vescalagin, the two major ellagitannins in sweet chestnut, as candidate compounds.

Accordingly, only one previous study showed that castalagin (50 mg/Kg) may prevent ethanol-induced gastric ulcer (Khennouf 2003), but the mechanism of action was not explained. Moreover, castalagin and vescalagin have never been investigated in the specific context of *H. pylori*-related inflammation before.

In our experiments, the two compounds exhibited similar strong inhibition of IL-8 release in AGS and GES-1 cells, with a concentration-response fashion: IC_{50}s against TNFα (**Fig. 22A**) ranged from 0.04 µM (AGS) to 0.2 µM (GES-1), while IC_{50}s against *H. pylori* (**Fig. 22B**) ranged from 10.9 to 15.6 µM.



	Ellagitannins	**IC_{50} (µg/mL)**	
		GES-1	**AGS**
TNFα	**Castalagin**	0.23	0.04
	Vescalagin	0.19	0.04
H. pylori	**Castalagin**	10.86	11.26
	Vescalagin	11.38	15.56

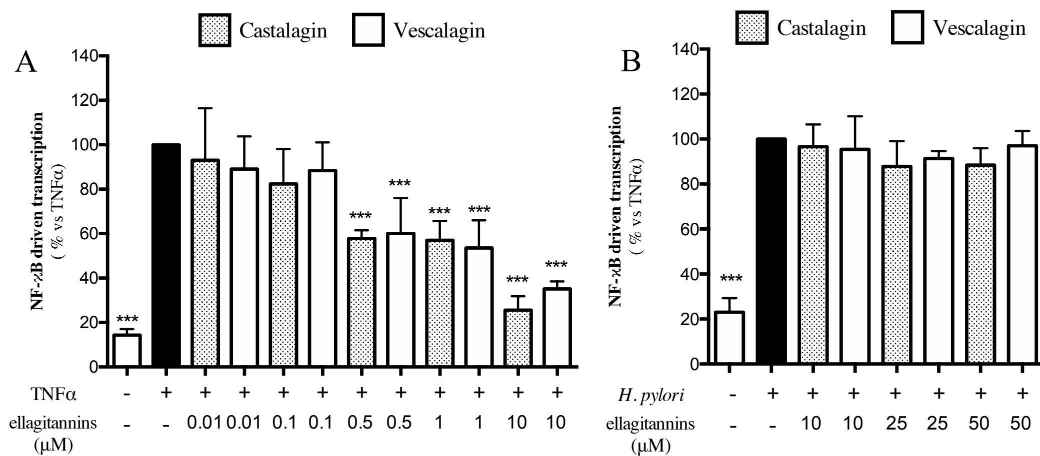
Fig. 22. *Effect of castalagin and vescalagin from sweet chestnut leaf extract on IL-8 release induced by TNFα (**A**) or H. pylori (**B**) in AGS and GES-1 cells.*

In analogy with related leaf extracts (**Fig. 19**), ellagitannins exhibited higher inhibitory potency against TNFα than *H. pylori*, in both cell lines. Another intriguing analogy among leaf extracts and ellagitannins is that the potency of inhibition against TNFα in AGS cells is higher than in GES-1, without a parallel with *H. pylori*. In fact, *H. pylori*-induced IL-8 release was impaired with similar IC_{50}s in the two cell lines.

From the point of view of cells, these observations paralleled with several aspects from previous PCR data: firstly, ellagitannins and leaf extract may act through NF-κB-dependent mechanisms, but not exclusively, thus involving a wide spectrum of regulatory factors which participate in the inflammatory response induced by TNFα, regardless of the gastric cell line; secondly, the possible presence of regulatory factors in GES-1, but not AGS, necessary to sustain the response to TNFα and thus reflecting in higher IC_{50}s against IL-8 in GES-1.

From the point of view of leaf extracts, these analogies may suggest that the inhibitory mechanism is acquired by related ellagitannins and should include NF-κB impairment, but not exclusively.

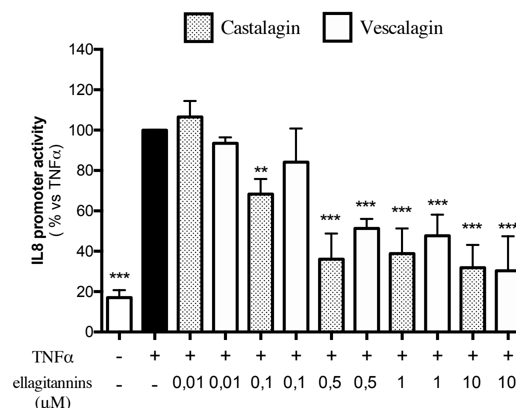
With the aim of understanding the effect of ellagitannins on crucial targets, the activity on NF-κB was assessed, focusing on GES-1 model. Castalagin and vescalagin were able to inhibit the NF-κB driven transcription in GES-1 induced by TNFα, with IC_{50s} of 0.71 and 0.85 μM, respectively (**Fig. 23A**), while the higher concentration tested of 50 μM was not able to inhibit *H. pylori*-induction (**Fig. 23B**). As a consequence, ellagitannins correlated with leaf extract for NF-κB activity inhibition, at least during TNFα induction. Of note, the IC_{50} was again higher than the ones on IL-8 release (0.2 μM), in analogy with leaf extracts.



	Ellagitannins	IC_{50} (μM)
TNFα	**Castalagin**	0.71
	Vescalagin	0.85
H. pylori	**Castalagin**	/
	Vescalagin	/

Fig. 23. *Effect of castalagin and vescalagin from sweet chestnut leaf extract on NF-κB driven transcription induced by TNFα (**A**) or H. pylori (**B**) in GES-1 cells.*

To obtain more details at transcriptional level, ellagitannins were assayed for TNFα-induced IL-8 promoter activity, involving NF-κB, AP-1 and C/EBP responsive elements. Once again, ellagitannins impaired IL-8 promoter activity in GES-1 cells (**Fig. 24)**. Of note, IC_{50}s were more representative for IL-8 release inhibition than NF-κB activity impairment, thus suggesting a secondary role for NF-κB-independent inhibitory mechanisms.



	Ellagitannins	IC_{50} (µM)
TNFα	**Castalagin**	0.18
	Vescalagin	0.34

Fig. 24. *Effect of castalagin and vescalagin from sweet chestnut leaf extract on IL-8 promoter activity induced by TNFα in GES-1 cells.*

For example, castalagin and vescalagin could impair IL-8 also by modulating transcription factors other than NF-κB or acting on IL-8 mRNA stability through p38 MAPK inhibition, as suggested by Iwatake and colleagues in different cellular models (Iwatake 2015). In analogy with these data, similar inhibitory values on NF-κB and IL-8 release were observed in a previous paper from our group concerning agrimoniin and sanguiin-H6 from *Fragaria* spp. (Fumagalli 2016).

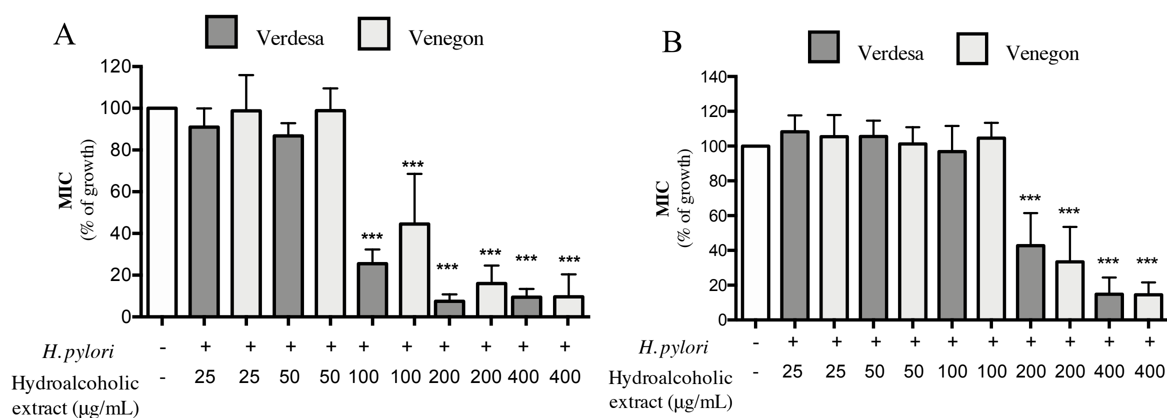
However, focusing on the main aims of the present work, the experiments demonstrated a correlation among the nature of the biological effect of leaf extracts and castalagin/vescalagin, thus corroborating the idea that ellagitannins play an important role for the inhibitory mechanism.

Conversely, the amount of ellagitannins in the extracts (till 2% in Verdesa variety) and their IC_{50s} on IL-8 and NF-κB could not account for the entire bioactivity of the extract, thus suggesting a role covered by other natural compounds and/or a synergistic effect.

Antibacterial and anti-adhesive effect of leaf extracts

Beside the anti-inflammatory effect, leaf extracts and related ellagitannins, castalagin and vescalagin, may also counteract bacterial infection acting on the interaction among *H. pylori* and cell. In fact, ellagitannins may exert direct anti-bacterial effect on *H. pylori*, according to previous studies from Funatogawa et al. (2004).

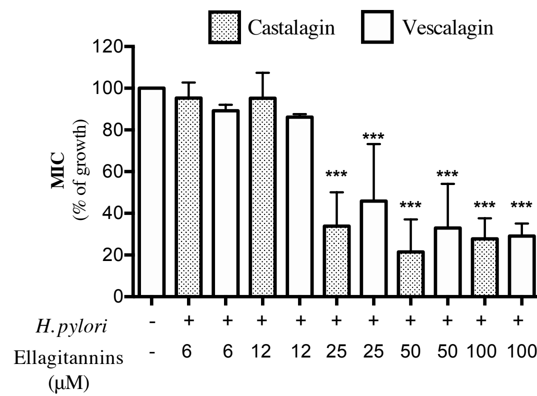
The inhibition of *H. pylori* growth by leaf extracts was assayed by dilution method obtaining a MIC of 100 µg/mL (**Fig. 25A**). The effect was maintained also after gastric simulated digestion with a relatively higher MIC of 200 µg/mL (**Fig. 25B**).



Hydroalcoholic extracts	MIC (µg/mL) before digestion	MIC (µg/mL) after digestion
Verdesa	100	200
Venegon	100	200

Fig. 25. *Effect of leaf extracts before (**A**) and after simulated gastric digestion (**B**) on H. pylori growth*

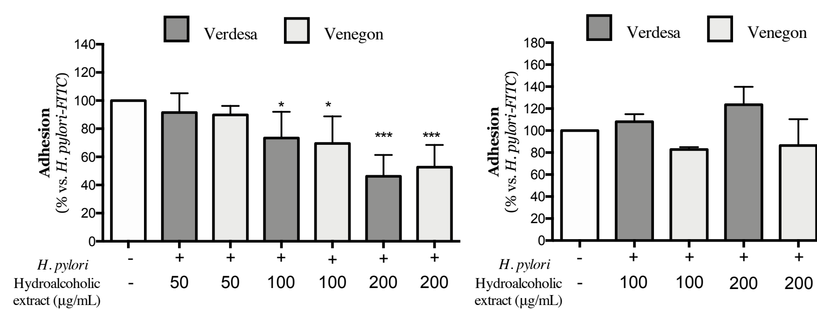
Following the previous approach, castalagin and vescalagin were evaluated in parallel, exhibiting a MIC of 25 µM (**Fig. 26**). Of note, this value reflects the experiments from Funatogawa et al. on casuarinin, a structural analog of castalagin and vescalagin, which showed a MIC among 12,5 and 25 µg/ml. Interestingly, the same paper suggested that ellagitannins may exert higher specificity against *H. pylori* than *E. coli* (MIC>100 µg/ml).



	MIC (μM)
Castalagin	25
Vescalagin	25

Fig. 26. *Effect of castalagin and vescalagin on H. pylori growth.*

Tannins from plants, especially from the class of condensed tannins, have been shown to counteract bacterial adhesion by interacting with bacterial proteins. However, less is known about the preventive effect involving host cells. The treatment of GES-1 cells with leaf extracts for 1h was able to prevent bacterial adhesion at concentrations of 79.18 and 97.26 μg/ml for Venegon and Verdesa varieties, respectively (**Fig. 27A**). The simulated digestion abolished this effect, at least till the higher concentration tested of 200 μg/ml (**Fig. 27B**).



Hydroalcoholic extract	IC_{50} (µg/mL) before digestion	IC_{50} (µg/mL) after digestion
Verdesa	97.26	/
Venegon	79.18	/

Fig. 27. *Effect of leaf extracts before (**A**) and after simulated gastric digestion (**B**) on H. pylori adhesion to GES-1 cells.*

Along with the parallel experiment, showing the failure of ellagitannins in bacterial adhesion impairment (**Fig. 28**), these data suggest that compounds susceptible of gastric degradation, different from ellagitannins, may account for the observed anti-adhesive effect.

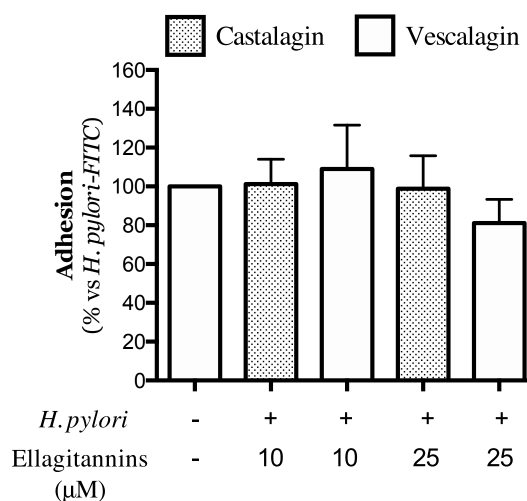


Fig. 28. *Effect of castalagin and vescalagin on H. pylori adhesion to GES-1 cells.*

Conclusions

Sweet chestnut (*Castanea sativa* Mill.) has been used from ancient times to treat ulcers and gastrointestinal disorders, basing on the astringent properties of tannins. Actually, different organs from the plant, such as bark, fruit (chestnut) and leaf are allowed ingredients for the formulation of food supplements in several European countries, including Italy. The poor knowledge about the phytochemistry of sweet chestnut limited the standardization of derived extracts and the development of new health claims.

Of note, extracts containing tannins are well-established remedies for epithelial and mucosal protection, ascribed to antioxidant and anti-inflammatory properties. However, the role of tannins in sweet chestnut extracts have been poorly investigated before. As a consequence, modern pharmacological studies were still needed to validate the traditional use and define the bioactive compounds. In particular, the main open questions regarded whether specific classes of tannins could account for the biological effects and whether the extracts from different varieties and organs of the plant may reflect differences in chemical and biological properties.

The present work aimed to answer those questions through the evaluation of sweet chestnut extracts in the specific context of gastric mucosa inflammation, which is mostly caused by *H. pylori* infection. Our *in vitro* study in gastric epithelial cells demonstrated that chestnut extracts from different varieties may display great differences in tannin content, thus leading to different degree of IL-8 inhibitory effect.

A second surprising result was that the main bioactive constituents of chestnut were concentrated in the outer part of the fruit and were represented by oligomeric proanthocyanidins, namely a well-known class of tannins with potential anti-*H. pylori* and anti-inflammatory properties.

Our data suggested the possible recovery of chestnut peel, which actually represent a by-product of chestnut flour production, for the development of anti-gastritis nutraceuticals like food supplements or a fortified chestnut flour. Of note, the extracts displayed biological stability after gastric simulated

digestion and after a certain degree of thermal stress (30 min, 100 °C), thus suggesting potential effects at gastric level following typical food processing. However, the organoleptic properties of a fortified flour should be elucidated by specific studies, since they may constitute a limit due to the potential bitter taste of the final product.

Curiously, the investigation of extracts from leaf revealed a completely different phytochemical composition, including ellagitannins, gallotannins and flavonols. The ellagitannins castalagin and vescalagin, previously documented in the bark by other authors, were further quantified in leaf and evaluated for their anti-inflammatory properties.

A peculiar effort was added during the investigation of ellagitannins, since less was known by literature about their potential anti-gastritis properties in respect to proanthocyanidins. In particular, the bioactivity was assessed in two gastric epithelial models, tumoral (AGS) and non-tumoral (GES-1) cells, characterized for their response to *H. pylori*. This methodological assessment allowed to verify the bioactivity in a more physiological context (GES-1) of infection.

The work demonstrated for the first time that leaf extracts and related ellagitannins exhibit inhibitory activity on *H. pylori*-induced IL-8 release in both cellular contexts. Moreover, according to our experiments, the potential anti-gastritis effect of leaf may include antibacterial and anti-adhesive properties, the latter not ascribable to ellagitannins.

The relevance of this work regards a number of aspects: the extracts in exam contained widespread dietary compounds, with high safety according to oral consumption with food. The metabolism of the identified tannins has been well-characterized by many authors and may have nor or even positive impact on gut microbiota. This aspect is particularly relevant in relation to the current eradication therapy against *H. pylori*, whose counterpart is represented by microbiota perturbance and increasing cases of antibiotic resistance. In this sense, the translation of our *in vitro* evidence to *in vivo* studies may support the use of sweet chestnut-based nutraceutical to adjuvate the eradication therapy, helping in lowering the courses of antibiotics and control the inflammatory state.

Finally, from an economic and ecological point of view, the valorization of by-products from chestnut production, such as chestnut peel and leaves, may increase the sustainability of sweet chestnut harvest, which is actually under crisis in Europe.

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