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TANNINS FROM *RUBUS* AND *FRAGARIA* BERRIES FOR THE CONTROL OF GASTRIC INFLAMMATION: *IN VITRO* AND *IN VIVO* STUDIES

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TABLE OF CONTENTS

1. INTRODUCTION

INFLAMMATION

Inflammation is a defense mechanism of innate immunity that protect higher organisms from infections and injury. Its purpose is to localize and eliminate the injurious agent removing damaged tissue components to promote the healing process. The inflammatory response consists of changes in permeability of blood vessels, and the migration of fluid, proteins, and white blood cells (leukocytes) from the circulation to the site of tissue damage. The classical signs of acute inflammation are pain, heat, redness, swelling, and loss of function. An inflammatory response during only a few days is called *acute inflammation*, while a response of longer duration is referred to as *chronic inflammation*. Chronic inflammation leads to a progressive shift in the type of cells occurring at the site of inflammation and is characterized by simultaneous destruction and healing of the tissue from the inflammatory process.

1.1 GASTRIC INFLAMMATION

The gastrointestinal tract represents an important barrier between the human hosts and microbial populations. One potential consequence of host-microbial interactions is the development of mucosal inflammation, which can lead to gastritis and ulcer. Gastritis has been considered a normal process of aging for different years, until the discover of the leading causative agent in 1982 [1], *Helicobacter pylori* (*H. pylori*). This turned the most common type of gastritis into a potentially curable disease. Gastric inflammation may occur with different characteristics, divided into five variables: chronic inflammation (presence of mononuclear cells), activity of gastritis (presence of neutrophils), atrophy defined as loss of specialized glands, intestinal metaplasia and *H. pylori* infection[2]. Based on etiology gastritis can be divided into three main categories: chemical, autoimmune gastritis and *H. pylori*-induced [3].

1.1.1 Chemical gastritis

Gastric mucosal damage caused by various drugs is variable, depending on the causative agents, but it is usually characterized by a low-grade inflammation[4]. For this reason this condition is currently defined as chemical gastritis or gastropathy. Exposure of the gastric mucosa to a noxious chemical environment accelerates the turnover of the gastric epithelium, and a concomitant histamine-mediated vascular response associated with the release of pro-inflammatory cytokines. Most chemical gastropathies are asymptomatic, but erosions and ulcers may develop, even with bleeding[3]. Among the main causes of gastropathy there are duodenal reflux, drugs including acetylsalicylic acid and alcohol consumption.

- Bile reflux, often as a consequence of partial gastrectomy, dysmotility or due to incompetent pyloric sphincter, induce inflammation and glandular atrophy at gastric level[5-7].
- Nonsteroidal anti-inflammatory drugs (NSAIDs), especially in cases of chronic administration, cause damage to the gastric mucosa by different mechanisms: they inhibit prostaglandin synthesis[8], enhance gastric motility[9], resulting in an increase in mucosal permeability, induce neutrophil infiltration[10] and oxyradical production[11]. All these mechanisms could bring to the formation of ulcers[12] and interfere with the healing of preexisting ones[13]. Acetylsalicylic acid[14] and indomethacin[15], two typical anti-inflammatory drugs, inhibit the secretion of mucus; acetylsalicylic acid can increase the pepsin-mediated proteolysis of the mucus, reducing its viscosity and increasing the permeability to hydrogen ions[16]. Indomethacin also inhibits the secretion of bicarbonate from the gastric mucosa[17], damaging the epithelial surface and causing necrosis in the gastric glands regions[18].
- Ethanol increases the risk of gastric mucosa erosion and ulcer formation[19], mainly by the generation of free radicals[20]. *In vivo* studies show that radicals induced by ethanol consumption influence lipid peroxidation and the activity of antioxidant enzymes, such as catalase and superoxide dismutase[21, 22]. Acetaldehyde formed by the gastric metabolism of ethanol could be responsible for the pathogenesis of chronic pathology of the stomach[23]. Pathology and symptomatology of gastritis induced by chronic consumption of ethanol are similar to those caused by *H. pylori* infection, alcoholic patients could also have higher incidence of chronic gastritis of the antrum[24], a lesion commonly associated with *H. pylori*. A prospective study revealed a statistically significant relationship between high alcohol consumption and the presence of *H. pylori* [25].

1.1.2 Autoimmune gastritis

Autoimmune gastritis is a chronic inflammatory gastric disease limited to the fundus and body of the stomach[26, 27], in which an immune response is directed against parietal cells and intrinsic factors. Parietal cell destruction results in hypochlorhydria or achlorhydria, hypergastrinemia and loss of pepsin activity[28]. The progression of this disease may eventually lead to vitamin B12 malabsorption followed by pernicious anaemia[29, 30] or iron-deficiency anaemia[31]. Some studies have shown a possible association between *H. pylori* infection and autoimmune gastritis [32, 33].

Other typologies of gastritis caused by virus, fungi, bacteria (with the exception of that caused by *H. pylori*) or parasites are associated with immunodeficiency[28] and are not significantly involved in the pathway of gastric cancer development.

1.1.3 *H. pylori* gastritis

H. pylori is a flagellated gram-negative bacterium (see Fig. 1), isolated for the first time in 1983 by Warren and Marshall[34], that can be converted in coccoid form under a hostile environment[35]. This bacterium colonizes the gastric mucosa of over 80% of human population in developing countries[36] and at least 50% of the world's human population[3], percentages which make it the leading cause of gastritis. In Western countries the prevalence of *H. pylori* infection decreased over the years to below 40%, probably as a result of sanitation and the use of antibiotics [37]. *H. pylori* infection is usually contracted during childhood and can be strongly influenced by the country of origin and socio-economic conditions[38]. The children living in developing countries are usually infected before 10 years old and the infection reflected the rate of acquisition in childhood[39]. *H. pylori* is a non-invasive bacterium, but it could induce a robust immune response [40]. Recently, this bacterium has been classified as type I carcinogen by the World Health Organization[41]. *H. pylori* influences early stages in gastric carcinogenesis and its eradication in infected individuals significantly decreases the risk of developing gastric adenocarcinoma[42]. The mode of transmission of infection still remains to be clarified, the most likely route of transmission is fecal-oral, while oral-oral transmission seems to be unlikely[43]. Despite the high prevalence of *H. pylori* infection, the majority of infected individuals (80-90%) appears to be completely asymptomatic (with a moderate inflammation detectable only by histological analysis), while only the remaining 10-20% goes towards the development of certain diseases such as atrophic gastritis, peptic ulcer, gastric adenocarcinoma and mucosa-associated lymphoma (MALT)[44]. Approximately 3% of patients also develop gastric cancer[45], characterized by disruption of the mucous layer, leading to exposition of gastric mucosa to the content of the stomach, like acid peptic.

1.2 *H. PYLORI* PATHOGENESIS

The steps of *H. pylori* colonization are the following: crossing the layer of gastric mucus, adhesion to the epithelium and the development of inflammation, tissue destruction and ulceration. The bacterium is able to establish a persistent infection leading to innate immune response that generates inflammation at gastric level, but the mechanisms are not fully elucidated[46]. *H.*

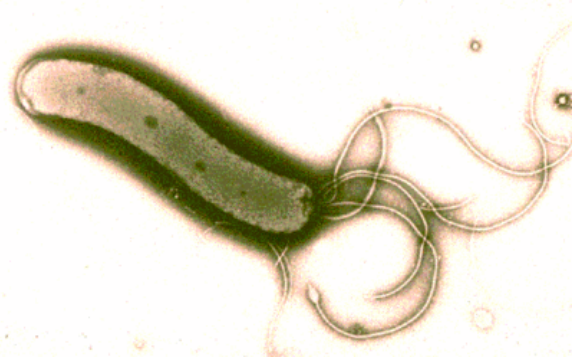


Fig. 1 Image of *H. pylori*. Image taken from Prof. D.J. Kelly at www.shef.ac.uk

pylori cell division-related gene A (*cdrA*) has a repressive role on cell division and is involved in the survival and antibiotics resistance of the bacterium[47]. *CdrA*-negative strains resulted in lower levels of IL-8 production by gastric epithelial cells *in vitro* and *in vivo* thus suggesting the mechanism by which the bacterium evade immune clearance[46].

To survive in the host, *H. pylori* should be able to tolerate the acidic environment of the stomach and evade the immune mechanisms of defense; its adaptability to the gastric mucosa is due to the production of urease. This enzyme converts urea into ammonia and CO_2 and this allows bacterium survival at low pH[48]. Urease also alters the viscosity of the gastric mucosa and this promotes bacterial motility[49]. Other features like the spiral shape and flagella help this bacterium to resist in the mucosa during gastric peristalsis[50]. In order to evade the defense mechanisms of the host organism, represented by the innate immune response, *H. pylori* antigens change, as the bacterial endotoxin lipopolysaccharide (LPS) present on the cell wall and flagella, making them relatively anergic[36, 50]. The genome of this bacterium encoding a variety of factors that facilitate the colonization expresses a number of membrane proteins, collectively known as *H. pylori* outer membrane porin (Hop), which mediate binding to gastric epithelial cells, the vacuolating cytotoxin VacA, a protein of 95 kDa responsible of the vacuolation of the mucosal cells, and the cytotoxin associated antigen CagA. CagA gene encodes a protein of 120-140 kDa responsible for alterations in the cytoskeleton of the affected cells. In addition to these exotoxins, *H. pylori* is provided with endotoxins with cytotoxic properties, like the lipopolysaccharide capsular, capable of inducing the release of toxins and the subsequent release of inflammatory cytokines such as IL-1β, IL-2, IL-6, IL-8 and TNFα[51-54]. The presence of these molecules causes the recall of polymorphonuclear cells, eosinophils, T and B lymphocytes leading to the formation of lymphoid aggregates[55].

1.2.1 Role of cytokines in *H. pylory* pathology – IL-8

H. pylori is able to induce an inflammatory response through the contact with the surface of epithelial cells, the mechanism of action is shown in Fig. 2. The local production of chemokines, a group of cytokines with chemoattractive activity, represents an important step for the recruitment and activation of inflammatory cells[56]; many chemokines have been identified, but the number is constantly growing. IL-8 is a potent pro-inflammatory chemokine, which promotes neutrophil infiltration in the gastric mucosa and is released through the signaling pathway of nuclear factor-kappa B (NF-κB). IL-8 secretion is induced by *H. pylori in vitro* and *in vivo* [57, 58]. Increased levels of IL-8 have been reported in various inflammatory conditions, including inflammatory bowel disease, psoriasis, rheumatoid arthritis, septic shock and cystic fibrosis[59]. IL-8 appears as a key point of *H. pylori* gastritis and epithelial cells are probably the main producers of this chemokine in

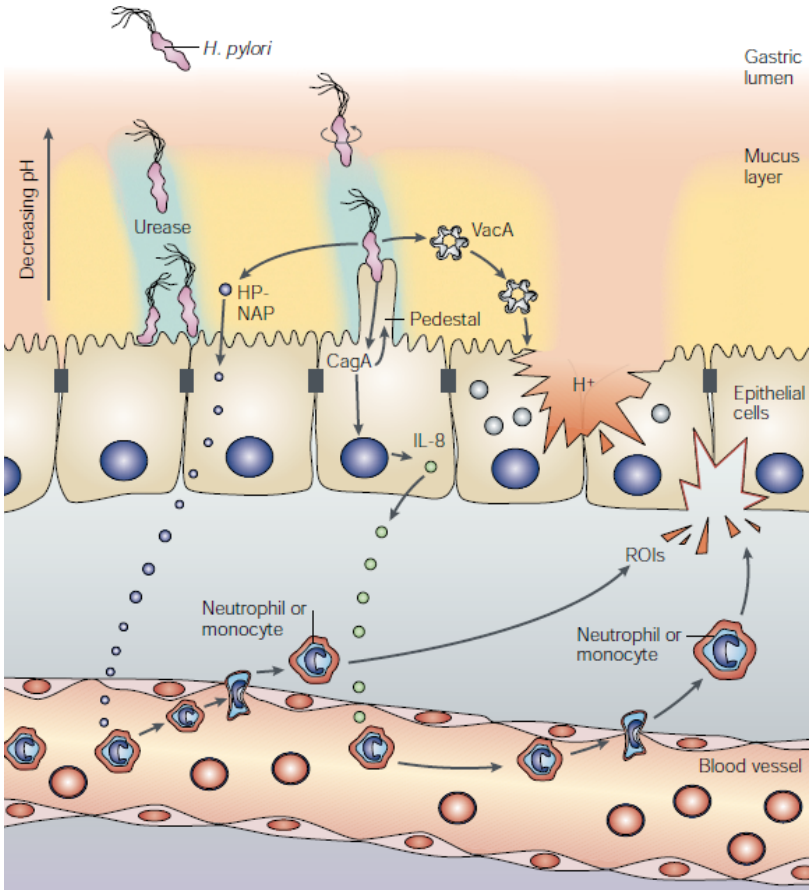


Fig. 2 Scheme of the gastric mucosa infection by *H. pylori*. During the infection, the bacterium gets into the gastric lumen and urease allows its survival in acid environment through the production of ammonia, creating a neutral layer around the bacterial surface. Use of flagellum allows the bacterium to move into the gastric lumen and pass through the mucus layer. Once reached gastric epithelium, *H. pylori* injects the CagA protein within the host cells through type IV secretion mechanism, also releases other toxic factors such as the HP-NAP (protein activating neutrophil) and VacA. VacA toxin induces the formation of alterations at the level of the tight junctions and the formation of vacuoles. HP-NAP protein crosses the epithelial cells causing tissue damage through the release of ROS. The CagA protein induces alterations of cytoskeleton and causes the release of pro-inflammatory cytokines. (Image from Montecucco C., 2001).

gastric mucosa[55]. In addition to IL-8, during *H. pylori* infection the expression of other pro-inflammatory cytokines like TNFα, IL-1β, interferon-γ (IFN-γ) and IL-6 also occurs[60, 61]. LPS and release of bacterial proteins stimulate production of IL-1β and TNFα by mononuclear cells in the lamina propria[62]; these cytokines are potent inducers of IL-8 expression in many cell type, including gastric epithelial cells[63]. TNFα is considered a key molecule in inflammation of the gastric mucosa; studies have shown that this cytokine is able to induce pro-inflammatory signals in gastric adenocarcinoma cells (AGS)[64]. AGS cell treated with TNFα in a concentration ranging between 0.1-10 ng/mL showed a marked induction of IL-8 and CCL20 (ligand Chemokine-20) secretion[65]. Gastric epithelial cells secrete increased amounts of IL-8 in response to strains of *H. pylori* CagA-positive compared to CagA-negative strains[66], therefore the infection with CagA-positive strains is related to more serious inflammation of the gastric mucosa[53]. The deletion of the gene PicB, located upstream CagA gene, leads to reduction of IL-8 production in gastric epithelial cells[67]. A pathogenicity island, which contains several genes (including CagA), has been identified; this set of genes is fundamental for the induction of expression of IL-8[68]. NF-κB, NF-

IL6 (a nuclear factor involved in the IL-6 gene expression) and activator protein-1 (AP-1) are involved in the regulation of IL-8 gene transcription [69], in particular both NF-κB and NF-IL6 are required for IL-8 promoter activity induction in human gastric epithelial AGS cells (AGS), in response to stimuli like TNFα and *H. pylori*. Another cytokine involved in *H. pylori* gastritis is IL-21, in fact bacterium eradication reduces the expression of this cytokine[70]. Treatment of AGS cell line with IL-21 increases the synthesis of MMP-9 and MMP-2, whereas MMP-1, MMP-3 and MMP-7 (present in the mucosa infected by *H. pylori*) are not affected[70].

1.3 NF-κB

NF-κB, firstly discovered in 1986[71] by Sen R. and Baltimore D., is a transcription factor involved in different physiological processes, including inflammation, cell growth and proliferation[72-74]. This dimeric transcription factor is formed by the combination of five members divided in two main groups (see Fig. 3): NF-κB1 (p50) and NF-κB2 (p52) belong to Class I proteins (or NF-κB) and are produced by proteolytic processing from their precursors (p105 and p100 respectively); c-Rel, RelB e RelA (p65) belong to Class II proteins (or Rel)[75]. All the members of the family possess a 300 amino acid long N-terminal Rel homology domain (RHD) responsible for DNA binding and dimerization[76, 77], while RelB can only form heterodimers, all the other proteins are also capable of homodimerization. Only Class II proteins have a C-terminal transcription activation domain (TAD) able to induce gene expression. NF-κB dimers bind

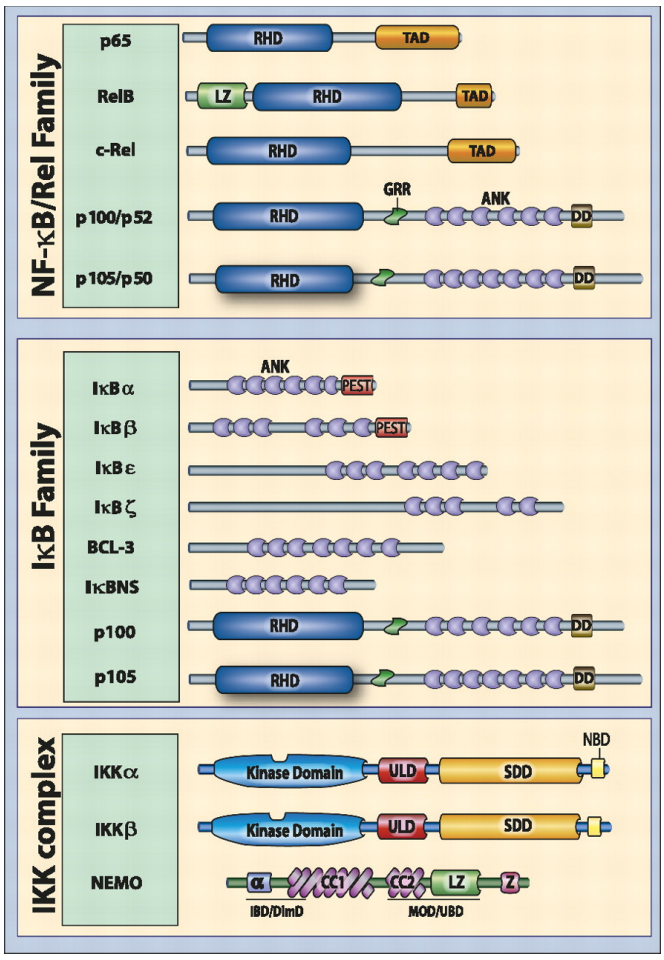


Fig. 3 Diagram of the protein family involved in NF-κB pathway in mammals. The class of Rel/NF-κB consists of five members: p65 (or RelA), RelB, and c-Rel (Rel), and proteins p100 (NF-κB2) and p105 (NF-κB1), precursors of mature forms p50 and p52, respectively. The IκB family consists of eight members, IκBa, IκBb, IκBe, IκBz, BCL-3, IκBNS, P100 and P105, characterized by the presence of multiple domains of ankyrin repeats. The IKK complex consists of IKKα (IKK1) and IKKβ (IKK2) and NEMO (IKKg). Image from Hayden and Ghosh, 2012

DNA in a specific region named κB in the promoters of different genes, whose possess the consensus sequence 5'GGGRXWYYCC3' (X: any base; R: purine; W: adenine or thymine; and Y: pyrimidine). Class I proteins p50 and p52 are characterized by the lack of TAD domains, and repress transcription unless associated, as heterodimers, with a member of Class II group[78]. In resting cells, most NF-κB/Rel dimers are held in the cytoplasm linked to a group of inhibitory proteins called IκBs. The IκBs is a gene family including seven members, IκBα, IκBβ, IκBε, IκBγ, Bcl-3, Class I precursor proteins p100 and p105[79]; all of them are characterized by multiple copies of ankyrin repeats, which interact with the RHD, thereby covering their nuclear localization sequence (NLS).

1.3.1 NF-κB activation

Three different mechanisms of NF-κB activation are known at present: classical (or canonical), atypical and alternative pathway[76, 80] as showed in Fig. 4. In the classical pathway IκBα, the most studied member of IκBs family, is rapidly degraded leading to the release of NF-κB dimers, especially p65:p50 heterodimers that are the primary targets of IκBα. Degradation depends upon the activation of the IκB kinase (IKK), a complex consisting of three different subunits: two highly homologous kinases (IKKα and IKKβ) and a regulatory subunit called IKKγ (or NEMO). The classical pathway is activated in response to several stimuli, including pro-inflammatory cytokines (TNFα, IL-1β), bacterial lipopolysaccharide (LPS), DNA damaging agents (camptothecin, daunomycin), Toll-like receptor (TLR) agonists, antigen receptor engagement (TCR or BCR) and, in some cell types, reactive oxygen species (ROS). Activation of IKK leads to the phosphorylation of IκBα on serine 32 (Ser 32) and 36 (Ser 36), which is followed by ubiquitination on a lysine residue. IκBα degradation by proteasome is required to allow nuclear translocation of p65:p50 heterodimers[81]. NF-κB activation promotes the transcription of IκBα and IκBε genes therefore establishing a negative feedback loop[77]. The newly synthesized IκBα is able to enter the nucleus, remove NF-κB from its DNA-binding sites[82, 83], and, thanks to the leucine-rich nuclear-export sequences (NES), transport it back to the cytoplasm[84, 85]. The complex IκBα-p65:p50 is constantly moved between the nucleus and the cytoplasm because IκBα protein masks only the nuclear localization sequence (NLS) of p65, whereas the NLS of p50 remains exposed. The crystal structure of IκBα bound to the p65:p50 heterodimer reveals that the IκBα protein masks only the nuclear localization sequence (NLS) of p65, whereas the NLS of p50 remains exposed[86]. The exposed NLS of p50 coupled with nuclear export sequences (NES) in IκBα and p65 leads to constant shuttling of IκBα/NF-κB complexes between the nucleus and the cytoplasm; however, the default location of this complex is the cytoplasm because the effect of the NES is dominant over that of the

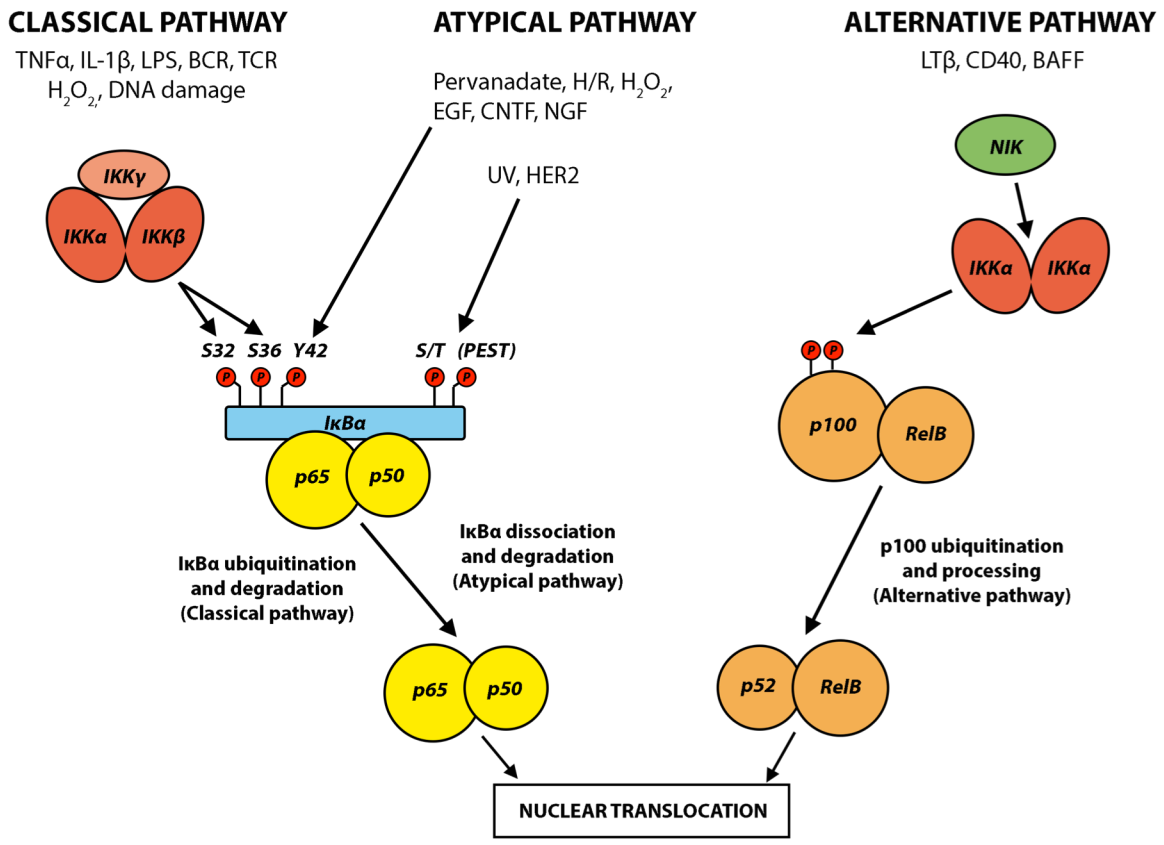


Fig. 4 Classical, atypical, and alternative pathways of NF-κB activation. The classical NF-κB pathway relies on IKK-mediated IκBα phosphorylation on Ser32 and 36, leading to its ubiquitination and degradation through the proteasome, which allows NF-κB nuclear translocation. Atypical pathways target IκBα Tyr42 or Ser and Thr in IκBα PEST region. The alternative pathway relies on NIK and IKKα-mediated p100 phosphorylation and processing to p52, which translocates into the nucleus with RelB.

NLS[87]. The atypical pathway is IKK-independent and is activated by hypoxia/reoxygenation, stimulation of tyrosine kinase receptor, pervanadate, UV irradiation and by H_2O_2[88, 89]. The activation of NF-κB is induced by phosphorylation of IκBα on Tyr 42 or on serine residues in the IκBα PEST domain which causes detachment of IκBα and its subsequent degradation[76]. In the alternative pathway the IKKα protein plays a pivotal role, and this type of activation is completely independent of IKKβ and IKKγ. This pathway is activated by a subset of tumor necrosis factor superfamily receptors (TNFSFRs) such as lymphotoxin β receptor (LTβR), B-cell activating factor receptor (BAFF) or CD40, and is dependent upon activation of IKKα homodimers by NF-κB-inducing kinase (NIK). NIK is continuously synthesized, but in resting cells it is continuously degraded by TRAF3 [90], ligand stimulation induces TRAF3 degradation resulting in NIK stabilization. TRAF3 potently suppresses canonical NF-κB activation[91]. Activation of NF-κB through the alternative pathway is generally slower than the activation of the classical pathway, but is important for secondary lymphoid organ development, B cell survival and homeostasis, adaptive immunity, and osteoclastogenesis[92].

1.3.2 Pathophysiological role of NF-κB

In humans, NF-κB participates in the transcription of more than 150 target genes, in particular it regulates the expression of genes involved in pivotal physiological processes such as: immune response, inflammatory response, cell adhesion, oxidative stress response and apoptosis. Thanks to the large variety of bacteria and viruses that are 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"[93]. There are different activators of NF-κB, its activity can be induced by various physiological stress conditions as ischemia or bleeding. Under these conditions, a large number of stress response genes are activated by NF-κB, such as cyclooxygenase-2 and nitric oxide synthase. In patients infected by *H. pylori* the activity of NF-κB reaches very high levels, probably due to the severity of the inflammation[94]. In gastric epithelial cells TNFα and IL-1β induce the expression of IL-8 and this mechanism is associated with the activation of NF-κB[95].

1.3.3 Inhibition of NF-κB pathway

NF-κB regulates inflammatory and immune responses, by increasing the expression of specific genes coding for different cytokines, chemokines, proteins involved in antigen presentation and receptor adhesion. This transcription factor also stimulates the expression of enzymes that may contribute to the pathogenesis of inflammatory processes, including nitric oxide synthase (iNOS), and cyclooxygenase 2 (COX-2). The mechanisms leading to the activation of NF-κB can be controlled at multiple levels:

- regulating IκB complex: increasing of the expression of inhibitory proteins IκB; reducing IKK-mediated phosphorylation or reducing IκB proteasomal degradation;
- blocking translocation of NF-κB at nuclear level;
- inhibiting NF-κB-DNA binding;
- controlling NF-κB gene transcription.

There are several types of inhibitors that are used to target NF-κB: biomolecular inhibitors, natural products (and their derivatives), and synthetic compounds.

Glucocorticoids

Glucorticoids, as dexamethasone and prednisone, are widely used for their anti-inflammatory and immunosuppressive properties, as they interact with the receptor of steroids to reduce the expression of genes involved in the inflammatory processes. The mechanisms proposed to explain

the inhibitory effects of these drugs on the NF-κB pathway are different. Firstly glucocorticosteroids could induce the expression of IκBα, which increases cytosolic permanence of NF-κB[96, 97]. Dexamethasone stimulates the synthesis of IκBα mRNA in Jurkat cells and in monocytes, resulting in cytoplasmic permanence of p65. Secondly dexamethasone could decreases transcriptional activity of NF-κB in the endothelial cells, without affecting the levels of IκB or NF-κB nuclear translocation.

Non-steroidal anti-inflammatory drugs (NSAIDs)

NF-κB regulates the expression of several genes involved in inflammatory responses, including cyclooxygenase 1 (COX-1) and 2 (COX-2). While COX-1 is constitutively expressed, COX-2 is an inducible enzyme, whose expression increases in response to inflammatory stimuli. COX-2 induces the synthesis of cyclopentenone prostaglandins (cyPGs), key mediators of inflammation in the late stages of the inflammatory response. cyPGs are capable of inhibiting NF-κB probably due to the activation of the PPAR-γ receptor, that can in turn antagonize the transcriptional activity of NF-κB. Pathway of NF-κB can be directly inhibited by cyPGs, by blocking the activity of IKKβ[98]. NSAIDs may block the pathway of NF-κB at different levels. Salicylic acid and sodium salicylate are examples of anti-inflammatory drugs whose molecular target is NF-κB. These agents suppress the synthesis of adhesion molecules VCAM-1 and ICAM-1 in endothelial cells; this inhibition prevents the trans-endothelial migration of neutrophils and the inflammatory process[99]. The inhibitory effect of sodium salicylate and aspirin is due to the specific inhibition of ATP- IKKβ binding[100]. Indomethacin and its derivatives are able to bind IKKβ, inhibit the catalytic activity and prevent the activation of NF-κB in response to stimulation with TNFα[101].

Immunosuppressive agents

cyclosporine and tacrolimus are immunosuppressants drugs used after organ transplantation to prevent rejection crisis. These drugs inhibit calcineurin activity, a calcium-calmodulin-dependent phosphatase, able to prevent degradation of IKBα thus promoting NF-κB activation[102]. Cyclosporine prevents proteasomal degradation of IκBα; however, tacrolimus blocks specifically translocation of c-Rel from the cytoplasm to the nucleus[103].

Proteasome inhibitors

Peptides and aldehydes, as MG101, MG132 and MG115, are able to inhibit proteasome activity by acylation of a threonine residue in the proteasomal subunit, and others act at IKK level blocking the nuclear transclocation of NF-κB[104-106].

Natural inhibitors

Several studies have suggested that the beneficial effects of polyphenols may result by inhibition of NF-κB pathway[107]. Antioxidants, including vitamin C, inhibit the phosphorylation of IκBα induced by TNFα and IL1β in endothelial cells DNA[108]. Several studies suggest that some flavonoids, like quercetin and myricetin, could mediate an inhibition in the signal transduction pathway of NF-κB[109, 110]. Resveratrol inhibits the activity of NF-κB, thus leading to apoptosis and inhibition of iNOS expression in a variety of cell lines. Treatment of macrophages with resveratrol blocks phosphorylation and degradation of IκBα reducing the activity of IKK [111].

1.4 Reactive oxygen species (ROS)

Molecular oxygen is essential for aerobic organisms, but in spite of its necessity for living, the respiration process could be harmful due to formation of reactive oxygen species (ROS). ROS are produced in living cells not only by normal metabolism, but also from pathophysiological processes and extracellular sources. Cells have developed a series of antioxidant mechanisms, which include non-enzymatic and enzymatic antioxidants[76] to counteract ROS damage. Several types of intracellular antioxidant molecules, such as glutathione (GSH), catalase (CAT), superoxide dismutases (SODs), thioredoxin (TRX) and thio-redoxin reductase protect cells from oxidative damage[112]. High levels of ROS are toxic for cells and could bring cells to apoptosis, when ROS production overcome the antioxidative defense and oxidative stress occurs[113].

1.4.1 ROS and NF-κB

The basis of the involvement of ROS in NF-κB pathway has been suggested when antioxidant dithiocarbamates have been shown to inhibit IκB phosphorylation at Ser 32 and Ser 36[114]. Since NF-κB can be activated in many cells by agents with redox regulation properties, ROS have been proposed to be involved in the activation of NF-κB pathway[115]. The relation between NF-κB activation and generation of intracellular reactive oxygen species seems to be highly cell type-dependent[116]. Although there are evidence that ROS do not mediate NF-κB activation[117], ROS are considered second messengers, implicated in NF-κB pathway modulation[89, 118-120]; however, NF-κB activation causes in turn the transcription of target genes that could affect ROS levels, such as SOD and CAT[78]. The mechanism by which ROS regulates NF-κB is still unclear, it seems that ROS are involved in phosphorylation/dephosphorylation, processes that are crucial for NF-κB pathway. Other studies have shown that H_2O_2 could act on IKK inducing or inhibiting this kinase depending on cell type[118, 121]. Antioxidants such as N-acetylcysteine (NAC), and vitamin E have been shown to block NF-κB activation[122]. A general consideration is that oxidative stress at low

levels can stimulate NF-κB activation, while at higher levels may lead to the inhibition of activation[123].

1.4.2 Endogenous antioxidant enzymes

Superoxide dismutases (SODs) are enzymes deeply involved in the first line of defense aimed to detoxify ROS[124]. SODs are a family of enzymes that catalyze the dismutation of superoxide radical anion to hydrogen peroxide and molecular oxygen. There are three distinct families of SOD, which differ in the metal ions complexed with the enzyme (Cu/Zn-SOD; Ni-SOD; Mn/Fe-SOD) and protein folding. In mammals, the classification is based on enzyme localization: SOD-1 (Cu/Zn-SOD) cytosolic, SOD-2 (Mn-SOD) mitochondrial and SOD-3 (Cu/Zn-SOD) extracellular. SOD 1 was the first to be characterized, it is a homodimer containing copper and zinc localized in the cytoplasm and in intracellular spaces, with a molecular weight approximately 32,000 Da[125-127]. SOD-2 contains manganese as cofactor and it localizes in mitochondria of aerobic cells[128], and possesses a tetrameric structure of approximately 23,000 Da. The most recently discovered isoform is SOD-3, a homotetramer of 135,000 Da, containing copper and zinc, which is located exclusively in the extracellular space. The three isoforms are able to catalyze the same reaction, converting two superoxide molecules to oxygen and hydrogen peroxide. In a recent study it was demonstrated a statistically significant increase in the DNA-binding activity of NF-κB in CuZn-SOD-deficient mice kidney[129]. A deficiency in various forms of SOD promotes oxidative damage in a wide range of organisms[130]. The SOD2 gene promoter is under the control of nuclear factor κB (NF-κB)[131]. Protein kinase D (PKD) plays an important role in the regulation of intracellular oxidative stress responses and under exposure to ROS this protein is highly phosphorylated and activated. Subsequently, PKD may dissociate from mitochondria and, through phosphorylation, can activate the IKK complex, resulting in NF-κB activation and SOD2 expression[131]. Hydrogen peroxide generated by the conversion of the superoxide anion by SODs is mostly degraded to H_2O and O_2 by CAT. CAT is a ubiquitous heme-protein, located at perossisomal level, belonging to the class of oxidoreductase; it has a tetrameric structure that confers resistance to changes of pH, temperature and proteolysis. Its catalytic activity is responsible for the conversion of two molecules of hydrogen peroxide to molecular oxygen and two molecules of water[132]. Catalase gene is controlled by nuclear factor-erythroid-2-related factor 2 (Nrf2), a member of the NF-E2 family of the basic leucine zipper transcription factors. Cross-talk between NF-κB and Nrf2 pathways seems to be possible because their upstream signaling pathways, such as MAPK, PI3K and PKC, are closely related[133], even if further studies are needed to better clarify this interaction.

1.4.3 Nutrition and health

Proper nutrition is essential for the maintenance of health status. In recent years the interest for the study of the composition in macro and micro nutrients contained in foods has grown more and more. Diet could now be used as a preventive strategy in the development of chronic pathologies. As reported by the Italian Ministry of Health, nutritionists recommend a daily intake from three to five portions of vegetables and two to three portions of fruits to maintain a good physical fitness and good health. Fruits and vegetables have demonstrated to exert multiple biological effects on the mucosa of the gastrointestinal tract due to their antioxidant content[134] and play a crucial role in maintaining gastric mucosa homeostasis by counteracting potential damage exerted by ROS[18]. Studies *in vitro* and *in vivo* demonstrate that the antioxidant and anti-inflammatory activities of some foods are due to their polyphenol content[18, 135]. Polyphenols provide protection against different diseases such as diabetes, obesity and stroke[136]. Among the natural sources rich in polyphenols there are wild berries. The aim of research included in the present thesis is to provide new insights on the protective role of these fruits against inflammatory and oxidative conditions occurring at gastric level.

WILD BERRIES

The term "wild berries" identifies some edible berries, with high organoleptic properties, distributed mainly in temperate areas and widely used both in cooking and in pharmacy (Fig. 5). Fruits belonging to this group are for example blackberry, raspberry, commercial and wild strawberry, bilberry, cranberry, lingonberry, black currant and alpine currant. Dietary guidelines recommend the increased consumption of fruits and vegetables, as good sources of dietary fiber, essential nutrients, and beneficial phytochemicals, to improve overall health and reduce chronic disease risk[137, 138]. Berries hold an important position among the fruits for their highly antioxidant phytochemicals[139]. Wild berries share, besides the organoleptic properties, the presence of two classes of molecules of particular interest for biological activities: flavonoids and tannins.

Fig. 5 Image of different berries

- Flavonoids are molecules with more than 4000 different structures, they are present in plants and consumed in high amount, in order of several grams, in diets providing adequate consumption of fruit and vegetables. From the chemical point of view flavonoids are flavone derivatives (2-phenyl-γ-benzopyrone). Depending on the structure, flavonoids are divided in several sub-classes and may have a variety of biological properties in humans, including antioxidant, anti-inflammatory, vasorelaxant (mostly anthocyanins), and phytoestrogenic activities. Anthocyanins are flavonoids very common in berries[140].
- Tannins are polyphenols, with a strongly bitter flavour, that possess astringent and tanning activities. They are used for processing the skin into leather, precipitating proteins and forming, with them, insoluble aggregates. The leather making activity of tannins is attributed to their capacity to form multiple hydrogen bonds to collagen in hide. From the chemical point of view, tannins can be classified into two main groups: hydrolysable tannins and condensed tannins. Hydrolysable tannins can be divided in two subgroups: gallotannins, if they release mainly gallic acid during hydrolysis, and ellagitannins, if they release mainly ellagic acid (EA). Proanthocyanidins, that are considered condensed tannins. are oligomers and polymers of flavonoids, mainly catechins or anthocyanins. Berry fruits contain two major types of proanthocyanidins: procyanidins and propelargonidins.

1.5 *Rubus fruticosus* L.

The fruit of *Rubus fruticosus* L. (Fig. 6) consists of a set of small red drupes, dark or black, called blackberry. Blackberries are characterized by a high content of anthocyanins, ellagitannins (1080 mg/kg; range: 704-1556 mg/kg) and ellagic acid conjugates (200 mg/kg; range: 112 − 346 mg/kg)[141]. Another study evaluating ellagitannins content in blackberries found a range between 80 and 700 mg/kg of fresh fruit[142]. The study identified 11 ellagitannins mainly in the seed and torus of blackberries: isomeric forms of pedunculagin, castalagin/vescalagin, galloyl-HHDP glucose, lambertianin C, lambertianin D, and galloyl-bis-HHDP glucose, sanguiin H-6/lambertianin A, and EA. This study also proposed that blackberry fruit may contain sanguiin H-10[142]. In addition to the high content of ellagitannins blackberries are also a rich source of anthocyanins, natural pigments with important antioxidant capacity[143-145]. A recent research focused on the content of anthocyanins in blackberries, revealing the presence of five anthocyanin: cyanidin-3-O-glucoside, the principal anthocyanin (constituting 80-90% of total content) and four secondary anthocianins: cyanidin-3-rutinoside, cyanidin-3-xyloside, cyanidin-3-O-β-(6"-malonilglucoside) and cyanidin-3-(6"-(3-Hydroxy-3-methylglutaryl) glucoside[146].

Fig. 6 Botanic table of *Rubus fruticosus* L.

1.5.1 Biological activities of blackberry

Antioxidant activities

Blackberries ranked at third position compared to 4 different berries (marionberries, boysenberries,

red raspberries and black raspberries) in a study that evaluate the antioxidant activity by Oxygen Radical Absorbance Capacity (ORAC) assay[147], this result can be attributed to the high amounts of acylated anthocyanins and cyanidin 3-glucoside. Berry phenolic compounds have shown protective effects against cardiovascular disease by inhibiting oxidation of LDL and by protecting LDL from hydrogen peroxide-induced oxidative stress in human endothelial cells *in vitro*[148]. A recent work has shown that the anthocyanin-enriched blackberries extract is able to reduce free radicals production and oxidative damage induced by UV radiation in keratinocytes; this extract is also able to regulate the expression of some antioxidant enzymes such as CAT, MnSOD, Gpx1/2 and Gstal[149].

Antitumoral activities

Blackberry extracts from eight varieties suppressed UV-induced mutagenesis in *Salmonella typhimurium*[150]. Blackberry plays a protective role against peroxynitrite-induced DNA strand breakage in cultured human vascular endothelial cells[151]. Blackberry juice showed a weak inhibition of proliferation of several tumor cell lines, including AGS[152], and anthocyanin-enriched blackberries extract was able to inhibit in a concentration-dependent manner the growth of HT-29 colon cancer cells[153]. Blackberry extracts have also demonstrated inhibitory properties in cancer-induced AP-1 and NF-κB activations and suppressed the expression of vascular endothelial growth factor and COX-2, two proteins involved in tumour promotion and progression.

Antiviral activities

An *in vitro* research showed that the ethanolic extract of blackberry, at concentrations ≥ 56 μg/mL is able to inhibit, by a percentage greater than 99%, the replication of the herpes simplex virus (HSV-1) in oral epithelial cells[154], but despite this result there are no *in vivo* study evaluating antiviral activity.

1.6 *Rubus idaeus* L.

The fruit of *Rubus idaeus* L. (Fig. 7) consists of a set of small red drupes called raspberry. Raspberries are characterized by a high content of ellagitannins (1041 mg/kg; range: 2175 − 662 mg/kg) and ellagic acid conjugates (242 mg/kg; range: 82-530 mg/kg)[141]. Sanguiin H-6 and lambertianin C constitute 81% (range: 73 – 86%) of total content of ellagitannins in these fruits [141]. The characterization of ellagitannins in raspberries was conducted also in another study reporting that ellagitannins ranged between 2600-3260 mg/kg and 37 mg/kg of ellagic acid of fresh fruit[155].

A third study reported 760 mg/kg for sanguiin H-6, 310 mg/kg for lambertianin C and 1.10 mg/kg for ellagic acid[156]. Raspberries have also a high content of fibers (6.5 g/100 g), minerals and vitamins and the fruit contains high levels of water-soluble vitamin C (26.2 mg/100 g of fresh weight) [157].

1.6.1 Biological activities of raspberry

Antioxidant activities

Raspberries obtained the highest antioxidant capacity compared to strawberries, kiwi, broccoli, leek, apple, and tomato[158]. This fruit was able to reduce oxidized-LDL formation via its antioxidant activity[159]. Red raspberry juice improved levels of glutathione and reduced DNA oxidative damage in healthy adult males[160].

Fig. 7 Botanic table of *Rubus idaeus* L.

Antitumoral activities

In vitro studies have found raspberry phytochemicals effective in reducing vascular endothelial growth factor (VEGF) expression[161, 162], a promoter of angiogenesis, which is a critical step for tumour metastasis. Raspberry juice inhibited the cell proliferation of PC-3 (prostate adenocarcinoma cells) and MDA-MB-231 (mammary gland adenocarcinoma) with an IC_{50} of 20 µL/mL on PC-3 cells and 32 µL/mL on MDA-MB-231[152]. Raspberries inhibited in a concentration-dependent manner the growth of several cancer cell lines, including KB cells, CAL-27, MCF-7, HT-29, HCT116 and LNCaP[163]. Freeze-dried raspberry extract, used in concentrations between 25 and 200 µg/mL was able to significantly inhibit the growth of cancer cells (HeLa, SiHa and C33A) in a concentration and time-dependent mechanism[164]. Freeze-dried blackberries given to patients diagnosed with Barrett's esophagus, a precancer condition, were able to significantly decrease oxidative DNA damage[165]. Raspberry gel applied on premalignant oral lesions

significantly reduced COX-2 protein levels and suppressed genes associated with RNA processing and growth factor recycling[166].

Antihyperglicemic activity

Raspberry components have shown to improve glucose control in diabetic by inhibiting carbohydrate digestion, and anthocyanins have been found to interact with α-amylases whereas ellagitannins with α-glucosidase[167, 168].

Antibacterial activities

Raspberry juice was found to significantly reduce the growth of several species of bacteria, including *Salmonella, Shigella* and *E. coli*[169]. The study of the antimicrobial activity of 12 berries against some human pathogens found that *H. pylori* and *Bacillus cereus* are sensible to raspberry acetone-water extracts[170]. The growth of *H. pylori* was significantly reduced by the incubation with a commercial extract of raspberries[171], while a polyphenolic enriched fraction of raspberries, obtained by solution of acetonitrile and water, inhibited the growth of *Giardia intestinalis*[172].

1.7 *Fragaria* X *ananassa* Duch.

Fragaria X *ananassa* Duch. (**Fig. 8**) is a hybrid species cultivated worldwide for its fruits, used in large quantities in both fresh food preparations such as fruit juices and smoothies. The plant is an hybrid originated from *Fragaria virginiana* Duch., native to North America, and *Fragaria chiloensis* L., native to Chile and Argentina. The fruit of this plant, the common strawberry, represents an important source of vitamin C and other bioactive compounds able to determine pharmacological effects[173,174]. Polyhenols are one of the main groups of molecules present in strawberries, which affect the organoleptic and nutritional qualities of this fruit. Polyphenols present in strawberries include anthocyanins (responsible for

Fig. 8 Image of Fragaria X ananassa Duch.

the red colour of fruits), flavonols, flavanols, hydroxycinnamic acid derivatives, ellagic acid, ellagic acid glycosides, ellagitannins and proanthocyanidins[163, 175-178]. In a study[178] the phenolic compounds of strawberries (*Fragaria* X *ananassa* Duch.) were analyzed and quantified from 15 different cultivars; the results showed that strawberries are an important source of polyphenols; in particular, proanthocyanidins are the predominant class of compounds, as shown in **Tab. 1**.

Phenolic compounds	mg/100 g of fresh fruits
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

Tab. 1 Composition of phenolic compounds in strawberries (*Fragaria* X *ananassa* Duch.)

The contents of ellagitannins found in another study is higher, 77.1 mg/100 g of fresh fruit, probably due to the different extraction method used by the two studies[155]. Polyphenolic composition of strawberries varies during the growth phase and the phase of maturation of the fruit, in most cases the pulp of unripe fruit has higher levels of phenolic compounds and antioxidant capacity compared to pulp of ripe fruit[179-181]. The profile of anthocyanins of strawberry also varies during maturation but with an opposite trend: in all cultivars considered in the study by Kosar et al. it was observed that anthocyanins accumulate in the red fruit, while smaller amounts were found in the earlier maturity stages[181]. Another study shows that during different phases of ripeness there is a strong decrease of the total concentration of ellagitannins and ellagic acid conjugates, this reduction is common in all the *cultivars* taken into consideration[182]. The genetic and environmental conditions play an important role in determining the characteristics of strawberry, in fact the content of micronutrients and phytochemicals can vary from *cultivar* to *cultivar*[177, 179, 183, 184]. The conditions in which the fruit is stored can affect the micronutrient and phytochemical profile, the storage temperature seems to be one among the key factors capable of influencing the stability of phenolic antioxidants[185, 186]. The content of flavonoids seems to be significantly higher as a result of the storage[187, 188], this result could be attributed to the phenolic post-harvest metabolism of the fruit. The antioxidant capacity increases during storage[186] or as a result of a long exposure to high temperature storage[187, 188] or remains stable during storage [189, 190]. It is important to underline that the information relating to the composition in literature are variable; the variability is due to many factors including genetic differences of *cultivars*, environmental factors (place of

cultivation, growth conditions, storage) as well as the methodology used for the extraction and quantification.

1.7.1 Biological activities of common strawberry

The beneficial effects on human health related to consumption of strawberries (*Fragaria* X *ananassa* Duch.) include the prevention of inflammation, oxidative stress, cardiovascular disease, cancers, diabetes mellitus type 2, obesity and neurodegeneration.

Antioxidant activities

Ellagitannins possess antioxidant properties; these molecules have shown effects comparable to that of phenols and other natural antioxidants such as ascorbic acid or α-tocopherol[191]. A study demonstrates the hypothesis that supplementation with strawberries leads to a reduction of oxidative stress caused by a diet rich in polyunsaturated fatty acids in pigs. In this study was observed a reduction of the formation of malondialdehyde, greater protection of blood mononuclear cells against DNA damage, increased total antioxidant status of plasma and a reduction of glutathione peroxidase in erythrocytes[192]. Another study noted a significant increase in red blood cell resistance to oxidative damage, following a prolonged intake of strawberries (2-3 weeks)[193]. In a recent study, rats were fed with 40 mg/Kg/day of strawberry extract for 10 days, then gastric damage was induced in animals by ethanol administration; strawberry extract had a significant antioxidant capacity. The results obtained in this study showed that strawberries are responsible for a gastroprotective effect against gastric damage caused by ethanol, reducing lipid peroxidation and preserving/activating some endogenous antioxidant enzymes (mainly SOD and CAT). The antioxidant activity shown in this study is probably related to polyphenolic content, and in particular to anthocyanins[135]. It has also been demonstrated *in vitro* that extracts of strawberry, from three different *cultivars* are able to prevent lipid peroxidation [194].

Antitumoral activities

One study evaluated the potential inhibitory effect of extracts from strawberry on the activation of AP-1 and NF-κB induced by tetradecanoilforbol-13-acetate (TPA) and UVB rays and assessed the inhibitory effect on proliferation and transformation of cancer cells. The results obtained are the following:

- • *Fragaria* extract inhibits the proliferation of A549 cell line (lung cancer epithelial cells) and reduces the neoplastic transformation of mouse epidermal cells (JB6 P +).
- • Pretreatment of mouse epidermal cells (JB6 P +) with strawberry extract, determine the inhibition of activation of AP-1 and NF-κB induced by TPA and UVB rays.

- • Extracts block the phosphorylation of ERKs induced by TPA and the phosphorylation of ERKs and JNK kinase induced by UVB, in JB6 cells P +.

Strawberries had effects on the reduction of AP-1 and NF-κB, the block of MAPK signal and the suppression of proliferation and transformation of cancer cells[195]. In another study an extract of strawberry was able to inhibit in a concentration-dependent manner the growth of several cancer cell lines, including KB cells, CAL-27, MCF-7, HT-29, HCT116 and LNCaP[163]. An extract of *Fragaria* X *ananassa* Duch. showed anti-proliferative activity against human cancer cells *in vitro*[196]. Extracts of strawberries showed photoprotective activity in human fibroblasts exposed to UVA irradiation[197].

Antimicrobial activity

A study demonstrates that a commercial strawberry extract was able to significantly reduce the growth of *H. pylori in vitro*; the combination of extract with clarithromycin was able to increase the susceptibility of *H. pylori* to clarithromycin[171]. An acetonitrile-water strawberry extract enriched in polyphenolic fraction was able to inhibit the growth of *Giardia intestinalis*, a parasite of the human intestine; the efficiency of strawberry extract was equal to the reference drug metronidazole[172].

Anti-hyperglicemic activity

Strawberry was recently investigated by *in vitro* studies, for its potential contribution to control hyperglycemia, linked to type 2 diabetes. Comparison between different *cultivars* of strawberry showed a high inhibitory activity towards α-glucosidase enzyme, this result might suggest a potential anti-hyperglycemic effect of strawberries[198]. In a subsequent study it was shown that ellagitannins from *Fragaria* X *ananassa* Duch. possess a high inhibitory activity against the ACE and α-amylase enzymes, suggesting that these compounds could be able to control hyperglycemia and hypertension related to type 2 diabetes[199].

Anti-inflammatory activity

The daily consumption, for 8 weeks, of a drink containing 50 g of freeze-dried strawberries, is able to reduce atherosclerotic risk factors, like total cholesterol, LDL and circulating levels of adhesion molecules VCAM-1[200]. Also ellagic acid has the ability to reduce the endothelial expression of adhesion molecules like ICAM-1, VCAM-1 and E-selectin, induced by TNFα[201]. A cross-over study of dietary intervention, conducted on 14 women and 10 men (average age: 50.9 ± 15 years; BMI: 29.2 ± 2.3) for 6 weeks, showed that subjects who consumed a strawberry drink before a meal rich in carbohydrates and fats, had a statistically significant reduction of postprandial concentrations of IL-1β and PAI-1[202]. In an *in vivo* study was evaluated the anti-inflammatory capability and the

ability to regulate blood glucose, by strawberries, in a mouse model of diet-induced obesity. The results showed a lower concentration of glucose in the blood of mice supplemented with strawberries, compared to mice receiving low or high fat diet, and a lower plasma concentration of C-reactive protein in relation to other groups. These results mark the possible role of strawberry in reducing the risks associated with obesity and diabetes, in non-obese subjects[203].

1.8 *Fragaria vesca* L.

The fruits of *Fragaria vesca* L. (Fig. 9), named wild straberries, received less attention by scientific studies compared to common strawberries. A recent study published in 2012 analyzed the variety of phenolic compounds present in four genotypes of *Fragaria vesca* L., providing for the first time a complete view of the polyphenolic composition of this species of *Fragaria*[204]. Another work discovered that fruits of *Fragaria vesca* L. are rich in ellagitannins and ellagic acid conjugates (658 – 1636 mg/Kg of fresh fruit)[182] and in the same study the authors noted a substantial drop in total concentration of these compounds to the increase of degree of ripeness.

Fig. 9 Botanic table of *Fragaria vesca* L.

1.8.1 Biological activities of wild straberry

Anti-inflammatory activity

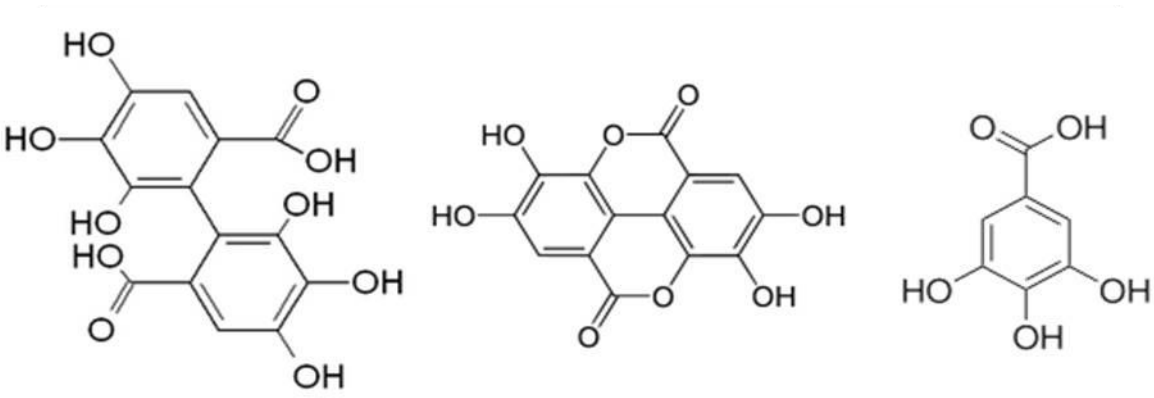
Ethanolic extract of *Fragaria vesca* L. (500 mg/kg) administered orally, in albino rats, was able to prevent the increase of markers commonly associated to colitis, at tissue level. The affected parameters were: myeloperoxidase (MPO), tissue catalase (CAT), glutathione and superoxide dismutase (GSH and SOD) and the reduction of microscopic and macroscopic lesions at intestinal

level[205]. A preparation rich in procyanidins derived from fermentation of tannins of *Fragaria vesca* L. improves the solubility of cimetidine and prevents adverse drug reactions such as the formation of nitrosamines.[206].

1.9 Hydrolizable tannins

1.9.1 Ellagitannins

Ellagitannins are a group of bioactive polyphenols belonging to the category of hydrolysable tannins. Compared to condensed tannins (proanthocyanidins) ellagitannins are more stable at gastric level. From the chemical point of view they are hexahydroxydiphenoyl esters of carbohydrates or cyclitols, while the definition of ellagitannins in a wider sense also cover compounds derived from further oxidative transformations, including oligomerization processes[207]. Ellagitannins are present especially in red fruits of the genus *Rubus* (blackberry, raspberry, cranberry, cloudberry) and *Fragaria* (strawberry)[208], as previously mentioned, and in other fruits such as pomegranate and walnuts[209-211]. In these foods, as well as ellagitannins, the presence of ellagic acid free was also assessed[209]. The hydrolysis of ellagitannins leads to the release of ellagic acid (or subsequent gallic acid) and the corresponding sugar molecules (see Fig. 10). This reaction is used for the quantification of ellagitannins, measured as ellagic acid equivalents, after acid hydrolysis of



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

Fig. 10 Chemical formulas of hexahydroxydiphenic acid, ellagic acid and gallic acid. When ellagitannins are exposed to acids, bases or specific enzymes (tannase) ester bonds are hydrolized and hexahydroxydiphenic group spontaneously rearrange to form ellagic acid. Ellagic acid is a dimer that can be further hydrolyzed to gallic acid, a derivative of benzoic acid.

samples[212-214]. Information regarding the contents of ellagitannins and ellagic acid in foods are still scarce, although in recent years the attention and the interest towards these compounds is increased

and various research teams have studies in deep the content in various foods commonly consumed and the biological activities of these compounds. A study conducted in the United States, has evaluated the contents of ellagic acid in various vegetable products: most relevant quantities are present in blackberries and raspberries (21.4 mg/100 g), followed by strawberries (9.0 mg/100 g), walnuts (8.4 mg/100 g) and cranberry (1.7 mg/100 g)[212]. A Finnish research group has investigated 33 food and the higher content of ellagitannins was found in some berries, specifically 315.1 mg/100 g in arctic cloudberries, 297.3 mg/100 g in raspberry, 109.6 mg/100 g in wild rose, followed by 77.1 mg/100 g in strawberries[155]. The most common ellagitannins in raspberries and blackberries are sanguiin H-6 and lambertianin C, whose chemical structures are shown in Fig. 11 and Fig. 12, while the most abundant ellagitannin found in strawberries is agrimoniin, Fig. 13.

One of the most notable activities of tannins and related polyphenols is their potent antioxidant activity [156, 215, 216]. In recent years ellagitannins and their derivatives have received increasing attention because of their large biological properties such as anti-inflammatory activities [217, 218], antitumoral activities[217, 219-223], antiviral activities[224, 225] and antiplasmodial activities[226].

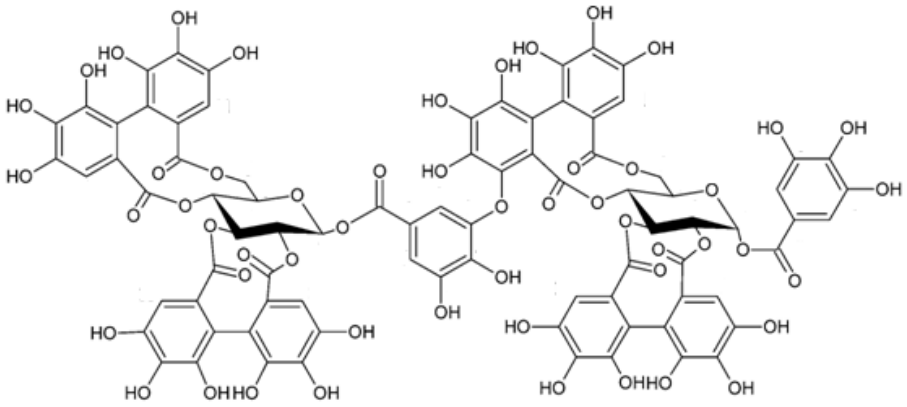


Fig. 11 Structure of sanguiin H-6. MW: 1871.3 g/mol

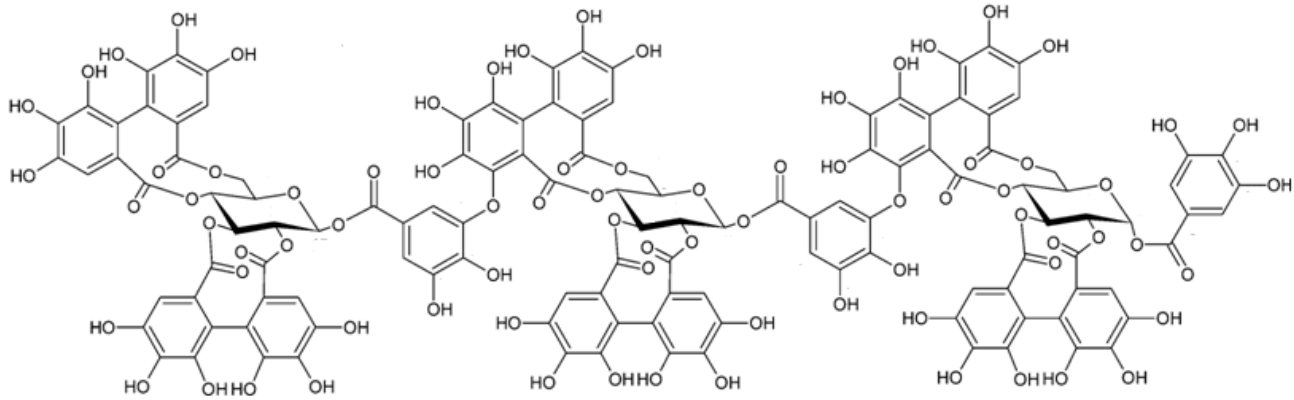


Fig. 12 Structure of lambertianin C. MW: 2804.0 g/mol

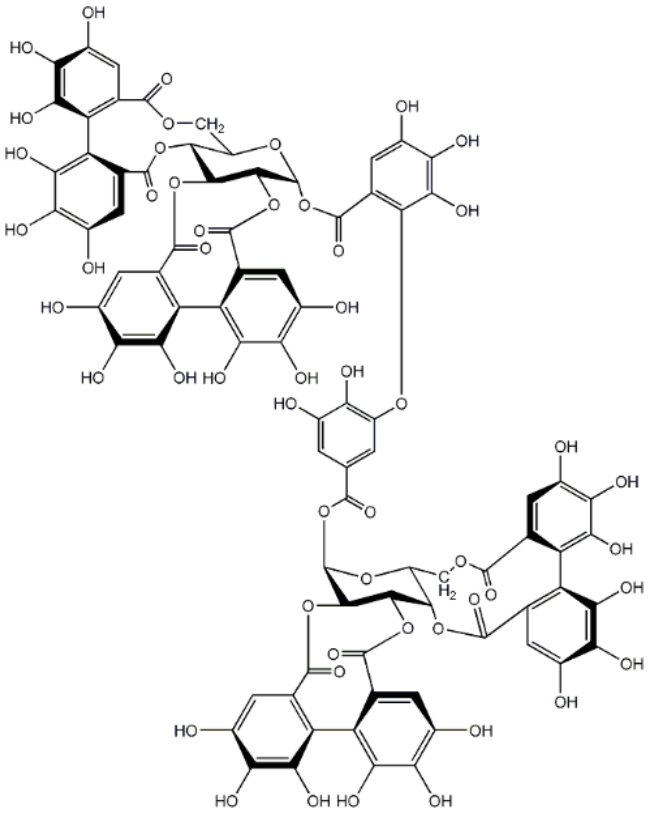


Fig. 13 Structure of agrimoniin. MW: 1871.3 g/mol

1.9.2 Absorption and metabolism of ellagitannins

The understanding of the mechanisms related to absorption and metabolism of ellagitannins and ellagic acid is essential in order to assess their potential beneficial effects in the gastrointestinal tract. The information concerning the intake of these compounds in the diet is limited, mainly because of the great variability in the content of ellagitannins and ellagic acid in foods and the different amount consumed by different populations, according to the availability of the products. *In vitro* studies have shown that ellagitannins are fairly stable at the physiological conditions of the stomach: gastric pH, between 1.8 and 2.0, and digestive enzymes are not able to hydrolyse or modify ellagitannins, and they are not absorbed in this district[227]. It is assumed therefore that, given the limited absorption, the action of ellagitannins takes place mainly *in situ* at gastric level. Ellagic acid that is introduced with the diet can be absorbed in the stomach, although concentrations reached in the bloodstream are small; the stomach is the first important site of absorption of this molecule.

On the contrary, under physiological conditions, ellagitannins are hydrolyzed to ellagic acid in the intestine and this seems to be due to the different pH, between 7.0 and 7.1[228]. Ellagic acid absorption remains limited: *in vivo* studies conducted on rats and mice have revealed the presence of this molecule in the faeces, urine, bile, blood and tissues, but in very low quantity[229]. Human studies (currently scarce and with a limited number of subjects) have confirmed the limited absorption of ellagic acid, derived from different food sources, by assessing plasma concentration

and urinary excretion, and emphasized a high degree of inter-individual variations[230-232]. If the bioavailability of ellagitannins and ellagic acid is very limited, however these compounds are extensively metabolized by the intestinal microbiota in related compounds with potential therapeutic activity, named urolithins (see Fig. 14)[141, 233]. 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 release of urolithin-A and urolithin-B[234]. Urolithins, once absorbed by intestinal cells, undergo a process of glucuronation, before entering the portal circulation. It has been estimated that these metabolites are present in significant concentrations in plasma and urine, reaching the maximum concentration after 24-48 hours after ingestion, being detected in the urine up to 48-72 hours[232, 235-237]. The composition of the intestinal microflora varies greatly between individuals and this means that each microbial community gives rise to different metabolic profiles[238, 239].

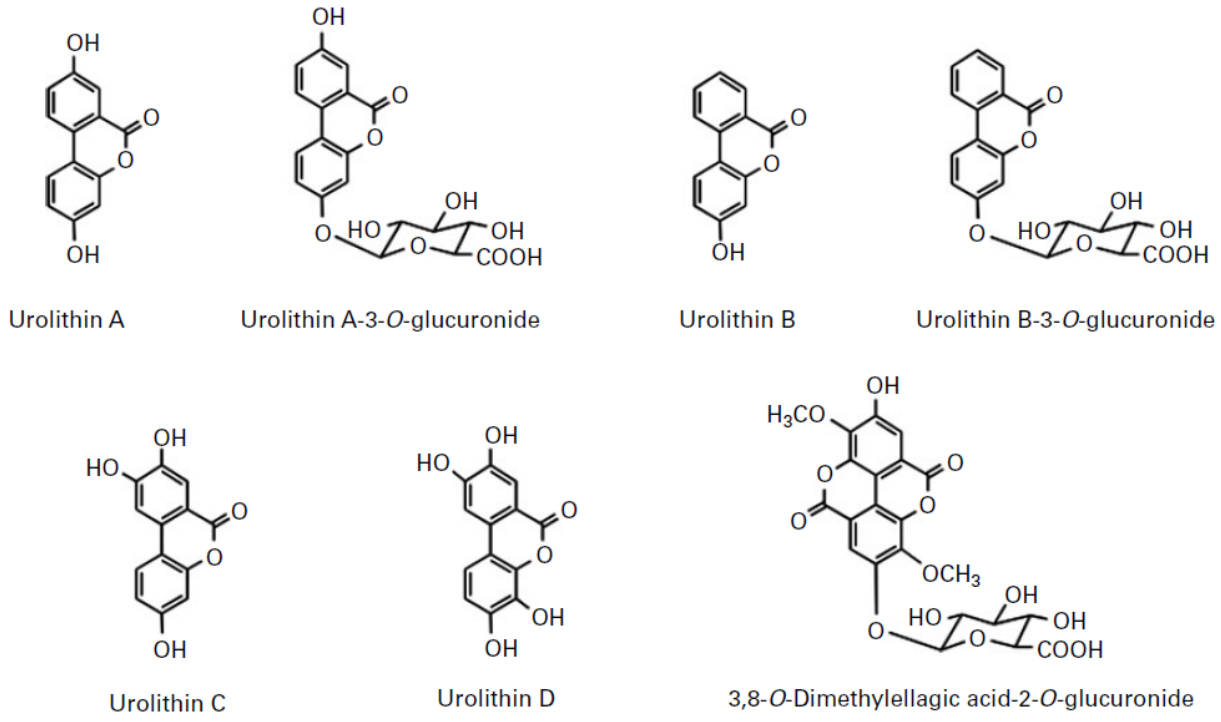


Fig. 14 Structures of urolithins

1.9.3 Biological activities of ellagic acid

Antioxidant activities

The antioxidant activity of ellagic acid has been evaluated *in vitro*, in several studies, on various cellular populations, like mammalian cells (KB), oral cells (CAL2A), colon (HT-29, HCT116, SW480, SW620), prostate (RWPE1, 22Rv1), keratinocytes, fibroblasts, and lung cells (V79-4)[240-243]. Ellagic acid was able to reduce levels of ROS and MDA and increase the levels of SOD on the

human cell line of bladder cancer T24; after treatment with H_2O_2 100 mM, it is observed a significant reduction of ROS and MDA of 53.3% and 42.2%, respectively, and a significant increase in SOD equal to 180%, relative to the control, in bladder cancer T24 cells treated with the compound[244]. The antioxidant activity of ellagic acid has been evaluated also in human keratinocytes (HaCaT) in a state of oxidative stress induced by exposure to ultraviolet radiation, ellagic acid increased intracellular levels of antioxidant enzyme, such as HO-1 and SOD, through the induction of Nrf2 pathway[245]. It was reported that ellagic acid has protective properties on A549 cells (human alveolar cells), through the activation of Nrf2 pathway which leads to the expression of HO-1 and NQO1 (Quinone Oxidoreductase 1); this activation causes a reduction in ROS levels and an increase of glutathione levels, which both reduce lipid peroxidation[246]. An *in vivo* study demonstrated a reduction of oxidative stress in brain and sciatic nerve tissues in diabetic rats treated with ellagic acid; the pure compound was able to decrease levels of MDA, nitric oxide and, in general, the oxidative status[247].

Anti-inflammatory activities

Ellagic acid, at 50 μM, was able to inhibit the activity of NF-κB, increase the phosphorylation of IκB and reduce the secretion of IL-8 induced by IL-1β, TNFα and LPS in intestinal Caco-2 cells[248]. Ellagic acid inhibited the release of PGE_2 induced by inflammatory stimuli (LPS followed by treatment with PMA), with an IC_{50} of 10 – 15 μM; the treatment with the compound reduce the expression of COX-2, mPGEs-1 and cPLA2, but not COX-1[249]. A study showed that components of an extract obtained from the peel of *Punica granatum* L., containing ellagic acid, were able to inhibit the inflammatory response in the early stages of malaria; ellagic acid inhibited MMP-9 release from THP-1 cells treated with hemozoin[218]. Ellagic acid (from walnut) was able to inhibit the activation and expression of adhesion molecules ICAM-1 and VCAM-1 induced by TNFα at endothelial level[201], this effect was probably due to the modulation of NF-κB activity, in particular through inhibition of nuclear translocation of p65 and p50 subunits[250]. In a recent study the ellagic acid-enriched extract from *Punica granatum* L. reduced the severity and extent of the damage of intestinal mucosa due to chronic inflammation induced by TNBS, in rats[251]. Ellagic acid reduced the effect of acute and chronic ulcerative colitis induced by dextran sulfate sodium (DSS), in rat; there was a reduction in levels of TNFα, IL-6 and IFN-γ and a significant inhibition of COX-2 and iNOS expression[252]. Ellagic acid reduced acute lung injury induced by acid in mice, by reduction of the activity of COX-2 and levels of IL-6 and increase of IL-10 levels[253]. Ellagic acid prevented the inflammatory response and the destruction of collagen in nude mice exposed to UVB radiation, reducing the levels of pro-inflammatory cytokines (IL-1β and IL-6) and macrophage infiltration[241].

Antitumoral activities

The antitumor activity of ellagic acid is well known and has been investigated in different cellular models, including esophagus, colon, skin, breast and prostate cell lines[228, 254-256], but the number of *in vivo* study is poor. Ellagic acid had effects on cell proliferation, induction of apoptosis and inhibition of tumour formation and growth[255, 257-259]. The treatment with ellagic acid reduced the proliferation of human prostate cancer (PC-3) and rats (PLS-10) cell lines, significantly affecting the proteolytic activity of collagenase and gelatinase secreted by PLS-10 and inhibiting in a concentration-dependent manner the activity of collagenase IV[260]. Anti-tumour activity of ellagic acid occurs also in Caco-2 cells by inhibition of proliferation (in a concentration- and time-dependent mechanism) and induction of apoptosis. Ellagic acid treatment increased the release of cytochrome c from mitochondria, the activation of procaspase-9 and procaspase-3, and reduced levels of bcl-XL protein[228]. Ellagic acid was able to reduce the accumulation of ROS by enhancing tissue antioxidant capacity in a model of colon neoplastic lesions induced by 1,2-dimethilhydrazine in rat, the activity was related to a chemopreventive action against colon cancer[261]. Ellagic acid exerted a concentration-dependent effect on metabolism of carcinogens acting on the enzymes involved in activation and detoxification of xenobiotics (phase I,II enzymes and antioxidant enzymes) in rat[262]. The results of clinical study revealed that ellagic acid may be an effective adjuvant therapy to chemotherapy in patients with hormone-refractory prostate cancer, reducing side effects and toxicity of classical therapy, particularly neuropathic pain[263].

1.10 Condensed tannins

1.10.1 Procyanidins

Proanthocyanidins, also named condensed tannins, are the result of flavanols condensation. Oligomers and polymers of proanthocyanidins can widely be found in the plant kingdom, as in fruits and berries, seeds, flowers, and leaves. Proanthocyanidins that consist exclusively of (epi)catechin units are called procyanidins and are the most abundant type in plants, see Fig. 15. In recent years, the role of dietary procyanidins as health protective agents has become an important area of human nutrition research[264-266]. Procyanidins are present as bioactive compounds in a wide range of foods, such as fruits, legume seeds, cereal grains, beverages such as red wine, tea, or cocoa; cocoa has the highest procyanidin content among all foods[267]. Epidemiological studies have indicated that the consumption of procyanidin-rich foods takes to lower incidence of inflammatory diseases and has beneficial effects on diseases of multifactorial pathogenesis, including metabolic syndrome, atherosclerosis, and cancer[268, 269].

1.10.2 Absorption and metabolism of procyanidins

The bioavailability of procyanidins depends on their absorption and metabolism at the gastrointestinal tract. An *in vitro* study demonstrated that procyanidins with high levels of polymerization are degraded to mixtures of epicatechin monomers and dimers under conditions similar to those in gastric juices[270], but an *in vivo* study demonstrated that procyanidins are stable during gastric transit in humans[271]. A second *in vivo* study on rats found that, after consumption of grapeseed, the small and large intestines contained (-)-epicatechin dimers, trimers, tetramers, and higher molecular weight procyanidins[272] and these molecules can be absorbed intact in the gastrointestinal tract of this animal[273]. Epicatechin dimer, trimer, and polymer are able to cross monolayers of human intestinal epithelial Caco-2 cells[274]. Once absorbed, procyanidins are conjugated to glucuronide, sulfate, and methyl groups, mainly in the gut mucosa and liver. In the plasma, monomers are present extensively as conjugated metabolites[275] .

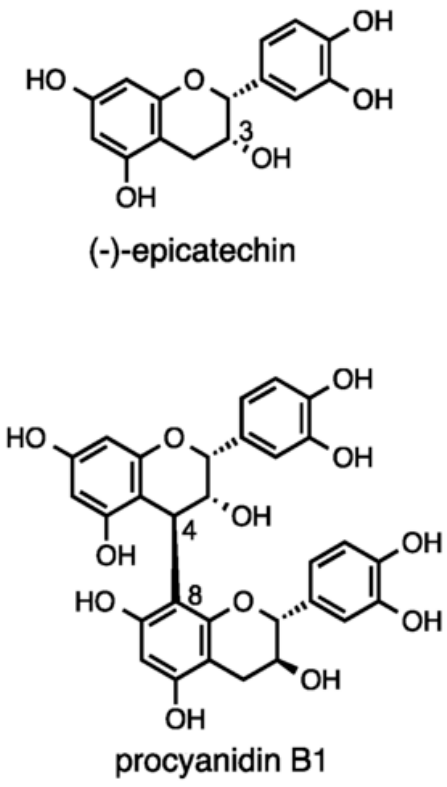


Fig. 15 Structure of epicatechin and example of dimer.

1.10.3 Biological activities of procyanidins

Anti-inflammatory activities

Several *in vitro* and *in vivo* studies have shown that procyanidins (extracts as well as monomers, dimers, or trimers) downregulate the transcription and secretion of proinflammatory cytokines, including IL-1β, IL-2, IL-6, and IL-8, TNFα and interferon-γ, and upregulate the secretion of anti-inflammatory cytokines such as IL-10, IL-4, also in *in vivo* models of inflammation[276-279]. Procyanidins were able to inhibit eicosanoid-generating enzymes, including phospholipase A2, cyclooxygenases, and lipoxygenases (LOXs), thereby reducing the secretion of prostanoids and leukotrienes (LTs)[280-282]. Procyanidins have been found to inhibit COX2; pre-treatment of mouse or human macrophages with procyanidin-rich extracts or with pure procyanidins inhibited the transcription of COX2 mRNA and the effect was mediated by regulation of COX2 transcription factors[283, 284]. Procyanidin-rich extracts and B2 procyanidin dimer are also inhibitors of COX2

protein expression in a dose dependent fashion in different cell inflammation models in both mice and humans[285, 286].

2. AIM

Gastritis and ulcers are very common inflammatory based diseases which can be caused by *H. pylori* infection, chemical factors or immunological disorders[3]. *H. pylori* is the leading cause of gastritis[1], it colonizes the gastric mucosa of over 80% of human population in developing countries[36] and at least 50% of the world's human population[3]. Recently, this bacterium has been classified as type I carcinogen by the World Health Organization[41], its eradication in infected individuals significantly decreases the risk of developing gastric adenocarcinoma[42]. *H. pylori* infection induces the release of several inflammatory cytokines in the gastric mucosa, such as IL-1β, IL-2, IL-6, IL-8 and TNFα[51-54]. In gastric epithelial cells TNFα and IL-1β induce the expression of IL-8, a potent chemokine which promotes neutrophil infiltration, and this mechanism is associated with the activation of NF-κB[57, 58, 95]. IL-8 appears as a key point of *H. pylori*-induced gastritis and epithelial cells are among the main producers of this chemokine in gastric mucosa[55]. Current therapies against *H. pylori* eradication involve the administration of proton pump inhibitors in combination with antibiotics; however, the development of resistant strains and poor compliance of patients have made the process of eradication complicated[287]; furthermore the use of gastric acid suppressors in *H. pylori* therapies has shown to increases the risk of pneumonia and hip fractures[288, 289]. For all these reasons it is very important to find new strategies for the treatment of gastric diseases.

Fruits and vegetables demonstrated to exert multiple biological effects on the mucosa of the gastrointestinal tract due to their antioxidant content[134] and they play a crucial role in the maintaining of gastric mucosa homeostasis by counteracting potential damage exerted by ROS[18]. ROS play an important role of second messengers in the NF-κB pathway modulation[89, 118-120]. *In vivo* and *in vitro* studies show that the antioxidant and anti-inflammatory activity of some foods is due to their polyphenols content[18, 135]. At this regard, among fruits, wild berries (blackberry, raspberry, common and wild strawberry) possess phytochemical contents with high antioxidant activity[139]. Berries contain two classes of molecules of particular interest for biological activities: flavonoids and tannins. Plants rich in tannins have a traditional use for treating gastric ulcer and tannins showed anti-bacterial activity against *H. pylori*[290, 291]. These molecules also revealed anti-inflammatory activity both *in vitro* and *in vivo*[217, 218, 220, 292, 293], but the anti-inflammatory activity of tannins derived from fruits of genus *Rubus* (blackberry, raspberry) and *Fragaria* (strawberry) has been poorly investigated[158]. Furthermore tannins are not absorbed and metabolized in the gastric district, therefore their biological activities can occur predominantly *in situ*[227, 271].

On this basis, the aim of this thesis was to clarify the anti-inflammatory effect of the extracts enriched in tannins from blackberry, raspberry (rich in ellagitannins) and strawberries (rich in ellagitannins and procyanidins) at gastric level.

For this purpose, the efficacy of tannins was evaluated in a rat model of ethanol-induced gastric ulcer. Tannins were also investigated *in vitro* in a model of gastric inflammation. AGS cells were stimulated by cytokines involved in *H. pylori* infection, TNFα and IL-1β, and then was evaluated:

- the inhibition of NF-κB translocation and driven transcription activity;
- the effect on IL-8 release;
- the effect on IL-8 promoter activity.

Furthermore the ability of tannins to inhibit pro-oxidant stimuli, like H_2O_2 and ethanol, was also evaluated *in vitro* on IL-8 secretion.

3. MATERIALS AND METHODS

3.1 Chemicals

All chemicals and solvents were of analytical ultra-pure grade. All the chromatographic solvents were HPLC grade or LC-MS grade for the MS experiments. Acetonitrile, acetone, methanol and diethyl ether were purchased from Sigma Aldrich (Milan, Italy). Hexane and formic acid were purchased from Carlo Erba (Milan, Italy). Ellagic acid standard (purity >98%) was purchased from Fluka (Steinheim, Germany). Sanguiin H-6, lambertianin C and agrimoniin were isolated as described in Gasperotti et al. 2010[141]. Quercetin and polyethylene glycol 400 (PEG 400) were purchased from Sigma-Aldrich (Milan, Italy).

3.2 Plant material and preparation of tannins enriched fraction (TEs)

The preparation of TEs and the isolation of single ellagitannins were conducted by the research group of Dr. Fulvio Mattivi, at "Fondazione Edmund Mach-Istituto Agrario di San Michele all'Adige" (Trento). Blackberry (cv. Lochness), raspberry (cv Tulameen), strawberry (cv Darselect) and wild strawberry were grown in an experimental field in Vigalzano (Trento, Italy). No specific permissions were required for these locations, since the experimental field belongs to Fondazione Edmund Mach, San Michele all'Adige (TN, Italy). Berries were harvested at maturity and transported to the laboratory for the extraction. Before the extraction the samples were maintained at -22°C. Fruits were extracted with a mixture acetone/water (70/30 v/v), as reported in Mattivi F. et al.[294]; the ratio fruit/solvent was 60 g/250 mL. Berries were homogenized with an 847-86 model Osterizer blender and centrifuged. Polyphenol-rich extracts 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 mixture methanol/water (30/70 v/v) and filtered using a Durapore 0.45 mm filter (Millipore, Vimodrone, Italy). The purification was carried out using an established method[141] with minor changes due to the high volume of the samples. Briefly, a column cartridge (1064 cm), connected to a vacuum line, was packed with Sephadex LH-20 resin, pre-washed with 50 mL methanol and then equilibrated with 100 ml methanol/water (30/70 v/v). The aqueous methanol extract (50 mL) was loaded and polyphenols, such as anthocyanins, were washed off with 500 mL methanol/water (30/70 v/v). The fraction containing the ellagitannins was eluted using 350 mL acetone/water (70/30 v/v). The tannin-rich extracts (TEs) were dried using rotary evaporation under reduced pressure at 37°C and reconstituted in 5 mL methanol, added to 350 mL diethyl ether and precipitated with hexane (700 ml). The TE fraction was recovered by filtration and dried. An aliquot of the precipitate was further quantified by UPLC-PDA-MS to determine the amount of the main ellagitannins present in the extract. The quantification method applied detected ellagitannins

at 260 nm[141].

3.3 Cell culture

Human adenocarcinoma cells (AGS, CRL-1739, LGC Standard S.r.l., Milano, Italy) were grown at 37°C in DMEM F12 (Gibco-Invitrogen) supplemented with 100 units penicillin/mL, 100 mg streptomycin/mL, and 10% heat-inactivated foetal calf serum (Euroclone S.p.A, Pero, Italy) (complete medium), in a humidified atmosphere containing 5% CO_2. For cell maintenance AGS were treated with tripsin 0.105 mM and EDTA 0.25% (Sigma-Aldrich, Milan, Italy).

3.4 Cytotoxicity assay

The integrity of the cell morphology before and after treatment was assessed by light microscope inspection. Cell viability of AGS was measured by MTT method[295]. Cells were plated in 24-well plates (BD FalconTM) at concentration of 30000 cells/well; after 36 hours, cells were treated with compounds and incubated for 6 hours. Medium was removed and cells were incubated with MTT solution (0.1 mg/mL) for 1 hour at 37°C. At the end of the incubation period, MTT solution was removed, and 200 µL of extraction solution (90% isopropanol; 10% DMSO) were added to the wells. 100 µL of the contents of each well were transferred to a 96 plate and the absorbance of each well was read at 550 nm using a microplate reader (Microplate Reader iMark™, Bio-Rad Laboratories S.r.l., Segrate, Italy).

For the trypan blue test, once the incubation with compounds was completed, medium was removed and cells were detached with 500 µL trypsin-EDTA solution. Trypsin activity was stopped with the addiction of complete medium (500 µL). The volume (20 µL) of cell suspension was added to 180 µL of trypan blue solution (Sigma-Aldrich, Milan, Italy). Ethanol 2% (342,5 mM) and H_2O_2 (500 µM) were not toxic to AGS cells at the concentration used in the experiments.

3.5 Evaluation of NF-κB driven transcription

To evaluate the NF-κB driven transcription, cells were plated in 24-well plates (BD Falcon™) at concentration of 30000 cells/well; after 48 hours, cells were transfected by calcium-phosphate method with a plasmid containing the luciferase reporter gene under the control of NF-κB promoter (NF-κBluc). Cells received a change with new complete medium one hour before transfection. Transfecting solution for one 24-well plate was prepared with the following procedure: 650 µL Hepes buffered saline 2X (NaCl 280 mM; HEPES 50 mM, Na_2HPO_4 1.5 mM; pH 7.0) were mixed in constant agitation with 650 µL of a solution containing $CaCl_2$ (250 mM) and 1300 ng of NF-κB luc plasmid in sterile H_2O. When complete 50 µL of transfecting solution (50 ng NF-kBluc per

well) were added to each well and incubated overnight before treatments with compounds. After 16 hours, cells were placed in a medium deprived of FCS, and stimulated with TNFα or IL-1β at 10 ng/ml. TEs were tested at 1–10 μg/ml; individual compounds at 0.5–10 μM. After 6 hours cells were harvested and luciferase assays were performed using Britelite™ Plus reagent (PerkinElmer Inc. Massachusetts, USA) according to manufacturer's instructions, and signal was read with Victor™ X3 (Perkin Elmer, Walthman MA, USA). Data were expressed considering 100% the luciferase activity related to the cytokine-induced NF-κB driven transcription. Parthenolide at 5 μM was used as reference inhibitor. Results are the mean ± s.d. of at least three experiments in triplicate.

3.6 Evaluation of IL-8 promoter activity

To evaluate the IL-8 promoter activity, cells were plated in 24-well plates (BD Falcon™) at concentration of 30000 cells/well; after 48 hours, cells were transfected by calcium-phosphate method with a plasmid containing the luciferase reporter gene under the control of IL-8 promoter (IL-8luc, containing one NF-κB responsive element) using the procedure described above, using a different amount of plasmid per well, 100 ng. The experiments with IL-8luc mutated in κB site were performed at the same conditions of native promoter.

3.7 Evaluation of NF-κB nuclear translocation

For the NF-κB (p65) nuclear translocation assay, AGS cells were plated at the concentration of 1.5 x 10^6 cells/mL in 60-mm plates. After 48 hours, cells were treated for 1 hour with the pro-inflammatory mediators and the extracts/compounds under study. Nuclear extracts were prepared using Nuclear Extraction Kit from Cayman Chemical Company (Michigan, USA) and stored at -80°C until assayed. The same quantity of total nuclear proteins, measured by the method of Bradford[296], was used to assess NF-κB nuclear translocation using the NF-κB (p65) transcription factor assay kit (Cayman) followed by spectroscopy at 450 nm, 0.1 s (Victor™ X3, Perkin Elmer, Walthman MA, USA). Data were expressed considering 100% the absorbance related to the cytokine-induced NF-kB nuclear translocation. Parthenolide at 5 μM was used as reference inhibitor of NF-κB translocation. Results are the mean ± sd of three experiments in triplicate.

3.8 Evaluation of IL-8 release by cytokines

Cells were grown in 24-well plates for 48 hours (30000 cells/well) before the cytokine treatment, TNFα or IL-1β for 6 hours. IL-8 was quantified by using Interleukin-8 High Sensitivity Human ELISA Set (Immunotools, Germany) using the method described below. Briefly, Corning 96 well EIA/RIA plates from Sigma-Aldrich (Milan, Italy), were coated with the antibody provided in the

ELISA Set, overnight at 4°C. After blocking phase, 100 ml of samples in duplicate, were transferred into wells at room temperature for 1 hour. The amount of IL-8 in the samples was detected by spectroscopy (signal read 450 nm, 0.1 s, by Victor^{TM} X3) by the use of biotinylated and streptavidin-HRP conjugate antibodies, evaluating 3,5,3,59-tetramethylbenzidine (TMB) substrate reaction. The quantification of IL-8 was done using an optimized standard curve supplied with the ELISA Set (1.0–240.0 pg/ml). Parthenolide (5 μM) was used as reference inhibitor of IL-8 secretion. Results are the mean ± s.d. of three experiments in triplicate.

3.9 Evaluation of IL-8 release by H_2O_2 and ethanol

Cells were grown in 24-well plates for 48 hours (30000 cells/well) and then incubated for 24 hours in the presence of 2% ethanol, or for 12 hrs in the presence of 500 μM H_2O_2, following the procedure described by Kim et al. 2011[297], with slight modifications. IL-8 was quantified as described above. Quercetin (10 μM) was used as reference inhibitor of IL-8 secretion. Results are the mean ± s.d. of three experiments in triplicate.

3.10 Protocol of *in vitro* gastric digestion

To evaluate the effect of *in vitro* digestion on the activity of the strawberry extract the following protocol was performed. First saliva and gastric juice solution were prepared according to the list of components in the following Tab. 2.

Saliva (pH 6.5 ± 0.1)	Gastric Juice (pH 1.1 ± 0.1)
896 mg KCl	2752 mg NaCl
200 mg KSCN	306 mg $NaH_2PO_4 \times H_2O$
1021 mg $NaH_2PO_4 \times H_2O$	824 mg KCl
570 mg Na_2SO_4	302 mg $CaCl_2$
298 mg NaCl	306 mg NH_4Cl
1.8 ml 1M NaOH	8.3 ml HCl (37%)
200 mg urea	650 mg glucose
145 mg amylase	20 mg glucuronic acid
15 mg uric acid	85 mg urea
50 mg mucin	330 mg glucosaminehydrochloride
nano pure H_2O	1 g BSA
	1 g pepsin
	3 g mucin
	nano pure H_2O

Tab. 2 Composition of juices from the *in vitro* fasted digestion model. Final amounts are based on 1000 mL juice. Adjustments to correct pH are made with NaOH (1M) or HCl (37%). Adapted from Oomen et al., 2003; Versantvoort et al., 2005; Hagens et al., 2007; Walczak et al., 2012.

The amount of 600 mg of dried strawberry extract was weighted in a glass vial at room temperature, then 6 mL was added and incubated for 5 min in constant shaking at 37°C. After this first step 12 ml of gastric juice were added and incubated for 2 hours at 37°C in constant agitation. The solution obtained was dried and frozen at -80°C until analyses. The digested extract obtained was referred to 600 mg of the original extract and tested on different biological activities comparing the effect to non-treated control adjusted with a mixture of saliva and gastric juice.

3.11 Animals

Thirty male Wistar rats (Charles River Laboratories, Calco, Lecco, Italy), weighing 175–200 g, were used. 3 Rats per cage were housed under constant environmental conditions (22 ± 1°C, 50 ± 5% relative humidity, 12-h light/12-h dark cycle), with free access to standard laboratory rat chow (014RF21C; Mucedola, Settimo Milanese, Milan, Italy) and tap water. Animals were acclimatized for a period of at least 7 days before the use. The study was approved (protocol number 16/2010) by the Animal Ethics Committee of University of Milan (Italy), and communicated to the Italian Ministry of Health, having regard to the article 7 of the D.L. 116/92. In addition, the study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All efforts were made to minimize animal suffering.

3.12 *In vivo* protocol

Before the experiment, the animals were randomly divided in 5 groups (6 rats in each group) and treated intragastrically (i.g.) by gavage. The dose of TEs was calculated on the basis of a daily consumption of 125 g of fresh fruit by a human healthy adult of 70 kg[135]. The day before the induction of gastric lesions, rats were placed in individual metabolic cages and deprived of food, with free access to tap water for 20 hours. The last administration of TEs extracts, quercetin (as positive control) or vehicle was given 120 minutes before ethanol treatment.

3.13 Assessment of gastric mucosal damage

One hour after the administration of 1 mL of ethanol, rats were sacrificed under ether anesthesia by cervical dislocation; the stomach was removed and opened along the greater curvature. The stomach was rinsed with water, pinned open for microscopic examination by a microscope (Opmi 6; Carl Zeiss S.p.A., Arese, MI, Italy) and for photo-documentation by a digital camera (EOS 1100D, Canon Italia S.p.A., Cernusco Sul Naviglio, MI, Italy). Gastric hemorrhagic lesions in the glandular part were examined under a dissecting microscope (X10). Gastric damage was assessed in

a blind manner. The Ulcer Index (UI) was obtained by a 0–3 scoring system based on the number and severity of the lesions[298, 299]. Severity was defined according to the length of the lesions: 0, no lesions; 1, lesions 1–2 mm; 2, lesions 2–3 mm; 3, lesions >3 mm. UI was calculated as the total number of lesions multiplied by their respective severity score.

3.14 Preparation of gastric mucosa homogenates

Samples of 50 mg from normal and ulcerated rat gastric mucosa were homogenized in buffer A [10 mM TRIS-HCl (pH 8), 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 μg/ml aprotinin, 2 μg/ml leupeptin, and 1% Triton X-100] using Tissue Lyser II (Qiagen) for 2 minutes at the highest frequency 30/s. The homogenates were centrifuged at 12,000 g for 10 min at 4°C and the supernatants collected, and stored at -80°C until use. Protein concentration was determined using Bradford protein assay (Bio-Rad) with bovine serum albumin (Sigma-Aldrich, Milan, Italy) as a standard.

3.15 Cinc-1 (rat IL-8) release from gastric mucosa

The quantity of 40 μg total proteins was used to assess Cinc-1 release using GRO/CINC-1 (rat) EIA kit (Enzo Life Sciences International, Inc., Plymouth Meeting, PA, USA). This kit uses a polyclonal antibody to rat GRO/CINC-1 labelled with the enzyme horseradish peroxidase. After a short incubation (10 minutes) the enzyme reaction was followed by spectroscopy (signal read 450 nm, 0.1 s). The concentration of rat GRO/CINC-1 in the samples was determined by interpolation with a GRO/CINC- 1 standard curve. The results are expressed as pg of CINC-1 per mL of sample.

3.16 Measurement of oxidative stress in rat gastric mucosa

The antioxidant capacity of the gastric mucosa homogenates was assessed by Oxygen Radical Absorbance Capacity (ORAC) assay. This method measures the oxidative degradation of fluorescein (Sigma-Aldrich Spa, Milan, Italy), after the addition of the free radical generator AAPH (2,29-azobis(2-methylpropanimidamide)-dihydrochloride) (Sigma-Aldrich S.p.a., Milan, Italy). The oxidation of fluorescein by free radicals leads to a decrease in fluorescence, prevented by the presence of antioxidant compounds. All reagents were prepared in 75 mM phosphate buffer, pH 7.4 and Trolox (4–160 μM) was used as the reference compound. Samples from gastric mucosa were suitably diluted in the phosphate buffer. Each well of a 96-well microplate contained 120 μL of fluorescein (0.07 μM) and 20 μL of the samples (corresponding to 5 μg protein) in a final volume of 200 μL assay solution. After the addition of AAPH (60 μL, 12 mM), the plate was shaken automatically for 2 seconds and the fluorescence was measured at 37°C every 2 min for 60 min

with emission and excitation wavelengths of 528 and 485 nm, respectively, by using a microplate fluorescence reader (Victor^{TM} X3, Perkin Elmer, Walthman MA, USA). The ORAC values were calculated as area under the curve and expressed as micromole of Trolox equivalent (TE) per gram of gastric mucosa sample (mmol TE/g of gastric mucosa sample).

3.17 Evaluation of CAT activity in rat gastric mucosa

CAT activity in gastric mucosa homogenates was determined by Catalase Assay Kit (Cayman Chemical, Ann Arbor, MI, USA), which utilizes the peroxidative function of CAT for the determination of the enzyme activity. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured colorimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogen compound, using a microplate reader (Microplate Reader iMark^{TM}, Bio-Rad Laboratories S.r.l., Segrate, Italy) at 540 nm absorbance. Before starting the reaction, each well of a 96 well microplate contained 100 μL of diluted assay buffer, 30 μL of methanol and 20 μL of diluted gastric mucosa homogenates (2.5 μg/well). The amount of formaldehyde was calculated by means of a calibration curve of formaldehyde standard. CAT activity is expressed as units (U) of CAT per mg of proteins. One unit of CAT is defined as the amount of enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25°C.

3.18 Evaluation of SOD activity in rat gastric mucosa

SOD activity was measured by using a SOD activity kit (Enzo Life Sciences International, Inc., Plymouth Meeting, PA, USA). This colorimetric assay evaluates the ability of SOD to reduce the superoxide ion concentration generated from the conversion of xanthine and oxygen to uric acid and hydrogen peroxide by xanthine oxidase. SOD activity was determined from percent inhibition of the rate of WST-1-formazan formation, a coloured product absorbing light at 450 nm. Each sample was loaded in a 96 well microplate to the final amount of 6.25 μg/well. Immediately after the addition of xanthine, the plate was transferred to a microtiter plate reader (Victor^{TM} X3, Perkin Elmer, Walthman MA, USA) and absorbance was read at 450 nm every minute for 10 minutes at room temperature, under 10 seconds orbital shake before each reading. The amount of SOD in the samples was calculated by correlating the inhibition percentage of WST-1-formazan formation with the logarithm of the SOD units in a standard calibration curve. SOD activity is expressed as U/mg of proteins.

3.19 Statistical analysis

All data are expressed as mean ± s.d., with the exception of the *in vivo* experiments expressed as mean ± s.e.. Differences between means were calculated using the unpaired t test or one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple group comparisons. Statistical analysis was done using GraphPad Prism 5.00 software (GraphPad Software Inc., San Diego, CA, USA); $p<0.05$ was considered statistically significant. IC_{50} was calculated using GraphPad Prism 5.00.

4. RESULTS

Rubus fruticosus L. and *Rubus idaeus* L.
(Blackberry and raspberry)

In vitro studies

4.1 Extracts composition

The quantification of tannins present in the extracts was performed by UPLC-PDA-MS. To determine the content of the main compounds, the analysis was performed at 260 nm according to the protocol of Gasperotti et al.[141]. In enriched fractions, tannins from blackberry (TE-black) corresponded to 343 mg/100 g of fresh fruits, while tannins from raspberry (TE-rasp) were 155 mg/100 g. The composition of TEs was as follows: in TE-black sanguiin H-6 represented 12%, lambertianin C 56%, and ellagic acid 1% of the precipitate, while in TE-Rasp sanguiin H-6 represented 19%, lambertianin C 35%, and ellagic acid 1% (**Fig. 16**). Sanguiin H-6 and lambertianin C, compounds belonging to the class of ellagitannins, account for more than 50% of both the extracts.

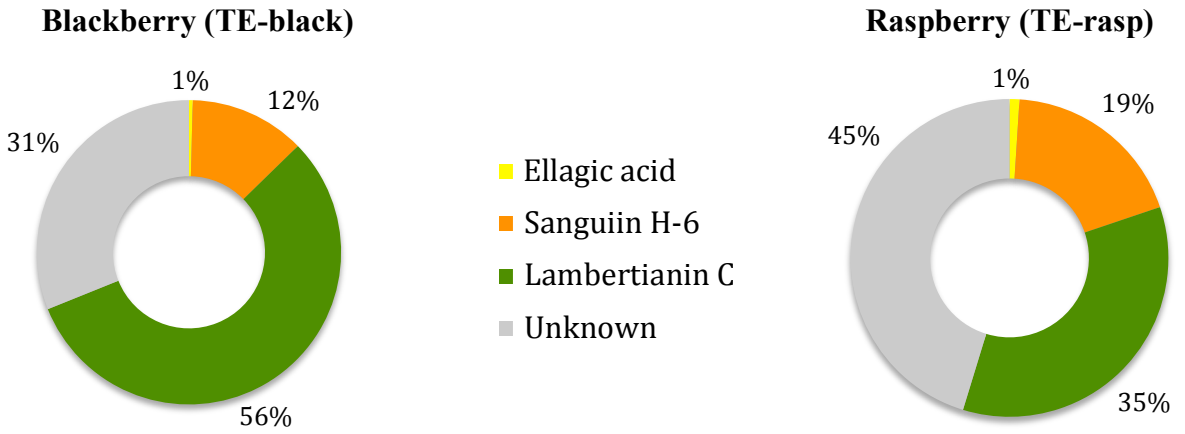


Fig. 16 Composition of blackberry (TE-black) and raspberry (TE-rasp) extracts. Berries tannins were extracted with acetone/water 70:30, isolated by Sephadex LH 20 column chromatography, precipitated with hexane and quantified by UPLC-PDA-MS. Tannins were detected at 260 nm.

4.2 Effect of cytokines on NF-κB driven transcription

Several experiments were conducted in AGS cell line in order to evaluate the effect on NF-κB driven transcription of the main pro-inflammatory cytokines involved in gastric inflammation (TNFα, IL-1β, IL-6, IL-21 and IL-8). The experiments of time-course were conducted at 3, 6, 24 and 30 hours, using cytokines at a concentration of 10 ng/mL. The transcription mediated by NF-κB was evaluated, in cells transiently transfected with NF-κBluc plasmid, by quantifying the luciferase activity as described in materials and methods. IL-6, IL-21 and IL-8 were not able to increase

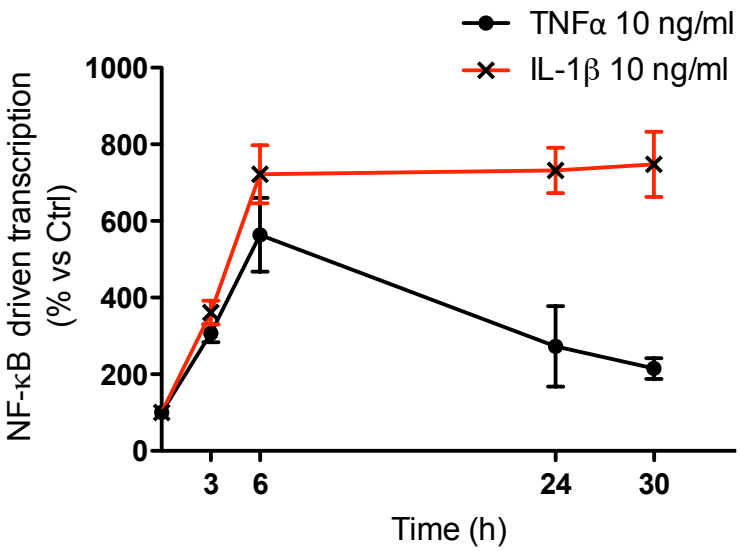


Fig. 17 Timecourse of NF-κB driven transcription. Effect of TNFα (black line) and IL-1β (red line) at 10 ng/mL on NF-κB driven transcription in AGS cells transfected with NF-κBluc plasmid. The analysis was performed using a luciferase assay and data are expressed in percentage, relative to the unstimulated control, which was arbitrarily assigned to the value of 100%.

significantly the transcription of luciferase compared to not stimulated cells (Data not shown). TNFα and IL-1β stimulated significantly the transcription of luciferase as shown in Fig. 17; the effect already appeared after 3 hours of stimulus with an increase of approximately 3 times compared to control. Both TNFα and IL-1β had the maximal effect after 6 hours treatment with an increase of 5.6 times for TNFα and 7.2 times for IL-1β. After 6 hours the stimulatory effect induced by TNFα decreased significantly, while the stimulus induced by IL-1β remains relatively constant up to 30 hours (maximum time considered).

4.3 Evaluation of cytotoxicity of blackberry and raspberry extracts and pure compounds.

The cytotoxic effect exerted by extracts on cell viability was assessed by quantifying the activity of mitochondrial succinate dehydrogenase (MTT test) and by the Trypan blue test. The results have shown that TE-black and TE-rasp had no cytotoxic effects on AGS cells in the range of concentrations of 0.05 –25 µg/mL. Isolated compounds sanguiin H-6, lambertianin C and ellagic acid were not cytotoxic at all the concentrations used in this study.

4.4 Effect of extracts and compounds on NF-κB driven transcription

TE-black and TE-rasp were able to inhibit significantly, in a concentration-dependent manner, the transcription driven by NF-κB in AGS cells subjected to stimulation with TNFα (panel A) and IL-1β (panel B) at 10 ng/mL as shown in **Fig. 18**. The inhibitory effect of TE-black was found to be slightly higher than that obtained by TE-rasp, as shown by the comparison between IC_{50} values, 0.52 and 0.95 µg/mL for TE-black and TE-rasp, respectively. In cells subjected to stimulation with IL-1β the inhibitory effect of the extracts was less pronounced and statistically significant starting from concentration of 5 µg/mL, TE-black IC_{50} was 6.83 µg/mL whereas for TE-rasp was 8.80 µg/mL. Lambertianin C and sanguiin H-6 inhibited NF-κB driven transcription stimulated by TNFα

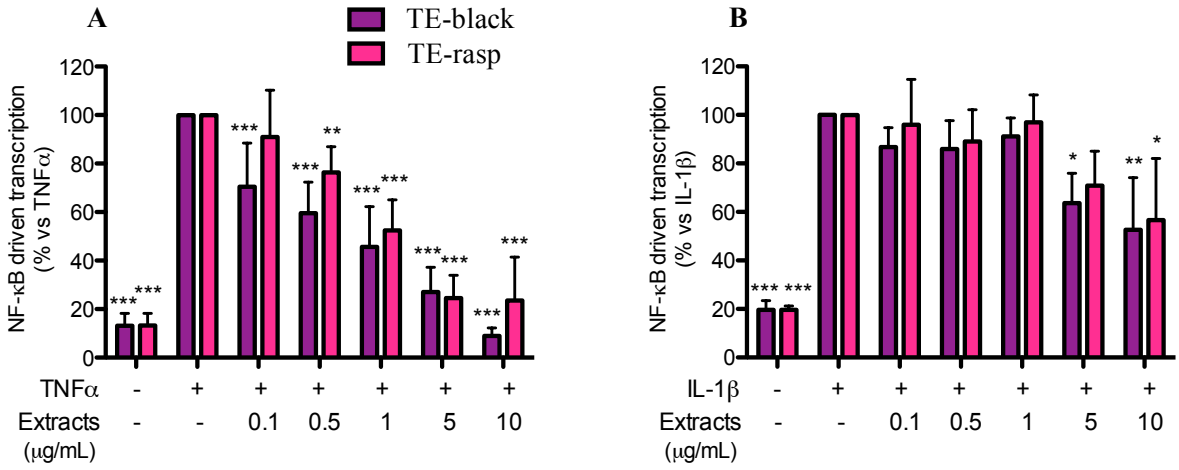


Fig. 18 Effect of TEs from blackberry and raspberry on NF-κB driven transcription induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 6 hours. TEs were tested at 1–10 μg/ml. Data are expressed as a relative percentage to the stimulated control, which is set to 100%. Inhibition by 5 μM parthenolide, used as reference inhibitor, was 72% on TNFα and 71% on IL-1β stimulus. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

with IC_{50} of 1.86 and 1.35 μM, respectively, however when the transcription was stimulated by IL-1β, the concentrations required for obtaining 50% inhibition were 5.89 μM and 2.52 μM for lambertianin C and sanguiin H-6, respectively (**Fig. 19**). Ellagic acid (0.25 μM) inhibited TNFα-induced transcription by 40%, while at the highest concentration tested (5 μM), the inhibitory effect was 80% (panel A, Fig. 20). The inhibitory effect of ellagic acid, following stimulation by IL-1β, was much less marked in comparison to TNFα, and the lowest concentration showing a significant reduction was 25 μM (about 20% inhibition, panel B, Fig. 20).

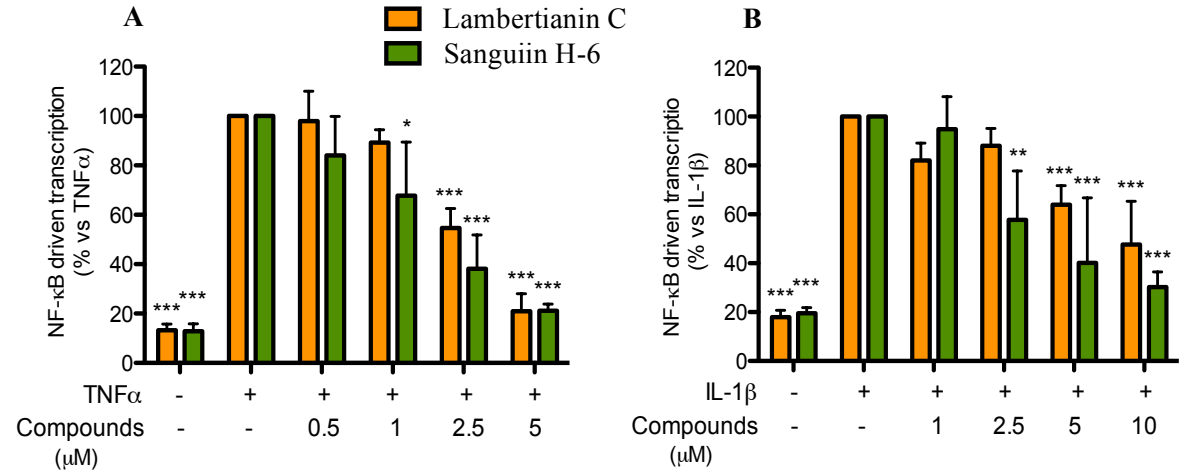


Fig. 19 Effect of sanguiin H-6 and lambertianin C on NF-κB driven transcription induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 6 hours. Compounds were tested at 0.5–10 μM. Data are expressed as a relative percentage to the stimulated control, which is set to 100%. Inhibition by 5 μM parthenolide, used as reference inhibitor, was 72% on TNFα and 71% on IL-1β stimulus. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

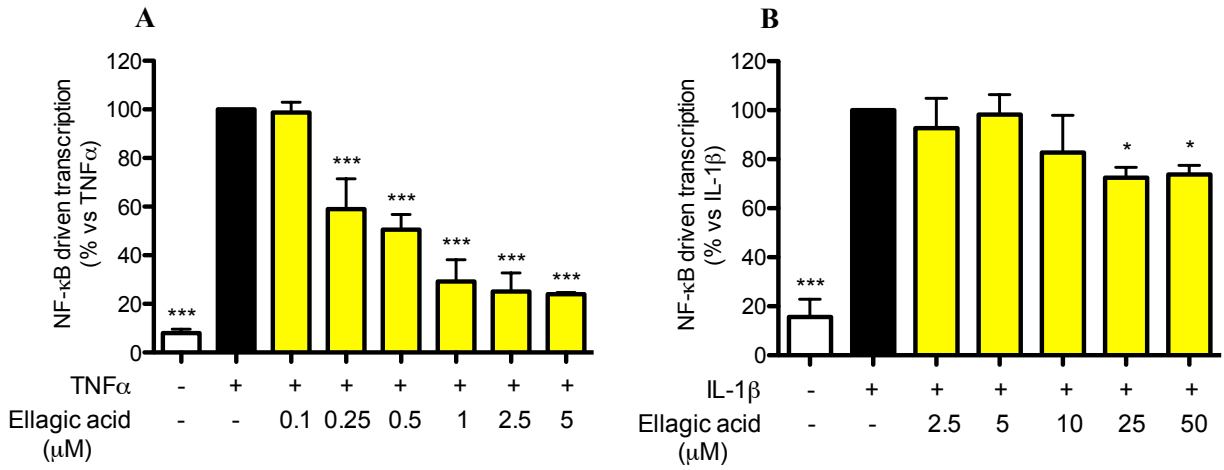


Fig. 20 Effect of ellagic acid on NF-κB driven transcription induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 6 hours. Ellagic acid was tested at 0.1–50 μM. Data are expressed as a relative percentage to the stimulated control, which is set to 100%. Inhibition by 5 μM parthenolide, used as reference inhibitor, was 72% on TNFα and 71% on IL-1β stimulus. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

4.5 Effect of cytokines on NF-κB nuclear translocation

In order to evaluate the effect of pro-inflammatory cytokines on the NF-κB nuclear translocation, AGS cells were treated with TNFα and IL-1β at 10 ng/mL for 1, 2, 3 and 6 hours; cells where then subjected to the nuclear extraction and a fixed quantity of nuclear proteins (10 μg protein/well) were loaded into an ELISA plate, as described in materials and methods. The effects of the cytokines on translocation are reported in Fig. 21. As can be seen from the figure TNFα and IL-1β were both able to induce the NF-κB translocation from the cytoplasm into the nucleus. The maximal effect was obtained after 1 hour treatment since the effect was increased about 7 times for

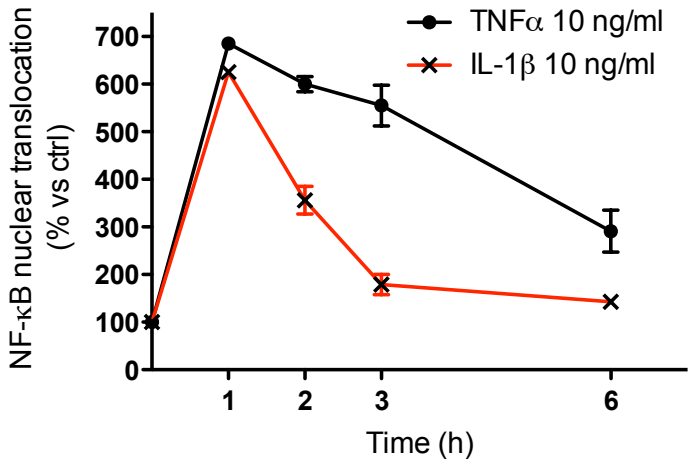


Fig. 21 Timecourse of NF-κB nuclear translocation. Effect of TNFα (black line) and IL-1β (red line) at 10 ng/mL on NF-κB nuclear translocation in AGS cells. The analysis was obtained by loading a fixed amount of nuclear lysates (10 μg protein/well) on an ELISA assay. Data are expressed in percentage, relative to the unstimulated control, which was arbitrarily assigned to the value of 100%.

TNFα and about 6 times for IL-1β. All the following experiments on nuclear translocation were therefore carried out in AGS cells after 1 hour treatment.

4.6 Effect of extracts and pure compounds on the NF-κB nuclear translocation

In order to better clarify the role of NF-κB, the ability of the extracts to interfere with nuclear translocation was also investigated. As shown in **Fig. 22** the extracts from blackberry and raspberry were able to inhibit TNFα-induced translocation, in a concentration-dependent manner (panel A). In particular TE-black and TE-rasp (0.5 μg/mL) inhibited nuclear translocation by 20.7% and 28.1%, respectively: the inhibitory effect reached 67% and 57% at the highest concentration tested (2 μg/mL). The inhibitory effect of the extracts on translocation induced by IL-1β (10 ng/mL) was much lower if compared to TNFα (panel B), as previously shown on the NF-κB driven transcription. The effect was statistically significant starting from 2 μg/mL (37% and 22.1% inhibition for TE-black and TE-rasp, respectively).

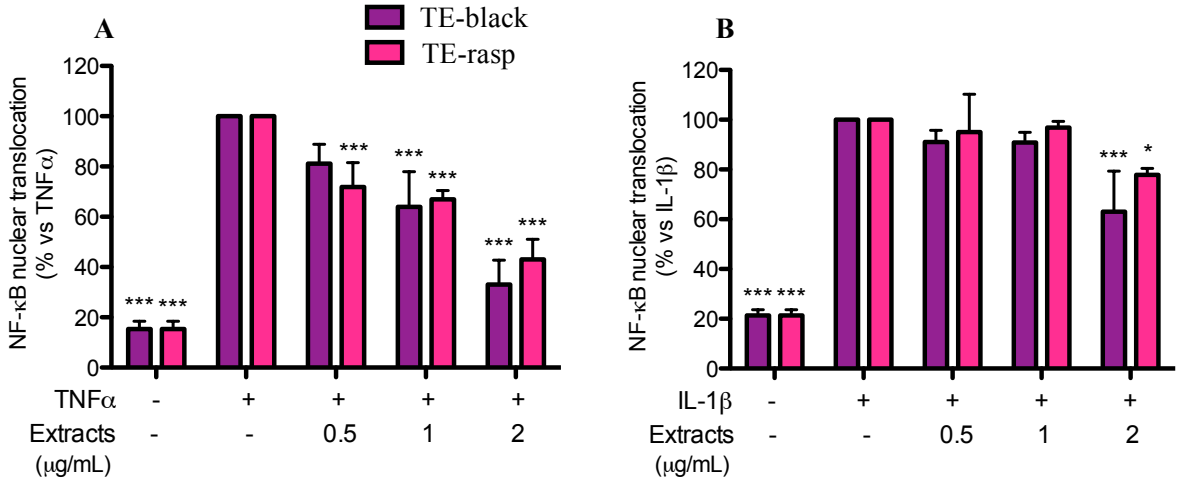


Fig. 22 Effect of TEs from blackberry and raspberry on NF-κB nuclear translocation induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 1 hour. NF- κB nuclear translocation was assessed by ELISA assay followed by spectrophotometric analysis (signal read 450 nm, 0.1 s). Inhibition by parthenolide at 5 μM, used as reference inhibitor, was 37% on TNFα and 40% on IL-1β stimulus. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

Pure compounds lambertianin C (5 μM) and sanguiin H-6 (2.5 μM) reduced NF-κB translocation induced by TNFα at the basal levels (control without stimulus), the effect was found to be concentration dependent (Fig. 23, panel A). IC_{50}s were 0.94 and 1.18 μM for lambertianin C and sanguiin H-6, respectively. When cells were treated with IL-1β, IC_{50}s were 1.51 and 1.06 μM for lambertianin C and sanguiin H-6, respectively (panel B). To evaluate the effect of ellagic acid, AGS cells were treated for 1 hour and the compound was tested at concentrations ranging between 0.5 – 2.5 μM. The data obtained are shown in Fig. 24. Ellagic acid inhibited the nuclear translocation of

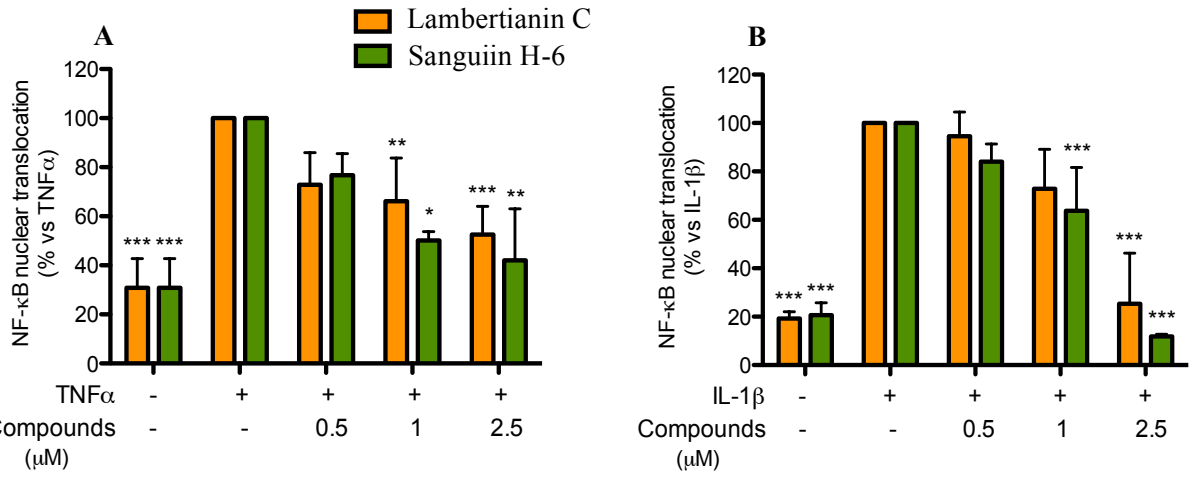


Fig. 23 Effect of sanguiin H-6 and lambertianin C on NF-κB nuclear translocation induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 1 hour. NF- kB nuclear translocation was assessed by ELISA assay followed by spectrophotometric analysis (signal read 450 nm, 0.1 s). Inhibition by parthenolide at 5 μM, used as reference inhibitor, was 37% on TNFα and 40% on IL-1β stimulus. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

NF-κB in AGS cells stimulated with TNFα, with a significant inhibition of approximately 50%, at the concentration of 2.5 μM. When IL-1β was used as pro-inflammatory stimulus ellagic acid showed a significant inhibition at concentration ten times higher tan that obtained with TNFα (2.5 vs 25 μM).

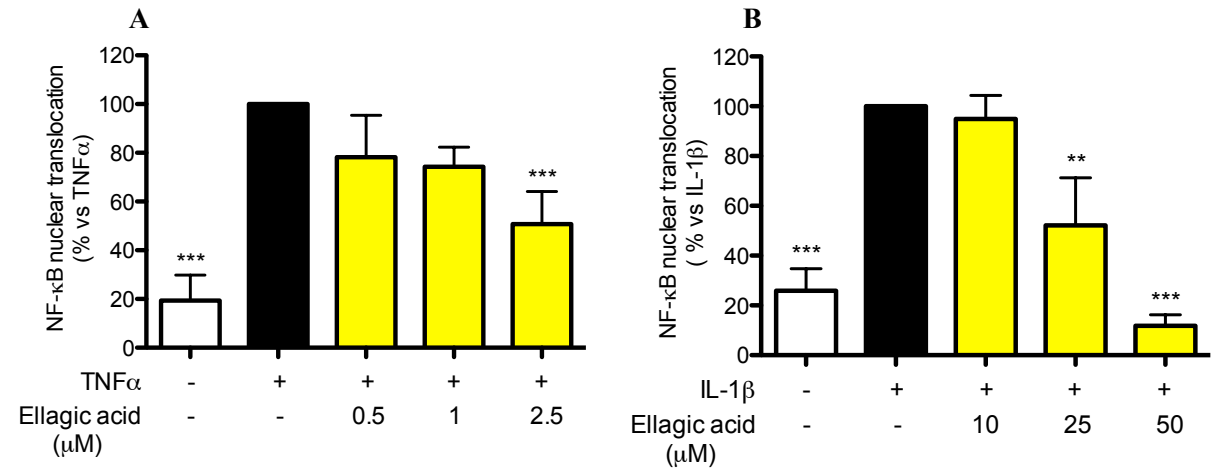


Fig. 24 Effect of ellagic acid on NF-κB nuclear translocation induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 1 hour. NF- κB nuclear translocation was assessed by ELISA assay followed by spectrophotometric analysis (signal read 450 nm, 0.1 s). Inhibition by parthenolide at 5 μM, used as reference inhibitor, was 37% on TNFα and 40% on IL-1β stimulus. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

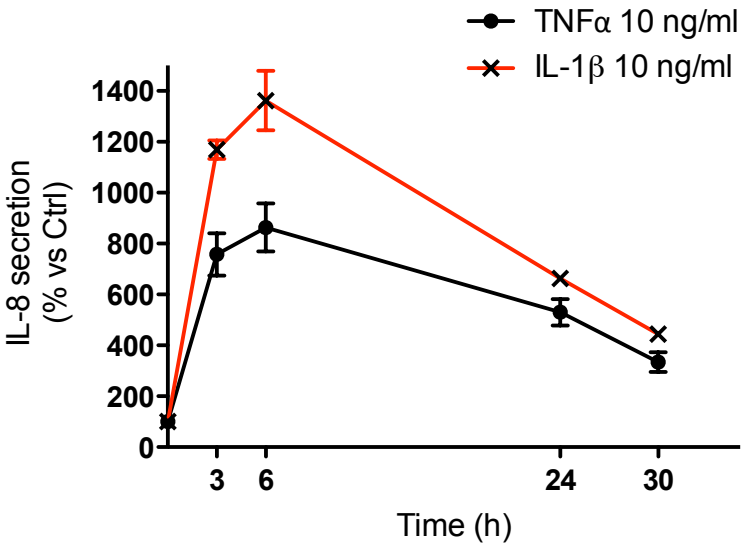


Fig. 25 Timecourse of IL-8 secretion. Effect of TNFα (black line) and IL-1β (red line) at 10 ng/mL on IL-8 secretion in AGS cells. The procedure has been described in materials and methods section. Data are expressed in percentage, relative to the unstimulated control, which was arbitrarily assigned to the value of 100%.

4.7 Effect of cytokines on IL-8 secretion

It is known that gastric inflammation is characterized by the release of IL-8 from the gastric epithelium. With the aim to characterize the release of IL-8 from AGS cell line, a time-course experiment was conducted at 3, 6, 24 and 48 hours, after induction with TNFα or IL-1β (both at 10 ng/mL). The results are shown in Fig. 25. The stimulation reached the highest effect after 6 hours of treatment (8 fold increase for TNFα; 15 fold increase for IL-1β); all the following experiments were conducted after 6 hours treatments.

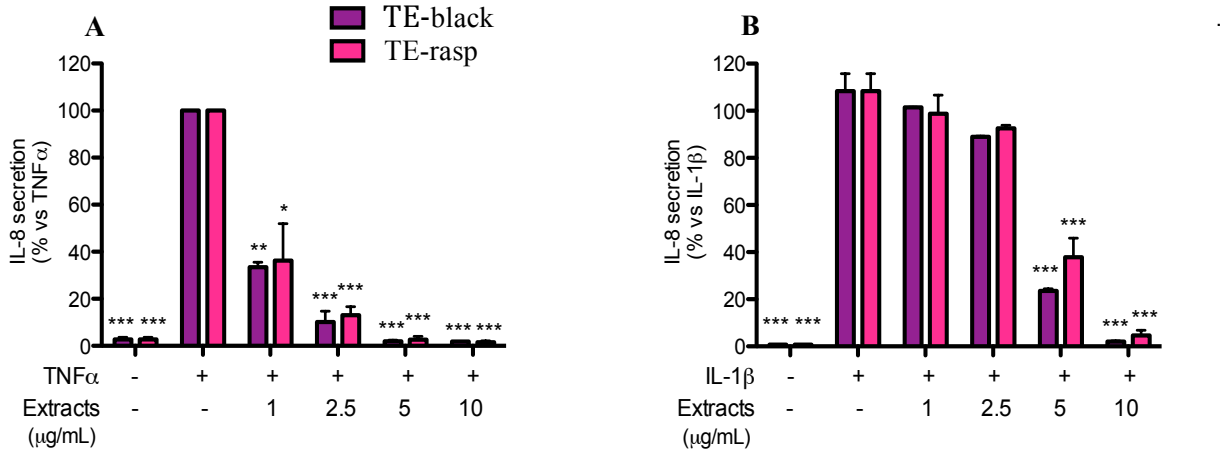


Fig. 26 Effect of TEs from blackberry and raspberry on IL-8 release induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 6 hours. IL-8 secretion was assessd by ELISA assay. Inhibition by 5 μM parthenolide, used as reference inhibitor, was 70% for both TNFα and IL-1β induced IL-8 secretion. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

4.8 Effect of extracts and pure compounds on IL-8 secretion

Tannin enriched extracts from blackberry and raspberry were able to reduce the IL-8 secretion in AGS cells, as shown in **Fig. 26**. Both the extracts inhibited the increase of IL-8 release induced by TNFα in a concentration dependent manner (panel A). IC_{50}s were 0.69 and 0.71 μg/ml for TE-black and TE-rasp, respectively. When IL-8 release was stimulated by IL-1β, inhibition of the extracts was lower and IC_{50}s were 3.62 μg/ml and 4.13 μg/ml for TE-black and TE-rasp, respectively. Ellagitannins showed greater inhibitory effect after treatment with TNFα than IL-1β, according to the effect showed by the extracts (Fig. 27). Lambertianin C and sanguiin H-6 started to produce a

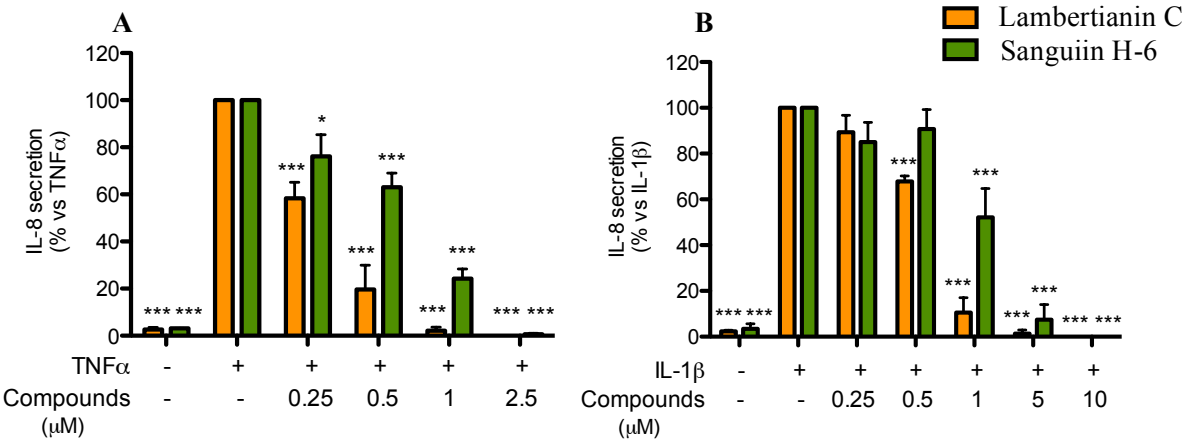


Fig. 27 Effect of sanguiin H-6 and lambertianin C on IL-8 release induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 6 hours. IL-8 secretion was assessd by ELISA assay. Inhibition by 5 μM parthenolide, used as reference inhibitor, was 70% for both TNFα and IL-1β induced IL-8 secretion. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

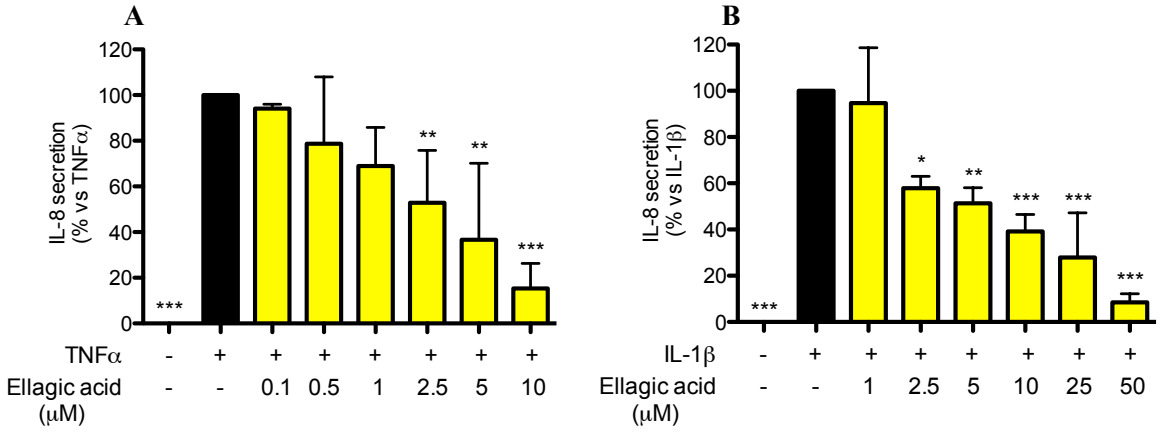


Fig. 28 Effect of ellagic acid on IL-8 release induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 6 hours. IL-8 secretion was assessd by ELISA assay. Inhibition by 5 μM parthenolide, used as reference inhibitor, was 70% for both TNFα and IL-1β induced IL-8 secretion. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

significant inhibition of TNFα induced IL-8 secretion at 0.25 μM. IC_{50}s calculated were 0.29 and 0.59 μM, respectively. According to the effect showed on the previous parameters, both ellagitannins needed higher concentrations to inhibit IL-1β-induced IL-8 secretion (IC_{50}:0.6 μM and and 1.04 μM for lambertianin C sanguiin H-6, respectively). Ellagic acid treatment was able to reduce IL-8 secretion in a concentration-dependent fashion (**Fig. 28**). Ellagic acid significantly inhibited TNFα –induced IL-8 secretion already at 2.5 μM; the inhibitory effect reached 80% at the highest concentration tested (10 μM). When cells were challenged with IL-1β, ellagic acid showed a statistically significant inhibition starting from 5 μM and the highest inhibitory effect was found to be at 50 μM (90%).

4.9 Antioxidant effect of the extracts

In order to assess whether the extracts were able to modulate IL-8 secretion, even in response to a pro-oxidant stimulus, AGS cells were treated with the pro-oxidant molecules H_2O_2 and EtOH (Fig. 29**)**. It has been widely reported in the literature that H_2O_2 (500 μM) causes a strong increase in IL-8 release after 12 hours in AGS cells[300], without cytotoxicity. According to these studies, cells have been treated with 500 μM H_2O_2 and IL-8 measured 12 hours after. When cells were treated with H_2O_2, IL-8 secretion increased ten folds with respect to control cells and secretion was inhibited by the extracts (IC_{50}s were 7.0 and 8.2 μg/mL for TE-black and TE-rasp, respectively, Fig. 29, panel A). Timecourse of IL-8 secretion induced by EtOH (2%, corresponding to 342,5 mM) revealed that 24 hours was the best treatment time to induce IL-8 release. This concentration was found to be

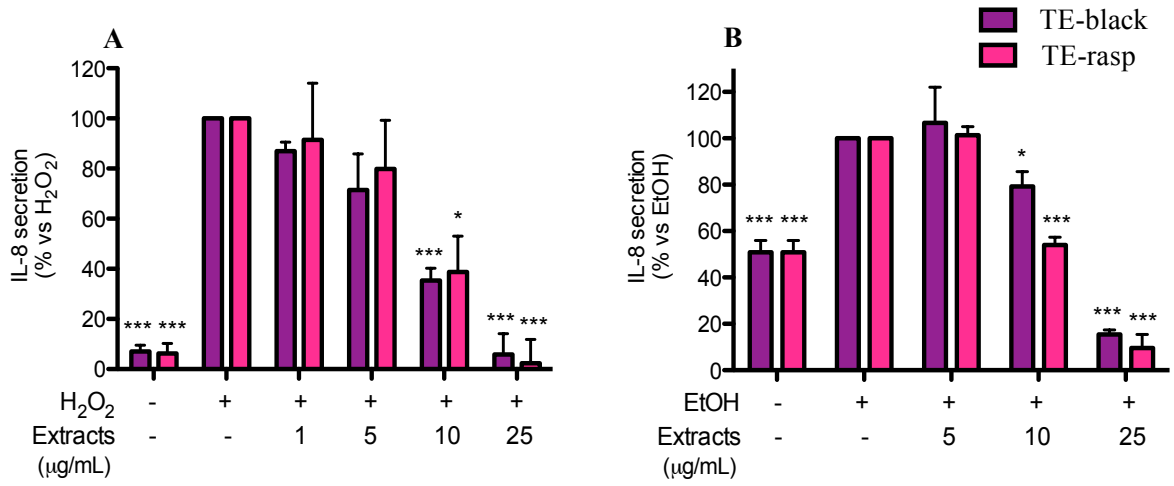


Fig. 29 Effect of TEs from blackberry and raspberry on IL-8 release induced by H_2O_2 and EtOH. AGS cells were stimulated with 2% EtOH (A) for 24h or 500 μM H_2O_2 (B) for 12 hours. IL-8 secretion was assessd by ELISA assay. Quercetin 10 μM, used as reference inhibitor, completely inhibited EtOH and H_2O_2 induced IL-8 secretion. Results are the mean±sd of three experiments in triplicate. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

without cytotoxicity in our cellular model. In cells treated with ethanol IL-8 secretion was twice higher than in control cells. Treatment with the extracts reduced the release of IL-8, although the effect was lower than that observed in cells challenged with TNFα. IC_{50}s were 11.5 and 9.8 μg/mL, respectively for TE-black and TE-rasp.

In vivo study

4.10 Protective effect of ETs on gastric injury in rats

The *in vivo* study aimed to demonstrate the protective effects of TE-black and TE-rasp was carried out on 30 Wistar rats, divided into 5 groups according to the different type of treatment, as follows:

- Group A: animals pre-treated for 10 days with only vehicle (10% polyethylene glycol 400) without administration of pure ethanol.
- Group B: animals pre-treated for 10 days with only vehicle (10% polyethylene glycol 400) followed by administration of pure ethanol for 1 hour.
- Group C: pre-treatment for 10 days with 100 mg/kg/day of quercetin dissolved in 10% polyethylene glycol 400, followed by administration of pure ethanol for 1 hour.
- Group D: pre-treatment for 10 days with 20 mg/kg/day of TE-black dissolved in 10% polyethylene glycol 400, followed by administration of pure ethanol for 1 hour.
- Group E: pre-treatment for 10 days with 20 mg/kg/day of TE-black dissolved in 10% polyethylene glycol 400, followed by administration of pure ethanol for 1 hour.

No difference in weight gain was observed in control and treated animals as shown in Tab. 3.

Group	Initial weight (g)	Final weight (g)
A	185.3 ± 6.7	214.8 ± 4.9
B	191.4 ± 5.3	221.3 ± 7.9
C	180.6 ± 8,2	211.7 ± 6.5
D	183.0 ±7.4	215.3 ± 5.3
E	190.2 ± 5.8	222.5 ± 8.4

Tab. 3 Effect of the treatment with the extracts on rat weight. No difference in weight gain was observed in the 4 groups of rats (group 2–5), as compared with controls animals (group 1) receiving only the chronic administration of vehicle (PEG 400).

Results are shown in **Fig. 30**: group 1 animals showed intact gastric mucosa without ulcerations, thus demonstrating that animals were healthy and not subjected to excessive stress during the treatment period. Group 2 animals showed a marked damage to gastric mucosa, visible also at

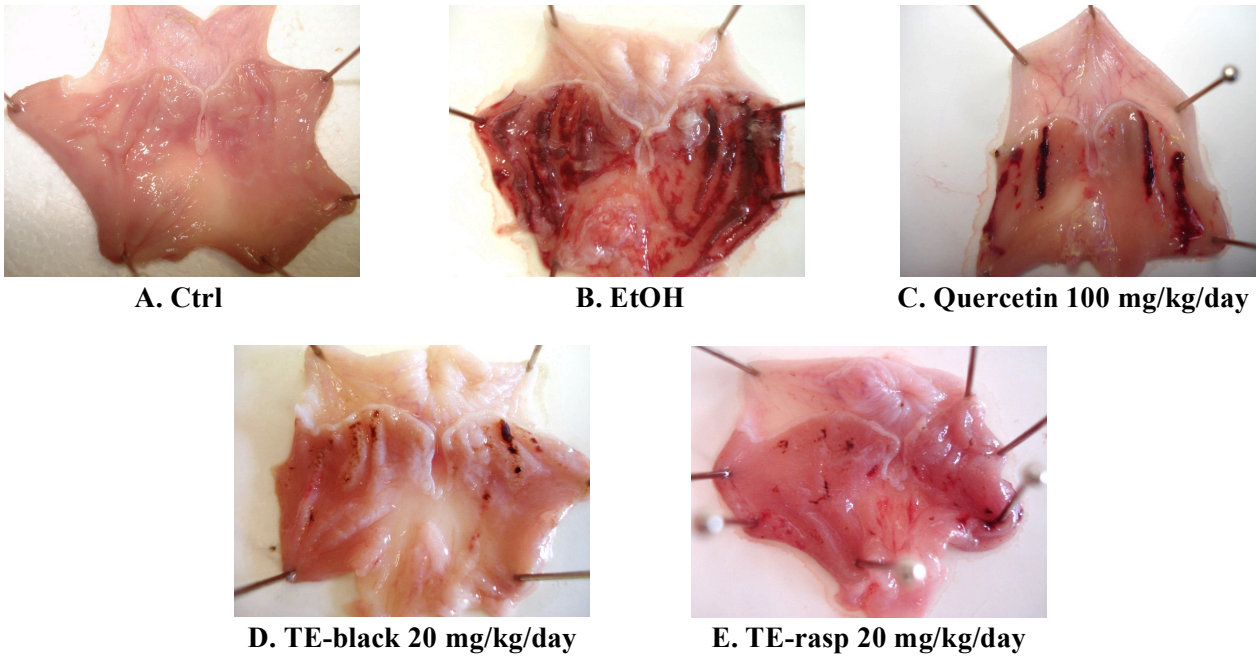


Fig. 30 Protective effect of TEs from blackberry and raspberry against ethanol induced gastric injury. Wistar rats were randomly divided in 5 groups (6 rats in each group). Controls were treated daily with vehicle (10% polyethylene glycol 400; PEG 400) for 10 days (A). Ethanol group received the vehicle (10% PEG 400) daily for 10 days, and then 1 ml of ethanol (B). TE-black group received 20 mg/kg of blackberry TEs dissolved in 10% PEG 400 for 10 days, and then 1 ml of ethanol (D). TE-rasp group received 20 mg/kg of raspberry TEs dissolved in 10% PEG 400 for 10 days, and then 1 ml of ethanol (E). Quercetin group (positive control) received 100 mg/kg of quercetin dissolved in 10% PEG 400 for 10 days, and then 1 ml of ethanol (C). The last administration of TEs, quercetin or vehicle was given 120 min before ethanol. Treatment was performed intragastrically by gavage.

INDEX	(A) Vehicle - Ethanol	(B) Vehicle + Ethanol	(C) Quercetin + Ethanol	(D) TE-black + Ethanol	(E) TE-rasp + Ethanol
Animal 1	0,0	16	4,0	5,0	9,0
Animal 2	0,5	19	1,5	7,0	3,0
Animal 3	0,0	20	1,0	2,5	9,5
Animal 4	0,0	18	3,5	2,0	3,5
Animal 5	0,0	15	1,0	4,0	2,5
Animal 6	0,5	19	1,5	6,0	2,5
Mean	0,2	17,8	2,1	4,4	5,0
Standard error	0,1	0,8	0,5	0,8	1,4

Tab. 4 Ulcer Index measured in all animal groups. Gastric damage was assessed in a blind manner by a scoring system based on the number and severity of the lesions: 0, no lesions; 1, lesions 1–2 mm; 2, lesions 2–3 mm; 3, lesions 3 mm. Ulcer Index was calculated as the total number of lesions multiplied by their respective severity score.

macroscopic level as elongated bands usually parallel to the long axis of the stomach. Pure ethanol induced the formation of gastric ulcers, measured by Ulcer Index (Tab. 4 and Fig. 31), and caused signs of necrosis, with marked infiltration of blood cells. Ulcers were located mostly in the corpus, the portion of stomach secreting acid and pepsin. No visible lesions were developed in the non-secretory part of the stomach. Pre-treatment with quercetin, a flavonoid used as reference inhibitor according to Alvarez-Suarez et al.[135], was able to prevent the damage to the gastric mucosa, reducing UI by 70%. TE-black and TE-rasp showed a high protective effect against ethanol injury: in fact, both the extracts were able to significantly reduced gastric lesions (88% and 75%, for TE-black and TE-rasp, respectively). Notably, a significant difference ($p \leq 0.05$) was found between the effect of TE-black and TE-rasp and between TE-black and quercetin.

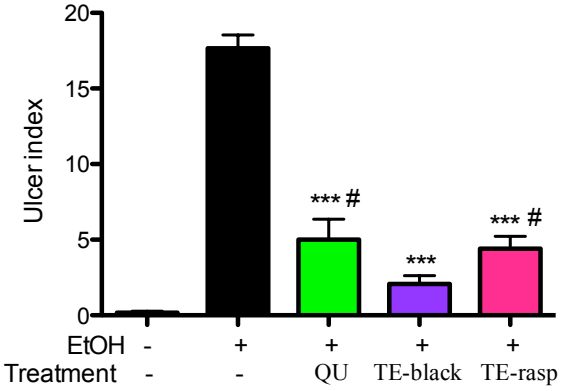


Fig. 31 Effect of ETs from blackberry and raspberry on prevention of EtOH-induced gastric lesions. Gastric damage was assessed in a blind manner by a scoring system based on the number and severity of the lesions: 0, no lesions; 1, lesions 1–2 mm; 2, lesions 2–3 mm; 3, lesions 3 mm. Ulcer Index was calculated as the total number of lesions multiplied by their respective severity score. Results are the mean ± s.e., n = 6. ***$p \leq 0.001$ vs EtOH, #$p \leq 0.05$ vs TE-black.

4.11 Effect of TEs on biochemical parameters

The gastric mucosa antioxidant capacity, which reflects the oxidation state of the mucosa, was evaluated using ORAC assay. Treatment with ethanol reduced in a statistically significant manner the anti-oxidant capacity of the tissue, compared to the control animals: reduction was probably due

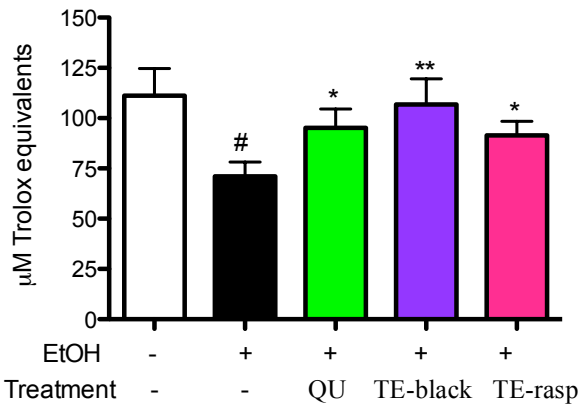


Fig. 32 Effect of ETs from blackberry and raspberry on antioxidant capacity of gastric mucosa. The antioxidant capacity was assessed by Oxygen Radical Absorbance Capacity (ORAC) assay and Trolox (4–160 µM) was used as the reference compound. ORAC values were calculated as area under the curve and expressed as micromole of Trolox equivalent (TE) per gram of gastric mucosa sample (µmol TE/g of gastric mucosa sample). *$p \leq 0.05$, **$p \leq 0.01$ vs EtOH, #$p \leq 0.05$ vs Unstimulated ctrl

to the increased production of ROS. Both quercetin and TE extracts were able to prevent oxidative stress in gastric mucosa, reporting the anti-oxidant capacity to values close to those obtained in control animals, as shown in **Fig. 32**. TE-black was more efficient in preventing damage caused by ethanol. Measurement of enzymatic activitiy of SOD in rat gastric mucosa after treatment with the extracts showed that both TE-black and TE-rasp were both able to preserve the activity of the enzyme in a statistically significant manner compared to ethanol treated group (**Fig. 33**, panel A), but TE-black was more efficient than TE-rasp. CAT activity was protected only by pre-treatment with TE-black (**Fig. 33**, panel B).

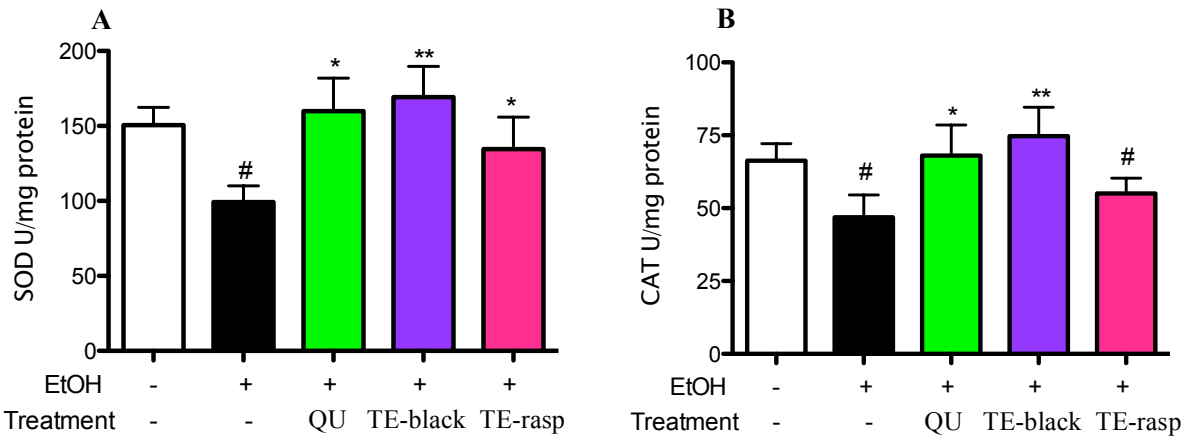


Fig. 33 Effect of TEs from blackberry and raspberry on enzymes activity ex vivo. The amount of SOD (A) in the samples was calculated by correlating the inhibition percentage of WST-1-formazan formation with the logarithm of the SOD units in a standard calibration curve. SOD activity is expressed as U/mg of proteins. CAT activity (B) was determined by the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured colorimetrically at 540 nm with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole as the chromogen using a microplate reader. CAT activity is expressed as units (U) of CAT per mg of proteins. One unit of CAT is defined as the amount of enzyme causing the formation of 1.0 nmol of formaldehyde per minute at 25°C. Results are the mean±se, n = 6. * $p \leq 0.05$, ** $p \leq 0.01$ vs EtOH, #$p \leq 0.05$ vs Unstimulated ctrl

The administration of ethanol caused a higher release of CINC- 1 (the rat homologue of human IL-8) from 12.8 pg/ml in the tissue from control animals to 28 pg/ml in ethanol treated rats, **Fig. 34**. In animals treated with TE-black and TE-rasp the amount of CINC-1 was significantly lower with respect to ethanol (16.5±1.9 and 22.2±2.3 pg/ml, respectively). In rats treated with quercetin as positive control CINC-1 levels were 15.9±0.72 pg/ml. The effect of TEs on CINC-1 was associated to a decrease of NF-κB translocation in TE-black and TE-rasp animals in comparison with control and ethanol treated animals. In the tissue of TE-black and TE-rasp animals, NF-κB translocation was inhibited by 38±0.11% (mean ± sd, n= 6, $p \leq 0.001$) and 72±1.6% (n= 6, $p \leq 0.0001$) respectively, with respect to ethanol. No difference was observed between control and ethanol group. No data are present in the literature as regards NF-κB nuclear translocation *in vivo* in the animal model of

ethanol-induced ulcer. An explanation is that the damage of the tissue does not allow properly isolation of the nuclear fraction.

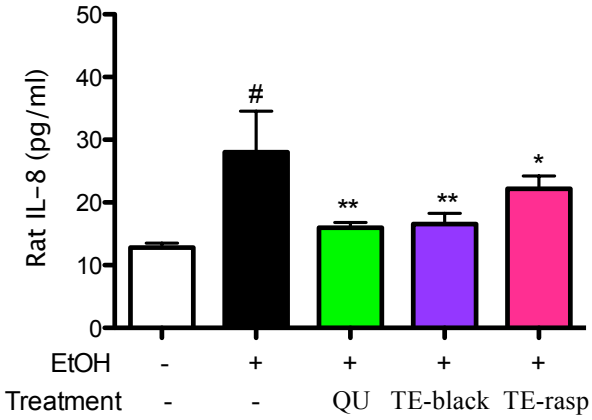


Fig. 34 Effect of TEs from blackberry and raspberry on CINC- 1 (IL-8 homologue) ex vivo. CINC-1 release was evaluated using GRO/ CINC-1 (rat) EIA kit. After a short incubation (10 minutes) the enzyme reaction was followed by spectroscopy (signal read 450 nm, 0.1 s). The concentration of rat GRO/CINC-1 in the samples was determined by interpolation with a GRO/CINC-1 standard curve. The results (mean ± se, n = 6) are expressed as pg of CINC-1 per mL of sample. * $p \leq 0.05$, ** $p \leq 0.01$ vs EtOH, # $p \leq 0.05$ vs Unstimulated ctrl

Fragaria X *ananassa* Duch. and *Fragaria vesca* L.
(Strawberry and wild strawberry)

In vitro studies

4.12 Extracts composition

The quantification of tannins present in *Fragaria* extracts was conducted by UPLC-PDA-MS as previously described[141]. The composition of TEs is shown in **Fig. 35**: in TE-straw agrimoniin represented 6% of the whole composition of the extract, whereas ellagic acid was 0.4%. Compounds belonging to the class of flavan-3-ols constituted 3.6% of the extract, while procyanidins represented 35%. In TE-wild agrimoniin was 8%, ellagic acid 2%, flavan-3-ols 2% and procyanidins 22%. More than 50% of compounds of both the extracts are actually unknown.

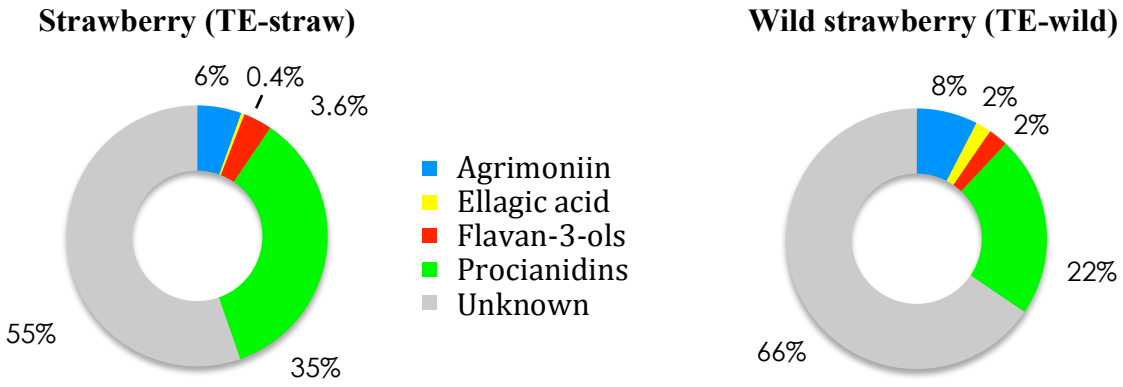


Fig. 35 Composition of strawberry (TE-straw) and wild strawberry (TE-wild) extracts. Berries TEs were extracted with acetone/water 70:30, isolated by Sephadex LH 20 column chromatography, precipitated with hexane and quantified by UPLC-PDA-MS. Tannins were detected at 260 nm.

For a detailed analysis of the compounds identified, see Tab. 5.

4.13 Evaluation of cytotoxicity of strawberry extracts and agrimoniin.

The cytotoxic effect exerted by extracts on cell viability was assessed by quantifying the activity of mitochondrial succinate dehydrogenase (MTT test) and by the Trypan blue test. The results have shown that TE-straw and TE-wild had no cytotoxic effects on AGS cells after 6 hours treatment in the concentration range of 0.05 –25 µg/mL after 6 hours treatment. Agrimoniin was not cytotoxic at all the concentrations used in this study.

4.14 Effect of extracts and pure compounds on NF-κB driven transcription

Strawberry extracts inhibited significantly, in a concentration-dependent fashion, the transcription

mediated by NF-κB in AGS cells, subjected to stimulation with TNFα and IL-1β at 10 ng/mL, **Fig. 36**. The inhibitory effect was greater for *Fragaria* extracts in comparison to *Rubus* extracts, IC_{50}s were 0.23 and 0.42 μg/mL under TNFα stimulus for TE-straw and TE-wild, respectively (panel A).

Method	Compounds	TE-straw %	TE-wild %
ellagitannins analysis (PDA 260 nm)	agrimoniin	5,5	7,6
	methyl ellagic acid rhamoniside	0,2	1,15
	ellagic acid	0,2	0,8
	sanguiin H-6	0,02	0,02
	lambertianin C	0	0,1
	total ellagitannins %	***5,92***	***9,67***
MRM polyphenols analysis (TQ MS)	gallic acid	0,0004	0,0008
	methyl gallate	0,0000	0,0000
	t-coutaric acid	0,0000	0,0000
	luteolin-7-O-Glc	0,0011	0,0003
	epigallocatechin	0,0001	
	gallocatechin	0,0001	0,0001
	catechin gallate + epicatechin gallate	0,0000	0,0004
	procyanidin B3	1,4309	0,7799
	taxifolin		1,8156
	kaempferol-3-Glc	0,0584	0,0265
	quercetin-3-glucuronide	0,0930	0,0068
	kaempferol-3-glucuronide	0,0935	0,0005
	chlorogenic acid	0,0003	
	t-piceide	0,0004	0,0001
	cis-piceide	0,0015	0,0002
	phlorizin	0,0111	0,0013
	catechin	1,6251	1,2022
	epicatechin	0,0068	0,0042
	procyanidin B1	0,5040	0,4489
	procyanidin B2 + B4	0,0166	0,0173
	quercetin-3-Glc + que3gal	0,0047	0,0456
	isorhamnetin-3-Glc	0,0001	0,0284
	fraxin		0,0000
	caffeic acid+catechin condensation	0,0022	0,0008
	phloretin	0,0000	
	trilobatin	0,0022	0,0002
	luteolin	0,0042	0,0050
	naringenin	0,0000	0,0001
	naringenin-7-glucoside	0,9382	0,1025
	procyanidin A2	0,0001	0,0001
	kaempferol pos	0,0011	0,0004
	total benzoic acid %	**0,0004**	**0,0008**
	total coumarins & phenylpropanoids %	**0,0003**	**0,0000**
	total stilbenes %	**0,0042**	**0,0011**
	total dihydrochalcones %	**0,0133**	**0,0015**
	total flavones & flavanones %	**0,9436**	**0,1076**
	total flavonols %	**0,2507**	**1,9238**
	total flavan-3-ols %	**3,5837**	**2,4530**
	total MRM polyphenols *%*	***4,7960***	***4,4881***
Vanillin 500	***Total procianidins %***	***35,17***	***22,5***
	Known compounds %	**45,89**	**36,66**

Tab. 5 Table of compounds analyzed in Fragaria extracts. The analysis reveals that the most abundant single compound in both the extracts is agrimoniin, which represents 5.5% of TE-straw and 7.6% of TE-wild.

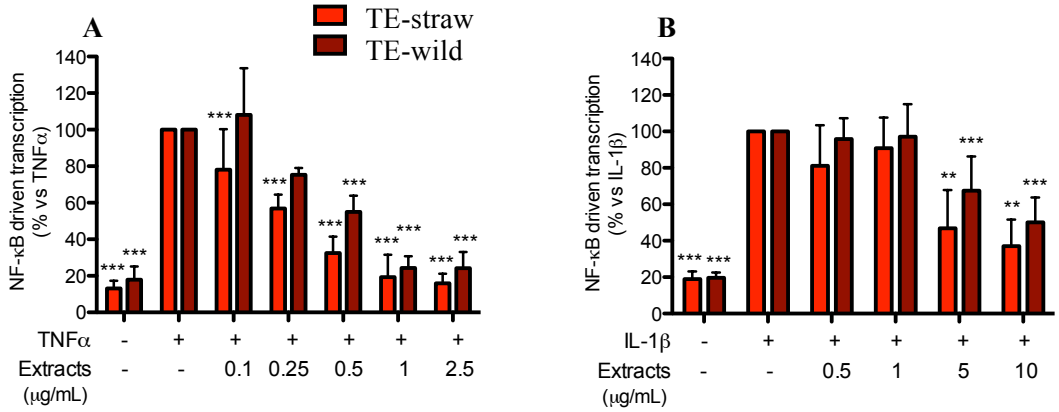


Fig. 36 Effect of TEs from strawberry and wild strawberry on NF-κB driven transcription induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/mL TNFα (A) or IL-1β (B) for 6 hours. TEs were tested at 0.1–10 μg/mL. Inhibition by 5 μM parthenolide, used as reference inhibitor, was 72% on TNFα and 71% on IL-1β induced NF-κB driven transcription. Results are the mean ± sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

Both the extracts showed a marked activity, bringing values to basal level at 1 μg/mL. In cells subjected to stimulation with IL-1β, however, the effect of extracts from strawberries on transcription mediated by NF-κB was less pronounced and statistically significant only from concentration above 5 μg/mL (panel B). The inhibitory effect of agrimoniin, the main ellagitannin identified in the extracts, on the TNFα-induced NF-κB driven transcription was statistically significant from 0.5 μM as shown in **Fig. 37**. IC_{50} of agrimoniin under TNFα stimulus was 0.50 μM (panel A), significantly lower with respect of IC_{50} obtained under IL-1β stimulus (13.34 μM, panel B).

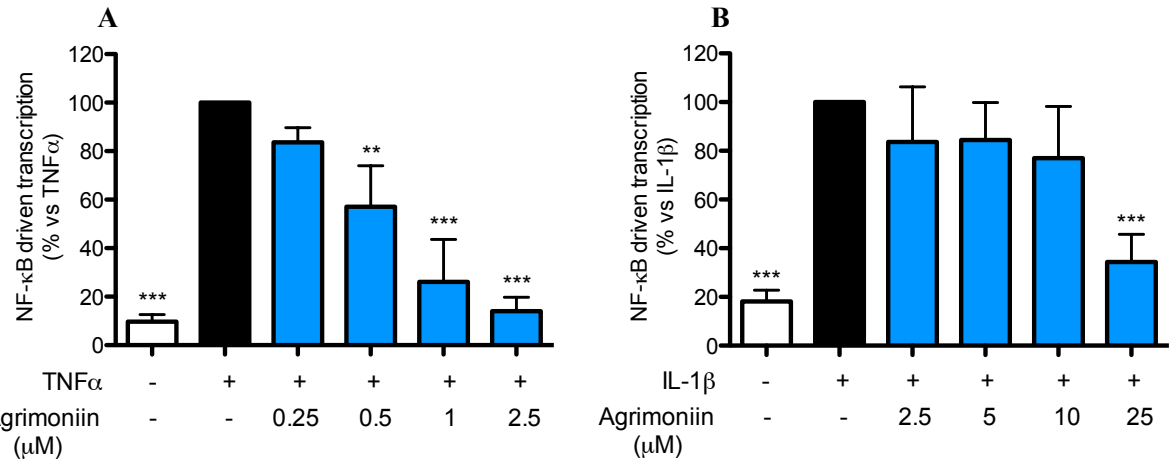


Fig. 37 Effect of agrimoniin on NF-κB driven transcription induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/mL TNFα (A) or IL-1β (B) for 6 hours. Agrimoniin were tested at 0.25–25 μM. Inhibition by 5 μM parthenolide, used as reference inhibitor, was 72% on TNFα and 71% on IL-1β induced NF-κB driven transcription. Results are the mean .± s.d. of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

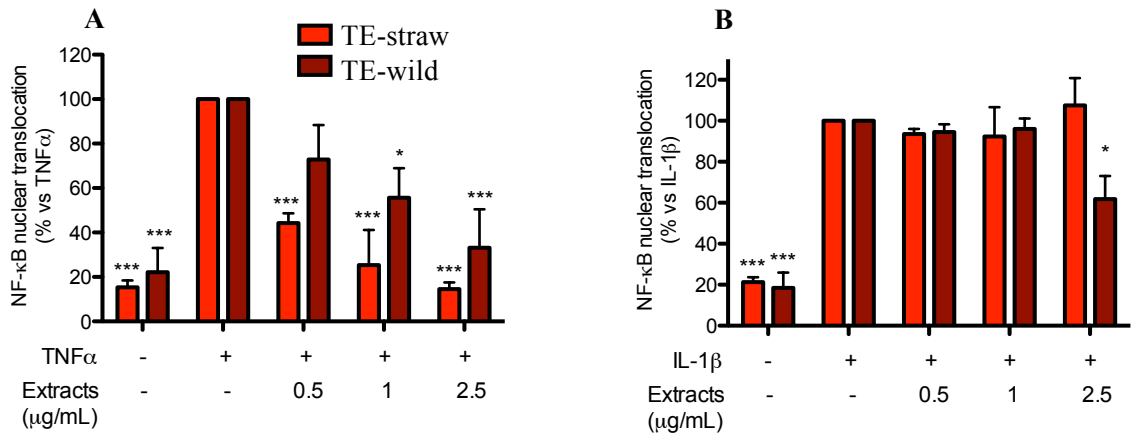


Fig. 38 Effect of TEs from strawberry and wild strawberry on NF-κB nuclear translocation induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 1 hour. NF- kB nuclear translocation was assessed by ELISA assay followed by spectrophotometric analysis (signal read 450 nm, 0.1 s). parthenolide at 5 μM, used as reference inhibitor, was 37% on TNFα and 40% on IL-1β stimulus. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

4.15 Effect of extracts and pure compounds on NF-κB nuclear translocation

The extracts were tested in a concentration range between 0.5-2.5 μg/mL after stimulation with TNFα and IL-1β. The results on translocation are shown in **Fig. 38**. TE-straw inhibited in a concentration-dependent way the nuclear translocation of NF-κB induced by TNFα, with a statistically significant inhibition already starting from 0.5 μg/mL; TE-straw activity was higher compared to TE-wild at each concentration tested (panel A). TE-wild showed statistically significant inhibition at concentrations below 1 μg/mL. Agrimoniin at concentrations ranging

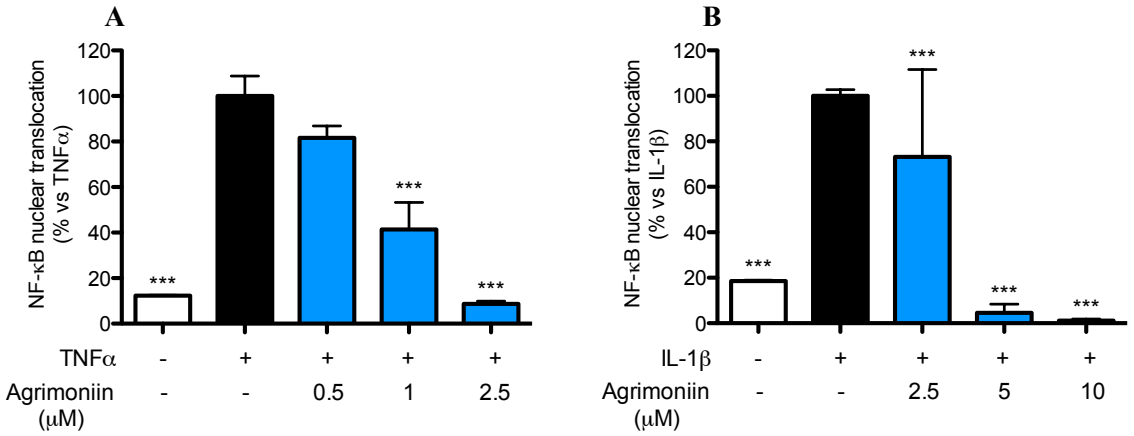


Fig. 39 Effect of agrimoniin on NF-κB nuclear translocation induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 1 hour. NF- κB nuclear translocation was assessed by ELISA assay followed by spectrophotometric analysis (signal read 450 nm, 0.1 s). Inhibition by parthenolide at 5 μM, used as reference inhibitor, was 37% on TNFα and 40% on IL-1β stimulus. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

between 0.5-2.5 µM (TNFα) and 2.5-10 µM (IL-1β), Fig. 39. showed a statistically significant inhibition at concentrations from 1 µM to 2.5 µM.

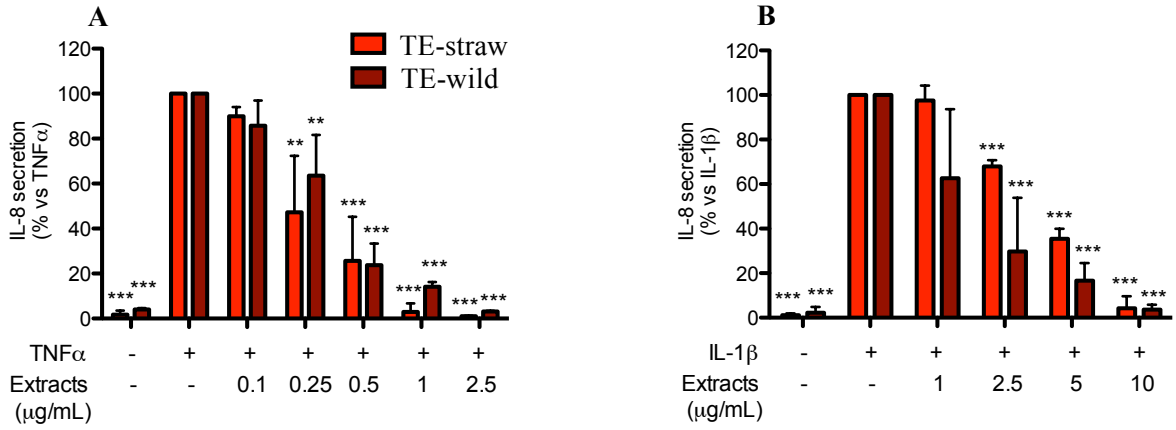


Fig. 40 Effect of TEs from strawberry and wild strawberry on IL-8 release induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 6 hours. IL-8 secretion was assessd by ELISA assay. Parthenolide (5 µM), used as reference inhibitor, showed 70% inhibition for both TNFα and IL-1β induced IL-8 secretion. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

4.16 Effect of extracts and pure compounds on IL-8 secretion

Extracts were tested at concentrations ranging between 0.1-10 µg/mL after stimulation with TNFα (10 ng/mL) or IL-1β (10 ng/mL). Both cytokines led to a significant increase of IL-8 secretion by AGS cells compared to non-stimulated control, Fig. 40. The inhibitory effect on TNFα was statistically significant starting at concentrations of 0.25 µg/mL and 0.5 µg/mL, for TE-straw and

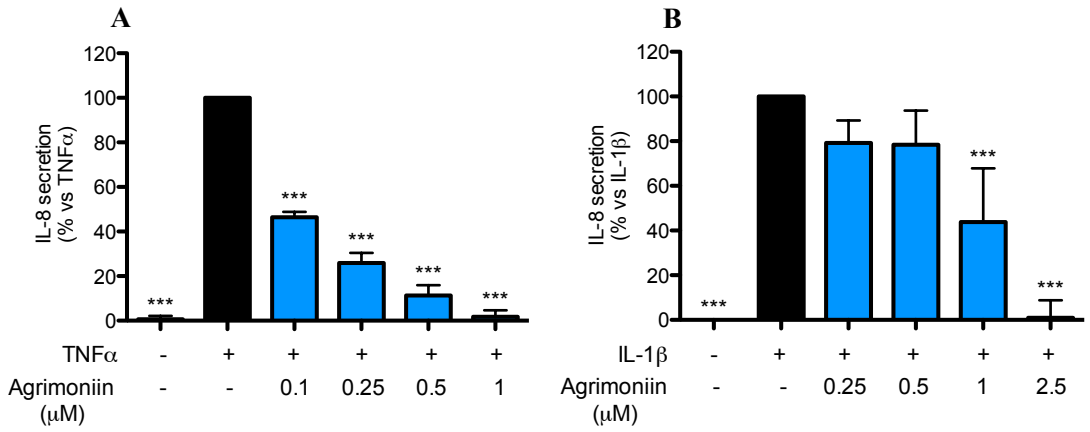


Fig. 41 Effect of agrimoniin on IL-8 release induced by TNFα and IL-1β. AGS cells were stimulated with 10 ng/ml TNFα (A) or IL-1β (B) for 6 hours. IL-8 secretion was assessd by ELISA assay. Inhibition by 5 µM parthenolide, used as reference inhibitor, was 70% for both TNFα and IL-1β induced IL-8 secretion. Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

TE-wild, respectively (panel A). The extracts inhibited the secretion of IL-8 induced by IL-1β in statistically significant way at 2.5 µg/mL (panel B). Both the extracts were able to restore the secretion of IL-8 to the basal level when used at higher concentrations (10 µg/mL). Agrimoniin inhibited in a concentration-dependent manner IL-8 secretion with statistically significant values starting from 0.1 µM under TNFα stimulus (**Fig. 41**, panel A).

4.17 Effect of *in vitro* gastric digestion on strawberry extract activity

In order to evaluate the effect of gastric digestion on the activity of extracts, TE-straw was subjected to an *in vitro* gastric digestion. The results obtained by the digested extract were compared under TNFα stimuls to the unprocessed one. Results are shown in Fig. 42. In all the

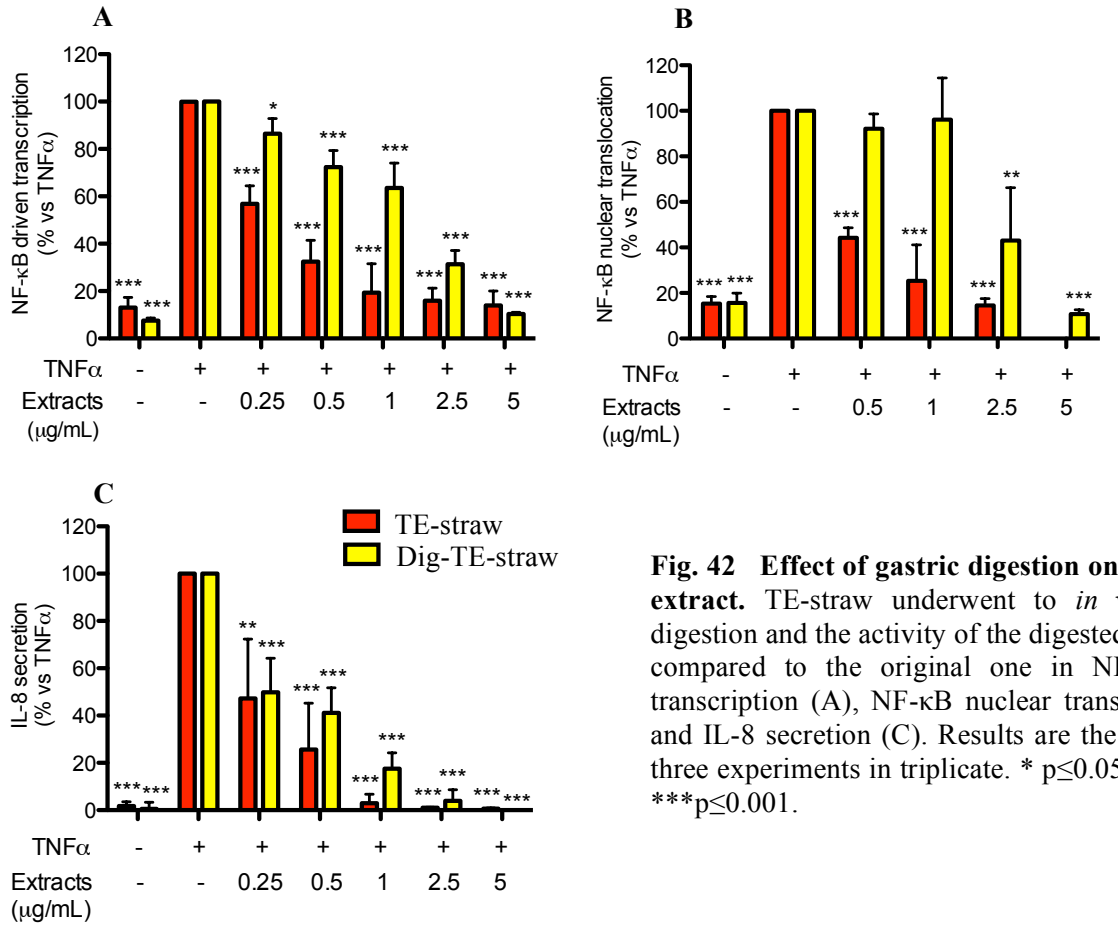


Fig. 42 Effect of gastric digestion on strawberry extract. TE-straw underwent to *in vitro* gastric digestion and the activity of the digested extract was compared to the original one in NF-κB driven transcription (A), NF-κB nuclear translocation (B) and IL-8 secretion (C). Results are the mean±sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

conditions, digestion decreased the activiy of TE-straw by approximately 50%. In case of NF-κB driven transcription (panel A), digested extract needed a concentration of 5 µg/mL to abolish the effect of TNFα stimulus, effect that was obtained at the concentration of 2.5 µg/mL by TE-straw. The concentration used to inhibit NF-κB nuclear transolcation induced by TNFα to basal level was 5 and 2.5 µg/mL for digested extract and TE-straw, respectively (panel B). Also the effect of TNFα

on IL-8 secretion was completely inhibited at 2.5 μg/mL by the digested extract, approximately a concentration double if compared to TE-straw (panel C).

4.18 Effect of extracts and pure compounds on IL-8 promoter activity

On the basis of previously results in IL-8 secretion the effect of TE-straw and digested extract were assessed on the inhibitory effect on IL-8 promoter activity, using TNFα (10 ng/mL) as pro inflammatory stimulus; furthermore, in order to ensure the effective involvement of NF-κB, the activity of TE-straw was also evaluated on the promoter of IL-8 mutated in the binding site for NF-κB, results are shown in Fig. 43. The pro-inflammatory stimulus increased the activity of the IL-8

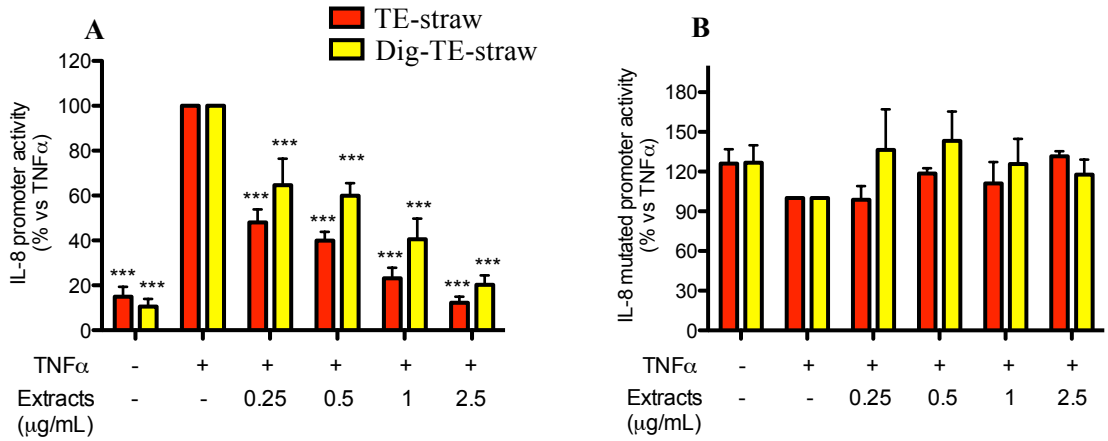


Fig. 43 Effect of TEs extracts from strawberry on IL-8 promoter activity induced by TNFα. AGS cells were stimulated with TNFα 10 ng/mL for 6 hours. TEs were tested at 0.25-2.5 μg/mL on IL-8 promoter activity (A) and on IL-8 promoter mutated at the NF-κB binding site (B). Results are the mean ± sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

promoter approximately 6 times compared to non-stimulated control cells (panel A). TE-straw and Dig-TE-straw had a significant inhibition of IL-8 promoter activity already at 0.25 μg/mL. Even in the evaluation of this parameter Dig-TE-straw required approximately a concentration double compared to TE-straw to obtain the same inhibitory effect. TNFα did not increased activity of IL-8 mutated promoter compared to non-stimulated cells; in addition, also TE-straw and Dig-TE-straw, at each concentration tested, did not induce the mutated promoter activity (panel B). Agrimoniin was tested on IL-8 promoter activity in a concentration range between 0.1-2.5 μM, using TNFα as pro-inflammatory stimulus. Results are shown in Fig. 44. Agrimoniin inhibited the activity of IL-8 promoter in a concentration-dependent manner; the effect was statistically significant starting at the concentration of 0.25 μM (panel A). The inhibition of 50% was obtained from agrimoniin at 1 μM. As previously reported, TNFα did not caused any increased activity of the promoter mutated in NF-κB binding site, with respect to non-stimulated control, and agrimoniin did not change the activity at any concentration tested (panel B).

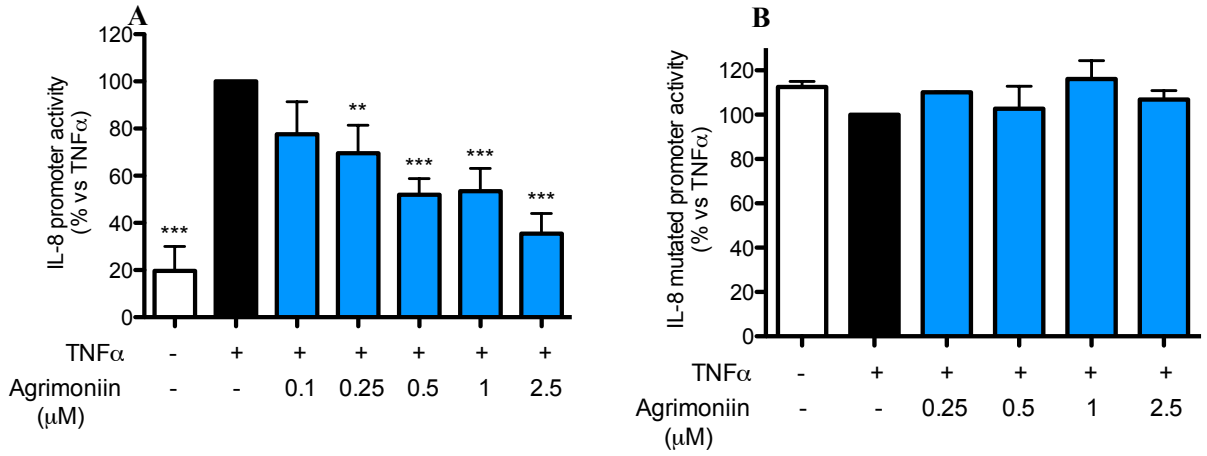


Fig. 44 Effect of agrimoniin on IL-8 promoter activity induced by TNFα. AGS cells were stimulated with TNFα 10 ng/mL for 6 hours. Agrimoniin was tested at 0.1-2.5 μM on IL-8 promoter activity (A) and on IL-8 promoter mutated at the NF-κB binding site (B). Results are the mean ± sd of three experiments in triplicate. * p≤0.05, ** p≤0.01, ***p≤0.001.

4.19 Summary of *in vitro* biological activity of berries extracts and pure compounds

The following tables summarized the inhibitory concentrations obtained by extracts and individual compounds on TNFα (**Tab. 6**) and IL-1β (**Tab. 7**).

Stimulus: *TNFα 10 ng/mL*	*NF-κB driven trascription 6h*	*NF-κB (p65) translocation 1h*	*IL-8 secretion 6h*
Extracts	IC_{50} values (μg/ml)	IC_{50} values (μg/ml)	IC_{50} values (μg/ml)
TE-black	0.52	1.11	0.69
TE-rasp	0.95	1.26	0.71
TE-straw	0.23	0.37	0.25
TE-wild	0.42	0.79	0.29
Dig-TE-straw	1.07	2.17	0.31
Compounds	IC_{50} values (μM)	IC_{50} values (μM)	IC_{50} values (μM)
LAMB-C	1.86	0.94	0.29
SANG-H-6	1.35	1.18	0.59
AGRI	0.50	0.81	0.09
EA	0.44	1.68	2.56

Tab. 6 Summary of results obtained after treatment with the extracts/pure compounds (TNFα stimulus). Values on NF-κB transcription, translocation and IL-8 secretion are expressed as IC_{50}.

Stimulus: *IL-1β 10 ng/mL*	*NF-κB driven trascription 6h*	*NF-κB (p65) translocation 1h*	*IL-8 secretion 6h*
Extracts	IC_{50} values (µg/ml)	IC_{50} values (µg/ml)	IC_{50} values (µg/ml)
TE-black	6.83	2.14	3.62
TE-rasp	8.80	2.79	4.13
TE-straw	3.26	No inhibition till 2.5 µg/ml	3.59
TE-wild	7.31	2.62	1.69
Compounds	IC_{50} values (µM)	IC_{50} values (µM)	IC_{50} values (µM)
LAMB-C	5.89	1.51	0.60
SANG-H-6	2.52	1.06	1.04
AGRI	13.34	2.94	0.82
EA	(-28% at 25 µM)	24.00	7.21

Tab. 7 Summary of results obtained after treatment with the extracts/pure compounds (IL-1β stimulus). Values on NF-κB transcription, translocation and IL-8 secretion are expressed as IC_{50}.

5. DISCUSSION

Gastritis are very common inflammatory based diseases which are mostly caused by *H. pylori* infection [1]. This bacterium colonizes the gastric mucosa of over 80% of human population in developing countries [36] and at least 50% of the world's human population[3]. *H. pylori* has been classified as type I carcinogen by the World Health Organization [41]; it is able to influence early stages in gastric carcinogenesis and its eradication in infected individuals significantly decreases the risk of developing gastric adenocarcinoma [42]. *H. pylori* infection induce a strong immune response in the host [40], which is characterized by the release of several inflammatory cytokines in the gastric mucosa, such as IL-1β, IL-2, IL-6, IL-8 and TNFα[51-54]. Gastric epithelial cells stimulated by cytokines, such as TNFα and IL-1β, induce the expression of IL-8, a potent chemokine which promotes neutrophil infiltration [57, 58, 95]. IL-8 secretion is strictly associated with the activation of NF-κB pathway in gastric epithelial cells [95, 301]; increased levels of IL-8 have been reported to be associated to several inflammatory conditions, including inflammatory bowel disease, psoriasis, rheumatoid arthritis, septic shock and cystic fibrosis, and gastric inflammation [59]. Gastric epithelial cells are probably the main producers of IL-8 during gastric inflammation [55].

In recent years, the study of medicinal and edible plants able to treat or prevent the development of various chronic diseases is attracting more and more interest. It has been demonstrated that fruits and vegetables occurring in the human diet, including berries, may exert a variety of health benefits mainly due to their antioxidants content [134]. The anti-oxidant and anti-inflammatory activity of these fruits can be attributed to their polyphenols content [18, 135]. Several works in the literature suggested the importance of the consumption of products rich in polyphenols in relation to gastritis induced by *H. pylori* [170, 302-306]. Recent studies have established that the intake of berries has a positive effect on human health, and this ability has been ascribed to the high phenolic content [174]. Among polyphenols, in the last few years anthocyanins and, to a lesser extent, condensed tannins, received more attention, whereas the biological effects of ellagitannins have been poorly investigated. Plant rich in tannins have a traditional use for treating gastric ulcer, and tannins showed anti-bacterial activity against *H. pylori* [290, 291]. The chemical composition of tannins depend on the fruit source, sanguiin H-6 and lambertianin C representing the main compounds in blackberry and raspberry [141], while procyanidins and agrimoniin are the main compounds in strawberries [307]. Different studies have demonstrated that tannins are stable at the physiological conditions of the stomach: gastric pH, between 1.8 and 2.0, and digestive enzymes are not able to hydrolyse or metabolize this class of molecules, and they are not absorbed in this district [227, 271]. Metabolism of tannins takes place in the intestine where the physiological pH of the small intestine causes the hydrolysis of ellagitannins and the release of ellagic acid. The latter is then metabolized by the gut microflora to urolithins [308], while procyanidins could be absorbed intact in the intestinal

tract [272, 273] although other studies are necessary to confirm this observation. Therefore the biological activities of tannins at the gastric level are fully associated to the unmodified structures, and are not related to the metabolic transformation.

This study reports for the first time that ellagitannins from blackberries and raspberries are able to protect the stomach against the gastric lesions caused by ethanol, whereas only one *in vivo* study previously demonstrated that a strawberry extract was able to inhibit gastric damage in the same animal model [135].

In the present study, TE-black and TE-rasp reduced the formation of gastric ulcers by 88% and 75%, respectively, in ethanol-treated rats. The anti-ulcer effect of blackberry, measured as UI, was higher compared to raspberry. This difference could be explained by the ellagitannins content of the two extracts; TE-black contained a higher amount of ellagitannins (343 mg/100 g of fresh fruits) than TE-rasp (155 mg/100 g of fresh fruit). Extracts were able to protect the antioxidant capacity of gastric mucosa, measured by ORAC, and preserve the activity of constitutive antioxidant enzymes such as SOD and CAT. In addition, the reduction of the severity of the lesions in ellagitannins-treated rats was also associated to a concomitant reduction of the release of CINC-1, the rat homologue of human IL-8. Since the IL-8 expression and secretion in gastric epithelial cells are mainly regulated by redox-sensitive transcription of NF-κB [300], it was proposed that NF-κB could be also modulated by anti-oxidant effect of tannins. To verify this theory *in vitro* experiments were performed on gastric epithelial cells (AGS). The anti-oxidant activity of TE-black and TE-rasp extracts was assessed in cells stimulated with H_2O_2 and ethanol, measuring IL-8 secretion, to mimic the condition used in the animal model. TE-black and TE-rasp inhibited IL-8 secretion induced by pro-oxidant stimuli confirming the supposed mechanism.

Since tannins have demonstrated to possess anti-inflammatory activities [201-203, 279, 283, 309], extracts enriched in tannins from blackberries, raspberries and strawberries were assayed evaluating anti-inflammatory activity *in vitro* at gastric level. The activity was tested in AGS cells exposed to TNFα and IL-1β since these cytokines are closely related to *H.pylori* infection. The results showed that TEs interfere with the metabolic cascade deriving from NF-κB activation. All the extracts inhibited IL-8 release from gastric epithelial cells induced by pro-inflammatory stimuli in a concentration dependent manner. In all cases the inhibitory effect of TE was higher when TNFα was used as stimulus. An explanation may reside in the role of ROS in the NF-κB activation induced by TNFα, thus suggesting also an involvement of the antioxidant mechanism of extracts [78, 310]. The signaling pathways of TNFα and IL-1β for the activation of NF-κB are different [311], and tannins could interact at different steps modulating different signaling cascades. *Rubus* extracts required more than double concentrations to produce the same inhibition on TNFα -induced IL-8

release exerted by *Fragaria* extracts; however IC_{50} were below 1 μg/mL for all the extracts (TE-black 0.69 μg/mL, TE-rasp 0.77 μg/mL, TE-straw 0.25 μg/mL and TE-wild 0.29 μg/mL). TEs were also able to interfere with NF-κB pathway, by inhibiting the NF-κB driven transcription and the nuclear translocation of this transcription factor. When TNFα was used as stimulus TEs inhibited NF-κB driven transcription with IC_{50} below 1 μg/mL, while at 2 μg/mL all the extract inhibited the translocation of this factor by more than 50%. Since NF-κB translocation is an upstream event compared to NF-κB driven transcription, the inhibitory effect of extracts could be explained mostly by reduced translocation.

The analysis of anti-inflammatory activity was also performed to the main individual compounds occurring in TEs. The main ellagitannins, sanguiin H-6, lambertianin C and agrimoniin, in addition to their hydrolysis product, ellagic acid, were tested on the previous inflammation parameters. All these compounds showed a strong inhibition of NF-κB activation (i.e. nuclear translocation and transcription), and IL-8 secretion thus confirming that these compounds contribute to the overall effect of the extracts. Individual procyanidins identified in the strawberry extract by UPLC-PDA-MS were present at low amount thus making impossible to associate biological activity of the extract to one of them. However, quantitative analysis of the extract is still under investigation.

To clarify the contribution of NF-κB on IL-8 secretion TE-straw, deriving from the most widely consumed strawberry fruit, was also assayed on IL-8 promoter activity. IL-8 promoter is characterized by having one site of NF-κB binding. TE-straw inhibited the activity of this promoter, induced by TNFα, with an IC_{50} of 0.17 μg/mL, comparable to that obtained on IL-8 secretion (0.25 μg/mL), thus indicating that the mechanism of IL-8 secretion is mainly regulated at transcriptional level. The role of NF-κB was also investigated by using cells transfected with the promoter of IL-8 mutate in the κB binding site. In this case TNFα was not able to induce a significant activity of the promoter and the use of TE-straw did not cause significant variations. Also agrimoniin, the main ellagitannin identified in the extract, did not influence the activity of the mutated promoter. These results confirmed that inhibition of the NF-κB pathway is the mechanism of action underlying inhibition of IL-8 secretion by the extracts under study.

Despite tannins are stable at the physiological conditions of the stomach, there were no studies related to the stability of tannins from *Rubus* and *Fragaria* spp at gastric level; for this reason the contribution of gastric digestion on the anti-inflammatory activity of extracts was also evaluated. The previous *in vivo* study on TE-black and TE-rasp already showed the anti-inflammatory effect of digested extracts: in fact, TE-black and TE-rasp were administered orally by *gavage*. For this reason the effect of an *in vitro* digestion was evaluated on TE-straw, the most widely consumed fruit containing tannins[307]. *In vitro* digestion halved the anti-inflammatory activity of TE-straw,

however the effect on NF-κB transcription, translocation, IL-8 secretion and IL-8 promoter activity remained significant at low concentrations. Digestion performed on TE-straw probably influenced concentrations of other class of compounds with biological activities present in the extract. Mucus and food *in vivo* could also affect the achievement of epithelial cells from the active molecules, but in pathological conditions epithelial cells could also be exposed directly to the gastric content.

This is the first study showing the anti-inflammatory activity of agrimoniin, which recent works have decribed as the main ellagitannin consumed in western diet, whose concentration could potentially reach high level in the stomach [307].

In conclusion, the anti-inflammatory activity exerted by tannins of blackberry, raspberry and strawberry occurs at extremely low concentrations, in many cases even less than 1 μg / mL, values that are considered easy to reach at gastric level as a result of the ingestion of a normal serving portion of berries (about 50 grams), considering a relative quantity of tannins: 100 mg for blackberry and raspberry [141] and 80 mg for strawberry [155, 178]. Considering a volume of human gastric juice of 20 mL, the consumption of 50 g of fresh berries by an human adult allows to reach a concentration of tannins in the stomach approximately around 2.6 mg/mL, which is 10^3 higher than the concentration required to inhibit NF-κB nuclear translocation and activation of gene transcription. Despite the effect of extracts was greater when pro-inflammatory was used, an antioxidant activity is also involved, since it occurred after pro-oxidant stimula.

Ellagitannins from *Rubus* berries efficiently protect against the onset of gastric ulcer in a rat animal model. TEs act through the inhibition of the NF-κB signaling cascade in response to pro-inflammatory or antioxidant agents. The effect of these fruits is not attributable only to anthocyanosides, whose effect on gastric inflammation has already been demonstrated for strawberry, but also to tannins. The effect *in vivo* might be higher consuming berries, which contains both classes of molecules. For these reasons, tannins deserve more attention; further studies could evaluate the effects of these extracts on inflammation directly induced by the presence of *H. pylori*, considering also the anti-bacterial properties of this class of compounds[169-171]. Tannins could also act in the intestine, but there are no *in vivo* studies to support this hypothesis, however, urolithins deriving from berries could be active in this district. Ellagic acid is an active molecule that is also present in the extracts, thus suggesting the possibility to have anti-inflammatory activity in the first part of the gut.

The outcome of this research will allow to draw the attention of the clinical/dietology community towards the benefits of fruits of *Rubus* and *Fragaria* spp. as integration in dietary regimens designed for inflammatory gastrointestinal diseases. Moreover, tannins could be used in association with drug therapy to treat gastric inflammatory diseases.

6. BIBLIOGRAPHY

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