UNIVERSITÀ DEGLI STUDI DI MILANO

Dottorato in Scienze Farmacologiche Sperimentali e Cliniche
XXXI ciclo

Dipartimento di Scienze Farmacologiche e Biomolecolari

Strawberry (*Fragaria* X *ananassa* Duch.) tannins as ingredients of nutraceuticals with potential anti-gastritis properties

Settore Scientifico Disciplinare Bio/14

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A.A. 2017 - 2018

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1. INTRODUCTION

1.1 INFLAMMATION

When a tissue receives an injurious stimulus causing death or suffering of some of its cells, it generates a series of tissue and vascular reactions leading to the eradication of the trigger agent, the removal of cellular debris and the repair of the damaged tissue. These processes, which determine an accumulation of fluids and cells in the extravascular space, are defined inflammation.

The inflammatory process aims to: circumscribe the lesion, eliminate the cause of damage and repair damaged tissues to restore their function.

The damaging agents are divided in exogenous factors (viral, bacterial or fungal infections, traumas, environmental toxic substances, etc.) and endogenous factors (tumors, autoimmune diseases, etc.).

Inflammation and tissue repair can become harmful to the organism, causing tissue damage, when the stimulation persists for a long time and the phases of destruction, inflammation and repair are activated simultaneously in an uncontrolled way.

On the basis of the vascular response, cellular infiltrate and nature of the injurious agent, inflammation can be distinguished in acute and chronic. The first is relatively short and characterized by movement of fluids and cells from the vessels to the extra vascular tissues, in order to circumscribe the lesion, to eliminate the causes of damage and to repair the tissues. Chronic inflammation, on the other hand, is a long-lasting inflammation in which active inflammation, tissue destruction and healing occur at the same time. Both are regulated and amplified by a wide range of soluble mediators having plasma or cellular origin, that participate as positive or negative signals to the development of the inflammatory response (Celotti F., 2013).

1.2 GASTRITIS

The stomach is anatomically divided in several portions, called: cardia, fundus, body and antrum. The gastric mucosa is the layer of the stomach which contains the glands and the gastric pits. In humans it is about 1 mm thick and it is constituted of a simple columnar epithelium, lamina propria (thin layer of connective tissue), and muscularis mucosae (a thin layer of muscle, located outside the lamina propria and separated from the submucosa).

The gastric epithelium consists of a monolayer of cells covered by mucus and that invaginate in order to form functional gastric glands or pits, which can be cardiac, oxyntic and pyloric. Cardiac glands are located closest to the oesophagus and lined mostly by mucus secreting

cells; the oxyntic glands in the fundus and corpus of the stomach have chief cells that produce pepsinogen, parietal cells which release hydrochloric acid and intrinsic factor and enterochromaffine-like cells responsible for histamine release; pyloric glands in the antrum contain G and D cells are responsible for gastrin and somatostatin production, respectively.
Gastritis is an inflammation of the gastric mucosa with different clinical, etiological and etiopathogenetic characteristics. They are generally divided in acute gastritis (with infiltration of neutrophils) and chronic gastritis (with infiltration of lymphocytes and plasma cells into the lamina propria) (Celotti F., 2013).

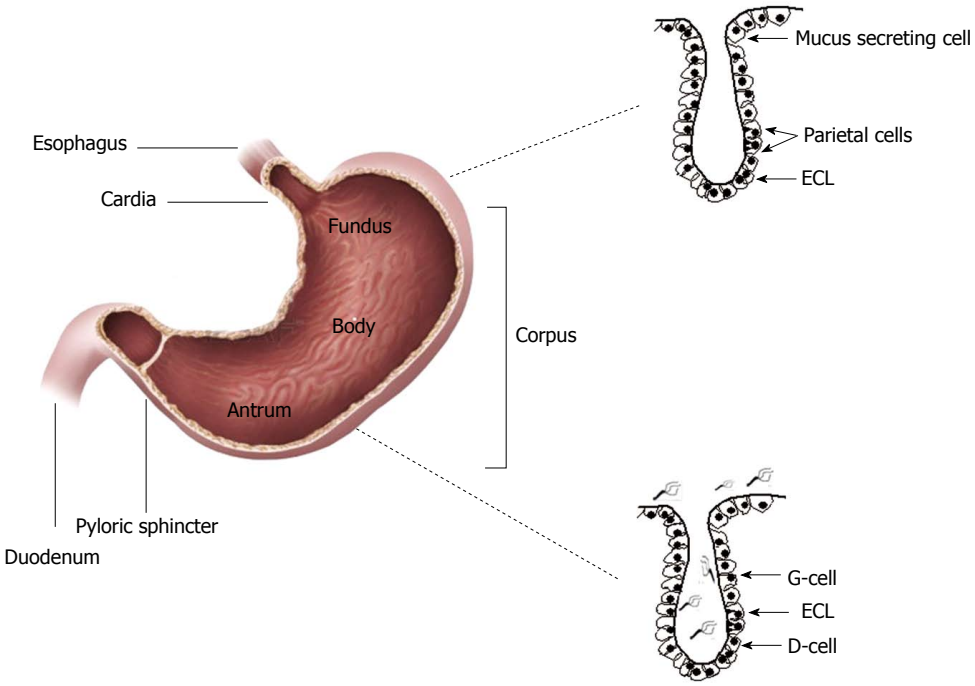


Figure 1. Human stomach and distribution of the different types of cells. (Image from Alzahrani et al. 2014).

1.2.1 Acute gastritis

Acute gastritis is characterized by a diffuse, but transient, inflammation of the mucosa, with possible erosion (loss of the superficial layer of the mucosa), which, although it does not exceed the muscularis mucosae, can cause bleeding.
The most significant clinical symptom is pyrosis (heartburn). The majority of these gastritis resolve spontaneously in a few days with the re-epithelialization of the mucosa. The most important pathogenetic elements are mucosa ischemia, direct damage to the mucosa and hyperchlorhydria. Although in some patients the cause of the disease is not known, it is often associated with stress, use of drugs (NSAIDs and chemotherapeutics), abuse of alcohol, smoking, ingestion of corrosive substances, presence of uremia, duodenal reflux or pathogen infections (mostly induced by *Helicobacter pylori*, in which gastritis evolves in chronic form).

- **Stress**: situations of severe physical stress, such as physical trauma, burns or serious infections, can cause gastritis.
- **Use of analgesic and anti-inflammatory drugs**: high use or abuse of non-steroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylic acid, ibuprofen, naproxen, may lead to the gastric mucosa damage, which can evolve to gastritis or ulcer. The elderly people are the most at risk due to increased vulnerability of the mucosa (senile atrophy) and prolonged contact between NSAIDs and gastric mucosa (delay in gastric emptying).
  These effects are explained by two main mechanisms: the first one is due to direct damage to the gastric mucosa, while the second one is due to the inhibition of COX-1 enzyme, which is responsible for prostaglandins (PGs) synthesis; PGs stimulate, at the gastric level, the mucus and bicarbonate production and participate in the maintenance of adequate local blood flow, thus carrying out a protective effect on the mucosa.
- **Alcohol Abuse:** ethanol is able to irritate and corrode the gastric mucosa, making the stomach more vulnerable to digestive juices.
- **Bile reflux:** bile, the substance that contributes to the absorption and digestion of fats, is produced by the liver and it is released after meals in the upper part of the small intestine. In normal conditions, the pyloric sphincter prevents the bile reflux into the stomach, but in the presence of an altered functionality of this sphincter, bile can reach the stomach causing irritation followed by gastric inflammation.

1.2.2 Chronic gastritis

Chronic gastritis is divided in two different types, called A and B. These two different kinds of chronic gastritis show different etiology, localization and associated symptomatology. Type A gastritis probably has an autoimmune origin, in 90% of cases there are anti-parietal cells antibodies and in 50% of cases anti-intrinsic factor antibodies; it is localized mainly at the level of the body and the fundus of the stomach and is characterized by hypo- or achlorhydria, pernicious anemia (due to the destruction of the parietal cells), hyperplasia of G cells and hypergastremia. Parietal cells produce hydrochloric acid and intrinsic factor, their progressive decrease may produce an increase of the pH of the stomach (hypochlorhydria and achlorhydria) and a loss of the intrinsic factor that compromises the absorption of vitamin B12, causing pernicious anemia.
Type B gastritis is the most common form, it is mainly localized in the antrum of the stomach but, if it persists for a long time (15-20 years), the entire stomach can be involved. Type B gastritis is associated with hypochlorhydria, but not with pernicious anemia. The etiology of

the disease is linked to the presence of *Helicobacter pylori* in the gastric mucosa and the eradication of the bacterium leads to the resolution of histological alterations (Celotti F., 2013).

1.3 GASTRITIS RELATED TO *HELICABACTER PYLORI*

1.3.1 *Helicobacter pylori* infection

Helicobacter pylori (*H. pylori)* is a Gram-negative bacterium, specialized in the colonization of the human stomach (Warren J.R. et al., 1983), a unique ecological niche characterized by acid condition lethal for most microbes. *H. pylori* is so well adapted to this unfriendly environment that, after the first infection which usually occurs early in life, it establishes a life-long chronic infection (Achtman M. et al., 2001). The selection of a niche with no competition and the ability to establish a chronic infection make *H. pylori* one of the most successful human bacterial parasites, which colonizes more than half of the human population (Parsonnet J., 1995).

H. pylori colonization is mainly limited to the antrum of the stomach. The most common consequence of *H. pylori* infection is chronic gastritis, but some infected people are asymptomatic, with moderate inflammation detectable only by biopsy and histology. However, chronic infection may lead to the development of severe gastroduodenal pathologies, including stomach and duodenal ulcers (around 10-15% of infected people), adenocarcinomas and stomach lymphomas (around 1-2% of infected people). The different outcomes of the infection are believed to be substantially influenced by an excessive or inappropriate reaction of the host, bacterial polymorphisms and environmental factors (Go M.F., 1997; Peek R.M. et al., 2006; Parsonnet J. et al., 1991; Moss S. et al., 1992; Ernst P.B. et al., 2000).

In south and east Europe, South America, Asia and Africa the prevalence of *H. pylori* in the population is often higher than 50%. Low socioeconomic conditions have been associated with the infection (Den Hollander W.J. et al., 2013), but the way of transmission is still unclear, probably interpersonal and environmental transmission, such as drinking contaminated water, appear to be the main routes.

The ability of the bacterium to colonize the harsh environment of the human stomach and to induce inflammation is achieved through a combination of virulence factors, including urease, adhesion molecules and toxic proteins (CagA and VacA). Different studies have shown the multiple effects that *H. pylori* has on gastric epithelial cells, such as induction of apoptosis, cell proliferation, and destruction of epithelial cell junctions (Xia H.H. et al., 2001).

❖ **Urease**

H. pylori dedicates several genes to the biosynthesis of a cytosolic urease, a Ni^{2+} containing enzyme (Hu L.-T. et al., 1990; Labigne A. et al., 1991), which hydrolyses urea into NH_3 and CO_2; this enzyme is a dodecamer of six UreA and six UreB subunits. Urea is taken up by *H. pylori* through a proton gated channel (Weeks D.L. et al., 2000). Its hydrolysis by urease generates ammonia that buffers the cytosol and periplasm generating a neutral layer around the bacterial surface. The essential role of urease as a virulence factor is confirmed since urease-defective *H. pylori* mutants cannot colonize the stomach (Eaton K.A. et al., 1991).

Urea is toxic to the bacterium at neutral pH because an unfavorable alkaline environment is generated (Clyne M. et al., 1995). Therefore, the urea channel is regulated positively by protons, opening at acidic pH values to allow more urea in to buffer cytosolic and surface pH, and closing at neutral pH to avoid over alkalinization.

Urease, among the soluble proteins released by *H. pylori*, might also help to recruit neutrophils and monocytes in the inflamed mucosa leading to production of pro-inflammatory cytokines. The local production of cytokines by urease-stimulated monocytes may play a central role in the development of *H. pylori* gastric inflammation (Harris P.R. et al., 1996).

❖ **Flagella**

Although well equipped to survive in strong acid, *H. pylori* is not an acidophile bacterium and needs to leave the lumen, also to avoid discharge in the intestine.

With its flagella, *H. pylori* can reach the thick mucus layer that covers and protects the epithelial lining of the stomach mucosa. Here, propelled by its flagella, the helicoidal shaped bacterium travels across the viscous mucus film.

Mucus has defined permeability properties and acts as a semipermeable barrier that allows the flow of protons from the H^+ releasing oxyntic cells to the stomach lumen, but not backwards. On the contrary, it is poorly permeable to bicarbonate anions. The net result is that there is a large pH gradient from the stomach lumen (strongly acid) to the apical surface of stomach mucosa (only slightly acidic). Non-motile mutants cannot colonize the stomach (Josenhans C. et al., 2001; Bhaskar K.R. et al., 1992).

❖ **Adhesion molecules**

An essential step in the colonization by *H. pylori* and its ability to mediate effects on the gastric epithelium is the establishment of intimate interactions with the epithelial surface. These interactions are largely mediated by outer membrane proteins (OMPs), that serve as

adhesins. The *H. pylori* genome has more than 30 genes which encode OMPs that are divided into Hop (*Helicobacter* outer membrane pro teins) and Hor (hop-related) subgroups. The Hop group of proteins contains *H. pylori* adhesion molecules such as BabA, SabA, AlpA/B, HopZ and OipA (Backert S. et al., 2011).

BabA binds to the Lewis^b antigen of human gastric epithelial cells; this binding enhances the type 4 secretion system's ability to exert the pathogenicity of *H. pylori* that includes triggering production of pro-inflammatory cytokines (Ishijima N. et al., 2011); SabA serves as a receptor for the bacteria, which uses sialic acid binding adhesion protein (SabA), an outer membrane protein, to bind to sialyl-Lewis^x (Mahdavi J. et al., 2002). In the early stages of infection, binding between BabA and Lewis^b is essential; however, with increased inflammation, the expression of sialyl-Lewis^x antigen also increases; thus, *H. pylori* SabA enhances the adherence to the inflamed gastric mucosa (Yamaoka Y., 2008).

❖ *H. pylori* neutrophil-activating protein (HP-NAP)

H. pylori does not invade the epithelial layer, and its components and metabolites have to permeate the epithelial barrier to induce the chemotaxis and activation of inflammatory cells.

A 150-kDa protein oligomer composed of identical 15-kDa subunits, called *H. pylori* neutrophil-activating protein (HP-NAP), was shown to promote the adhesion of human neutrophils to endothelial cells and the production of reactive oxygen radicals (Yoshida N. et al., 1993; Evans D.J. et al., 1995). Purified recombinant HP-NAP, free of LPS, is chemotactic for human neutrophils and monocytes, indicating that it might have a role in the accumulation of these cells at the site of infection. HP-NAP is a powerful stimulant of the production of reactive oxygen radicals and acts through a cascade of intracellular activation events: it includes the increase of cytosolic Ca^{2+} and the phosphorylation of proteins (cytosolic subunits of the NADPH oxidase), leading to the assembly of functional NADPH oxidase on the neutrophil plasma membrane (Satin B. et al., 2000). NADPH oxidase produces superoxide anions and reactive oxygen intermediates (ROIs).

❖ Vacuolating cytotoxin A (VacA)

Vacuolating cytotoxin A (VacA) is a 95-kDa protein of *H. pylori* that induces the formation of large cytoplasmic vacuoles (Cover T.L. et al., 1992). It is an important antigen in the human immune response to *H. pylori*, playing a key role as virulence factor of *H. pylori*, which confers a strong competitive advantage to wild-type strains with respect to VacA-defective mutants in the colonization of the stomach (Salama N. et al., 2001). The toxin is an oligomer.

VacA inhibits the stimulation of T-cell clones, specific for epitopes generated in the antigen processing compartment. *H. pylori* does depress the local immune response because VacA-specific CD4+ T cells are found at low levels in the stomach mucosa of *H. pylori*-infected subjects (D'Elios M.M. et al., 1997); moreover, persistent *H. pylori* infection down-modulates specific CD8+ cytotoxic T cell response, prolonging viral infection (Shirai M. et al., 1998). The inhibition of local antigen processing by VacA could be part of a strategy of survival for *H. pylori* that could significantly contribute to chronic infection of the human stomach.

It binds to the apical portion of epithelial cells and inserts into the plasma membrane, forming a hexameric anion-selective channel of low conductance. These channels release bicarbonate and organic anions from the cell cytosol to support bacterial growth. The toxin channels are slowly endocytosed and eventually reach late endosomal compartments, increasing their permeability to anions with enhancement of the electrogenic vacuolar ATPase (v-ATPase) proton pump. In the presence of weak bases, including the ammonia generated by the *Helicobacter pylori* urease, osmotically active acidotropic ions will accumulate in the endosomes. This leads to water influx and vesicle swelling, an essential step in vacuole formation (Ricci V. et al., 1997; Molinari M. et al., 1997; Papini E. et al., 1997).

VacA toxin alters tight junctions and increases the paracellular route of permeability providing iron, nickel and other nutrients, essential for *H. pylori* growth, from the underlying mucosa (Pelicic V. et al., 1999).

VacA associates with mitochondria and, alone or in cooperation with endogenous proteins, it promotes cytochrome *c* release, leading to cell death by apoptosis (Galmiche A. et al., 2000).

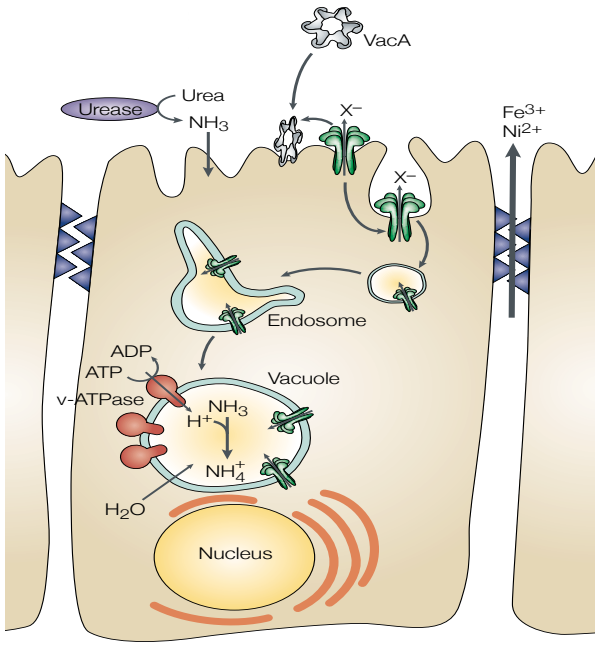


Figure 2. Cellular alterations induced by VacA cytotoxin. (Image from Montecucco and Rappuoli 2001).

❖ **Pathogenicity island (CagPAI)**

H. pylori isolates are classified as *cag*+ and *cag*–, depending on the presence of a pathogenicity island (PAI) of 40 kb, containing ~30 genes, termed cagPAI (Censini S. et al., 1996).

Pathogenicity islands seem to increase the fitness of bacteria in a given environment by providing them with environment-specific functions.

The cagPAI provides *H. pylori* with at least two unique properties: an increased transmission probability and the transformation of what would be an almost commensal into a potential pathogen. Many epidemiological studies show that, in humans, severe gastric diseases are always associated with infection by *cag+* strains (Perez-Perez G.I. et al., 1999; Parsonett J. et al., 1997; Webb P.M. et al., 1999). A major pathogenetic event contributed by the cagPAI is the induction of host cells to release pro-inflammatory chemokines (Crabtree J.E. et al., 1995; Cover T.L. et al., 1992).

The cagPAI region codes for the building blocks of a secretion apparatus, called type IV secretion system, that delivers bacterial proteins directly into the cytosol of host cells (Christie P.J. et al., 2000). Cytotoxin associated gene A (*cagA*), a 128–145-kDa protein that becomes phosphorylated in the cytosol of host cells (Asahi M. et al., 2000; Odenbrait S. et al., 2000), is the only protein known so far to be injected by *H. pylori* into host cells (Covacci A. et al., 1993). It is likely that other *H. pylori* proteins are delivered in a similar way, as the intact type IV secretion system, but not CagA, is required to induce the release of IL-8 from gastric epithelial cells (Crabtree J.E. et al., 1995), although it cannot be excluded that the insertion of the *H. pylori* type IV needle into the host plasma membrane is sufficient to activate endogenous signals. The type IV injection system is composed of a pilus-like structure, which crosses the inner and outer membrane of the bacterium and, like a needle, inserts into the membrane of a host cell to inject *cagA*, and possibly other bacterial proteins, into the cytosol.

The proteins translocated by type IV secretion system trigger several intracellular signaling pathways that result in epithelial cell gene expression as well as the production of pro-inflammatory cytokines and chemokines. A central mediator in the expression of these cytokines/chemokines is NF-κB, a transcription factor that is the convergence point for multiple pathways activated by *H. pylori*. In addition to its role in inducing pro-inflammatory cytokines/chemokines, the target gene Bcl-XL can suppress the mitochondrial apoptosis pathway which may lead to unregulated proliferation. Crabtree and Naumenn summarized

the overall effects of *cag*PAI products in the sequential activation of the IKK complex, JNK, p38 kinase, NF-κB, and AP-1 in gastric epithelial cells (Crabtree J.E. et al., 2006).

1.3.2 *H. pylori* and oxidative stress

The presence of *H. pylori* results in reactive oxygen species (ROS) and reactive nitrogen species (RNS) production by the host in the gastric mucosa; this has been reported to impact upon gastric inflammation and carcinogenesis. Excessive ROS/RNS production has been reported in *H. pylori*-infected human gastric mucosa (Suzuki H. et al., 1996), and correlates well with histological mucosal damage (Davies G.R. et al., 1994) and with bacterial load (Zhang Q. et al., 1997).

Although there are many cell types that can contribute to the production of ROS/RNS, including the epithelial cells, neutrophils primarily contribute at the greatest amount.

Gastric epithelial cells are one source of ROS in the *H. pylori*-infected stomach. Upon stimulation by bacterial cytotoxic factors of *H. pylori* or cytokines (Handa O. et al., 2004), gastric epithelial cells passively produce ROS as by-products of increased mitochondrial respiration rather than as active products. Recently, not only phagocytic cells but also non-phagocytic epithelial cells of the alimentary tract have been reported to express Nox (Sumimoto H. et al., 2005). *H. pylori* LPS not only activate neutrophils to produce superoxide anion, but also increase Nox and Toll-like receptor 4 expression in gastric epithelial cells leading to further production of superoxide anion (Kawahara T. et al., 2001). Bacterial cytotoxic factors of *H. pylori*, such as vacuolating cytotoxin, cytotoxin associated gene product (CagA), and peptidoglycan, also stimulate gastric epithelial cells to cause oxidative stress. In addition, increased ROS production might be involved in acceleration of the cell cycle and subsequent cell proliferation (Handa O. et al., 2007). It has been shown that IL-8 expression, is up-regulated by oxidative stress in gastric epithelial cells (Watanabe N. et al., 1997).

Gastric epithelial cells protect themselves from oxidative stress by activating antioxidant defense mechanisms, including oxygen scavenger enzymes such as SOD, catalase and glutathione peroxidase (Mori M. et al., 1997), as well as vitamins (Vit) E and C (Calvino Fernandez M. et al., 2010).

Although antioxidant systems seem to be involved in infection to counteract the increased ROS (Bagchi D. et al., 1996), when *H. pylori*-induced excessive ROS generation on the epithelium is augmented by reduced effectiveness of antioxidant defenses, the risk of cytotoxicity from oxidation, and DNA damage is potentially increased (Smoot D.T. et al., 2000). An impaired redox balance may then result in cell death, which would increase the

proliferation rate of the remaining cells and thereby increase the chance of mutations leading to increased oncogene expression, which ultimately results in gastric cancer (Obst B. et al., 2000).

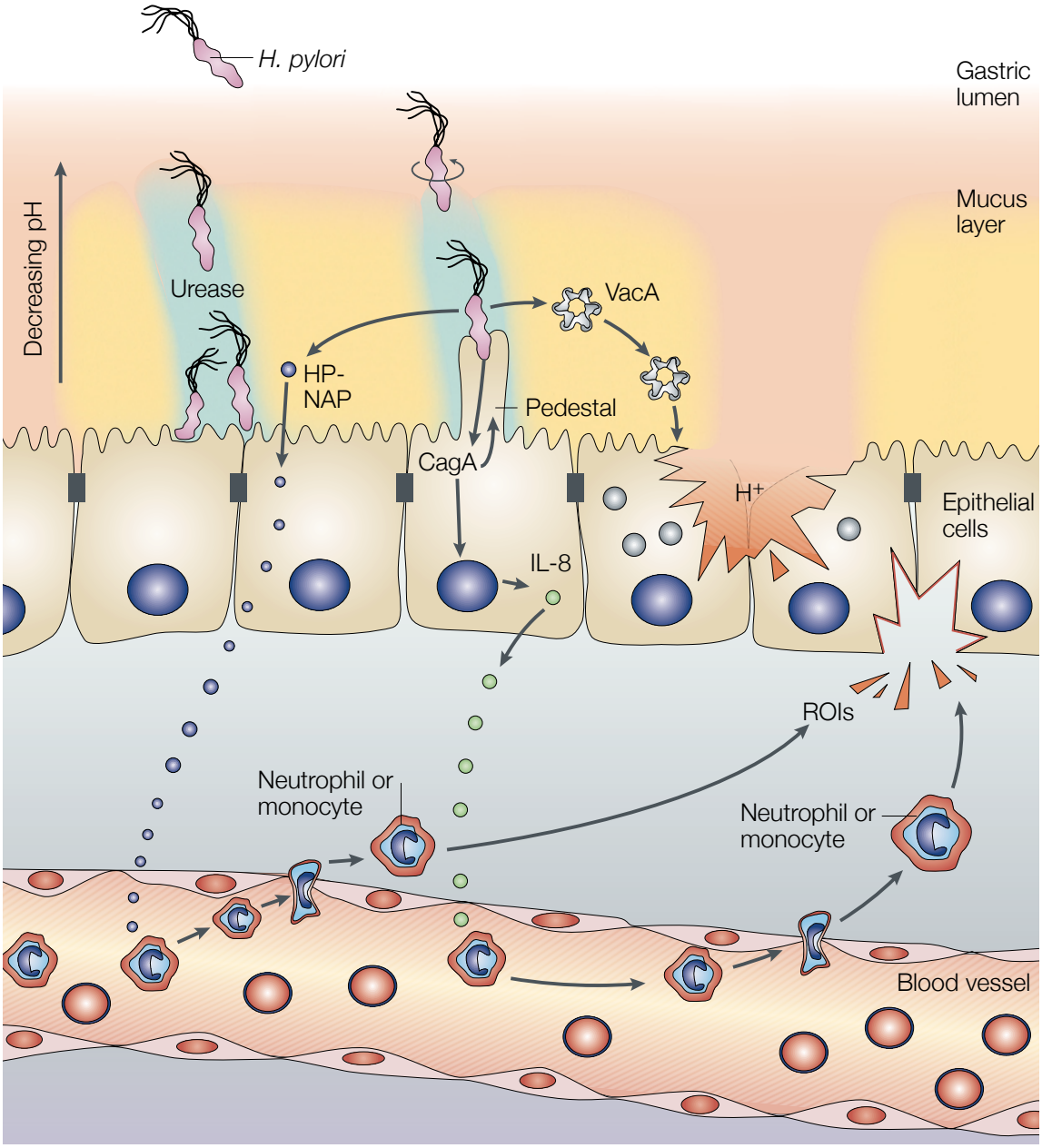


Figure 3. Representation of the stomach mucosa colonized by *Helicobacter pylori*, showing the main virulence factors involved in colonization and disease. During infection, the bacterium is able to enter in the gastric lumen where the urease allows survival in the acidic environment by producing ammonia molecules that buffer cytosolic and periplasmic pH as well as the surface layer around the bacterium. Through its flagella, the bacterium moves into the mucus layer reaching the apical domain of gastric epithelial cells, to which it sticks using specialized adhesins. *H. pylori* then injects the cagA protein into the host cells by a type IV secretion system and releases other toxic factors such as *H. pylori* neutrophil-activating protein (HP-NAP) and VacA. VacA induces alterations of tight junctions and the formation of large vacuoles. The neutrophil-activating protein HP-NAP crosses the epithelial lining recruiting neutrophils and monocytes, which can cause tissue damage by releasing reactive oxygen intermediates (ROIs). Injected Cag proteins cause alteration of the cytoskeleton, pedestal formation and signal the nucleus to release pro-inflammatory lymphokines, which amplify the inflammatory reaction with recruitment of lymphocytes and further induce the release of ROIs. (Image from Montecucco and Rappuoli 2001).

1.4 ROLE OF CYTOKINES IN THE PATHOLOGY

H. pylori is a Gram-negative bacterium strictly involved in the pathogenesis of major gastrointestinal diseases, such as peptic ulcer disease, gastric carcinoma and gastric lymphoma (Parsonnet J. et al., 1994); moreover, as documented in the previous paragraph, this microorganism is associated with gastritis, which is characterized by the infiltration of neutrophils, monocytes, lymphocytes in the gastric epithelium and in the lamina propria (Crabtree J.E. et al., 1996). This bacterium is not invasive, but it is able to induce an inflammatory response through the contact with the surface of the epithelial cells. The local production of chemokines, a group of cytokines with chemoattractant activity, is a very important factor for the recruitment of inflammatory cells at the site of inflammation (Harada A. et al., 1996); many chemokines have been identified, however the number is constantly growing. One of the earliest cytokines reported to be produced by the infected gastric epithelium is interleukin IL-8 (Crabtree J.E. et al., 1994). It is well known that *H. pylori* increases the IL-8 expression both *in vitro* and *in vivo* (Crabtree J.E. et al., 1994). Crabtree et al. reported an increase in IL-8 content in the gastric mucosa infected with *H. pylori*, moreover they reported a study showing the localization of IL-8 in the epithelium of a histological preparation of healthy gastric mucosa and the effective increase of the expression of this cytokine in the infected mucosa (Crabtree J.E. et al., 1993; Crabtree J.E. et al., 1994). Thus, IL-8 production appears as a key point in the gastritis associated with *H. pylori* and epithelial cells are probably the main producers of this cytokine in the gastric mucosa (Shimada T. et al., 1998). Early investigations correlate IL-8 production with CagA protein expression by *H. pylori* (Crabtree J.E. et al.,1994; Odenbreit S. et al., 2002). In a detailed comparison of *H. pylori* strains lacking CagA protein with those expressing the protein, CagA positive strains were reported to induce higher levels of IL-8 mRNA and protein expression by gastric epithelial cells (Crabtree J.E. et al., 1994). Following studies, unexpectedly, showed that deletion of the CagA gene has no effect on the degree of IL-8 induction, suggesting that probably this protein is not directly involved in the mechanism underlining chemokine release. In addition, the presence of a pathogenicity island, which contains several genes (including CagA) has been identified; this set of genes is considered important for the induction of IL-8 expression (Censini S., et al., 1996).

The transcription factor NF-κB, NF-IL6 and the activating protein AP-1 are involved in the regulation of the IL-8 gene transcription (Matsusaka T. et al., 1993); in particular, Sharma et al. demonstrated that IL-8 induction induced by *H. pylori* in AGS gastric cells is regulated via an NF-κB-dependent transcriptional process (Sharma S.A. et al., 1998).

Other cytokines reported to be produced by the infected gastric epithelium include IL-6, tumor necrosis factor alpha (TNFα) (Tanahashi T. et al., 2000), IL-1β, IL-1α, granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1) (Jung H.C. et al., 1997), MIF (Beswick E.J. et al., 2006) and TGF-β (Beswick E.J. et al., 2006).

The lipopolysaccharide (LPS) of *H. pylori* and the release of bacterial proteins stimulate immune cells to produce IL-1β and TNFα (Mai U.E. et al., 1991); these, in turn, are potent inducers of IL-8 expression in many cell types. *In vitro* studies have shown that the IL-8 expression in gastric epithelial cells is up-regulated by these cytokines (Watanabe N. et al., 1997).

Various studies have examined the response of human gastric epithelial cells to purified *H. pylori* urease, since it is the most abundant protein produced by the bacteria. These studies reported that *H. pylori* urease induces the production of IL-6, TNFα and IL-8 by gastric epithelial cells (Tanahashi T. et al., 2000; Beswick E.J. et al., 2006). Interestingly, mucosal IL-6 and TNFα increase in patients suffering from *H. pylori* chronic gastritis (Crabtree J.E. et al., 1991). In a recent study the c*ag* PAI and OipA were examined for their role in IL-6 secretion by gastric epithelial cells and both virulence factors were found to increase the levels of IL-6 production by MKN-28 gastric epithelial cells through different pathways (Lu H. et al., 2005).

Interleukin-17 (IL-17) RNA transcription and secretion have been found to increase in the human gastric mucosa during *H. pylori* infection and its expression is up-regulated in animal stomach after 3 weeks of infection (Kabir S., 2011). Since gastric epithelial cells express IL-17 receptors, IL-17 interaction with its receptor stimulates the epithelial cells to produce IL-8, thus confirming that gastric epithelial cells serve as important contributors for IL-17 and IL-8 synthesis during *H. pylori* infection (Kabir S., 2011). Furthermore, IL-21 and IL-23, which are cytokines that induce and sustain IL-17 production, are found to be upregulated in the gastric mucosa of patients infected with *H. pylori* (Kabir S., 2011).

Another cytokine related to the gastritis caused by *H. pylori* is IL-21. In *H. pylori*-negative patients affected by gastritis, the level of expression of this cytokine is not different from healthy patients, indicating a relationship between the bacterium and the production of IL-21. This relationship is further supported by the fact that the eradication of the bacterium significantly reduces the expression of the cytokine. The treatment of AGS and MKN-28 cells with IL-21 produced an increase of the metalloprotease synthesis, such as MMP-9 and MMP-2 (Caruso R. et al., 2007). Metalloproteases are a family of endopeptidases able to degrade

extra-cellular matrix proteins and remodeling connective tissue (Nagase H. et al., 1999). Patients with *H. pylori*-associated gastritis exhibit an elevated mucosal MMP-9 content, the level of which decreases dramatically following successful *H. pylori* eradication therapy (Bergin P.J., et al. 2004; Kubben F.J.G.M. et al., 2007).

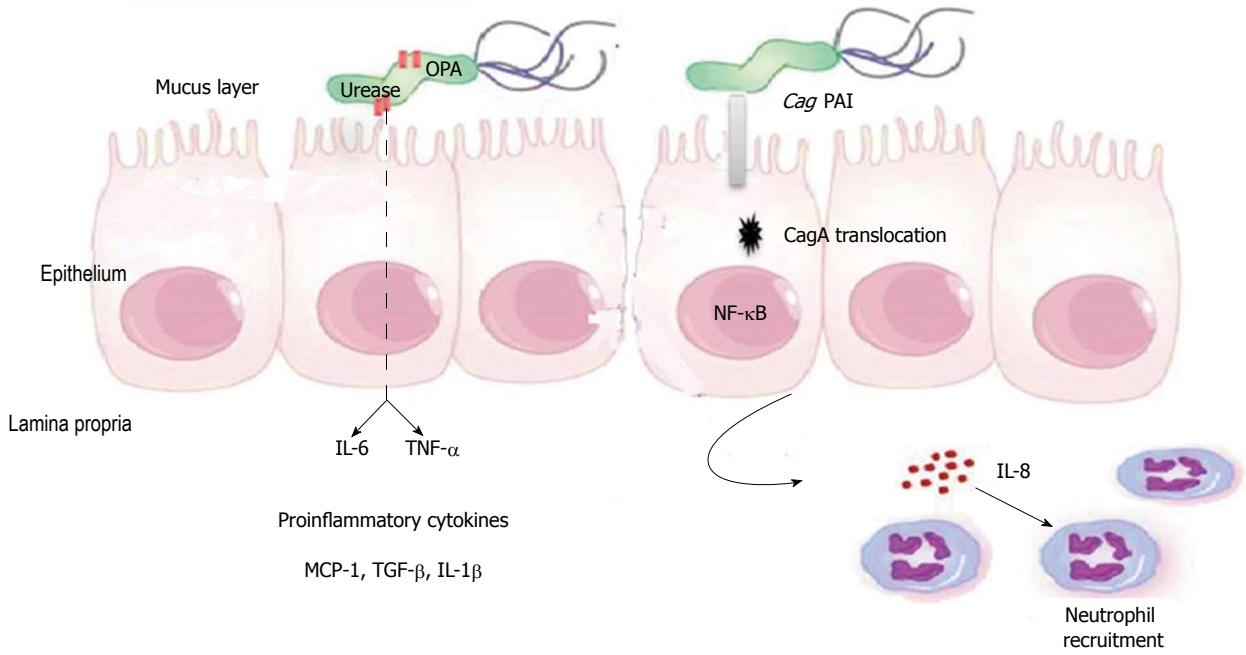


Figure 4. Cytokines production by gastric epithelial cells during *H. pylori* infection. (Image from Alzahrani et al. 2014).

1.5 NF-κB TRANSCRIPTION FACTOR

1.5.1 Structure

Nuclear Factor-kappa B (NF-κB) is a transcription factor family that regulates a large number of genes that are involved in important physiological processes, including survival, inflammation, and immune responses.

Five members of this transcription factor family have been identified, designated as p65 (RelA), RelB, c-Rel, NF-κB1 and NF-κB2. NF-κB1 and NF-κB2 are expressed as the precursors p105 and p100, which are cleaved to the functional transcription factors p50 and p52, respectively (Hoesel B. et al., 2013). The NF-κB members are ubiquitously expressed, but their functionality might depend on specific cellular stimuli (Gilmore T.D. et al., 2011). These transcription factors generally act as homo- or heterodimers, and the combination of every member is possible, although some of them have greater affinity. For instance, due to their structure, p65/p50 and RelB/p52 are the most stable dimers (Huang B.D. et al., 1997; Huang B.D. et al., 2005).

The five members of NF-κB family share an evolutionarily conserved N-terminal region, known as the Rel homology domain (RHD), which is essential for homo- and heterodimerization, nuclear targeting, and binding to DNA or IκB. The C-terminal region, where the transcriptional activation domain (TAD) is located, is not conserved among the NF-κB members because p50 and p52 are products of partial proteolysis, and thus have a glycine-rich region instead of TAD (Nolan G.P. et al., 1993; Wang J. et al., 2011).

In most quiescent cells these dimers are bound to inhibitory molecules of the IκB family of proteins (inhibitors of NF-κB); the IκB family of proteins consists of four members: IκBα, IκBβ, IκBε and BCL-3. These inhibitors are characterized by ankyrin repeats, which associate with the DNA-binding domains of the transcription factors thereby making them transcriptionally inactive. Binding of NF-κB dimers to IκB molecules does not only prevent binding to DNA, but also shifts the steady-state localization of the complex to the cytosol. Nevertheless, shuttling between cytosol and nucleus does occur (Birbach A. et al., 2002; Huang T.T. et al., 2000), which might be a basis for a low basal transcriptional activity of NF-κB given that the IκB/NF-κB complex is subject to dissociation and re-association processes.

Activation of the NF-κB pathway can be initiated by a large number of extracellular stimuli, but they have a similar signal transduction cascade resulting basically from phosphate transfer. This signaling comprises canonical (classical) and non-canonical (alternative) pathways.

1.5.2 Canonical pathway

In the canonical pathway, NF-κB dimers are regulated by inhibitory molecules of the IκB family, which prevent their translocation into the nucleus forming a stable complex. To release the NF-κB complex, signaling pathways are activated by pro-inflammatory cytokine receptors, such as tumor necrosis factor receptor (TNFR), IL-1 receptor (IL-1R) and toll-like receptor (TLR) family members (TLR3, TLR4, TLR7); antigen receptors, such as T cell receptor (TCR) and B cell receptor (BCR); growth factors, such as epidermal growth factor receptor (EGFR) family members. These receptors are able to activate the IκB kinase (IKK) complex, an enzyme complex containing IκB kinases (IKK1/IKKα and IKK2/IKKβ) and at least one non-catalytic accessory protein (NF-κB Essential Modulator, NEMO or also termed IKKγ), which phosphorylates and facilitates the ubiquitination of IκBα (primarily by IKKβ) and its subsequent degradation by the 26s proteasome. The dimers p65/p50 and c-Rel/p50 are then translocated into the nucleus and activate target gene expression (Gupta S.C. et al., 2010; Chen J. et al., 2013).

In the nucleus, the NF-κB complex binds to sequence-specific target DNA, known as κB sites, which are present in promoters of the target genes, assemble with the basal transcriptional machinery, and might associate with other transcription factors, including AP-1 (c-Jun/c-Fos complex) and chromatin remodeling proteins, such as CREB-binding protein (CBP) and p300. In this way NF-κB factor can activate the transcription of different target genes (Hoesel B. et al., 2013).

1.5.3 Non-canonical pathway

Non-canonical pathway originates from different classes of receptors including B-cell activation factor (BAFFR), lymphotoxin β-receptor (LTβR), CD40, receptor activator for nuclear factor kappa B (RANK), TNFR2 and Fn14 (Sun S.C., 2010). These lead to activation of the NF-κB inducing kinase NIK, which phosphorylates and activates predominantly the homodimer IKKα/IKKα. The activity of the latter enzyme induces phosphorylation of p100 resulting in its ubiquitination and partial degradation to p52 (Xiao G. et al., 2001). The mechanisms leading to activation of the non-canonical pathway are thus independent of the activity of IKKβ and NEMO (Dejardin E. et al., 2002).

In many cases, p100 is associated with RelB, so that its proteolytic processing induces the formation of a transcriptionally active RelB/p52-complex (Solan N.J. et al., 2002; Senftleben U., et al., 2001), which translocates into the nucleus regulating the expression of several target genes.

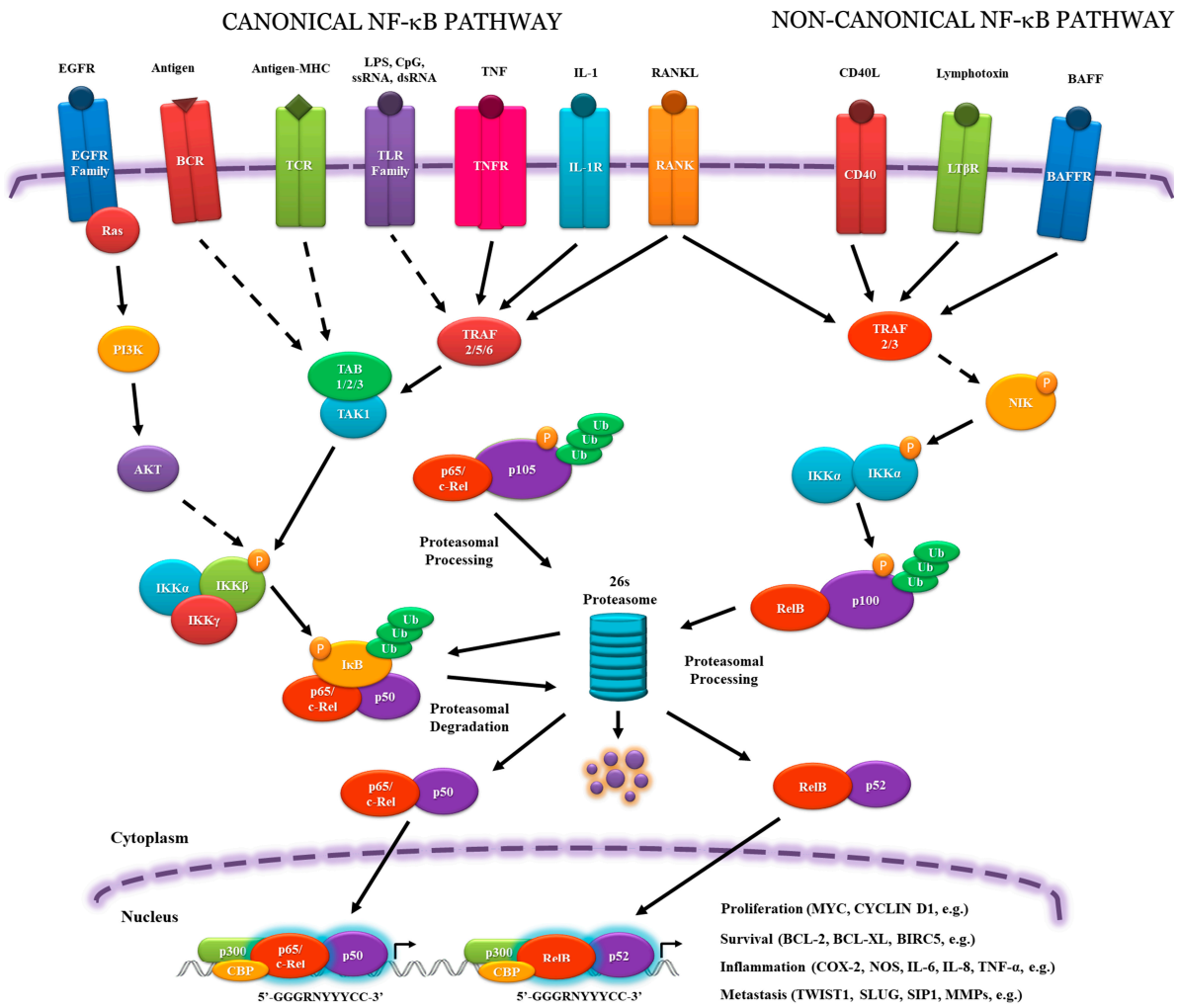


Figure 5. Canonical and non-canonical NF-κB pathways. (Image from Pires R.B. et al. 2014).

1.5.4 Role of the NF-κB in gastric inflammation

Nuclear Factor-kappa B (NF-κB) is a transcription factor family that regulates a large number of genes (more than 150) involved in important physiological processes, including survival, inflammation, oxidative stress response, immune responses and apoptosis. Thanks to the large variety of stimuli able to activate NF-κB and to its ability in regulating the expression of pro-inflammatory cytokines, chemokines, immunoreceptors and adhesion molecules, this transcription factor is generally indicated as "The central mediator of the immune response in humans" (Pahl L.H. et al. 1999).

NF-κB, nuclear factor-IL6 (NF-IL6) and activator protein AP-1 are involved in the transcriptional regulation of the IL-8 gene (Mukaida N. et al., 1990; Yasumoto K. et al., 1992), with the role of NF-κB appearing to be the most important. NF-κB regulates the expression of a wide variety of inducible genes (Baueuerle P.A. et al., 1996). In gastric epithelial cells, induction of IL-8 expression by pro-inflammatory cytokines, such as TNFα or IL-1β, is associated with the NF-κB activation (Yasumoto K. et al., 1992). Aihara et al. demonstrated

that co-culturing *H. pylori* with MKN45, a gastric epithelial cell line, there is NF-κB activation and an increased IL-8 transcription. In their study, mutating the NF-κB site completely abrogated *H. pylori*-simulated epithelial IL-8 expression, suggesting that NF-κB plays a pivotal role in IL-8 release and expression (Aihara M. et al., 1997). Keates et al. also reported similar NF-κB activation by *H. pylori* infection, using the gastric epithelial cell lines AGS and KATOIII (Keates S. et al., 1997). NF-κB is also involved in the expression of MMP-9 and MMP-2 in AGS cells, two gelatinases possessing an important role in the gastric inflammation (Caruso R. et al., 2007).

During *H. pylori*-induced gastritis, oxidative stress in gastric epithelial cells is increased; ROS are known to over-express IL-8 by activating oxidant-sensitive transcription factors such as NF-κB in gastric epithelial cells (Kim Y. et al., 2011). Several target genes of NF-κB are involved in the detoxification of ROS (such as SOD and CAT) but some of them that can also have a pro-oxidant function such as nitric oxide synthases (NOS) pointing at a complex interplay between ROS and NF-κB (Morgan M.J. et al., 2010).

1.5.5 Natural inhibitors of NF-κB

The central role of NF-κB in the inflammatory process makes this factor an important molecular target to search for new anti-inflammatory drugs.

Several natural compounds able to inhibit NF-κB transcription factor have been identified, among these molecules a large number belong to the class of polyphenols, such as curcumin, resveratrol, epigallocatechin-3-gallate, quercetin and some lignans (Nam N.H., 2006). Tsai et al. demonstrated that resveratrol is able to inhibit iNOS expression and NO production in macrophages blocking LPS-induced IκBα phosphorylation thus reducing the binding between NF-κB and DNA. This suggests that the anti-inflammatory activity of the resveratrol could be due, at least in part, to the inhibition of NF-κB-dependent NO synthesis (Tsai S.H. et al., 1999). Sangiovanni et al. showed that some phenols extracted from olive oil (oleuropein aglycone, ligstroside aglycone, hydroxytyrosol and tyrosol) were able to reduce NF-κB driven transcription in TNFα-treated AGS cells (Sangiovanni E. et al., 2012). The same research group demonstrated that the two major ellagitannins contained in blackberry and raspberry, sanguiin H-6 and lambertianin, inhibited NF-κB driven transcription and nuclear translocation in gastric epithelial cells stimulated with TNFα or IL-1β (Sangiovanni et al., 2013). Jiang and colleagues demonstrated that epigallocatechin-3-gallate inhibited TNF-α-induced nuclear factor-κB (NF-κB) activity in macrophage foam cells (Jiang J. et al., 2012).

1.6 Nrf2 TRANSCRIPTION FACTOR

1.6.1 Structure

Reactive oxygen species (ROS) are biological molecules produced naturally as a by-product of oxygen metabolism by aerobic organisms. Under physiological conditions, the ROS level will be in equilibrium in the system. Oxidative stress is the overproduction or imbalanced production of ROS, which disturbs the normal antioxidant mechanism in the system.

The transcription factor Nrf2 (NF-E2-related factor 2) is a potent transcriptional activator and plays a central role in inducible expression of many cytoprotective genes in response to oxidative and electrophilic stresses (Itoh K. et al., 1997; Motohashi H. et al., 2004). Target genes of Nrf2 are involved in the glutathione synthesis, inactivation of ROS, xenobiotic metabolism and drug transport (Okawa H. et al., 2006; Yates M.S. et al., 2009).

Nrf2 belongs to the bZIP factors of the CNC family. The CNC family proteins regulate gene expression, tissue differentiation and development in a variety of organisms. Nrf2 is the most studied CNC family member and is responsible for the expression of constitutive and inducible levels of phase II enzymes and endogenous antioxidants.

The Nrf2 protein is comprised of six highly conserved Neh (Nrf2–ECH homology) domains, Neh1–Neh6. The Neh1 domain contains the CNC-type bZIP region which is crucial for DNA binding and dimerization with other transcription factors (Nioi P. et al., 2005). The Neh1 domain is required for homo- or heterodimerization with Maf proteins (MafF, MafG and MafK) and also with leucine zipper containing protein domains (Motohashi H. et al., 2002). The Neh3 domain lies at the C-terminal region of Nrf2, acts as a transactivation domain to promote the transcription of antioxidant response element (ARE)-dependent genes by means of interacting with the chromo-ATPase/helicase DNA binding protein family member CHD6 (Nioi P. et al., 2005). The Neh4 and Neh5 domains of Nrf2 coordinate with co-activators CBP (CREB/ATF4) and BRG1 (brahma-related gene 1), respectively (Moi P. et al., 1994). The Neh6 domain plays a key role in the Keap1-independent degradation pathway of Nrf2. The degradation of Nrf2 in stressed cells is predominantly mediated by the redox-insensitive Neh6 domain (McMahon M. et al., 2004). The Neh2 domain is present at the N-terminal region of Nrf2. It possesses two motifs, namely, DLG and ETGE motifs. These two motifs of Neh2 are mainly responsible for the direct interaction with the negative regulator, Keap1, which subsequently guide the degradation of an excess of Nrf2 factor to maintain homeostatic conditions (McMahon M. et al., 2004).

Keap1 is a dimeric protein consisting of 624 amino acid residues. Keap1 acts as a substrate adapter protein for the E3 ubiquitin ligase complex formed by Cul3 and Rbx1 and targets Nf-

E2/Nrf2 for ubiquitination and degradation by the proteasome (McMahon M. et al., 2004; Bryan H.K. et al., 2013). The Keap1 protein is mainly located in the cytoplasm; however, it also shuttles between cytoplasm and nucleus (Sun Z. et al., 2011).

1.6.2 Mechanism of Keap1-Nrf2 pathway

Under normal conditions, Nrf2 is constantly degraded via the ubiquitin–proteasome pathway in a Keap1-dependent manner (Sekhar K.R et al., 2002; McMahon M. et al., 2003; Kobayashi A. et al., 2004). In the presence of electrophiles or ROS, Nrf2 dissociates from Keap1 (its degradation is blocked), stabilized Nrf2 translocates into the nucleus, heterodimerizes with small Maf proteins and activates target genes for cytoprotection through antioxidant response element (ARE) / electrophile response element (EpRE) (Itoh K. et al., 2004). Cul3 gets dissociated from the Keap1–Cul3 complex in the presence of ROS. Hence, ubiquitination of Nrf2 is halted, which leads Nrf2 to escape from the proteasomal degradation and results in its subsequent nuclear translocation (Niture S.K. et al., 2010).

Thus, the level of Nrf2 protein is regulated by degradation processes, and the inducible stabilization of Nrf2 is the essence of the cellular response to oxidative and electrophilic stresses. The stress conditions lead to the suspension of Keap1–Nrf2 interactions and cause transcription of cytoprotective genes, which in turn, counteract the cellular oxidative stress (Kansanen E. et al., 2013). Nrf2 regulates the expression of various cytoprotective genes by binding specifically to the antioxidant response element (ARE) in the promoters of the corresponding genes, including those encoding xenobiotic metabolizing enzymes (NAD(P)H:quinone oxidoreductase 1 (NQO1) and glutathione S-transferase (GST)), stress response proteins, growth factors, and nucleotide synthesis enzymes that enhance cell proliferation (Itoh K. et al., 1997; Thimmulappa R.K. et al., 2002; Mitsuishi Y. et al., 2012).

1.6.3 Benefits and risks of Keap1-Nrf2 pathway

Nrf2 activation upregulates the various set of enzymes for the detoxification of chemical carcinogens and confers protection against carcinogenicity, mutagenicity and other types of toxicity (Yu X. et al., 2005). Several studies have shown that Nrf2 protects against oxidative stress, chemotherapeutic agents and radiotherapy (Lau A. et al., 2008; Kensler T.W. et al., 2010). However, Nrf2 disruption has enabled the cells towards carcinogens, which lead to the progression of inflammation and, finally, cancer formation (Slocum S.L. et al., 2011; Takahashi H. et al., 2015). This dual action of Nrf2 has been termed as a ‘double-edged sword’ with respect to the benefits or risks of the Keap1–Nrf2 pathway in cells (Lau A. et al., 2008). The Nrf2 transcription factor is associated with the phase II enzymes gene regulation, as it

maintains the appropriate level of these enzymes inside the cell. Excessive and uncontrolled Nrf2 expression leads to the survival of both normal as well as cancerous cells. Hence, the Nrf2 down-stream gene expression balance is required to obtain the clinical benefits and with less side effects. The regulation of Nrf2 levels by Keap1 is abrogated in some human cancers (Padmanabhan B. et al., 2006; Singh A. et al., 2006). Mutations in Nrf2 and Keap1 genes have been found frequently in various cancer cases. These mutations are sufficient to lead to constitutive activation of Nrf2 by disrupting the Nrf2–Keap1 interaction. Nrf2 activation in cancers contributes to drug resistance by efflux of anticancer drugs and cancer cell proliferation (Ohta T. et al., 2008; Singh A. et al., 2008). Nrf2 overexpression accelerates cancer cell proliferation, because Nrf2 regulates genes involved in metabolic reprograming and cell cycles (Mitsuishi Y. et al., 2012).

In this context, the development of Nrf2 inhibitors is challenging for cancer treatment. The maintenance of proper homeostatic conditions by the development of Nrf2 inhibitors/activators is a vital therapeutic strategy.

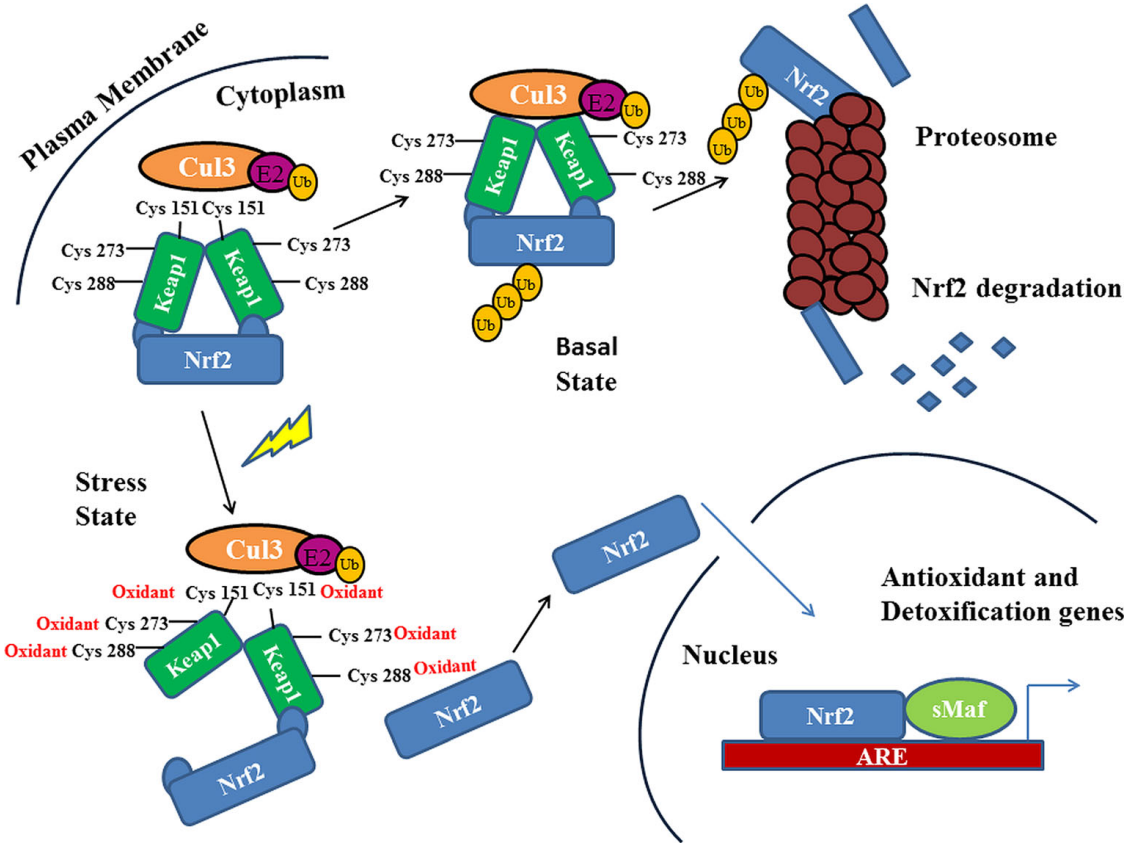


Figure 6. Keap1-Nrf2 pathway. Under normal conditions, Nrf2 is constantly degraded via the ubiquitin–proteasome pathway in a Keap1-dependent manner. In the presence of electrophiles or ROS, Nrf2 dissociates from Keap1 and translocates into the nucleus, where it binds specific sequences called ARE (Antioxidant Responsive Elements). Nrf2 regulates the expression of cytoprotective genes involved in the response to oxidative stress. (Image from Deshmukh P. et al. 2017).

1.7 PHARMACOLOGICAL THERAPIES

1.7.1 Different pharmacological treatments

Various combinations of proton pump inhibitors (PPIs) and antimicrobial agents have been designed to treat *H. pylori* infection. These regimens include triple therapy, bismuth-containing quadruple therapy, sequential therapy, concomitant therapy (non-bismuth quadruple therapy), and hydrid therapy.

Triple therapy: this therapy consists in a conventional PPI with amoxicillin and clarithromycin. The efficacy of standard triple therapy is decreasing in many regions worldwide (Thung I. et al., 2016); triple therapy is still the most commonly used treatment against *H. pylori*, but a fast decrease in efficacy has forced gastroenterologists to find other regimens.

Quadruple therapy: this regimen contains bismuth subcitrate, a PPI, tetracycline and metronidazole (Zullo A. et al., 2017).

Sequential therapy: this simple regimen is a dual therapy including a PPI plus amoxicillin 1 g (both twice daily) given for the first 5 days, followed by standard triple therapy including a PPI, clarithromycin 500 mg and tinidazole 500 mg (twice daily) for the next 5 days.

The principle for this regimen was that following initial exposure to amoxicillin the bacterial cell wall will be disrupted, facilitating penetration of the subsequent drugs (Zullo A. et al., 2000; Gerrits M.M. et al., 2006; Lee H. et al., 2017). Moreover, there is some evidence indicating the role of amoxicillin in changing the permeability of efflux pumps of target bacteria (Webber M.A. et al., 2003).

Concomitant therapy: This regimen contains a standard dose of PPI, metronidazole (500 mg twice daily), clarithromycin (500 mg twice daily) and amoxicillin (1 g twice daily), administrated for the whole duration. The main preference for concomitant therapy rather than standard therapy is its lower complexity compared with sequential therapy.

Hybrid therapy: Hybrid therapy is a regimen that is a combination of sequential therapy and concomitant therapy. This regimen contains a conventional PPI and amoxicillin for 10days or 14 days and metronidazole and clarithromycin for the last 7 days of therapy as a quadruple (hybrid) therapy.

1.7.2 Therapy failure

Graham et al. reported three reasons for failure of anti-*H. pylori* therapy (Graham D.Y. et al., 2010):

I. poor patient compliance;
II. antimicrobial resistance;
III. fast metabolism of conventional PPIs.

It has been widely declared that clarithromycin resistance is the crucial factor affecting the success of triple therapy (Gong E.J. et al., 2014; Kawai T. et al., 2014). As such, the susceptibility profile to clarithromycin is an important factor before prescription (Abadi A.T.B., 2017). Use of clarithromycin in therapeutic regimens against *H. pylori* should be cautious in regions with a resistance rate >20% (Sugano K. et al., 2015; Thung I. et al., 2016).

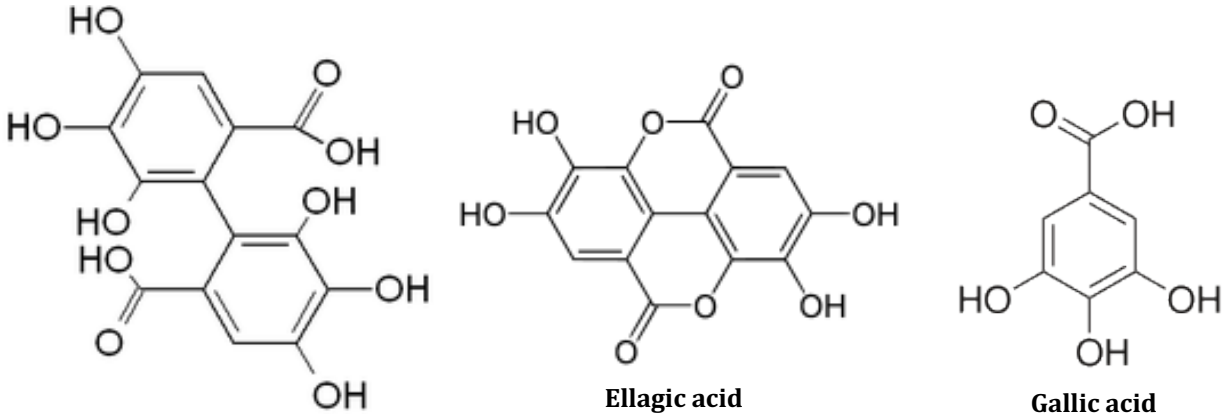
1.8 TANNINS

Tannins are polyphenolic compounds contained mainly in the vacuoles of the cells of the barks, leaves and galls of some plants; tannins are divided in hydrolizable and condensed tannins.

1.8.1 Hydrolizable tannins

Hydrolizable tannins are constituted by a molecule of sugar linked to molecules of gallic acid (gallotannins) or ellagic acid and gallic acid (ellagitannins). Chemically, ellagitannins are considered hexahydroxydiphenoyl esters of carbohydrates. In presence of specific enzymes (such as tannase), ellagitannins are hydrolyzed leading to the release of the ellagic acid and the sugar molecule.

These compounds are contained only in few fruits, in particular red fruits of the genus *Rubus* (blackberry, raspberry, cranberry and cloudberry) and *Fragaria* (strawberry) (Häkkinen S.H. et al., 2000), and in other fruits such as pomegranate and walnuts (Zafrilla P. et al., 2001; Gil M.I. et al., 2000; Fukuda T. et al., 2003).



Hexahydroxydiphenic acid **Ellagic acid** **Gallic acid**

Figure 7. Chemical structure of hexahydroxydiphenic acid, ellagic acid and gallic acid.

In vitro studies show that, at the gastric level, ellagitannins are not absorbed and they are quite stable, gastric acidity (pH 1.8 -2.0) and digestive enzymes are not able to hydrolyze ellagitannins to free ellagic acid, which can be absorbed in this district (the stomach is the first place for the absorption of free ellagic acid) (Larrosa M. et al., 2010). The limited absorption of the ellagitannins, at the gastric level, suggests that the activity of these compounds, introduced with the diet, takes place mainly *in situ*. The ellagitannins, however, in the intestinal tract are partially hydrolyzed to ellagic acid (absorption of ellagic acid in the gut is very limited) and they can be further metabolized by the intestinal microflora to

urolithins, a family of compounds characterized by the presence of a dibenzopyran-6-one structure, with different hydroxyl substituents (Gasperotti M. et al., 2010; Cerda B. et al., 2005). Generally speaking, the synthesis of urolithins begins in the small intestine with the release of urolithin D and urolithin C and ends in the distal part of the colon, with the release of urolithin A and urolithin B (Espín J.C. et al., 2007). Urolithins, once absorbed by intestinal cells, are subjected to a process of glucuronation before entering the portal circulation. However, the type of urolithins released in the gut is strictly dependent on the source of ellagitannins. REF

It has been shown, however, that these molecules can reach concentrations around 20 μM, in humans following a consumption of pomegranate juice, a food rich in ellagitannins (Cerda B. et al., 2004). It is important to consider that there are significant inter-individual differences regarding the bioavailability of ellagitannins; this variability is probably dependent to the composition of the microbiota in the human digestive tract (Puupponen-Pimia R. et al., 2013). The composition of the intestinal microflora is different among individuals, and this means that each microbial community gives rise to different metabolic profiles (Tappenden K.A. et al., 2007; Forester S.C. et al., 2009); therefore, the metabolism of the constituents contained in foods, by the intestinal microbiota, is one of the key factors for the biological effects exerted by these constituents (Tomas-Barberan F.A. et al., 2012). Recently, the biological properties of the ellagitannins and their derivatives have been studied with increasing attention, showing anti-inflammatory (Adams L.S. et al., 2006; Dell'Agli M., 2010), anti-proliferative (Adams L.S. et al., 2006), antiviral (Puupponen-Pimiä R. et al., 2005), and antiplasmodial activities (Dell'Agli M. et al., 2009).

1.8.2 Condensed tannins

Condensed tannins (proanthocyanidins) are formed by molecules of catechin and epicatechin linked by strong carbon-carbon bonds. Proanthocyanidins characterize exclusively of (epi)catechin units are called procyanidins. Procyanidins are present in a wide range of foods, such as fruits, legume seeds, cereal grains, beverages including red wine and tea. Cocoa contains the highest procyanidins content among all foods (Martinez-Micaelo N. et al., 2012). Rios et al. demonstrated that cocoa procyanidins are stable during gastric transit in humans (Rios L.Y. et al., 2002), therefore it is possible that they can act, in the stomach, in their unaltered form. Once absorbed, procyanidins are conjugated to glucuronide, sulfate and methyl groups, mostly in the mucosa and liver (Fraga C.G. et al., 2011). Epidemiological studies demonstrate that consumption of procyanidins-rich foods reduce the incidence of inflammatory diseases and exert beneficial effects on several

pathologies, including metabolic syndrome, atherosclerosis and cancer (Khan N. et al., 2010; Engler M.B. et al., 2006).

1.9 *FRAGARIA* X *ANANASSA* DUCH.

1.9.1 Botanical description

Fragaria X *ananassa* Duch. is a hybrid species cultivated all over the world for its fruits, used in large quantities as fresh fruits or in food preparations such as fruit juices, smoothies, jams etc.

The plant is a hybrid originated from *Fragaria virginiana* Duch., native to North America, and *Fragaria chiloensis* L., native to Chile and Argentina. It is a perennial herbaceous plant belonging to the family of *Rosaceae*, rhizomatous, with long rooting stolons. Leaves consist of three oval toothed leaflets. Flowers, arranged at the top of the stem, are constituted of five white petals. The fruit is aggregated and consists of a red fleshy receptacle with numerous small achenes, which are the true fruits of the strawberry, scattered along the entire surface.

Figure 8. Image of *Fragaria* X *ananassa* Duch.

1.9.2 Nutritional profile

On the basis of its nutritional profile, strawberry is a healthy food choice; fiber and fructose content may help to regulate blood sugar levels by slowing the digestion; moreover, the fiber content can also contribute to the control of the energy intake thanks to their satiating effect. Strawberries are a source of essential fatty acids, since strawberry seed oil is rich in unsaturated fatty acids.

Among the main carotenoids and tocopherols identified in the fruit we can find α-tocopherol, β-tocopherol, γ-tocopherol, δ-tocopherol, lutein, etc.

A very interesting aspect of strawberry is its high content of vitamin C; in addition to vitamin C, the presence of folic acid can contribute to enhance the importance of this fruit, considering that strawberry is one of the main natural sources of this essential micronutrient (the content is estimated in the range 20-25 μg /per 100 g of fresh product).

Strawberry, although not in high quantities, is also a source of other vitamins, such as vitamin A, E, K, B6, niacin, riboflavin and thiamine.

It has been estimated that a consumption of about 144 g of strawberries can provide an amount of manganese able to satisfy more than 20% of daily intake of this mineral; other important minerals contained are: potassium, iodine, magnesium, copper, iron and phosphorus.

In addition to these nutrients, strawberry also contains several non-nutritive components such as phenolic and polyphenolic compounds, including flavonoids, phenolic acids, lignans and tannins.

1.9.3 Polyphenolic content

Strawberry contains different class of polyphenols, including: anthocyanins, responsible for the red color of the fruit, flavonols, flavanols, hydroxycinnamic acid derivatives, ellagic acid, ellagic acid glycosides, ellagitannins and proanthocyanidins (Aaby K. et al., 2005; Maatta-Riihinen K.R. et al., 2004; Seeram N.P. et al., 2006; Clifford M.N., 2000; Buendia B. et al., 2010).

The study performed by Buendia et al., analyzed and quantified polyphenolic compounds from 15 different cultivars of strawberry, obtaining the following results:

Polyphenolic compounds	**mg/100 g of fresh fruit**
Anthocyanins	20.2 – 47.4
Flavonols	1.5 – 3.4
Proanthocyanidins	53.9 – 163.2
Ellagitannins	9.67 – 22.86
Ellagic acid glycosides	0.88 – 2.06

This study shows that strawberries are an important source of polyphenols and proanthocyanidins represent the most abundant component (Buendia B. et al., 2010). Regarding the ellagitannins content, a study carried on by Koponen and colleagues suggests that strawberries contain larger amount of these compounds (77.1 mg / 100 g of fresh product) (Koponen J.M. et al., 2007) compared to that reported by Buendia et al. This

difference, as suggested by the authors, is probably ascribable to the different extraction method used in the two studies. It is important to underline that the information concerning the ellagitannins content reported in the literature is very variable. This variability is due to numerous factors including genetic differences of the cultivars, environmental factors (place, conditions of growth, storage of the fruit and its ripeness) as well as the method used for the extraction and for the quantification.

In a study performed by Gasperotti et al., the authors have been discovered that agrimoniin is the main ellagitannin contained in *Fragaria* X *ananassa* Duch., as suggested by the same research group in a previous work (Vrhovsek U. et al., 2012); moreover, casuarictin, identified for the first time as a component of strawberry, is another important ellagitannin of *Fragaria* X *ananassa* Duch., reaching, in some cultivars, a concentration similar to that of agrimoniin.

The total content of ellagitannins and ellagic acid conjugates found in this study is quite variable, 84.8 - 1636 mg/kg of fresh product, depending on the cultivars considered and the fruit ripening phase (Gasperotti M. et al., 2013).

Ellagitannins	**mg/Kg of fresh fruit**
Agrimoniin	25 – 510
Casuarictin	19 – 386

Anthocyanins: the anthocyanins (anthocyanosides) are water-soluble pigments responsible for the color of many flowers and fruits. On the chemical point of view, they are glycosides, whose aglycone (called anthocyanidin) derives from 2-phenylbenzopyril cation (flavylium cation). The most abundant anthocyanoside contained in strawberry is pelargonidin-3-O-glucoside (Buendia B. et al., 2010).

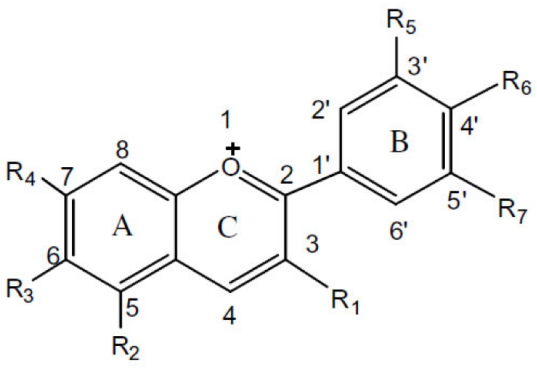


Figure 9. Flavylium cation

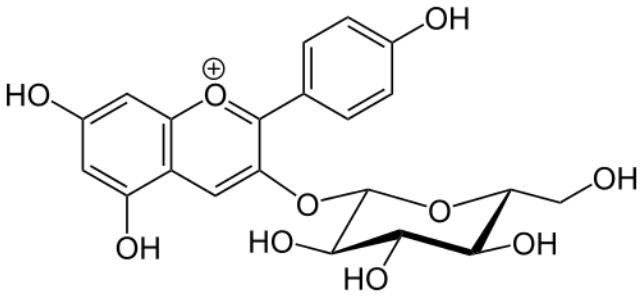


Figure 10. Pelargonidin-3-O-glucoside

Ellagitannins: as described in the previous paragraph, ellagitannins are formed by a central molecule of sugar linked to molecules of gallic and ellagic acid. The two most abundant ellagitannins find in strawberry are agrimoniin and casuarictin (Gasperotti M. et al., 2013).

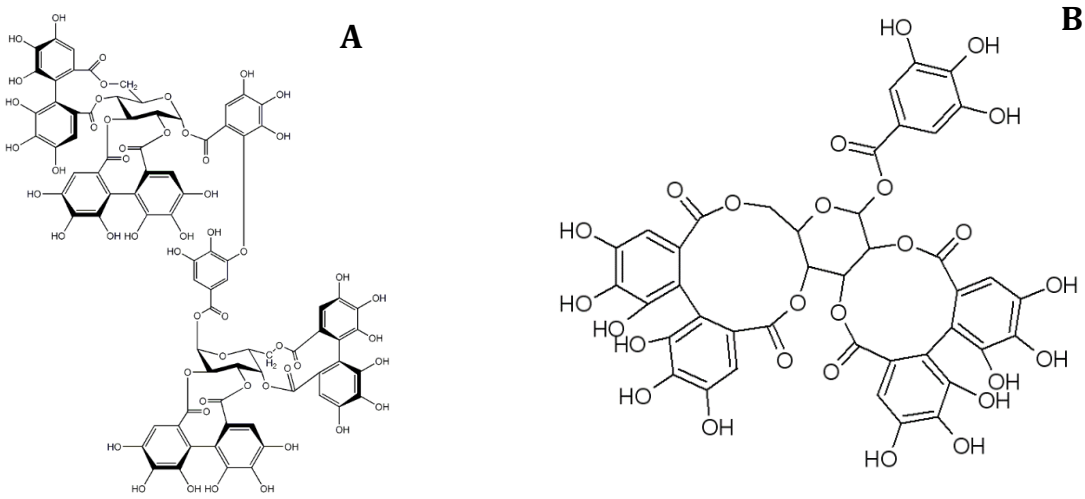


Figure 11. Chemical structure of agrimoniin (A) and casuarictin (B)

Proanthocyanidins: these compounds are polymers formed by the condensation of flavan-3-ols. They are composed by molecules of catechin and epicatechin linked by strong carbon-carbon bonds. An example of procyanidins contained in strawberry is procyanidins B1 (Fumagalli M. et al., 2016).

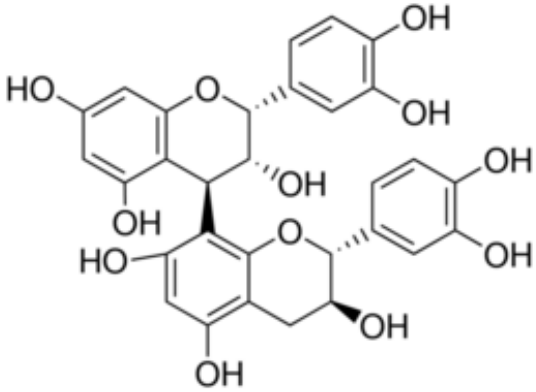


Figure 12. Chemical structure of procyanidin B1

1.9.4 Factors affecting micronutrients and polyphenols content

Several factors such as development and ripening stage, environmental conditions, genetic factors and storage can influence the polyphenolic content and the antioxidant capacity of the strawberry fruit (Wang S.Y. et al., 2000; Tulipani S. et al., 2011; Kosar M. et al., 2004).

The anthocyanin profile of strawberry also varies during ripening but in an opposite way. In all the cultivars, it has been observed that the anthocyanins accumulate in the fruit when it is red, while low quantities have been found in the previous ripening stages (Wang S.Y. et al., 2000; Tulipani S. et al., 2011; Kosar M. et al., 2004). According to several studies, the antioxidant capacity of strawberry decreases gradually during ripening and decrease is associated with a strong reduction of the tannic component, while the non-phenolic polar antioxidants (ex. vitamin C) slightly increase during ripening (Tulipani S. et al., 2011; Kosar M. et al., 2004; Olsson M.E. et al., 2004).

Gasperotti et al. show that during the ripening phases of the fruit (*Fragaria* X *ananassa* Duch.) the concentration of ellagitannins and ellagic acid conjugates rapidly decrease, observation that is common in all the cultivars evaluated in the study. The total concentration of ellagitannins and ellagic acid conjugates in the fruit at the end of ripening is about half of that found in the green fruit (Gasperotti M. et al., 2013). This decline in ellagitannins reported by Gasperotti et al. is comparable with the results published in other papers (Tulipani S. et al., 2011; Pinelli L.D.D. et al., 2011).

Genetic factors and environmental conditions play a key role in the chemical profile of strawberry, micronutrients content and phytochemical compounds can change in the different cultivars (Wang S.Y. et al., 2000; Clifford M.N., 2000; Castro I. et al., 2002; Garcia-Viguera C. et al., 1998; Lopes-da-Silva F. et al., 2002).

The conditions in which the fruit is stored can influence the phytochemical profile and the micronutrient content. The storage temperature is a key factor able to influence the stability of the phenolic compounds in the post-harvest phases (Olsson M.E. et al., 2004). The flavonoid content of strawberry increases during the storage phase (Jin P. et al., 2011; Ayala-Zavala J.F. et al., 2004), this result could be attributed to the phenolic metabolism of the fruit after harvest.

The storage positively influences the antioxidant capacity of the strawberries, because the complex reactions that take place inside them during the post-harvest period facilitate the formation of compounds with a great antioxidant capacity, even if the organoleptic qualities of the fruit are already deteriorated significantly (Piljac-Zegarac J. et al., 2011).

Therefore, in general, the antioxidant capacity increases during storage (Tulipani S. et al., 2010) or remains stable (Piljac-Zegarac J. et al., 2011; Kevers C. et al., 2007), in addition the antioxidant capacity also increases following a long exposure to a high storage temperature (Jin P. et al., 2011; Ayala-Zavala J.F. et al., 2004). Although strawberries are consumed as fresh fruits, many strawberry-based products such as fruit juices, nectars, purees, jams and jellies are available on the market. It has been shown that fruit processing affects the content of antioxidant substances. Several studies have shown that the antioxidant content of strawberry-based products is lower than the fresh fruit, the reduction degree is strictly linked to the time and processing method (such as heat treatment) (Hartmann A. et al., 2008; Bursac Kovacevic D.L. et al., 2009).

1.9.5 Biological activities of *Fragaria* X *ananassa* Duch.

Antioxidant activity: in a study performed by Pajk et al., the authors assessed the antioxidant effect of strawberries in an animal model (pig), investigating the ability of the fruit to reduce oxidative stress induced by the intake of a large amount of polyunsaturated fatty acids. The animals were fed daily with a basal diet supplemented with seeds oil and strawberries; the oxidative stress and the antioxidant status of the animals were evaluated at the beginning and at the end of the experiment, measuring the following parameters: the degree of DNA damage in mononuclear blood cells, the concentration of malonildialdehyde (MDA) in the blood plasma, 24-h urine MDA excretion rate, plasma total antioxidant status, the concentration of tocopherols in the blood plasma and the concentration of glutathione peroxidase in the erythrocytes. The results obtained suggest that supplementation with strawberries can decrease diet-induced oxidative stress; indeed, the authors observed: a reduction of MDA, a greater protection of the blood mononuclear cells against DNA damage, an increase of the antioxidant state of the plasma and a reduction of the concentration of glutathione peroxidase in the erythrocytes (Pajk T. et al., 2006).

Henning et al. have demonstrated that the consumption of strawberries, for a long time (3 weeks), delays LDL peroxidation (Henning S.M. et al., 2010), while Tulipani et al. observed a significant increase in the resistance of erythrocytes to oxidative damage, following a prolonged strawberry intake (2-3 weeks) (Tulipani S. et al., 2011).

In a study of Alvarez-Suarez et al., several groups of rats were fed with strawberry extracts (40 mg/kg of body weight) for 10 days. Subsequently, a gastric lesion was induced, in the animals, using ethanol. The strawberry extracts contained high amounts of anthocyanins showing an important antioxidant capacity. The results obtained in this study showed that strawberry extracts were able to exert a gastroprotective effect against ethanol-induced

damages, reducing lipid peroxidation and preserving/activating some endogenous antioxidant enzymes (SOD and cat); the antioxidant activity shown in this work was probably related to the polyphenolic content of the extracts and, in particular, to the anthocyanins (Alvarez-Suarez J.M. et al., 2011).

In a further study, published in 2012 by Ozsahin et al., the authors have been shown that strawberry extracts (rich in flavonoids), from three different cultivars, were able to prevent lipid peroxidation in an *in vitro* environment (Ozsahin A.D. et al., 2012).

Another work, performed by Gasparrini et al., aimed to evaluate the effects of strawberry extracts on inflammation evoked by *E. Coli* lipopolysaccharide in human dermal fibroblast, by measuring reactive oxygen species production, apoptosis rate, antioxidant enzymes activity, mitochondria functionality and also investigating the molecular pathway involved in inflammatory and antioxidant response. The results demonstrated that strawberry pre-treatment reduced intracellular reactive oxygen species levels, apoptotic rate, improved antioxidant defence and mitochondria functionality in lipopolysaccharide-treated cells. Strawberry exerted protective activities through the inhibition of the NF-κB signalling pathway and the stimulation of the Nrf2 pathway, with a mechanism AMPK-dependent. These results confirm the health benefits of strawberry in the prevention of inflammation and oxidative stress condition in lipopolysaccharide-treated cells (Gasparrini M. et al., 2018).

Anti-inflammatory activity: Basu et al. demonstrated, in 27 patients with metabolic syndrome, that a daily consumption (for 8 weeks) of a beverage containing 50 g of lyophilized strawberries, was able to decrease several risk factors related to the development of atherosclerosis, such as total and LDL cholesterol and the levels of the adhesion molecules in the blood (Basu A. et al., 2010).

A cross-over study, performed on 14 women and 10 men (mean age: 50.9 ± 15 years; BMI: 29.2 ± 2.3) for 6 weeks, showed that the consumption of a strawberries-based beverage before a meal rich in carbohydrates and fats (associated with an increase of the inflammatory markers and oxidative stress) reduced, in statistically significant manner, the post-prandial concentration of IL-1β and PAI-1, and, although in not statistically significant manner, the concentration of IL6, compared to the group who received placebo (Ellis C.L. et al., 2011).

The aim of the study of Parelman et al. was to evaluate the anti-inflammatory capacity and the ability, of strawberries, to regulate blood glucose in a mouse model of diet-induced obesity. Thirty-six male mice (C57BL/6J) were divided in 4 groups, each of these groups was fed with a different diet: one group was fed with a low-fat diet (LF, 13% fat), a group with a

low-fat diet supplemented with strawberry freeze-dried (LFSB), a group with a high-fat diet (HF, 44% fat) and a group with a high-fat diet supplemented with strawberry freeze-dried (HFSB). The results showed a lower blood glucose concentration in mice supplemented with strawberries compared to non-supplemented mice, and a lower plasma concentration of C-reactive protein in the LFSB group compared to the other groups; these results suggest the possible role of strawberry in reducing the risks associated with obesity and diabetes and in regulating the levels of some inflammatory markers in non-obese individuals (Parelman M.A. et al., 2012).

Antimicrobial activity: in a study performed in 2004, Chatterjee and collaborators showed that the incubation of the strain 49503 of *Helicobacter pylori* for 18 hours with anthocyanins-enriched extracts, of some berries (including strawberry), was able to significantly reduce the growth of the pathogen compared to the controls. They also showed that the use of strawberry extract with clarithromycin increased the susceptibility of *Helicobacter pylori* to the antibiotic (Chatterjee A. et al., 2004).

Anthony and colleagues showed that the strawberry extract enriched in the polyphenolic fraction inhibited the growth of *Giardia intestinalis*, a parasite able to colonize the human intestine. The efficacy of the strawberry extract was similar to the conventional drug, metronidazole (Anthony J.P. et al., 2011).

Anti-hyperglycemic activity: strawberry has been investigated, for its potential contribution to the control of hyperglycemia related to type 2 diabetes.

Strawberry extracts (*Fragaria* X *ananassa* Duch.), from different cultivars, showed that the fruit was able to inhibit the activity of the enzyme α-glucosidase, this could suggest a potential anti-hyperglycemic effect of the strawberry (Da Silva Pinto M. et al., 2008).

In a following study performed by Da Silva Pinto et al., it has been shown that the ellagitannins contained in *Fragaria* X *ananassa* Duch. possessed a high inhibitory activity against the enzyme ACE and α-amylase, suggesting that these compounds may be able to control hyperglycemia and hypertension related to type 2 diabetes (Pinto M.D. et al., 2010).

Anti-proliferative activity: an important study concerning the anti-proliferative activity of *Fragaria* X *ananassa* extracts was developed by Wang S. et al. The aim of this study was to test the potential inhibitory effect of strawberry extracts on the activation of AP-1 and NF-κB transcription factors, induced by tetradecanoylforbol-13-acetate (TPA) and UVB rays, and assessed the inhibitory effect on the proliferation and transformation of cancer cells.

The results obtained are as follows:

- *Fragaria* extracts inhibited the proliferation of the A549 cell line (tumor lung epithelial cells) and reduced the neoplastic transformation of mouse epidermal cells (JB6 P^+).
- Pre-treatment of mouse epidermal cells (JB6 P^+) with strawberry extracts inhibited AP-1 and NF-κB activation induced by TPA and UVB rays.
- The extracts blocked the phosphorylation of ERKs kinases induced by TPA and the phosphorylation of ERKs and JNK kinases induced by UVB, in JB6 P^+ cells.

Taken together, these results suggest that strawberry probably possess an antiproliferative activity, reducing: AP-1 and NF-κB activity, MAPK signal, proliferation and transformation of cancer cells (Wang S.Y. et al., 2005).

Seeram and colleagues have shown that the extracts obtained from some berries (including *Fragaria* X *ananassa* Duch.) were able to inhibit, in a concentration-dependent manner, the growth of several tumor cell lines, including KB cells, CAL-27, MCF-7, HT-29, HCT116 and LNCaP (Seeram N.P. et al., 2006).

Zhang et al., according to the previous study performed by Seeram et al., confirmed that the *Fragaria* X *ananassa* extracts exert an anti-proliferative activity against some human tumor cell lines (Zhang Y. et al., 2008).

1.10 *FRAGARIA VESCA* L.

1.10.1 Botanical description

Wild strawberry (*Fragaria vesca* L.) is a diploid species (2n = 14) belonging to the family of Rosaceae. Wild strawberry is a low-growing, deciduous perennial herb, with petioles and flowering stems typically arising from a single crown in rosette form. Occasionally a single crown may split into 2 or more crowns by the development of an axillary meristem, but production of leaves and flowers is generally restricted to a single meristematic axis in each ramet. Petioles are generally 0.3 to 6.9 inches (0.8-17.5 cm) long, with flowering stems often shorter. Leaves are basal and palmately trifoliate, with leaflets 0.5 to 2.6 inches (1.3-6.5 cm) long and 0.5 to 2.8 inches (1.3- 7.0 cm) wide, the terminal leaflet being largest. Fleshy fruits are up to 0.4 inch (1 cm) thick and covered with 0.05 inch (1.3-1.4 mm) long achenes. Crowns arise from short rhizomes, spreading and forming colonies by stolons that root and produce plantlets at the nodes.

Figure 13. Image of *Fragaria* vesca L.

1.10.2 Polyphenolic content

Del Bubba et al. analyzed the variety of phenolic compounds contained in four genotypes of *Fragaria vesca* L., providing for the first time a complete view of the polyphenolic composition of this species of *Fragaria*. About 60 phenolic compounds have been identified, including phenolic acids, ellagitannins, ellagic acid derivatives, flavonols, flavanols, dihydrochalcons and anthocyanins (Del Bubba M. et al., 2012).

In the work done by Gasperotti et al., two different cultivars of *Fragaria vesca* L. were used; they observed that *Fragaria vesca* L. is rich in ellagitannins and ellagic acid conjugates (658

- 1636 mg/kg of fresh product) and, as demonstrated in *Fragaria* X *ananassa* Duch., the main ellagitannin is agrimoniin. In addition, a significant presence of 3-O-methyl-3'-O-α-ramnopyranoside ellagic acid (33 - 132 mg/kg of fresh product), which is the main ellagic acid conjugate present in *Fragaria vesca*, was also observed.

Even in *Fragaria vesca* L., a substantial decrease in the total concentration of ellagitannins and ellagic acid conjugates during the last ripening stages was assessed (Gasperotti M. et al., 2013).

1.10.3 Biological activities of *Fragaria vesca* L.

Antioxidant activity: a work published by Kanodia and colleagues, showed that the ethanol extract from *Fragaria vesca* L., administered orally in albino rats (used as animal model of colitis), at the dose of 500 mg/kg, was able to counteract the oxidative stress associated to colitis, at tissue level. The extract showed significant prevention of increase in colon weight and disease activity index along with decrease in macroscopic and microscopic lesion score as compared to control group. Significant improvement was observed in the levels of myeloperoxidase (MPO), catalase (CAT) and superoxide dismutase (SOD), except glutathione (GSH). *Fragaria vesca* extract showed significant amelioration of experimentally induced intestinal colitis, which may be attributed to its antioxidant and anti-inflammatory properties (Kanodia L. et al., 2011).

2. AIM OF THE WORK

Gastritis is an inflammatory-based disease which can be divided in acute or chronic form. Acute gastritis is caused by several risk factors, such as stress, use of anti-inflammatory drugs (NSAIDs), alcohol abuse and bile reflux, while the chronic form is due to *Helicobacter pylori* infection (*H. pylori*).

H. pylori is a Gram-negative bacterium, specialized in the colonization of the human gastric epithelium through some virulence factors. It is one of the most successful human bacterial parasites which can persist lifelong if not treated efficiently (Graham D.Y. et al., 2010). During *H. pylori* infection, gastric epithelial cells release several pro-inflammatory cytokines, which play a key role in development of gastric diseases. One of the earliest cytokines produced by the infected gastric epithelium is IL-8, a potent chemokine involved in the recruitment of immune cells (Crabtree J.E. et al., 1994; Crabtree J.E. et al., 1994). This response is highly dependent on the NF-κB activation, a transcription factor deeply involved in the control of the expression of several pro-inflammatory mediators, including IL-8.

The bacterium causes also an increase of oxidative stress in the human stomach, and gastric epithelial cells are a source of ROS in the *H. pylori*-infected stomach. ROS can also contribute to the inflammatory process, acting as common mediators of NF-κB activation (Handa O. et al., 2004).

Currently, there are different therapeutic approaches for *H. pylori* eradication based on the administration of proton pump inhibitors in association with antibiotics; although antimicrobial therapy appears the best way to eliminate the bacterium and related inflammation, it is characterized by some factors leading to therapy failure, including bacterial and host-related factors, resistance to the antibiotics and side effects (Graham D.Y. et al., 2010). Common anti-inflammatory drugs (NSAIDs) can't be used in this context, because they, in turn, are responsible for the development of gastric inflammation.

For the reasons mentioned above, the search for new strategies, also derived from natural products, as adjuvant to the conventional pharmacological approach, is needed. Several classes of polyphenols present in fruits and vegetables have demonstrated to possess, in form of phytocomplex or as pure compounds, anti-inflammatory and antioxidant activity at the gastric level, including tannins (Graziani G. et al., 2005; Lai S. et al. 2009; Sangiovanni E. et al., 2013).

Strawberry is one of the most commonly consumed fruits in the world and it is appreciated for its organoleptic and nutritional properties. Moreover, this fruit contains several polyphenols, including anthocyanosides (mostly pelargonidin and cyanidin glycosides) and

tannins, both condensed (procyanidins) and hydrolysable tannins (especially ellagitannins, such as agrimoniin) (Buendia B. et al., 2010; Gasperotti M. et al., 2013). Strawberry is one of the few fruits, of our diet, containing ellagitannins, since these compounds are uncommon in fruits and vegetables. Recently, our group demonstrated that ellagitannin-enriched extracts from fruits of raspberry and blackberry inhibit ethanol-induced gastritis in rats acting on the NF-κB pathway; the anti-inflammatory effect was ascribed, at least in part, to the presence of the ellagitannins sanguiin H-6 and lambertianin C. (Sangiovanni E. et al., 2013). Regarding strawberry, Alvarez-Suarez et al demonstrated that administration of a strawberry extract was able to inhibit ethanol-induced gastritis in rats; notably, the protective effect was ascribed to anthocyanosides (Alvarez-Suarez J.M. et al., 2011). Although tannins are important components of strawberries, until now the activity of strawberry tannins on gastric inflammation, has not been investigated.

Another issue that should be taken into consideration is that these compounds are not absorbed and metabolized in the acid environment of the stomach, suggesting that their activity may be exerted *in situ* (Quideau Editor., 2009; Rios L.Y. et al., 2002).

Therefore, strawberry appears as a valuable source of bioactive components that can exert protective effects on the gastric epithelium and that deserve further investigation.

On these premises, the aim of this study was to investigate the anti-inflammatory and antioxidant properties of strawberry tannins in *in vitro* models of gastric inflammation. Tannin-enriched extracts (obtained from *Fragaria* X *ananassa* and *Fragaria vesca*) and pure compounds were assessed using two gastric cell lines, AGS (tumor gastric epithelial cells) and GES-1 (normal gastric epithelial cells). AGS cell line is one of the mostly used *in vitro* models to study the effects of natural products at gastric level and to study the interaction between gastric epithelial cells and *H. pylori*, while GES-1 cell line is less used and allows to reproduce an experimental model closer to a normal gastric epithelium.

The cells were stimulated with TNFα (a cytokine strictly involved in the *H. pylori*-induced gastritis), at the concentration of 10 ng/mL, or with *H. pylori*.

In brief, the experiments described in this thesis were devoted to:

- Obtain the chemical characterization of the tannin-enriched extracts from two species of *Fragaria* (*Fragaria* X *ananassa* Duch. and *Fragaria vesca* L.) and the evaluation of their antioxidant and anti-inflammatory activities. The attention was focused on several biological parameters related to gastric inflammation, such as IL-8 release and NF-κB signaling pathway.

- ❖ Assess the impact of the *in vitro* gastric digestion on the chemical and anti-inflammatory properties of strawberry tannins.
- ❖ Dissect the molecular mechanisms underlying the effect of pure tannins.
- ❖ Compare the effects of strawberry tannins between normal and tumor gastric epithelial cells (GES-1 and AGS).
- ❖ Evaluate the effect of the strawberry extract in a co-culture with gastric epithelial cells and *H. pylori.*

By accomplishing the over mentioned objectives it is conceivable to obtain in vitro robust evidence of the potential anti-gastritis properties of strawberry bioactive components. This evidence could represent a preliminary but indispensable basis to develop food supplements or adjuvants for the treatment of gastritis and, potentially, other inflammatory diseases of the stomach.

3. MATERIALS AND METHODS

3.1 CELL CULTURE

The experiments were performed using two different gastric epithelial cell lines, AGS (tumor gastric epithelial cells) and GES-1 (normal gastric epithelial cells).

3.1.1 AGS cells

AGS cells (ATCC American Type Culture Collection) are polyploid cells derived from a human gastric adenocarcinoma. These cells were obtained from a tumour resection of a 54-year-old caucasian woman. Although these cells show chromosomal abnormalities compared to the corresponding normal gastric cells, they represent a well-established *in vitro* model to study the inflammation of the human gastric epithelium. Human adenocarcinoma cells were grown at 37°C in Modified Eagle's Medium F-12 (DMEM-F12, Gibco, Thermofisher Scientific, United States) supplemented with 10% heat-inactivated foetal calf serum (FCS) (Euroclone S.p.A, Pero, Italy) (complete medium), 100 units penicillin/mL, 100 mg streptomycin/mL, 2 mM L-glutamine (Gibco, Thermofisher Scientific, United States), in a humidified atmosphere containing 5% CO_2.

3.1.2 GES-1 cells

GES-1 cells derived from 9-month human foetal gastric epithelium. These cells were infected with the SV40 virus (immortalized cells) and represent a cell culture of normal gastric epithelium (Ke Y. et al., 1994, Li S. et al., 2016). Gastric epithelial cells were grown at 37°C in RPMI 1640 medium (Gibco, Thermofisher Scientific, United States) supplemented with 10% heat-inactivated fetal calf serum (FCS) (Euroclone S.p.A, Pero, Italy) (complete medium), 100 units penicillin/mL, 100 mg streptomycin/mL, 2 mM L-glutamine (Gibco, Thermofisher Scientific, United States), in a humidified atmosphere containing 5% CO_2.

3.1.3 Maintenance of cell culture

Every three days the culture medium was removed from the 75 cm^2 flask (Euroclone S.p.A, Pero, Italy) and 3 mL of 0.105 mM trypsin with 0.25% EDTA (Sigma-Aldrich, Milan, Italy) were added in order to promote the detachment of the cells from the support. After few minutes, the enzymatic action of trypsin was inhibited by the addition of 5 mL of complete medium. Subsequently, the cells were rapidly counted under a microscope using a Burker chamber to calculate the volume of cell suspension needed to prepare the new flask and the new plates/petri for the next experiments.

In each flask were seeded 1.5 × 10^6 cells in a total volume of 15 mL of complete medium (DMEM-F12 for AGS and RPMI 1640 for GES-1), while in the plates and petri the cells were distributed as follow:

- in order to evaluate the NF-κB driven transcription, IL-8 promoter activity (wild-type and mutated) and the release of pro-inflammatory mediators (IL-8, IL6 and MMP9), the cells were seeded in 24-well plates at the density of 3 x 10^4 cells/well in a total volume of 500 μL/well of DMEM-F12 (AGS) or RPMI 1640 (GES-1) complete medium.
- in order to evaluate NF-κB and Nrf2 nuclear translocation, the cells were seeded in 100 mm petri at the density of 1 x 10^6 cells/petri in a total volume of 10 mL of DMEM-F12 (AGS) or RPMI 1640 (GES-1) complete medium.

Flask, petri and plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2 for 72 hours.

3.2 BACTERIAL CULTURE

H. pylori strain (ATCC American Type Culture Collection) was cultured in petri containing Mueller Hinton Broth (BBL™, BD, Franklin Lakes, U.S.A.) medium supplemented with agar and 5% sheep blood defibrinated (LifeTechnologies, Monza, Italy). The bacteria were grown for 72 hors under microaerophilic conditions (5% O_2, 10% CO_2, and 85% N_2 at 37°C with 100% humidity). Before co-culture experiments, the bacterium was recovered from each petri and the bacterial concentration was estimated by using the value of optical density (O.D. value = 5 correspond to 2*10^8 bacteria/mL).

3.3 CELL TREATMENTS

AGS and GES-1 cells were co-treated with the stimulus TNFα (10 ng/mL) or the bacterium *H. pylori* (bacterium/cell ratio of 50:1) and *Fragaria* extracts (from *Fragaria* X *ananassa* or *Fragaria vesca*) or single compounds (agrimoniin, casuarictin, procyanidin B1 and ellagic acid) at different concentrations. Treatments were performed using serum-free medium (containing 1% L-glutamine and 1% penicillin/streptomycin) or using serum-free and antibiotic-free medium (containing 1% L-glutamine) during the co-culture with the *H. pylori.* The same percentage of solvent (DMSO) was added in each experimental condition. During the treatment, cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. The time of treatment was established, for every parameter, on the basis of *time course* experiments in which the cells were exposed to the stimulus TNFα (10 ng/mL) at different

hours in order to identify the maximal induction of each parameter (IL-8 release, IL-8 promoter activity, NF-κB driven transcription and nuclear translocation). Regarding IL-8 release, IL-8 promoter activity and NF-κB driven transcription, the cells were treated with TNFα for 3, 6, 24 and 30 hours; the cytokine TNFα showed the maximal induction after 6-hours treatment. The cells were stimulated with TNFα for 1, 2, 3 and 6 hours to assess the NF-κB nuclear translocation; the maximal effect was reached after 1-hour treatment.

T*ime-course* experiments allowed to choose the best times to investigate the effects of the extracts and pure compounds.

Concentrations of the extracts or individual compounds were selected according to previous results performed by our group on the same cell model with other natural sources of ellagitannins (Sangiovanni E. et al., 2012 PlosOne). Of note, concentrations in the micromolar order are easily reachable in the gastric environment following consumption of a portion (5—75 g) of strawberry; all the concentrations used in the experiments did not show any cytotoxicity in the MTT assay (described below).

At the end of the treatment, medium or cells were collected in order to evaluate the corresponding parameters.

Epigallocatechin gallate 20 μM (EGCG) was used as reference compounds in our experiments.

3.4 PREPARATION OF *FRAGARIA* EXTRACTS AND ISOLATION OF PURE COMPOUNDS (AGRIMONIIN AND CASUARICTIN)

One kilogram of woodland strawberries (*Fragaria vesca*) and 60 g of strawberries (*Fragaria* X *ananassa* D. cv. Darselect) were grown in an experimental field in Vigalzano (Trento, Italy). All of the plants were grown under the same conditions to minimize the effect of environmental and agronomic factors. Strawberries were harvested at maturity and were frozen at −20°C and then transported to the laboratory for solvent extraction. The extraction of polyphenols was carried out with an acetone/water mixture (70:30 v/v). Before extraction, the fruit and extraction solution were cooled to 4 °C to limit enzymatic and chemical reactions. Sixty grams of fresh fruit was homogenized in an 847-86 model Osterizer blender at speed 1, in 2 × 100 mL of a mixture of acetone/water (70:30 v/v) for 1 min and made up to 250 mL with the same solvent.

Aqueous acetone strawberry extracts (1 kg of fruits extracted in 4 L of acetone/water mixture (70:30 v/v)) were evaporated until dryness in a pear-shaped flask, using rotary

evaporation under reduced pressure at 37°C. The sample was diluted to 1 L with methanol/water mixture (30:70 v/v) and filtered using a Durapore 0.45 μm filter (Millipore, Vimodrone, Italy).

Then, the extracts were subjected to chromatography Sephadex LH-20 in order to remove anthocyanosides. A column cartridge (10 × 4 cm) was packed with Sephadex LH-20 resin, connected to a vacuum line to speed elution, prewashed with 50 mL of methanol, and equilibrated with 100 mL of methanol/water (30:70 v/v). An aliquot of 50 mL of the aqueous methanol extracts was loaded, and anthocyanins were washed off with 500 mL of methanol/water (30:70 v/v). The yellowish fraction containing the ellagitannins was eluted from the cartridge using 350 mL of acetone/water (70:30 v/v). The ellagitannin fraction was dried using rotary evaporation under reduced pressure at 37°C and reconstituted in 50 mL of methanol for the next isolation step.

Only the ellagitannin fraction from woodland strawberries was used to isolate single compounds (agrimoniin and casuarictin). The chromatographic isolation of agrimoniin and casuarictin was performed using preparative HPLC with a 250 × 50 mm, 10 μm, Discovery HS C18 column (Supelco, Bellefonte, PA, USA), After separation, these compounds were dried using rotary evaporation under reduced pressure and then dissolved in the smallest possible volume of methanol, diluted with diethyl ether and hexane. The pure isolated compounds (ca. 200 mg) were recovered by filtration and precipitation from n-hexane as an amorphous pale rose powder, which was further characterized by NMR and MS. The experiments were conducted in collaboration with the research group of Dr. Fulvio Mattivi (Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige, Italy).

3.5 QUANTIFICATION OF TANNIN-ENRICHED EXTRACTS AND *IN VITRO* DIGESTED EXTRACT

The quantification of the ellagitannins and the other polyphenols were performed using a Waters Aquity system coupled with a triple quadrupole (TQ) mass spectrometer Waters UHPLC Xevo TQ (Millford, Massachusetts, USA). In addition, proanthocyanidins contained in tannin-enriched extracts and *in vitro* digested extract were quantified by the vanillin-HCl method (or vanillin index). Proanthocyanidins were expressed as equivalent of (+)-catechin.

3.6 CYTOTOXICITY ASSAY

The integrity of the morphology before and after treatment was assessed by light microscope inspection. The cytotoxicity of the *Fragaria* extracts and the single compounds was evaluated by the 3,4,5-dimethylthiazol-2-yl-2-5-diphenyltetrazolium bromide (MTT) method (Sigma-Aldrich, Milan, Italy) (Denizot F. et al., 1986). This assay is able to evaluate to activity of the mitochondrial enzyme, called succinate dehydrogenase. The activity of this enzyme is an index of the viability and proliferation of the cells. This test is based on the conversion of the MTT compound (yellow color) into violet formazan salt, through the activity the mitochondrial enzyme succinate dehydrogenase. AGS and GES-1 cells were seeded in 24-well plates (DB Falcon™) (30000 cells/well), after 48 h the cells were co-treated with the stimulus and the extracts/single compounds. At the end of the treatment, the medium was removed and 200 μL of MTT solution (0.1 mg/mL) were added in each well, for 45 minutes at 37°C and in dark conditions. Then, MTT solution was removed from each well and the formazan was extracted from the cells using a mixture isopropanol:DMSO (90:10). Formazan solution was transferred in to a 96-well plate to read the absorbance at 550 nm (Victor™ X3, Perkin Elmer, Walthman MA, USA).

The results, about the influence of the extracts or single molecules (agrimoniin, casuarictin, procyanidin B1 and ellagic acid) and the stimulus TNFα (10 ng/mL) on cell viability, show that the extracts and pure compounds are not cytotoxic on AGS and GES-1 cells till the maximal concentration tested. The concentrations range tested are:

- 0.05 – 10 μg/mL for *Fragaria* X *ananassa* extract.
- 0.1 – 2.5 μg/mL for *Fragaria vesca* extract.
- 0.1 – 5 μM for agrimoniin.
- 0.05 – 5 μM for casuarictin.
- 0.1 – 50 μM for procyanidins B1.
- 0.05 – 5 μM for ellagic acid.

In *H. pylori*-treated AGS and GES-1 cells, *Fragaria* X *ananassa* extract doesn't show cytotoxic effects till the concentration of 100 μg/mL.

3.7 PLASMIDS AMPLIFICATION

The plasmids amplification was carried out through the use of *Escherichia coli* bacteria (*E. coli*), belonging to DH5α strain, transformed with the NF-κB-Luc or IL-8-Luc (native or mutated) plasmids. The plasmids were extracted from the bacteria using a commercial kit (Plasmid DNA Purification, NucleoBond® Xtra Maxi, Macherey-Nagel). The first step was a 6 hours pre-inoculation phase (at 37°C under stirring), in order to allow to the transformed bacteria to reach an exponential growth condition at the time of the inoculum.

The bacteria were grown in LB medium (Luria-Bertani Broth culture medium, containing 1% NaCl, 1% Bacto tryptone and 0.5% Bacto yeast extract) with the addiction of ampicillin (100 ug/mL); the antibiotic contained in the culture medium allowed the growth of the transformed bacteria only, since the plasmid conferred resistance to ampicillin.

At the end of the pre-inoculation phase, the growing bacteria were transferred in 250 mL of LB medium containing ampicillin (100 ug/mL) and incubated overnight at 37°C under stirring. The next day, the bacterial culture was centrifuged at 5000 rpm for 10 min in order to obtain a bacterial pellet, which was suspended in a lysis buffer (an alkaline solution containing sodium hydroxide and SDS) to allow the lysis of the bacterial membrane and the release of the bacterial content, including the plasmids.

A solution composed by potassium acetate and acetic acid to buffer the pH was added to block the lysis allowing, at neutral pH, the DNA renaturation.

The last step was the detachment of the plasmids from the filter by elution with sterile water. The plasmids were quantified by spectrophotometric reading (Nanodrop - Spectrophotometer ND-1000, Euroclone).

3.8 TRASIENT TRANSFECTION

3.8.1 Calcium phosphate method

In order to evaluate NF-κB driven transcription and IL-8 promoter activity, AGS cells were seeded in 24-well plates (DB Falcon™) (30000 cells/well). After 48 hours, the cells were transiently transfected by the calcium-phosphate method with different reporter plasmids (NF-κB-LUC, 50 ng/well; IL-8-LUC, native or mutated, 100 ng/well):

- NF-κB-LUC, a reporter plasmid containing luciferase gene under control of a promoter characterized by the presence of three responsive elements -κB.
- IL-8-LUC, a reporter plasmid containing luciferase gene under control of a fragment of the native promoter of the human IL-8 gene, which is characterized by different

responsive sequences for transcription factors such as activator protein-1 (AP-1), CCAAT- enhancer-binding protein-β (C/EBPβ), and NF-κB.

- Mutated IL-8-LUC, the same plasmid previously described mutated in the responsive sequence for NF-κB transcription factor.

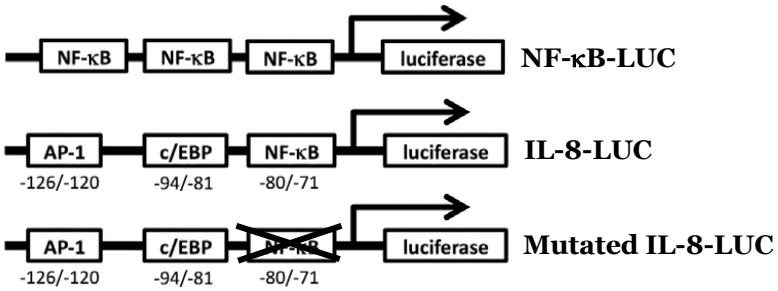


Figure 14. Reporter plasmids containing luciferase gene (NF-κB-LUC, IL-8-LUC and mutated IL-8-LUC)

The transfection mix was prepared with the following procedure: 650 μL Hepes buffer saline 2X (Na_2HPO_4 1.5 mM, NaCl 280 mM, HEPES pH 7.15, 50 mM) were mixed in constant agitation with 650 μL of a solution containing calcium chloride (250 mM), 1300 ng of NF-κB-LUC plasmid or 2600 ng IL-8-LUC plasmid (native or mutated) and sterile water. 50 μL of this transfection mix was distributed in each well of the 24-well plates.

After 16 hours, the cells were treated with the stimulus and the extract/single compound for 6 hours; at the of the treatment 100 μL of BritelitePlus reagent (PerkinElmer Inc. Massachusetts, USA), a lysis buffer containing D-luciferin, were added in each well of the plates. The luminescence, produced by the reaction between luciferase and D-luciferin, was read with Victor™ X3 (Perkin Elmer, Walthman MA, USA).

$$\text{D-LUCIFERIN} + \text{ATP} + O_2 \xrightarrow{\text{LUCIFERASE, } Mg^{2+}} \text{OXYLUCIFERIN} + \text{PPi} + \text{AMP} + CO_2 + \text{Light}$$

3.8.2 Lipofectamine method

In order to evaluate the NF-κB driven transcription, GES-1 cells were seeded in 24-well plates (30000 cells/well). After 48 hours, the cells were transiently transfected by the lipofectamine method with NF-κB-LUC (50 ng/well) reporter plasmid. The transfection was performed using Lipofectamine® 3000 (LifeTechnologies, Monza, Italy) in the ratio 2 μl lipofectamine: 1 μg DNA, according to the manufacturer's instructions. After 16 hours, cells were treated with the stimulus and the extracts/single compounds for 6h; at the end of the treatment, luciferase assay was performed as previously described.

3.9 NF-κB AND Nrf2 NUCLEAR TRANSLOCATION

3.9.1 Nuclear and cytoplasmatic extraction

To assess the effect of the extracts and individual compounds on the NF-κB (p65) and Nrf2 nuclear translocation, AGS and GES-1 cells were plated at the density of 1.5×10^6 cells/mL in 100 mm plates.

After 48 hours, the cells were treated in the following way:

- 1 hour treatment with the pro-inflammatory mediator (TNFα 10 ng/mL) and the extracts/compounds to evaluate NF-κB (p65) nuclear translocation.
- 1, 2, 3, 6 hours treatment with the single compounds to evaluate Nrf2 nuclear translocation.

Nuclear extracts were prepared using Nuclear Extraction Kit from Cayman Chemical Company (Michigan, USA). The cells were detached from the support by enzymatic treatment with trypsin, which was blocked after few minutes through the addition of complete DMEM-F12 medium. The cells were centrifuged for 5 minutes at 300 g and subsequently suspended in PBS (Phosphate Buffered Saline) containing phosphatase inhibitors. After performing several washing steps with PBS, the cells were dissolved in a hypotonic buffer in order to increase cell swelling and the fragility of the cell membranes. The addition of 10% Nonidet P-40, a detergent, produced the breakdown of cell membranes (maintaining the integrity of nuclear membranes), thus allowing the recovering of the cytoplasmic fraction as a supernatant, following separation of the cytoplasmic fraction from nuclei through centrifugation (30 seconds to 14000 g). The nuclei were lysed with an extraction buffer containing a mixture of protease and phosphatase inhibitors; after a centrifuge (10 minutes at 14,000 g), the supernatants containing the nuclear extracts were collected in 0.5 mL tubes and store at -80°C until assayed.

3.9.2 Protein quantification

The protein concentration of the nuclear extracts was evaluated by the Bradford assay (Bio-Rad Protein Assay). Bradford reagent (composed of Coomassie® Brilliant Blue G-250, phosphoric acid and methanol) was added in each sample and the measurement of the absorbance was performed at 595 nm using a spectrophotometer.

A standard curve, prepared with bovine serum albumin (BAS), was used to quantify the unknown protein content of the nuclear extracts.

3.9.3 ELISA assay

Nuclear extracts were used to evaluate the translocation of NF-κB (p65) and Nrf2 transcription factors by a ELISA kit commercially available (Cayman Chemical, Michigan, USA). The 96-well plate used for this assay contained, on the bottom of each immobilized well, double-strand DNA sequences characterized by the presence of NF-κB or Nrf2 responsive elements, respectively. The transcription factor (NF-κB or Nrf2), contained in the sample, binds specifically these sequences and, in this way, it can be detected by the addition of a specific primary antibody. The addition of a secondary antibody conjugated with horseradish peroxidase (HRP), allows the development of a measurable colorimetric reaction. After stopping the reaction with sulfuric acid, the absorbance was read through spectrophotometer at 450 nm, 0.1s (Victor™ X3, Perkin Elmer, Walthman MA, USA).

3.10 *IN VITRO* GASTRIC DIGESTION OF *FRAGARIA* X *ANANASSA* EXTRACT

Gastric digestion was simulated using an *in vitro* approach (Oomen A.G. et al. 2003; Versantvoort C.H. et al., 2005).

Strawberry extract (200 mg) was mixed with 2 mL of saliva juice solution (pH 6.5 ± 0.1; 12 mM KCl, 2 mM KSCN, 7.4 mM NaH_2PO_4, 4 mM Na_2SO_4, 5 mM NaCl, 1.8 mM NaOH, 3.3 mM urea, 89 µM uric acid, 145 mg L^{-1} amylase and 50 mg L^{-1} mucin) and incubated for 5 min under constant shaking at 37°C. After this first step, 4 mL of gastric juice (pH 1.1 ± 0.1; 47 mM NaCl, 2.2 mM NaH_2PO_4, 11 mM KCl, 2.7 mM $CaCl_2$, 5.7 mM NH_4Cl, 8.3 mL L^{-1} HCl, 3.6 mM glucose, 0.1 mM glucuronic acid, 1.4 mM urea, 1.5 mM glucosamine hydrochloride, 1 g L^{-1} BSA, 1 g L^{-1} pepsin and 3 g L^{-1} mucin) were added and incubated for 2 h at 37°C in constant agitation. Simulated gastric digested extract was frozen, lyophilized and store at -20°C until use. The digested extract was tested on different biological activities comparing the effect to non-treated control adjusted with a mixture of saliva and gastric juice.

3.11 MEASUREMENT OF THE RELEASE OF PRO-INFLAMMATORY MEDIATORS

3.11.1 IL-8 release

For measurement of IL-8 secretion, AGS and GES-1 cells were grown in 24-well plates (DB Falcon™) (30000 cells/well) for 48 hours; then, cells were treated with the pro-

inflammatory stimulus (TNFα at 10 ng/ml) and the extracts or single compounds. After 6 h treatment, the medium was removed and stored at −20 C until the assay.

IL-8 was quantified by an enzyme-linked immunosorbent assay (ELISA) Kit (Peprotech, London, UK). Corning 96-well EIA/RIA plates (Sigma Aldrich, Milan, Italy) were coated with the antibody provided in the ELISA Kit and incubated overnight at room temperature to allow the binding between the antibody and the bottom of the wells. After blocking phase, the samples were transferred into wells at room temperature for 2 hours. The IL-8 in the samples was detected by the use of a biotinylated antibody and of HRP-conjugated avidin (horseradish peroxidase). The colorimetric reaction between HRP enzyme and 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma Aldrich, Milan, Italy) was read using a spectrophotometer at 450 nm, 0.1s (Victor™ X3, Perkin Elmer, Walthman MA, USA). The quantification of IL-8 was performed through a standard curve supplied with the ELISA Kit (0 – 1000 pg/mL). Data were expressed considering 100% the absorbance related to the TNFα-induced IL-8 release.

3.11.2 IL-6 release

For measurement of IL-6 secretion, AGS and GES-1 cells were grown in 24-well plates (DB FalconTM) (30000 cells/well) for 48 hours; then, cells were treated with the pro-inflammatory stimulus (TNFα at 10 ng/ml) and the extract or single compounds. After 6 hours treatment, the medium was removed and stored at −20 C until the assay.

IL-6 was quantified by an enzyme-linked immunosorbent assay (ELISA) Kit (Peprotech, London, UK). Corning 96-well EIA/RIA plates (Sigma Aldrich, Milan, Italy) were coated with the antibody provided in the ELISA Kit and incubated overnight at room temperature to allow the binding between the antibody and the bottom of the wells. After blocking phase, the samples were transferred into wells at room temperature for 2 hours. The IL-6 in the samples was detected by the use of a biotinylated antibody and of HRP-conjugated avidin (horseradish peroxidase). The colorimetric reaction between HRP enzyme and 3,3′,5,5′-Tetramethylbenzidine (TMB) (Sigma Aldrich, Milan, Italy) was read using a spectrophotometer at 450 nm 0.1s (Victor™ X3, Perkin Elmer, Walthman MA, USA). The quantification of IL-6 was performed through a standard curve supplied with the ELISA Kit (0 – 1500 pg/mL). Data were expressed considering 100% the absorbance related to the TNFα-induced IL-6 release.

3.11.3 MMP-9 release

For measurement of MMP9 secretion, AGS and GES-1 cells were grown in 24-well plates (DB FalconTM) (30000 cells/well) for 48 hours; then, cells were treated with the pro-inflammatory stimulus (TNFα at 10 ng/ml) and the extract or single compounds. After 24 hours treatment, the medium was removed and stored at −20 C until the assay. MMP-9 was quantified by an enzyme-linked immunosorbent assay (ELISA) Kit (Raybiotech, Georgia, USA). The samples were transferred in the 96-well plate supplied in the Kit, containing a specific antibody for MMP-9, and incubated for 2.5 hours at room temperature under slight stirring. Following, the wells were washed, and a biotinylated antibody was added for 1 hour (at room temperature under slight stirring); after washing unbound antibody, HRP-conjugated streptavidin was added in the wells for 45 minutes (at room temperature under slight stirring). The color developed through the reaction with TMB substrate was read using a spectrophotometer at 450 nm 0.1s (Victor™ X3, Perkin Elmer, Walthman MA, USA). The quantification of MMP-9 was performed through a standard curve supplied with the ELISA Kit (0 – 6000 pg/mL). Data were expressed considering 100% the absorbance related to the TNFα-induced MMP-9 release.

3.12 ORAC ASSAY (OXYGEN RADICAL ABSORBANCE CAPACITY)

ORAC assay is a well-known method used in order to evaluate the antioxidant capacity of extracts and molecules derived from natural products (Ou B. et al., 2001; Dávalos A. et al., 2004). The ORAC assay measures the ability of extracts/compounds under study to counteract, in the time, oxidation of a fluorescent probe (loss of fluorescence) by peroxyl radicals. In this context, fluorescein is used as fluorescence probe, while AAPH (2,2'-Azobis(2-amidinopropane) dihydrochloride) is commonly used as free radical generator. The *Fragaria* X *ananassa* extract and the single ellagitannins were mixed with fluorescein and AAPH in 96-well plate, the fluorescence (excitation wavelength: 485 nm; emission wavelength: 535 nm) was read for 60 minutes with an interval of 2 minutes maintaining the plate reader to 37°C. The antioxidant capacity of the samples correlates to the fluorescence decay curve, which is usually represented as the area under the curve (AUC). The AUC derived from each sample was used in order to calculate the antioxidant capacity of the extract or single compounds, using the equation obtained from the standard curve of the Trolox (Sigma Aldrich, Milan, Italy), which was considered as reference compound. Data were expressed as μM Trolox equivalents.

3.13 GENE EXPRESSION

3.13.1 RNA extraction

AGS and GES-1 cells were grown in 24-well plates (DB FalconTM) (30000 cells/well) for 48 hours; then, the cells were treated with the pro-inflammatory stimulus (TNFα at 10 ng/ml) and the pure compounds (agrimoniin and casuarictin). After 6 hours treatment, the medium was removed. The cells were lysed through the addition of the Qiazol lysis buffer (QIAGEN GmbH, Germany) according to the indications provided by the manufacturer, in order to obtain cell lysis and inactivation of the endogenous RNases. The lysates were frozen at -80°C until the following RNA purification steps.

Total RNA was isolated from the cell lysates using the miRNeasy® Mini Kit (QIAGEN GmbH, Germany), according to the manufacturer's protocol. A set of RNase-free DNase (QIAGEN GmbH, Germany) was used to ensure the complete elimination of genomic DNA. Total RNA was eluted in 35 µL of 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 optical densities of the samples at 260 nm and 280 or 230:

- Ratio A260/A280 → protein contamination, expected value 1.8-2
- Ratio A260/A230 → solvent contamination, expected value 2-2.2

3.13.2 cDNA synthesis

cDNA was synthetized, after elimination of any residual genomic DNA, using the RT² First Strand kit (QIAGEN, GmbH, Germany), according to the manufacturer's indications. 400 ng of total RNA, of each sample, were used to produce cDNA.

3.13.3 qPCR

Quantitative analysis of gene expression was performed using a 384-well PCR array, related to human genes involved in the inflammatory process and coding for chemokines, cytokines, receptors, TNFα family members and other pro-inflammatory mediators (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 (in total 84 different target genes), or housekeeping gene for data normalization (5 different housekeeping genes). Moreover, the array included some 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.

A diluted aliquot of cDNA, equivalent to 400 ng total RNA, was mixed with the SYBR® Green Master Mix RT² reagent (QIAGEN Sciences, USA) according to the manufacturer's instructions and loaded into the 384-well array. The real-time PCR was performed using the CFX384™ Real-Time PCR Detection System (coupled to C1000TM Thermal Cycler) (Bio-Rad Laboratories Srl, Segrate, Italy), heating the plate at 95°C for 10 min., followed by 40 cycles of 15 s at 95°C and 1 min. at 60°C.

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 ΔΔCt method (Livak & Schmittgen, 2001), through the web portal SABiosciences company (QIAGEN Sciences, USA) (www.SABiosciences/pcrarraydataanalysis.php). The C_t cut-off was set to 35.

Data were normalized on the basis of housekeeping genes: hypoxanthine-guanine phosphoribosyl transferase 1 (HPRT1), beta-2-microglobulin (B2M), ribosomal protein lateral stalk subunit P0 (RPLP0), beta-actin (ACTB) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In each experiment, the housekeeping genes with a variability higher than ±1 threshold cycle among the different experimental conditions, were excluded to the analysis.

3.14 STATISTICAL ANALYSIS

All data were expressed as mean ± s.d. of at least four experiments. The number of experiments for each assay is specified in the figure legends; data were analyzed by unpaired one-way analysis of variance (ANOVA) followed by Bonferroni as post-hoc test. Statistical analyses were done using GraphPad Prism 6.0 software (GraphPad Software Inc., San Diego, CA, USA). $p < 0.05$ was considered statistically significant. IC_{50}s were calculated using GraphPad Prism 6.00 software.

4. RESULTS

4.1 INHIBITORY ACTIVITY OF *FRAGARIA* EXTRACTS ON IL-8 RELEASE AND NF-κB SIGNALING IN TNFα-TREATED AGS CELLS

IL-8 is released in high amount by gastric epithelial cells during *H. pylori* infection and NF-κB is a transcription factor involved in the expression of several pro-inflammatory genes, including IL-8. For these reasons, the first part of the work was aimed to study the effects of tannin-enriched extracts from *Fragaria* X *ananassa* and *Fragaria vesca* on IL-8 and NF-κB signaling. The activity of *Fragaria* X *ananassa* extract was also assessed after an *in vitro* gastric digestion, in order to simulate the digestive processes occurring in humans.

4.1.1 Inhibitory effect of *Fragaria* X *ananassa* extract on IL-8 and NF-κB pathway in TNFα-treated AGS cells

In order to evaluate the effect of *Fragaria* X *ananassa* extract on IL-8 secretion, IL-8 promoter activity, NF-κB driven transcription and nuclear translocation, AGS cells were co-treated with the stimulus TNFα (10 ng/mL) and the extract at different concentrations (0.1 – 5 μg/mL).

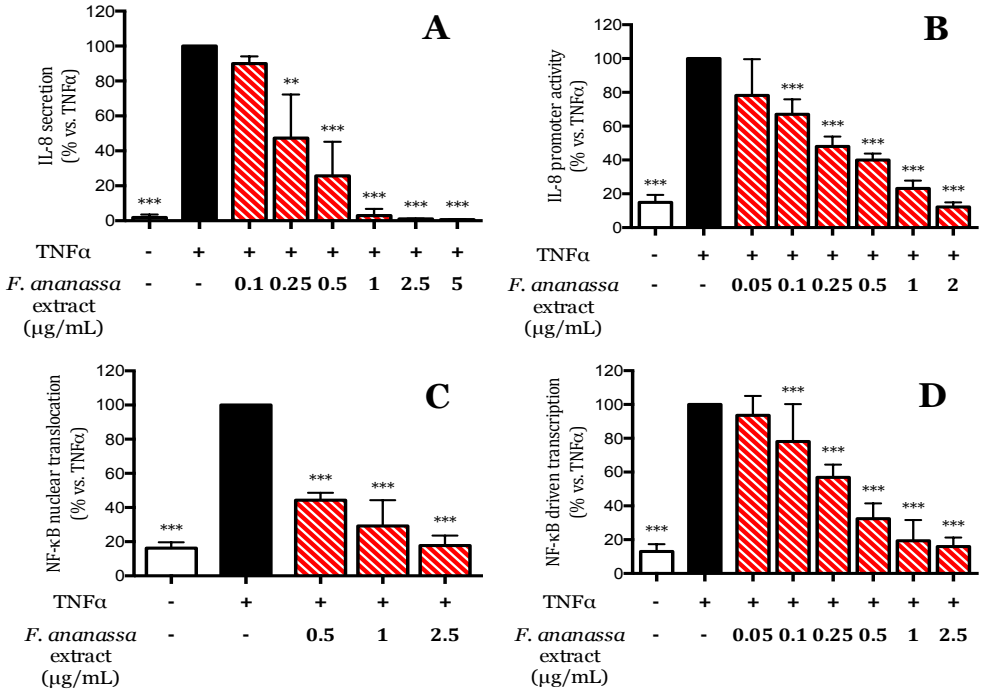


Figure 15. Effect of *Fragaria* X *ananassa* extract on IL-8 release (A), IL-8 promoter activity (B), NF-κB nuclear translocation (C) and NF-κB driven transcription (D) in TNFα-treated AGS cells. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. **p < 0.01, ***p < 0.0001 versus TNFα.

Figure 15 shows that tannin-enriched extract from *Fragaria* X *ananassa* is able to inhibit all the parameters evaluated in a concentration dependent manner, with IC_{50}s below 1 μg/mL.

In detail, the IC_{50}s on IL-8 release and expression are 0.25 μg/mL and 0.17 μg/mL respectively, while the IC_{50}s on NF-κB nuclear translocation and transcription are 0.30 μg/mL and 0.23 μg/mL respectively.

To evaluate the real involvement of NF-κB in the IL-8 expression, an IL-8 promoter carrying a mutation at the NF-κB binding site was used. It loses responsiveness to TNFα and, consequently, to strawberry extract suggesting that inhibition of IL-8 promoter activity operates through impairment of NF-κB signaling.

The treatment with the reference compound (20 μM EGCG) yielded the expected inhibition of the tested parameters: >70% inhibition of IL-8 secretion, >80% inhibition of IL-8 promoter activity, >85% inhibition of NF-kB driven transcription and >90% inhibition of p65 translocation.

4.1.2 Inhibitory effect of *Fragaria vesca* extract on IL-8 and NF-κB pathway in TNFα-treated AGS cells

Tannin-enriched extract from *Fragaria vesca* species was tested on IL-8 expression and release and NF-κB signaling pathway (NF-κB driven transcription and nuclear translocation). AGS cells were co-treated with the stimulus TNFα (10 ng/mL) and the extract at increasing concentrations (0.1 – 2.5 μg/mL).

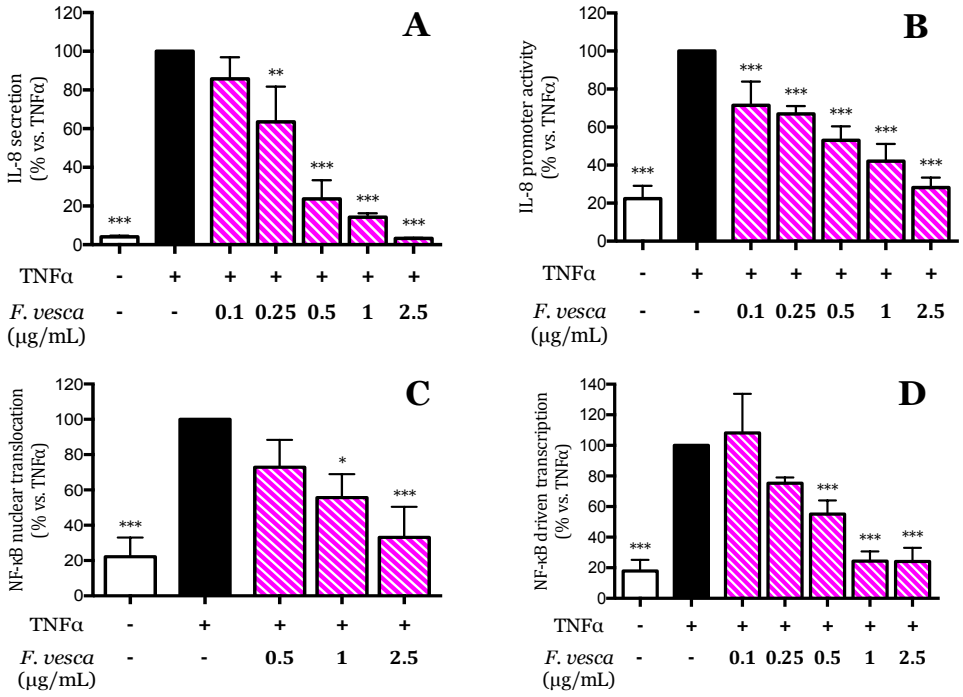


Figure 16. Effect of the *Fragaria vesca* extract on IL-8 release (A), IL-8 promoter activity (B), NF-κB nuclear translocation (C) and NF-κB driven transcription (D) in TNFα-treated AGS cells. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. *p < 0.05, **p < 0.01, ***p < 0.0001 versus TNFα.

The graphs in **Figure 16** demonstrate that *Fragaria vesca* extract inhibits all the parameters tested in a concentration dependent way. The wild strawberry extract shows the following IC_{50}s: 0.29 μg/mL on IL-8 release, 0.27 μg/mL on IL-8 promoter activity, 0.42 μg/mL on NF-κB driven transcription and 0.79 μg/mL on NF-κB nuclear translocation.

Although below 1μg/mL, these IC_{50}s are slightly higher compared to those obtained by testing *Fragaria* X *ananassa* extract, suggesting that the extract from *Fragaria vesca* is less active in comparison to that from *Fragaria* X *ananassa*.

As mentioned above, EGCG was used as reference compound exerting the expected inhibitory activity on each parameter.

4.1.3 Inhibitory effect of *Fragaria* X *ananassa* extract, after *in vitro* gastric digestion, on IL-8 and NF-κB pathway in TNFα-treated AGS cells

To test the chemical stability and biological activity of strawberry tannins in the harsh environment of the human stomach, *Fragaria* X *ananassa* extract was subjected to an *in vitro* gastric digestion, as described in the Material and Method section. Then, the digested extract was assessed on the same parameters analyzed with undigested extract.

AGS cells were co-treated with the stimulus TNFα (10 ng/mL) and the extract at different concentrations (0.25 – 5 μg/mL).

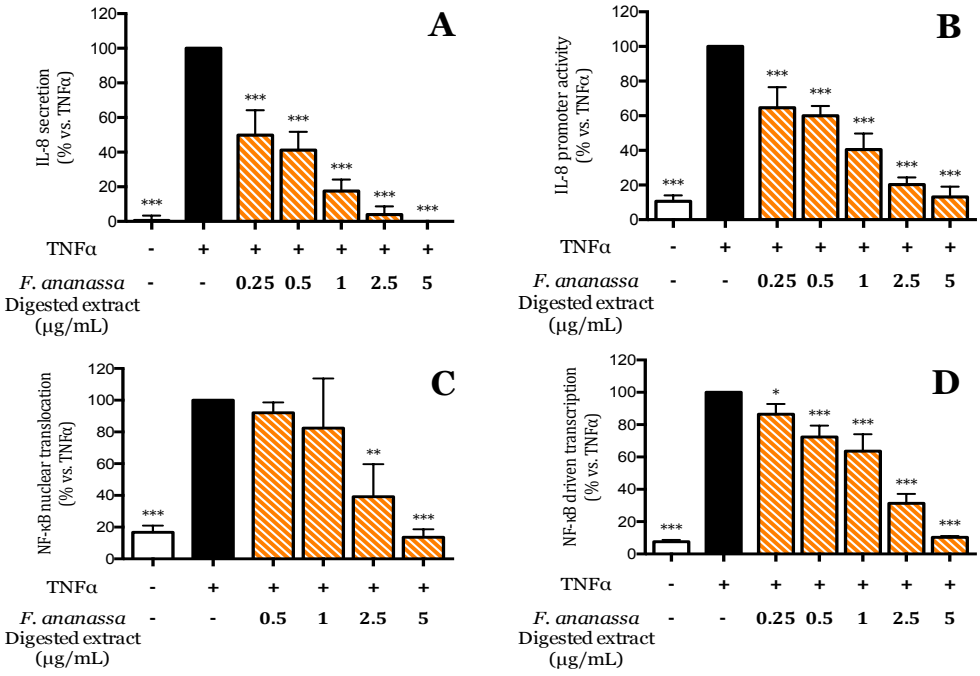


Figure 17. Effect of the *Fragaria* X *ananassa* extract, after *in vitro* gastric digestion, on IL-8 release (A), IL-8 promoter activity (B), NF-κB nuclear translocation (C) and NF-κB driven transcription (D) in TNFα-treated AGS cells. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. *p < 0.05, **p < 0.01, ***p < 0.0001 versus TNFα.

The activity of strawberry extract, subjected to simulated gastric digestion, on IL-8 release is unchanged, the IC_{50}s of digested and undigested extracts are similar, 0.25 and 0.31 μg/mL respectively. While considering the other parameters, a slight loss of activity is observed with the digested extract. The IC_{50}s of the digested extract on the NF-κB driven transcription and nuclear translocation are around 5 or 6-fold higher than those calculated with undigested extract. Moreover, the complete inhibition of TNFα-induced IL-8 promoter activity, NF-κB driven transcription and nuclear translocation is reached at higher concentrations with respect to the native extract.

EGCG was used as reference compound exerting the expected inhibitory activity on each parameter.

4.2 CHEMICAL COMPOSITION OF TANNIN-ENRICHED EXTRACTS FROM STRAWBERRIES

Chemical characterization of strawberry extracts was performed in collaboration with the research group of Dr. Fulvio Mattivi (Department of Food Quality and Nutrition, Research and Innovation Centre, Fondazione Edmund Mach, San Michele all'Adige, Italy).

Compound	Tannins enriched extract		*in vitro* digested extract
	strawberry	wild strawberry	strawberry
	μg/g	μg/g	μg/g
methyl gallate	0.5	0.1	2.0
fraxin	0.1	0.2	0.0
chlorogenic acid	1.6	2.2	2.7
trans-coutaric acid	0.2	0.1	0.4
trans-piceide	6.0	4.8	11.2
cis-piceide	28.6	1.0	15.1
phloretin	0.7	0.2	20.1
phlorizin	366.2	31.8	281.5
trilobatin	39.4	2.5	29.9
luteolin	64.8	118.4	45.3
luteolin-7-*O*-glucoside	8.8	1.3	5.8
naringenin	1.0	1.9	6.8
catechin	19223.4	17242.4	21486.1
epicatechin	346.0	653.0	317.1
procyanidin B1	15630.6	15553.1	21082.8
procyanidin B2 + B4 *(as eq. Procyanidin B2)*	1660.8	2510.5	667.4
procyanidin B3 *(as eq. Procyanidin B1)*	27270.7	18310.6	17710.4
kaempferol	112.7	12.5	77.1
quercetin	24.2	6.8	35.2
taxifolin	7.3	10673.2	5.1
kaempferol-3-glucoside	1164.9	494.6	1023.9
kaempferol-3-rutinoside	99.1	2.4	84.4
dihydrokaempferol	78.8	49.9	70.2
quercetin-3-glucuronide	1815.4	175.0	1824.9
kaempferol-3-glucuronide	1197.8	19.9	935.3
4-hydroxybenzoic acid	229.1	156.1	248.0
p-coumaric acid	11.1	0.2	14.3
gallic acid	2.1	6.8	28.0
caffeic acid	2.4	0.1	0.8
trans-ferulic acid	1.1	0.0	2.5
urolithin A	0.4	1.2	8.3
ellagic acid	1675.9	2378.5	1845.6
pyrocatechol	116.6	106.9	97.6
quercetin-3-glucoside + quercetin-3-galactoside *(as eq. quercetin-3-glucoside)*	148.7	798.3	116.9
isorhamnetin-3-glucoside	15.4	317.8	10.3
methyl ellagic acid rhamnoside	19.9	7697.2	13.5
agrimoniin	42903.8	52297.8	31563.8
casuarictin	46471.3	23169.2	42866.3
sanguiin H6	2241.0	2069.6	3078.3
Total polyphenols (MS/MS analysis)	162988.4	154868.1	145635.2
proanthocyanidins	485576.0	360983.2	349877.7
Total polyphenols (MS/MS analysis and proanthocyanidins)	648564.4	515851.3	495513.0

Table 1. Chemical composition of tannin-enriched extracts from strawberries.

In **Table 1** the chemical characterization of the tannin-enriched strawberry extracts before and after *in vitro* digestion is reported. A total of 41 different polyphenols were detected in both undigested extracts. Agrimoniin and casuarictin are the main ellagitannins occurring in strawberry extracts, while the second most abundant class of polyphenols is procyanidins (procyanidin B1-B4). About 40-50% of strawberry extracts are composed by oligomeric and polymeric proanthocyanidins. Other classes of polyphenols, such as flavonones, flavanones, flavonols, dihydrochalcones are present in lower amounts. Anthocyanins, instead, are not present most likely because due to lower molecular weight they were removed during chromatography Sephadex LH-20, which allows to obtain extracts enriched in tannins.

Only *Fragaria* X *ananassa* extract was subjected to an *in vitro* gastric digestion since this species is more active than *Fragaria vesca*, moreover its consumption is much more diffused among the population.

The chemical analysis of the material subject to simulated gastric digestion reveals that among ellagitannins, agrimoniin is the most affected (26% loss with respect to 8% loss of casuarictin). Even the procyanidin fraction undergoes chemical modification, in fact the content of procyanidin B1 increases by 26% while that of procyanidin B3 decreases by 35%. This increase in procyanidins B1 is probably due to the degradation of other condensed tannins. Almost negligible degradation of ellagitannins to urolithins is observed since only minor amounts of urolithin A (7.9 μg/g) are formed, starting from about 90 mg/g of agrimoniin and casuarictin.

The relative stability of casuarictin, agrimoniin and sanguiin H-6 to hydrolysis can be inferred also from the modest release of gallic acid and ellagic acid following *in vitro* digestion (25.9 μg/g and 169.7 μg/g, respectively).

4.3 INHIBITORY ACTIVITY OF PURE TANNINS AND ELLAGIC ACID ON IL-8 RELEASE AND NF-κB SIGNALING IN TNFα-TREATED AGS CELLS

Agrimoniin and casuarictin, belonging to the class of ellagitannins, are the most abundant compounds occurring in both *Fragaria* extracts (as reported in **Table 1**) and they are present at comparable concentrations. Moreover, they are chemically stable after simulated gastric digestion. On the basis of these findings, I examined the effects of these pure compounds on the NF-κB signaling and IL-8 expression and release. The experiments were aimed to investigate the contribution of these compounds to the activity observed with the extracts and to identify the underlying molecular mechanisms.

The strawberry extracts contain also significant amount of condensed tannins, such as procyanidin B1 and B3. Therefore, the biological activity of procyanidin B1 was evaluated, in AGS cells stimulated with TNFα, on the same pro-inflammatory parameters previously indicated. This compound was chosen as a representative member of condensed tannins, procyanidin B3 was not tested since it is not commercially available.

In the extracts, free ellagic acid is present at biologically significant concentrations, 1.7 and 2.4 mg/g of strawberry and wild strawberry extract, respectively. Hence, I inferred that ellagic acid might contribute to the biological activities exhibited by the tannin-enriched

extracts shown above. Moreover, from the structure-activity point of view, the moiety of ellagic acid is a common feature of many ellagitannins found in strawberries and other natural sources. For these reasons I decided to investigate the biological activities of ellagic acid in TNFα-treated AGS cells.

4.3.1 Inhibitory effect of agrimoniin on IL-8 and NF-κB pathway in TNFα-treated AGS cells

Agrimonin was evaluated on IL-8 release, IL-8 promoter activity, NF-κB driven transcription and nuclear translocation in AGS cells. The cells were co-treated with the stimulus TNFα (10 ng/mL) and the pure molecule at different concentrations (0.1 – 2.5 μM).

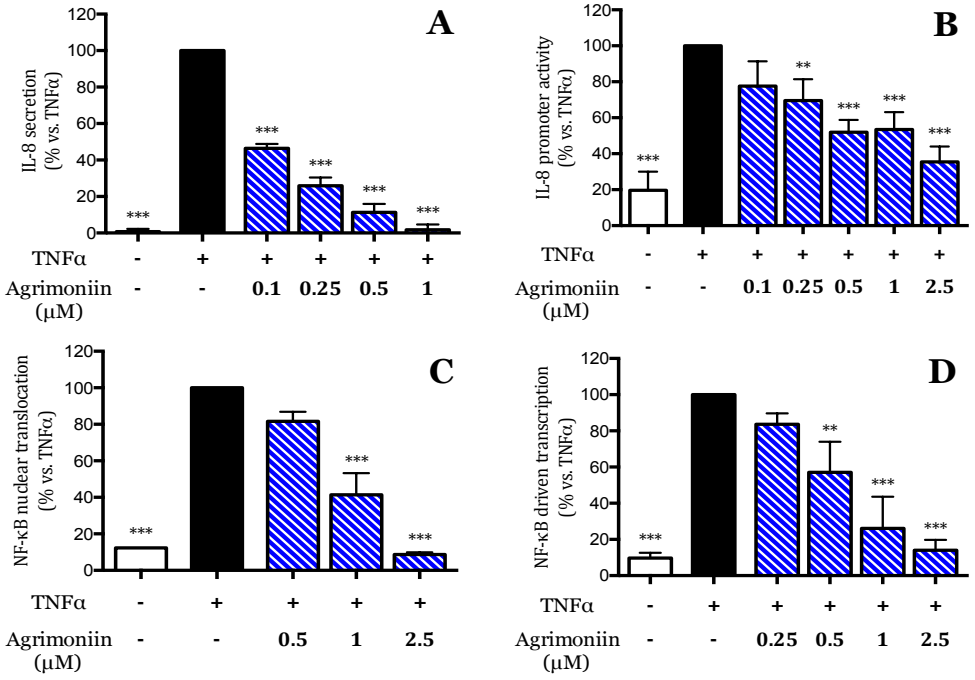


Figure 18. Effect of agrimoniin on IL-8 release (A), IL-8 promoter activity (B), NF-κB nuclear translocation (C) and NF-κB driven transcription (D) in TNFα-treated AGS cells. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. **p < 0.01, ***p < 0.0001 versus TNFα.

The pure compound was able to inhibit all the parameters in concentration dependent way (**Figure 18**). Inhibition of TNFα-induced IL-8 secretion by agrimoniin occurs with an IC_{50} (0.09 μM) much lower than those observed in the analysis of the other parameters (0.42 μM on IL-8 promoter activity, 0.50 μM on NF-κB driven transcription and 0.81 μM on NF-κB nuclear translocation). These results suggested that agrimoniin inhibits IL-8 release acting on NF-κB pathway, as demonstrated by the inhibitory effects observed on NF-κB driven transcription and p65 translocation, but also on other biological targets. EGCG was used as reference compound exerting the expected inhibitory activity on each parameter.

4.3.2 Inhibitory effect of casuarictin on IL-8 and NF-κB pathway in TNFα-treated AGS cells

Casuarictin was evaluated on IL-8 release, promoter activity, NF-κB driven transcription and nuclear translocation in AGS cells. The cells were co-treated with the stimulus TNFα (10 ng/mL) and the pure molecule at different concentrations (0.05 – 5 μM).

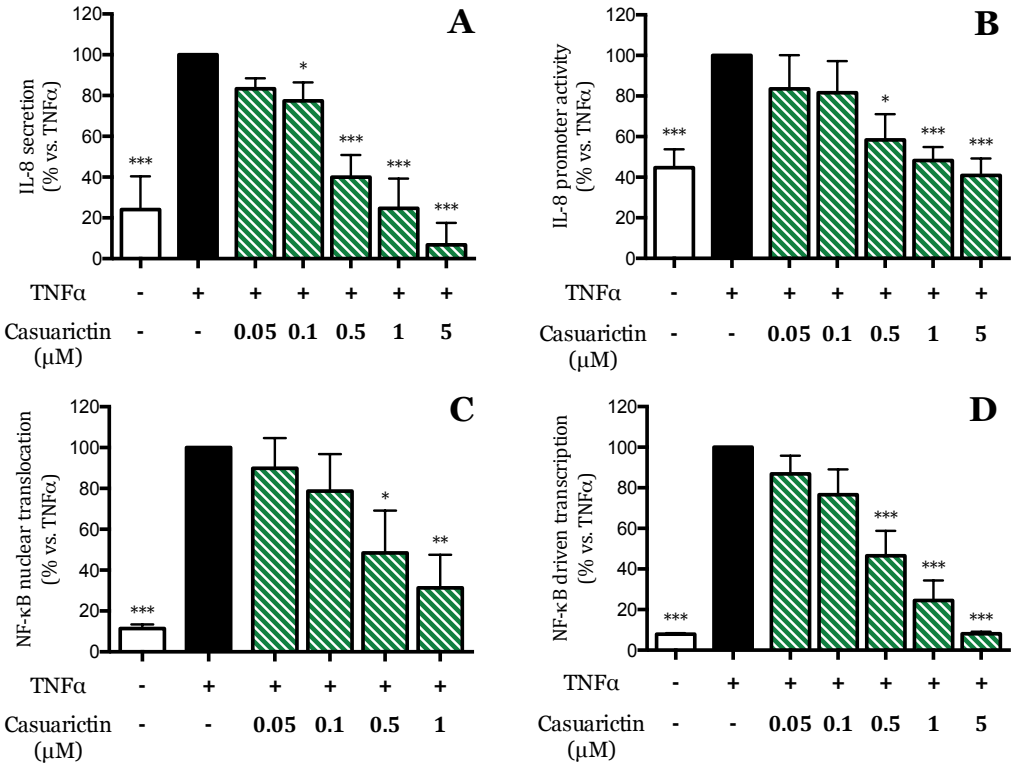


Figure 19. Effect of casuarictin on IL-8 release (A), IL-8 promoter activity (B), NF-κB nuclear translocation (C) and NF-κB driven transcription (D) in TNFα-treated AGS cells. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. *p < 0.05, **p < 0.01, ***p < 0.0001 versus TNFα.

Casuarictin is able to inhibit all the parameters in a concentration dependent manner (**Figure 19**). This pure ellagitannin reduces IL-8 secretion (IC_{50}: 0.29 μM) at the same concentrations required to dampen p65 translocation (IC_{50}: 0.33 μM), NF-κB driven transcription (IC_{50}: 0.29 μM) and IL-8 promoter activity (IC_{50}: 0.19 μM). These results indicate that casuarictin affect IL-8 release acting solely as a pure NF-κB inhibitor.

EGCG was used as reference compound exerting the expected inhibitory activity on each parameter.

4.3.3 Inhibitory effect of procyanidins B1 on IL-8 and NF-κB pathway in TNFα-treated AGS cells

Procyanidin B1 was tested on IL-8 release, promoter activity, NF-κB driven transcription and nuclear translocation in AGS cells. The cells were co-treated with the stimulus TNFα (10 ng/mL) and the pure molecule at different concentrations (0.1 – 50 μM).

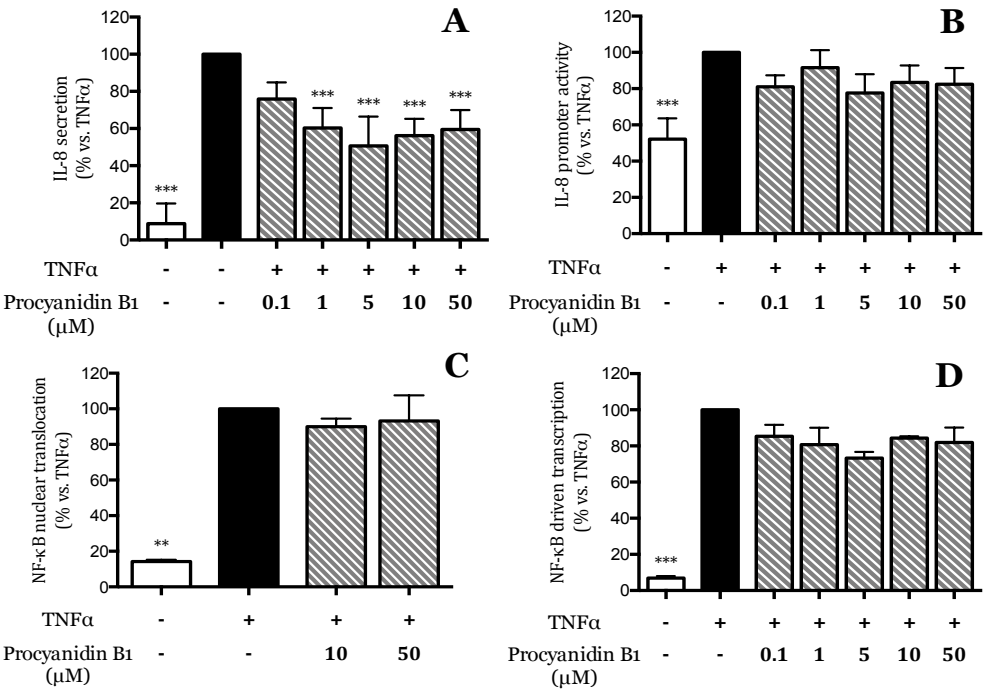


Figure 20. Effect of procyanidin B1 on IL-8 release (A), IL-8 promoter activity (B), NF-κB nuclear translocation (C) and NF-κB driven transcription (D) in TNFα-treated AGS cells. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. **p < 0.01, ***p < 0.0001 versus TNFα.

As shown in **Figure 20**, procyanidin B1 reduces TNFα-induced IL-8 secretion in non-concentration dependent manner, exerting a statistically significant inhibition around 44% at the concentration of 1 μM, while no effect on p65 translocation and NF-κB-driven transcription is observed at concentration as high as 50 μM. These effects suggest the ability of this representative condensed tannin to counteract TNFα-induced IL-8 release without interfering with the NF-κB pathway.

EGCG was used as reference compound exerting the expected inhibitory activity on each parameter.

4.3.4 Inhibitory effect of ellagic acid on IL-8 and NF-κB pathway in TNFα-treated AGS cells

Ellagic acid was tested on IL-8 release, promoter activity, NF-κB driven transcription and nuclear translocation in AGS cells. The cells were co-treated with the stimulus TNFα (10 ng/mL) and the pure molecule at different concentrations (0.05 – 10 μM).

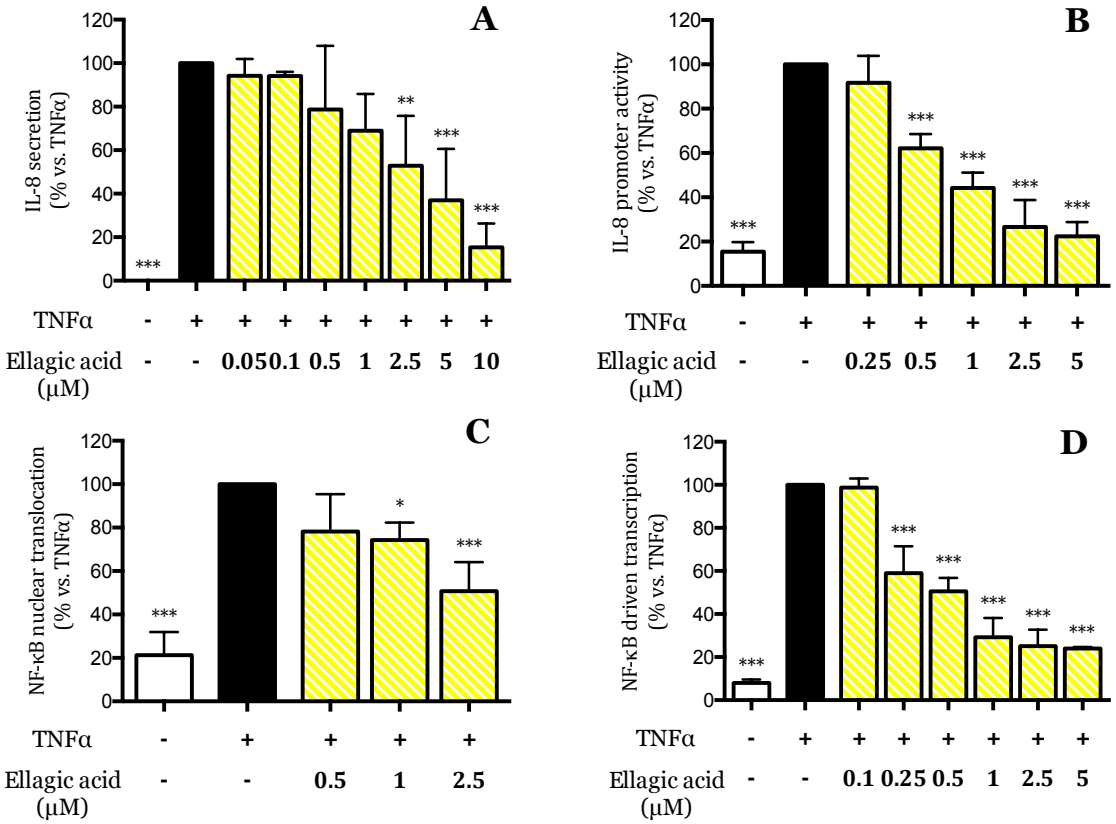


Figure 21. Effect of ellagic acid on IL-8 release (A), IL-8 promoter activity (B), NF-κB nuclear translocation (C) and NF-κB driven transcription (D) in TNFα-treated AGS cells. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. *p < 0.05, **p < 0.01, ***p < 0.0001 versus TNFα.

In **Figure 21** it is shown that ellagic acid inhibits all the parameters in a concentration dependent manner, the IC_{50}s calculated are:

IL-8 release = 2.56 μM

IL8 promoter activity = 0.62 μM

NF-κB nuclear translocation = 1.68 μM

NF-κB driven transcription = 0.44 μM

These IC_{50} values suggest that ellagic acid moiety contributed, at least in part, to the biological activity of ellagitannins and similarly to agrimoniin and casuarictin, ellagic acid counteract the activation of IL-8 secretion elicited by TNFα in an NF-κB-dependent manner.

However, other chemical and stereochemical features seem to impact its ability to interact with biological targets.

EGCG was used as reference compound exerting the expected inhibitory activity on each parameter.

4.4 SUMMARY OF IC50 VALUES

Biological process	**NF-κB signaling pathway**		**IL-8 expression and secretion**	
Biological assay	**p65 translocation**	**NF-κB driven transcription**	**IL-8 promoter activity**	**IL-8 secretion**
strawberry	0.30 μg/ml	0.23 μg/ml	0.17 μg/ml	0.25 μg/ml
wild strawberry	0.79 μg/ml	0.42 μg/ml	0.27 μg/ml	0.29 μg/ml
strawberry (digested)	1.71 μg/ml	1.07 μg/ml	0.48 μg/ml	0.31 μg/ml
Agrimoniin	0.81 μM	0.50 μM	0.42 μM	0.09 μM
Casuarictin	0.33 μM	0.29 μM	0.19 μM	0.29 μM
Ellagic acid	1.68 μM	0.44 μM	0.62 μM	2.56 μM
Procyanidins B1	inactive	inactive	inactive	44% inhibition at 1 μM

Table 2. IC_{50} of tannin-enriched extracts and strawberry tannins for the tested biological activities. Results are the mean of at least three experiments performed in triplicate.

4.5 CHARACTERIZATION OF GES-1 CELL LINE

Despite AGS, a tumour gastric epithelial cell line, is a well-established *in vitro* model and one of the mostly reported in literature to study the effects of natural products on gastric inflammation, it appears appropriate to investigate the biological activities of extracts or pure compounds derived from plants, using an *in vitro* model closer to a normal gastric epithelium.

Indeed, GES-1 cell line represents a model of normal gastric epithelial cells (Ke Y. et al., 1994). It has been used in less works, compared to AGS cells, reported in literature; however, this cell line is less characterized and only fragmented information about the release of pro-inflammatory mediators and the activation of transcription factors involved in the gastric inflammation are available.

For these reasons, before testing the *Fragaria* extracts, I decided to investigate the ability of this cell line to respond to TNFα, evaluating: the release of several pro-inflammatory mediators (IL-8, IL-6 and MMP-9), the mRNA levels of several pro-inflammatory genes and the activation of NF-κB pathway.

4.5.1 TNFα-induced IL-8, IL-6 and MMP-9 release and NF-κB driven transcription in GES-1 and AGS cells

GES-1 and AGS cells were stimulated with TNFα (10 ng/mL) at different times, in order to evaluate the release of some NF-κB-dependent pro-inflammatory mediators (IL-8, IL-6 and MMP-9) exerting a key role in the gastric inflammation, and the activation of the NF-κB transcription factor.

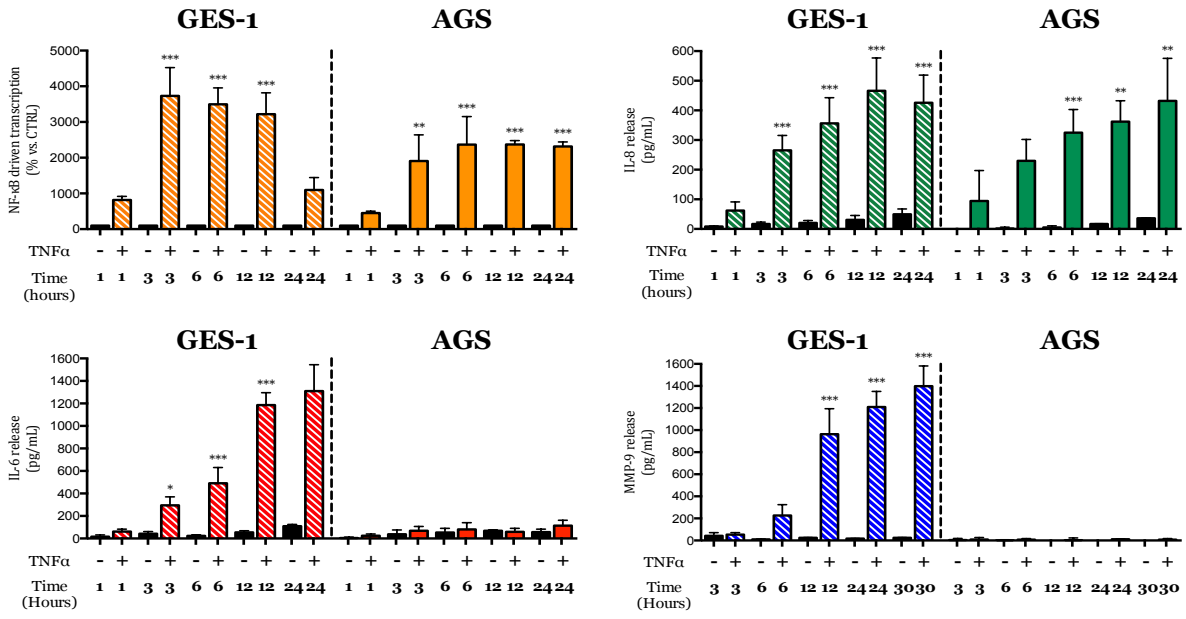


Figure 22. Effect of TNFα on the release of IL-8 (green bars), IL-6 (red bars) and MMP-9 (blue bars) and on the activation of NF-κB transcription factor (orange bars) in GES-1 and AGS cells. Data are expressed in pg/mL. *p < 0.05, **p < 0.01, ***p < 0.0001 versus TNFα.

As shown in **Figure 22**, NF-κB driven transcription and IL-8 release are induced by TNFα in both cell models. Differences are observed in IL-6 and MMP-9 release; in fact, the secretion of these two pro-inflammatory mediators is increased (in time-dependent manner), by the stimulus TNFα, in GES-1 cells only. Despite NF-κB factor is involved in IL-6 and MMP-9 expression and it is activated, by TNFα, in our cell models, this pro-inflammatory stimulus is not able to induce the release of IL-6 and MMP-9 in AGS cells.

4.5.2 TNFα-induced pro-inflammatory genes in GES-1 and AGS cells

In order to better characterize GES-1 cell model, the expression of 84 key genes mediating the inflammatory response was evaluated using an array (Human Inflammatory Cytokines and Receptors RT² Profiler PCR Array) commercially available.

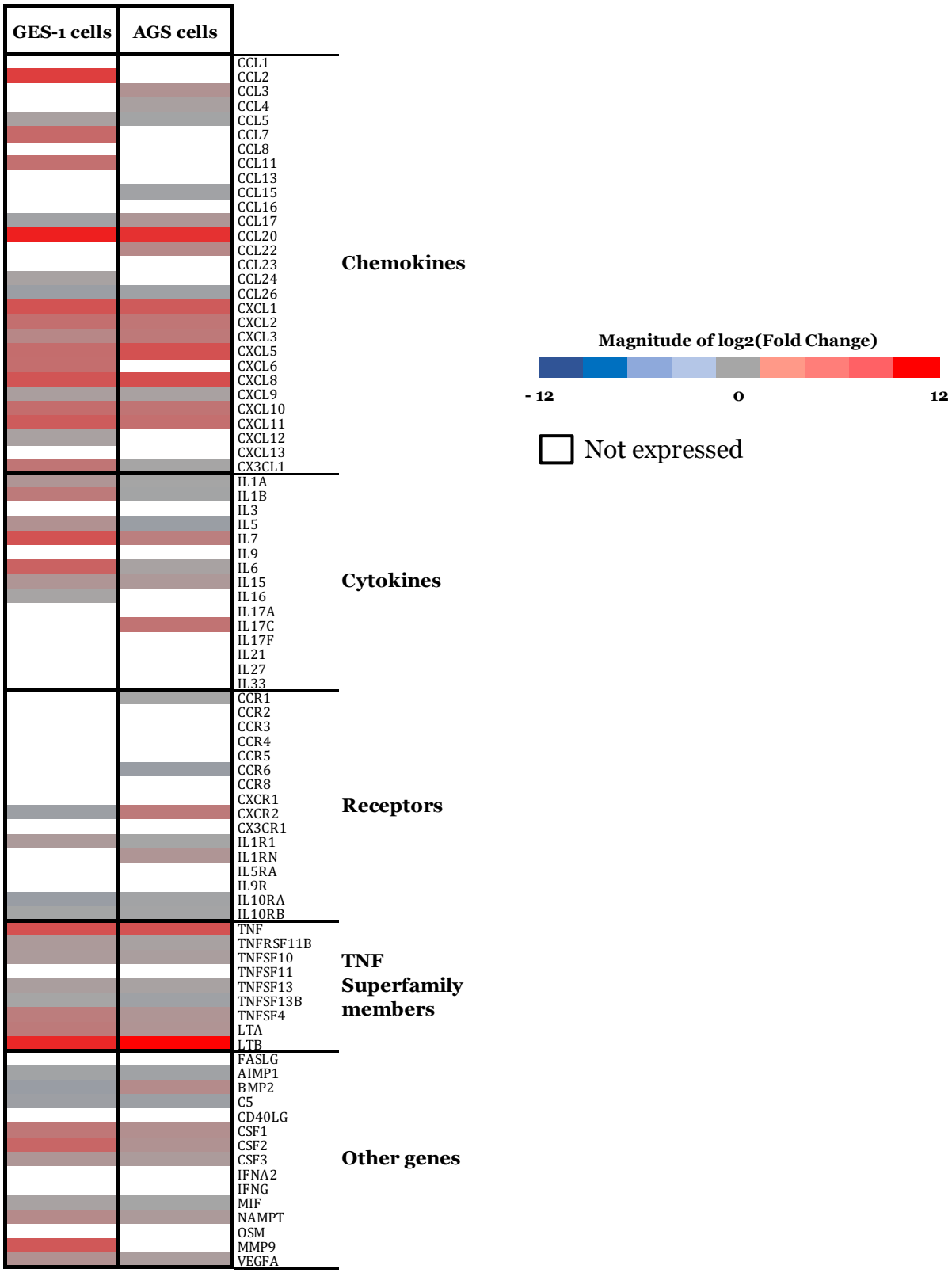


Figure 23. Heat map. Effect of TNFα on the expression of 84 pro-inflammatory genes in GES-1 and AGS cells. The cells were treated with TNFα (10 ng/mL) for 6 h. Data are expressed as $\log_2$ (Fold Change).

GES-1 and AGS cells were treated with TNFα (10 ng/mL) for 6 hours in order to evaluate the expression of 84 pro-inflammatory genes, coding mostly for chemokines, cytokines, receptors and TNF superfamily members, in the two cell models. The results reported in **Figure 23** show that TNFα up-regulates several chemokines in both GES-1 and AGS cells, for example IL-8 (CXCL8) and CCL20 are up-regulated in both cell lines while CCL2 only in GES-1 cells. Among TNF superfamily members, TNF (TNFα) and LTB (TNFC) are highly expressed in both cell models.

IL-6 and MMP-9 expression is induced, by TNFα, in GES-1 cells but not in AGS cells: IL-6 expression is unchanged compared to the basal level, while MMP-9 is not expressed in this cell model, reflecting what previously observed on the release of these pro-inflammatory mediators. These data show that the ability of GES-1 and AGS cells to respond to TNFα is characterized by differences in the expression of some pro-inflammatory genes, some genes are upregulated in both cell models, while others in GES-1 or AGS cells; GES-1 cells could be an interesting model to study the activity of natural extracts on the major mediators involved in the gastric inflammation.

The heat map shows the mean of three experiment, data are expressed as $\log_2$ (Fold Change). Fold Change are calculated as TNFα vs. CTRL. The C_t cut-off is set to 35.

4.6 INHIBITORY ACTIVITY OF STRAWBERRY TANNINS ON NF-κB PATHWAY AND THE RELEASE OF PRO-INFLAMMATORY MEDIATORS IN GES-1 CELLS

Fragaria X *ananassa* extract and pure ellagitannins were tested on NF-κB driven transcription and on the release of some NF-κB-dependent pro-inflammatory mediators (IL-8, IL-6 and MMP-9) in GES-1 cells. Since NF-κB driven transcription and IL-8 release were induced, by TNFα, in both GES-1 and AGS cells, the activity of the extract and single compounds, on these parameters, was compared in the two cell models in order to verify if the effect observed in tumor gastric epithelial cells was maintained also in normal gastric epithelial cells.

4.6.1 *Fragaria* X *ananassa* extract inhibit NF-κB driven transcription and the release of IL-8, IL-6 and MMP-9 in GES-1 cells

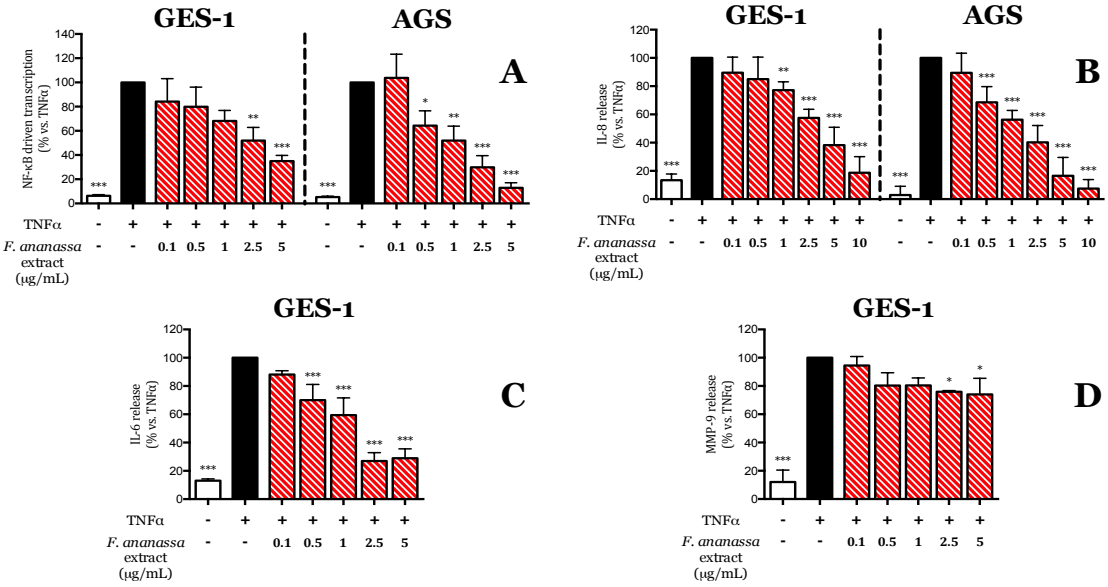


Figure 24. Effect of *Fragaria* X *ananassa* extract on NF-κB driven transcription (A), IL-8 release (B), IL-6 release (C) and MMP-9 release (D) in TNFα-treated GES-1 and AGS cells. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. *p < 0.05, **p < 0.01, ***p < 0.0001 versus TNFα.

In order to investigate the effects of tannin-enriched extract from *Fragaria* X *ananassa* species, on the parameters previously indicated, tumor and normal gastric cells were co-treated with the stimulus TNFα (10 ng/mL) and the extract at different concentrations (0.1 – 10 μg/mL).

As demonstrated in **Figure 24**, strawberry extract is able to inhibit NF-κB driven transcription (IC_{50}: 2.15 μg/mL), IL-8 release (IC_{50}: 2.23 μg/mL) and IL-6 release (IC_{50}: 0.92

μg/mL) in concentration dependent manner in GES-1 cells, while a mild activity is observed on MMP-9 release (IC_{50} > 5 μg/mL). The extract appears more active in AGS cells, in fact the IC_{50}s obtained in AGS cells on NF-κB driven transcription and IL-8 release (0.93 μg/mL and 1.15 μg/mL, respectively) are lower than those calculated, on the same parameters, in GES-1 cells (2.15 μg/mL and 2.23 μg/mL, respectively).

EGCG 20 μM was used as reference compound: >70% inhibition of IL-8 secretion, >50% inhibition of IL-6 release, >80% inhibition of MMP-9 release and >50% inhibition of NF-κB driven transcription.

4.6.2 Pure ellagitannins inhibit NF-κB driven transcription and the release of IL-8, IL-6 and MMP-9 in GES-1 cells

In order to investigate the effects of agrimoniin and casuarictin, the cells were co-treated with the stimulus TNFα (10 ng/mL) and the extract at different concentrations (0.1 – 5 μM).

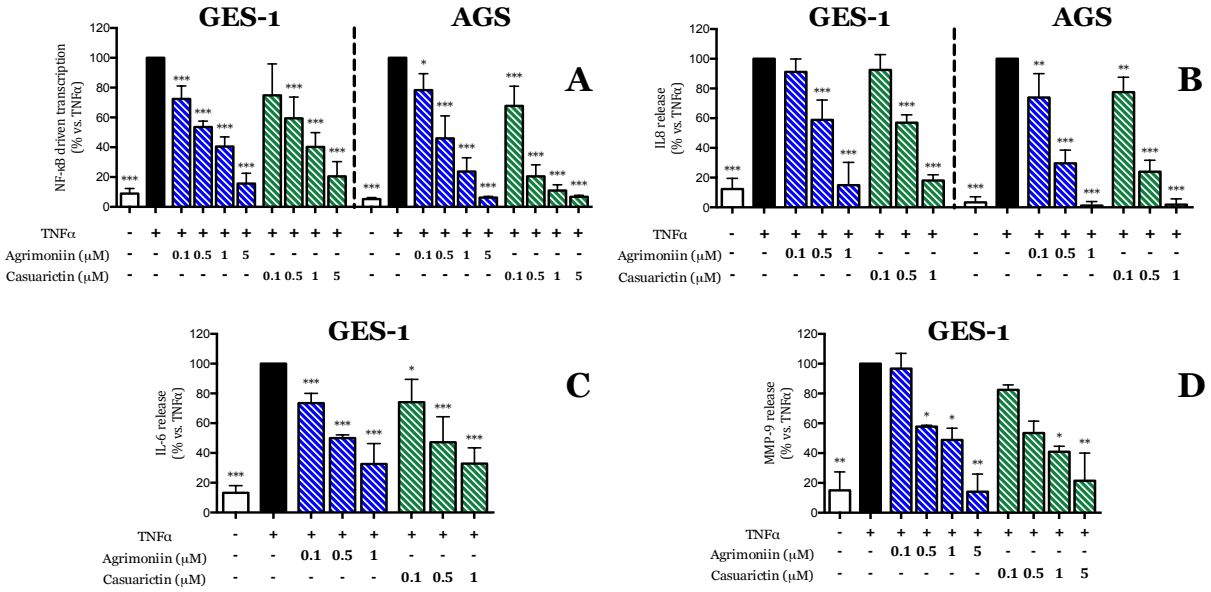


Figure 25. Effect of agrimoniin (blue bars) and casuarictin (green bars) on NF-κB driven transcription (A), IL-8 release (B), IL-6 release (C) and MMP-9 release (D) in TNFα-treated GES-1 and AGS cells. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%. *p < 0.05, **p < 0.01, ***p < 0.0001 versus TNFα.

The two compounds are able to reduce, in GES-1 cells, each parameter in concentration dependent manner, showing the following IC_{50}s:

NF-κB driven transcription → 0.39 μM (agrimoniin), 0.49 μM (casuarictin)

IL-8 release → 0.50 μM (agrimoniin), 0.51 μM (casuarictin)

IL-6 release → 0.28 μM (agrimoniin), 0.28 μM (casuarictin)

MMP-9 release → 0.60 μM (agrimoniin), 0.41 μM (casuarictin)

These values suggest that the two ellagitannins, in GES-1 cells, affect each pro-inflammatory parameter with very similar IC_{50}s. Similarly to strawberry extract, as described in the previous paragraph, the two ellagitannins appear more active in AGS cells compared to GES-1 cells; this is confirmed comparing the IC_{50} values:

	GES-1 cells	**AGS cells**
NF-κB driven transcription	0.39 μM (agrimoniin) 0.49 μM (casuarictin)	0.33 μM (agrimoniin) 0.16 μM (casuarictin)
IL-8 release	0.50 μM (agrimoniin) 0.51 μM (agrimoniin)	0.21 μM (agrimoniin) 0.21 μM (casuarictin)

EGCG 20 μM was used as reference compound: >70% inhibition of IL-8 secretion, >50% inhibition of IL-6 release, >80% inhibition of MMP-9 release and >50% inhibition of NF-κB driven transcription.

4.7 AGRIMONIIN AND CASUARICTIN INHIBIT THE EXPRESSION OF SOME PRO-INFLAMMATORY GENES IN GES-1 AND AGS CELLS

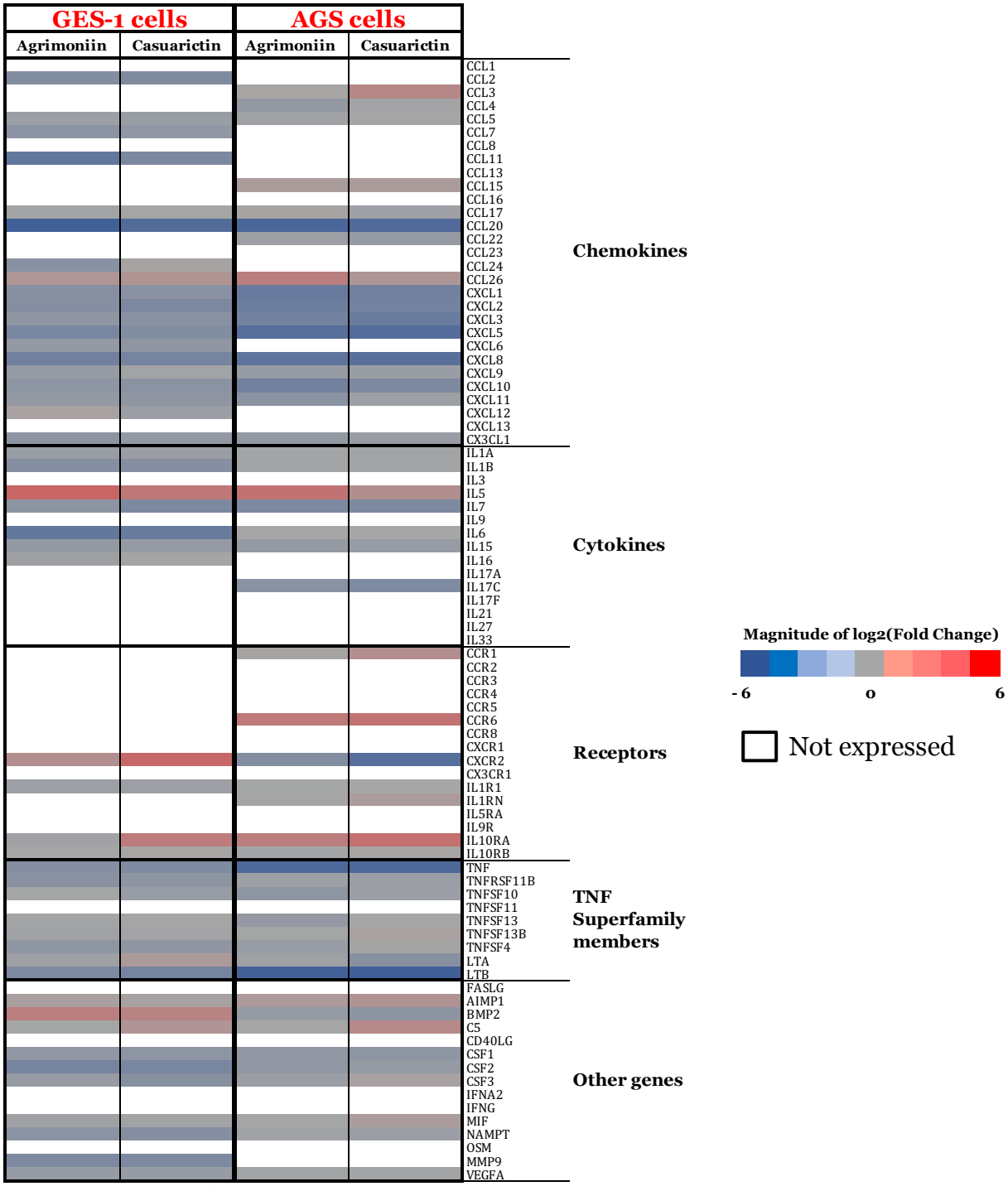


Figure 26. Heat map. Effect of agrimoniin and casuarictin on TNFα-induced gene expression of 84 pro-inflammatory genes in GES-1 and AGS cells. The cells were treated with TNFα (10 ng/mL) and agrimoniin and casuarictin for 6 h. Data are expressed as $\log_2$ (Fold Change).

GES-1 and AGS cells were treated with TNFα (10 ng/mL) and agrimoniin or casuarictin (1 μM) for 6 hours. The effect of these compounds on the expression of 84 pro-inflammatory genes was evaluated using a real-time PCR, as reported in Materials and Methods section. In both cell lines, agrimoniin and casuarictin are able to decrease the mRNA levels of most up-

regulated genes. The two molecules appear more active in AGS cells compared to GES-1 cells; for example, on IL-8, TNF and LTB genes, agrimoniin and casuarictin clearly show a higher ability to reduce the mRNA levels of these genes in AGS cells than in GES-1 cells.
The heat map shows the mean of three experiment, data are expressed as $\log_2$ (Fold Change). Fold Change are calculated as MOLECULE vs. TNFα. The C_t cut-off is set to 35.

4.8 INHIBITORY ACTIVITY OF *FRAGARIA* X *ANANASSA* EXTRACT ON *H. PYLORI*-INDUCED IL-8 RELEASE

GES-1 and AGS cells were treated with *H. pylori* (ratio *H. pylori*:cells 50:1) and *Fragaria* X *ananassa* extract at increasing concentrations (10 – 100 μg/mL). After 6 hours treatment the culture medium was collected in order to measure the amount of IL-8. *H. pylori* was used to reproduce an experimental setting closer to the pathological condition.

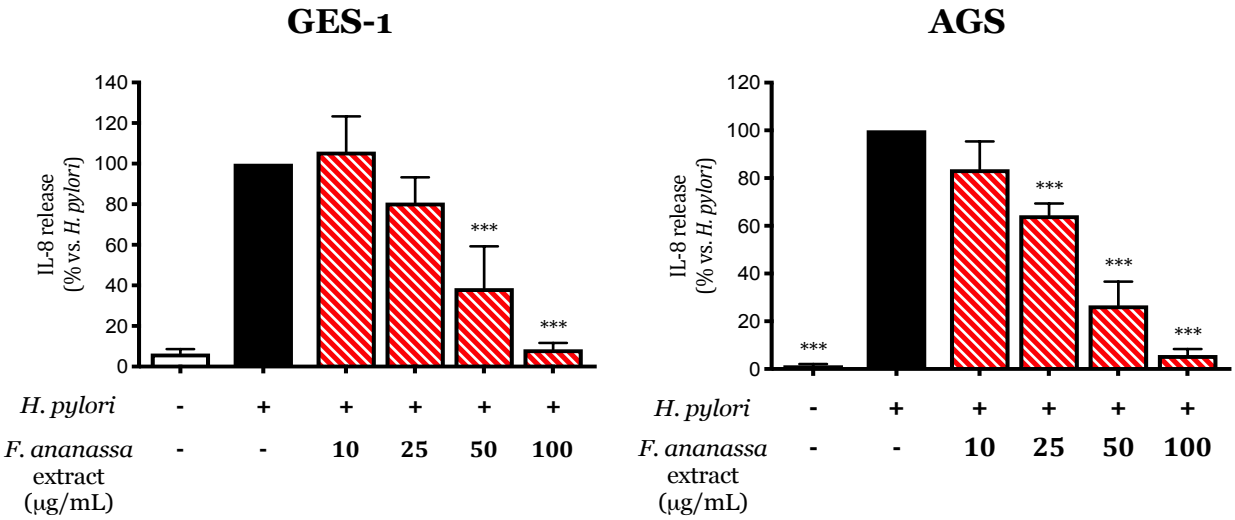


Figure 27. Effect of *Fragaria* X *ananassa* extract on *H. pylori*-induced IL-8 release in GES-1 and AGS cells. Data are expressed in percentage, relative to the stimulated control, which is arbitrarily assigned to the value of 100%.

As shown in **Figure 27**, *Fragaria* X *ananassa* extract is able to reduce IL-8 release in both cell lines. The IC_{50} of the extract in AGS cells is lower than in GES-1 cells (30.8 and 40.0 μg/mL, respectively), thus confirming, as observed in the previous results, that the extract is more active in AGS model. At the highest concentration tested (100 μg/mL) the release of the chemokine is reported at the basal level. This is a preliminary data, it needs to be confirmed with other experiments. EGCG 50 μM was used as reference compound, showing around 50% of inhibition.

4.9 ANTIOXIDANT ACTIVITIES OF STRAWBERRY TANNINS

Oxidative stress is increased in the gastric mucosa infected with the bacterium *H. pylori*, enhancing the risk of damage to the gastric mucosa. Since gastric epithelial cells contribute to ROS production, the antioxidant capacity of the strawberry extract and pure ellagitannins was assessed. Moreover, I tested the effect of the single ellagitannins on Nrf2 nuclear translocation, this transcription factor is activated in presence of stress oxidative conditions, regulating the expression of cytoprotective genes (including NQO1 and GSTs).

4.9.1 Antioxidant capacity of the strawberry extract and pure ellagitannins

The antioxidant capacity of the extract and pure ellagitannins was measured by ORAC method, as described in Materials and Methods. This method measures the oxidative degradation of fluorescein, after the addition of the free radical generator AAPH in a cell-free system, leading to a decrease in fluorescence, prevented by the presence of antioxidant compounds. This method allows to evaluate the scaveging activity of extracts and pure compounds against peroxyl radicals in a cell-free system.

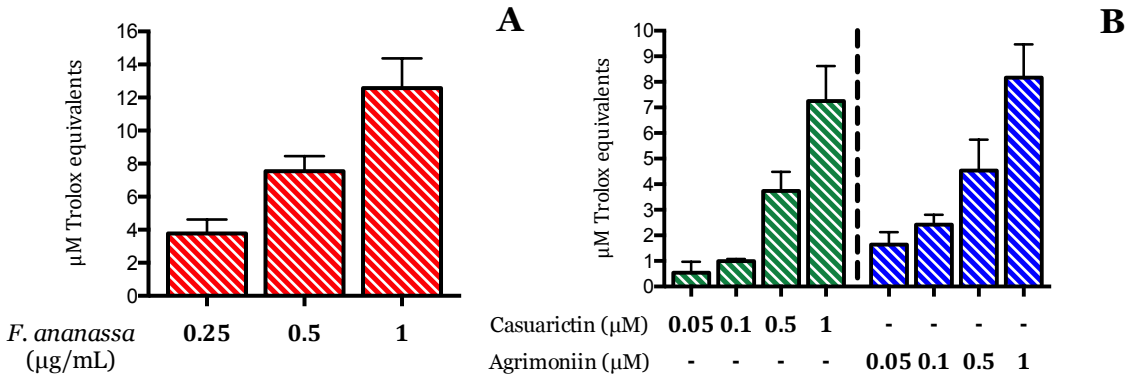


Figure 28. Antioxidant capacity of the strawberry extract (A) and pure ellagitannins (B) measure by ORAC method. Data are expressed as µM Trolox equivalents.

Fragaria X *ananassa* extract was tested in the concentration range 0.25 - 1 µg/mL, while the two ellagitannins in the concentration range 0.05 – 1 µM. **Figure 28** shows that *Fragaria* extract (A) and single compounds (B) are able to counteract peroxyl radicals (acting as scavengers) in a concentration dependent manner.

4.9.2 Agrimoniin and Casuarictin inhibit Nrf2 nuclear translocation in GES-1 and AGS cells

Nrf2 is a transcription factor able to respond to oxidative stress, regulating the expression of cytoprotective genes, including antioxidant endogenous enzymes. Since oxidative stress is increased during *H. pylori* infection, agrimoniin and casuarictin were evaluated for their

contribution to promote Nrf2 nuclear translocation and, consequently, its transcriptional activity.

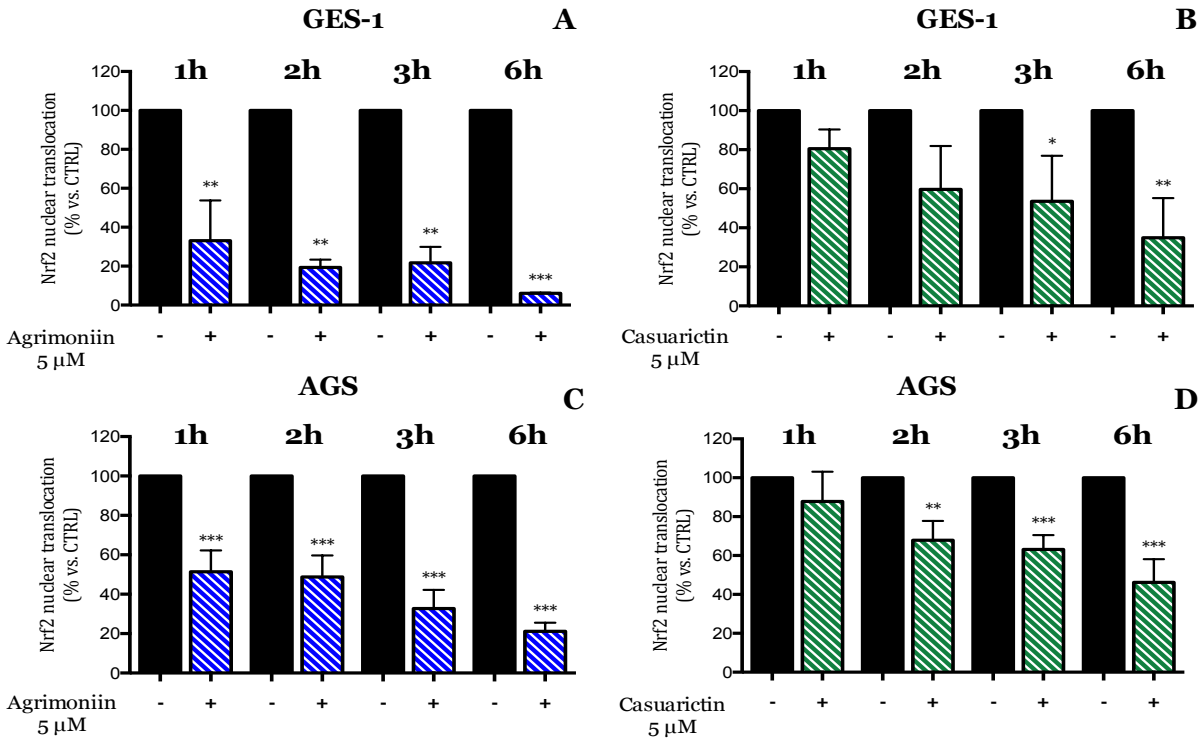


Figure 29. Effect of agrimoniin (A, C) and casuarictin (B, D) on Nrf2 nuclear translocation in GES-1 and AGS cells. Data are expressed in percentage, relative to the unstimulated control, which is arbitrarily assigned to the value of 100%. *p < 0.05, **p < 0.01, ***p < 0.0001 versus CTRL.

Since it has been observed that TNFα is not able to induce Nrf2 nuclear translocation in GES-1 and AGS cells, I tested the activity of agrimoniin and casuarictin, on this parameter, in basal conditions.

The two ellagitannin (5 μM) were tested at different times (1, 2, 3 and 6 hours) in GES-1 and AGS cells. These molecules reduce the basal levels of Nrf2 nuclear translocation in time dependent manner, both in GES-1 and AGS cells. In GES-1, agrimoniin exerts a statistically significant inhibition after 1-hour treatment, while casuarictin after 3 hours treatment. In AGS cells, agrimoniin exerts a statistically significant inhibition after 1-hour treatment, while casuarictin after 2 hours treatment. These results suggest that the two ellagitannins are not able to induce the expression of the genes regulated by Nrf2.

5. DISCUSSION

Gastritis is an inflammatory-based disease involving millions of people in the world, acute gastritis is caused by several risk factors, such as stress, use of anti-inflammatory drugs (NSAIDs), alcohol abuse and bile reflux, while the chronic form is mostly due to *Helicobacter pylori* infection. *H. pylori* is a non-invasive pathogenic bacterium able to induce an inflammatory response through the contact with the surface of gastric epithelial cells (Harada A. et al., 1996); it is one of the most successful human bacterial parasites which can persist lifelong if not treated efficiently (Graham D.Y. et al., 2010). It colonizes more than half of the human population (Parsonnet J., 1995). Despite some infected people are asymptomatic, with moderate inflammation detectable only by biopsy and histology, the most common consequence of *H. pylori* infection is chronic gastritis; chronic infection may lead to the development of severe gastric pathologies, including gastric ulcers (10-15% of cases), gastric adenocarcinoma and gastric lymphoma (1-2% of cases) (Parsonnet J. et al., 1991; Moss S. et al., 1992), This bacterium has been classified as a Type 1 carcinogen by World Health Organization. During *H. pylori* infection, gastric epithelial cells release several pro-inflammatory cytokines, which play a key role in development of gastric diseases.

This response is highly dependent on the activation of NF-κB in gastric epithelial cells, a transcription factor involved in gastric inflammatory processes. Several studies demonstrated that *H. pylori* infection induces macrophages to release pro-inflammatory cytokines, such as TNFα, which in turn bring the activation of NF-κB in gastric epithelial cells. Once activated by pro-inflammatory stimuli, including TNFα, NF-κB translocates from the cytoplasm into the nucleus where it promotes the expression of numerous genes involved in inflammation, including IL-8 (Yasumoto K. et al., 1992; Keates S., 1997).

Currently, there are different therapeutic approaches for H. pylori eradication based on the administration of proton pump inhibitors in association with antibiotics; although antimicrobial therapy appears the best way to eliminate the bacterium and related inflammation, it is characterized by some factors leading to therapy failure, including bacterial and host-related factors, resistance to the antibiotics and side effects (Graham D.Y. et al., 2010).

The search for new strategies able to interfere with these mechanisms by preventing a prolonged inflammation could bring benefit to a large number of subjects. In this respect, botanicals are widely consumed all over the world for health purposes, as different types of products, including herbal products. An emerging research provides substantial evidence to classify strawberries as a functional food with several preventive and therapeutic health benefits (Basu A. et al., 2014; Afrin S. et al., 2016).

Strawberry is one of the most commonly consumed fruits in the world and it is an important source of sugars, vitamins, fibers, micronutrients and polyphenols, including anthocyanosides (pelargonidin and cyanidin glycosides) and tannins, in particular condensed tannins (procyanidins) and hydrolysable tannins (especially ellagitannins, such as agrimoniin) (Buendia B. et al., 2010; Gasperotti M. et al., 2013). Recently, our group demonstrated that ellagitannin-enriched extracts from fruits of raspberry and blackberry inhibit ethanol-induced gastritis in rats acting on the NF-κB pathway; the anti-inflammatory effect was ascribed, at least in part, to the presence of the ellagitannins sanguiin H-6 and lambertianin C. (Sangiovanni E. et al., 2013). Regarding strawberry, it has been shown the ability of the extract to inhibit ethanol-induced gastritis in rats and this was ascribed to anthocyanosides (Alvarez-Suarez J.M. et al., 2011). Although tannins are important components of strawberries, until now the activity of these strawberry compounds, on gastric inflammation, is unknown.

An interesting point is that these compounds are not absorbed and metabolized in the acid environment of the stomach, suggesting that their activity is exerted *in situ* (Bioavaibility and metabolism of ellagic acid and ellagitannins, in Chemestry and Biology of Ellagitannins, Quideau Editor, 2009; Rios L.Y. et al., 2002).

The results obtained in this study, in AGS cell model of gastric inflammation, clearly show that tannins from *Fragaria* X *ananassa* and *Fragaria vesca* significantly attenuate the release of IL-8, a key mediator in gastric diseases, at concentrations lower than 1 μg/ml. Considering the average volume of gastric content in adult individuals during a meal (0.6 L), this concentration can be easily achieved upon consumption of a regular serving of fresh strawberries (100 g). Coherently with their chemical composition, tannin-enriched extracts from both *Fragaria* species inhibit IL-8 secretion by TNFα-treated cells in a similar fashion. Moreover, the biological activities of strawberry tannins are maintained upon simulated digestion, consistently with the slight changes in the chemical profile.

This study demonstrates that the anti-inflammatory activity of strawberry tannins is strongly, although not exclusively, related to inhibition of the NF-κB pathway, a central player in inflammatory diseases, including gastritis. AP-1 complex was unaffected by the inflammatory stimulus TNFα and by strawberry tannins in AGS cells, however, the involvement and modulation of other pathways cannot be excluded.

On the basis of the characterization of the extracts, I selected the major components of strawberry tannins to dissect their contribution to the anti-inflammatory activities observed with the extracts. Investigation was focused on agrimoniin and casuarictin, as the main

ellagitannins found in these berries, ellagic acid, as a common chemical determinant of ellagitannins, and procyanidin B1, as representative of condensed tannins.

The results indicate that agrimoniin dampen IL-8 secretion by inhibiting the NF-κB pathway and by acting on other targets, except AP-1. Findings are consistent with previous reports on the anti-inflammatory and immunomodulatory use of plants containing agrimoniin, such as *Potentilla spp*. (Bazylko A. et al., 2013), and *Agrimonia spp*. (Miyamoto K. et al., 1988; Murayama T. et al., 1992), and the ability of pure agrimoniin to prevent LPS-induced secretion of IL-8 by human neutrophils (Granica S. et al., 2015). Casuarictin exhibits anti-inflammatory properties with high selectivity for the NF-κB pathway, at least in TNFα-treated AGS cells. This observation was not previously reported in the literature.

Ellagic acid inhibits the NF-κB pathway and IL- 8 secretion in epithelial gastric cells at concentrations as low as 1–2 μM that could be easily reached upon moderate consumption of strawberries. Being the ellagic acid moiety present in all ellagitannins, it is conceivable that it may contribute to the biological activities of these compounds. Since condensed tannins are abundant in the extract, they could contribute to the anti-inflammatory activity. Procyanidin B1 was selected as representative condensed tannin and evaluated on IL-8 release and NF-κB pathway. This molecule is active only on IL-8 secretion and inactive on the other parameters tested, suggesting that this condensed tannin attenuated IL-8 secretion without affecting the NF-κB pathway in AGS cells subject to TNFα treatment.

This first part of the project demonstrates that strawberry tannins, either as enriched extract or as pure compounds, are quite stable after simulated gastric digestion and they were able to exert anti-inflammatory effects on gastric epithelial cells by inhibiting the inflammatory response to TNFα through NF-κB-dependent and independent mechanisms.

AGS cell line, a tumour gastric epithelial cells, is a well-established *in vitro* model and one of the mostly used in literature in order to study the effects of natural products on gastric inflammation; thus, it appears interesting to investigate the biological activities of extracts or pure compounds derived from plants, using an *in vitro* model closer to a normal gastric epithelium. GES-1 cell line represents a model of normal gastric epithelial cells. It is used in some works reported in literature, however this cell line is not well-characterized and only few information, about the release of pro-inflammatory mediators and the activation of transcription factors involved in the gastric inflammation, are available.

Before testing the strawberry extract and pure tannins, I deeply investigated and compared the ability of GES-1 and AGS cells to respond to TNFα stimulus, in terms of release and expression of some pro-inflammatory mediators. Differences were observed between the

two cell lines, for example IL-6 and MMP-9 expression and release were induced, by TNFα, in GES-1 cells but not in AGS cells. However, NF-kB activity was induced in both cell lines, suggesting that TNFα is not able to activate, in AGS cells, other transcription factors involved in the expression of these two pro-inflammatory mediators.

According to what reported in literature, pro-inflammatory mediators like IL-8, IL-6, MMP-9, TNFα, IL-1β, CSF-2, CCL2, CCL20 and LTβ, produced by gastric epithelium, possess a key role in gastric inflammation (Tanahashi T. et al., 2000; Jung H.C. et al., 1997; Shimada T. et al., 1998; Caruso R. et al., 2007; Yoshida A. et al., 2009; Mejías-Luque R. et al., 2016); moreover, their expression is NF-κB-dependent (Hiscott J. et al., 1993; Shimizu H. et al., 1990; Matsusaka T. et al., 1993; Mori N. et al., 2003; Shakhov A.N. et al., 1990; Ueda A. et al., 1994; Kuprash D.V. et al., 1996; Schreck R. et al., 1990; Zhao L. et al., 2014). These mediators are involved in different processes, like recruitment of immune cells, degradation of extracellular matrix components and activation of different pathways (including canonical and non-canonical NF-κB pathway). *Fragaria* X *ananassa* extract and pure ellagitannins exert anti-inflammatory activities in normal gastric epithelial cells (GES-1), inhibiting the release of IL-8, IL-6 and MMP-9, the expression of the genes previously mentioned and NF-κB activity. Although around 1-2 μg/ml for *Fragaria* extract and below 1 μM for pure ellagitannins, the IC_{50}s obtained in GES-1 cells were slightly higher compared to those calculated in AGS cells.

The activity of strawberry tannins was also maintained in normal and tumor gastric epithelial cells after infection with the bacterium *H. pylori*, however the IC_{50}s were higher compared to those obtained using TNFα as stimulus (30.8 μg/mL in AGS and 40.0 μg/mL in GES-1) but again reachable after consumption of strawberry.

The presence of *H. pylori* results also in reactive oxygen species (ROS) and reactive nitrogen species (RNS) production by the gastric mucosa, there are many cell types that can contribute to the production of ROS/RNS, including the epithelial cells (Naito Y. et al., 2002). ROS has a role in the activation of NF-κB pathway and if produced in excess they can damage gastric mucosal cells and cause the peroxidation of membrane lipids, thereby increasing the level of lipid peroxide (LPO) in damaged tissues (Suzuki M. et al., 1994). Nuclear factor-E2-related factor 2 (Nrf2) is a transcription factor which control cellular redox homeostasis and protect cells from oxidative stress (Itoh K. et al., 1997; Kensler T.W. et al., 2007). In response to oxidant stimuli, Nrf2 proteasomal degradation induced by Keap1 is suppressed, which results firstly in an increased nuclear accumulation of Nrf2 and secondly in a transcriptional induction of cytoprotective genes (Kobayashi A. et al., 2006).

Agrimoniin and casuarictin, unexpectedly, reduce the basal levels of Nrf2 nuclear translocation in GES-1 and AGS cells, suggesting a reduced production of antioxidant enzymes. This effect could be explained with the ability of these compounds to counteract the basal reactive oxygen species of the cells acting as radical scavengers, in this way the cell can decrease its basal antioxidant defenses. However, our interpretation of these results is just speculative and needs further investigations. Moreover, Nrf2 inhibition in tumor gastric epithelial cells could increase the effects of anticancer drugs; in fact, it has been demonstrated in literature that Nrf2 is stably overexpressed in cancer cells where enhances cell proliferation (Singh A. et al., 2008; Chian S. et al., 2014). Overexpression of Nrf2-regulated detoxifying enzymes contributes to advantages on cancer cells for resistance to cytotoxicity of anticancer drugs (Ohnuma T. et al., 2011; Singh A. et al., 2008).

In conclusion, this study demonstrates that strawberry tannins, either as enriched extract or as pure compounds, act on gastric epithelial cells by inhibiting the inflammatory response to TNFα, with concentrations easily reachable *in vivo*. They were quite stable in the acid environment, suggesting that their activity could be maintained also *in vivo*. While procyanidins are widely present in natural sources and their biological activities have been well documented, ellagitannins can be found in some foods only and represent an emerging class of phytochemicals. Agrimoniin and casuarictin, at the moment, are not commercially available. According to available data, agrimoniin and casuarictin could be considered the most consumed ellagitannins in the world because of the large presence of strawberries in the human diet (Vrhovsek U. et al., 2012). The anti-inflammatory action of strawberry tannins at the stomach level, if supported to further investigation in a co-culture epithelial gastric cells/bacterium, coupled with the previously reported anti-*H. pylori* activity (Funatogawa K et al., 2004), makes these compounds exploitable as preventive or co-adjuvant agents in gastric diseases.

This work shows the anti-inflammatory activity of strawberry tannins and their stability during simulated gastric digestion; however, the experimental setting, characterized by the use of two gastric epithelial cell models, is not able to reproduce all the processes that take place during gastric digestion in humans. For example, the mucus layer produced in the stomach by specific cells could be a factor affecting the activity of the molecules under investigation in this study. For these reason, clinical studies are needed in order to confirm the activity of these compounds. Despite the bacterium *H. pylori* is able to colonize the stomach of humans, inducing a strong inflammation, there are evidences in the literature

reporting lower virulence in rodents than in humans, thus limiting the usage of animal models to study the effect of these extracts/compounds.

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Ringraziamenti

Al termine di questo importante traguardo voglio dedicare uno speciale ringraziamento al Prof. Mario Dell'Agli per avermi accolto in tutti questi anni nel suo laboratorio e per la fiducia dimostrata in me, dandomi la possibilità di svolgere la tesi di Laurea e successivamente il percorso di Dottorato.

Ringrazio la Prof.ssa De Fabiani per aver contribuito, attraverso numerosi e preziosi suggerimenti, alla realizzazione di questo lavoro di tesi.

Un particolare ringraziamento ad Enrico per avermi supportato e aiutato con grande pazienza, disponibilità e competenza fin dai primi giorni in laboratorio sino ad oggi.

Ringrazio Giulia, Stefano, Andrea e Saba per i bellissimi momenti condivisi dentro e fuori l'ambiente di lavoro e per il grande contributo e sostegno fornito quotidianamente.

Ringrazio tutti gli studenti che hanno scelto, con grande impegno, di completare il loro percorso di studi nel laboratorio di Farmacognosia.

Infine, un ringraziamento alla mia famiglia e a tutti gli amici per avermi dimostrato grande conforto, sostegno e vicinanza durante i difficili momenti di questi ultimi anni.