THE ACTIVITY OF GARLIC EXTRACTS TO THE EPITHELIAL DAMAGE CAUSED BY SODIUM TAUROCHOLATE IN A CELL CULTURE MODEL OF ULCER DISEASE

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Co-mentor of Doctoral dissertation: Prof. Catherine H. Wu, Ph.D.

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Preface:

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Lucija Kuna

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List of abbreviations used in the text :

ABCG2	ATP-binding cassette, sub-family G, member 2
AGS	Adenocarcinoma epithelial gastric cell line
ATP	Adenosine triphosphate
BSA	Bovine serum albumin
CGRP	Somatostatin-associated calcitonin peptides
CH	Cholesterol
CNS	Central nervous system
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
CYP1A2	Cytochrome P450 1A2
CYP2C9	Cytochrome P450 2C9
CYP2D6	Cytochrome P450 2D6
DAPI	4′,6-diamidino-2-phenylindole
DAS	Diallyl disulfide
FBS	Fetal bovine serum
GE	Garlic extracts
GI	Gastrointestinal
GSH	Glutathione
GSSG	Glutathione disulfide
GPx	Glutathione peroxidase
H2O2	Hydrogen peroxide
H2RAs	H2 receptor antagonists
H.Pylori	Helicobacter pylori
IFN- γ	Interferon gamma
IL-8	Interleukin 8
IL-17	Interleukin 17
IL1β	Interleukin 1β
LPZ	Lansoprazole
LR	Lipid rafts
MIP-1	Macrophage Inflammatory Protein 2

MTT	3-(4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H- tetrazolium bromide
NaT	Sodium taurocholate
NFκB	Nuclear factor kappa B
NFKB2	Nuclear factor kappa B subunit 2
NSAID	Non-steroidal anti-inflammatory drugs
PG	Prostaglandin
PGE2	Prostaglandin E2
PL	Phospholipids
PPI	Proton pump inhibitors
PUD	Peptic ulcer disease
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
ROS	Reactive oxygen species
SAC	S-allyl-L-cysteine
SNP	Single nucleotide polymorphism
SOD	Superoxide dismutases
TNF-α	Tumor necrosis factor alpha
TRX1	Thioredoxin

1. INTRODUCTION

1.1. Peptic ulcer disease

Peptic ulcer disease (PUD) is a disease of gastrointestinal (GI) tract that appears as a result of mucosal damage that extends to the muscularis propria layer. The effect of two aggressive factors, gastric acid or pepsin causes PUD. It most commonly appears in the stomach and post-pyloric part of duodenum, but can also be located in any part of GI tract that is exposed to gastric acid or pepsin action (1). In the past, it was postulated that excess gastric acid secretion in combination with dietary habits are the cause of the most cases of PUD, but the discovery of *Helicobacter pylori* (*H. pylori*) infection and the frequent use of nonsteroidal anti-inflammatory drugs (NSAIDs) in the 20th century have changed this point of view (2).

1.1.1. Epidemiology

The lifetime prevalence of PUD is found in 5-10% of the world's population, with a mortality rate of 4.08 million per year (incidence 0.1–0.3% per year) (1–3). However, literature data suggest significant reduction in the incidence, rates of hospital admissions and mortality associated with PUD in recent years (1, 3). The reason behind it is thought to be improved hygiene and advent of new treatment options which resulted in decreased number of *H. pylori* infections (4).

Mortality caused by PUD achieved it's highest rate in generations born in the late 19th century, and a significant reduction was recorded in those born in the 20th century. Even though the observed reduction involves different types of ulcers, such as idiopathic and ulcers associated with *H. pylori* infection or NSAID use (2).

The etiology of ulcers is not fully elucidated, but it is generally accepted that gastric ulcers occur due to impaired homeostasis of gastroprotective factors such as mucosal-bicarbonate barrier, prostaglandin secretion and aggressive factors such as gastric acid, pepsin, *H. pylori* infection and bile salts (5). Traditionally, mucosal damage in patients with PUD is considered to be a result of excess gastric acid secretion in combination with dietary habit or

stress. Risk factors for developing peptic ulcer include *H. pylori* infection, alcohol, cigarette smoking and NSAIDs usage (6). Consequently, proinflammatory cytokines Interleukin 17 (IL-17), Interleukin 1β (IL-1β), Tumor necrosis factor alpha (TNF-α), Interferon gamma (IFN-γ) and chemokines Macrophage Inflammatory Protein 2 (MIP-2) and Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES) are released at the site of inflammation and ulcerative process exacerbation (7).

Peptic ulcer is associated with functional polymorphisms in various cytokine genes, such as polymorphisms of Interleukin 1 beta (IL-1β). It affects the production of mucosal Interleukin-1 (IL-1), resulting in gastroduodenal diseases associated with *H. pylori* (8). On the other hand, it is well known that the risk of complications of PUD is increased twice in users of aspirin and four times in users of nonsteroidal drugs (9).

Co-administration of NSAIDs or Aspirin with anticoagulants, corticosteroids, and selective serotonin reuptake inhibitors increases the risk of upper gastrointestinal bleeding (10). However, even though most people using NSAIDs have co-infection with *H. pylori*, their combinatorial effect in the pathogenesis of PUD remains unknown.

Recently, some meta-analyses have shown that NSAIDs or aspirin usage and *H. pylori* infection independently contribute to development of PUD (11). PUD is considered to be idiopathic in about 20% of cases and it can not be linked to *H. pylori* infection and aspirin or NSAID use (12). Idiopatic ulcer is thought to develop due to impairment of mucosal integrity, but the underlying mechanism is not clear (6).

1.1.2. Pathogenesis

Infection by *H. pylori* has been shown to be one of major causes of PUD and nearly 50% of world's population is infected by this pathogen (13). The prevalence of *H. pylori* infection is more prevalent in more developed countries of Africa and also countries of Central America, Central Asia and Eastern Europe (14). This bacteria is usually acquired in early stages of life if the child is raised in poor sanitary conditions. *H. pylori* causes epithelial damage in gastric mucosa, most commonly in the antrum leading to inflammatory response involving neutrophils, lymphocytes, plasma cells and macrophages. The mechanism by which *H. pylori* causes the development of specific types of lesions in the gastroduodenal mucosa has not been

fully elucidated. However, it is known that *H. pylori* infection may result in hypochlorohydria or hyperchlorohydria, thus determining the underlying mechanism of PUD. Cytokines play a significant role in *H. pylori* infection by inhibiting parietal cell secretion. In addition, *H. pylori* can directly affect the H^+/K^+ ATPase subunit and inhibit gastrin production (15).

The development of gastric ulcers has been associated in most cases with hyposecretion, but in 10–15% of patients with *H. pylori* infection it has led to gastric secretion caused by hypergastrinemia and decreased level of somatostatin in the antrum. As a consequence, secretion of histamine is stimulated, followed by secretion of pepsin or gastric acid from parietal cells (16). In addition, it was shown that eradication of *H. pylori* leads to increased somatostatin mRNA expression and decreased gastrin mRNA expression (17).

In some patients, gastric ulcer is associated with mucosal atrophy. Inhibition of constitutively active Cyclooxygenase-1 (COX-1), an enzyme responsible for prostaglandin synthesis, is a major mechanism of gastroduodenal mucosal damage by NSAIDs. Consequent imbalance in prostanglandine levels leads to vasoconstriction in gastric mucosa, decreased secretion of bicarbonate and mucus and reduced cell proliferation. The concomitant usage of exogenous prostaglandins and Cyclooxygenase-2 (COX-2) – selective NSAIDs decreases mucosal damage and the risk of ulcers (18).

Specific biochemical properties of NSAID cause distinction in their adverse effects (19). NSAIDs damage mucus phospholipids and lead to the impairment of mitochondrial oxidative phosphorylation, resulting in damage of gastric mucosa. NSAIDs as acids remain unionized in acidic gastric fluid (pH 2) and as such easily diffuse through lipid membranes into epithelial cells (pH 7.4) where they ionize and release a proton. In this form, NSAIDs become trapped in epithelial cells and cannot cross the lipid membrane leading to oxidative phosphorylation separation, increased cell permeability and decreased mitochondrial energy production. Geriatric population, patients with history of GI hemorrhage and ones who use anticoagulants or steroids in combination with NSAIDs are at the highest risk of developing NSAID-induced ulcers (4).

1.2. Medical therapy for peptic ulcer disease

1.2.1. Proton pump inhibitors

Proton pump inhibitors (PPIs) such as lansoprazole, omeprazole, pantoprazole, rabeprazole and esomeprazole are one of the most commonly prescribed medications worldwide. Until recently they were considered to have a good safety profile. However, it has been observed that chronic use of PPIs is associated with a variety of side effects, such as a risk of cardiovascular, cerebrovascular events and an increased risk of bone fracture. It is well known that omeprazole interacts with numeorus drugs, and therefore clinicians must be careful when prescribing drugs concomitantly. Many of these findings resulted from observational studies, hence more investigation is needed to elucidate the mechanisms behind the findings (20). The most common adverse effects of PPIs are: headaches, diarrhea, constipation and abdominal discomfort. However, it was noticed that prolonged PPI use increases the risk of osteoporosis due to its interference with absorption of calcium and magnesium ions. Consequently, long term PPI use is linked to an increased risk of bone fractures, which particularly in the elderly can have serious complications (21). By suppressing acid production and creating more basic environment, PPIs inactivate pepsin, block peptide degradation and absorption therefore exposing them to small intestine milieu where they can cause allergic reaction (4, 22). Some cases of eosinophilic esophagitis are also attributed to PPI use (23).

Additionally, PPIs may lead to hepcidin upregulation and consequent ferroportin inhibition in duodenum, resulting in reduced iron absorption (20). Also, PPIs also may cause gynecomastia (24).

One of the main disadvantages of long-term use of PPI is the increased risk of bone fractures. Hip fracture is characteristic in the elderly population, and it is most concerning because it significantly contributes to overall mortality worldwide. In addition, PPIs reduce bone mineral density causing hypochlorhydria and consequently reduced absorption of vitamin B_{12}, Mg^{2+} and Ca^{2+}. PPIs also cause hypergastrinemia, which leads to increased osteoclast activity and hyperparathyroidism (25).

1.2.2. NSAID-Associated peptic ulcer disease and the use of PPIs

Nowadays, there are a several strategies to prevent NSAIDs and aspirin - associated PUD and its complications. For instance, concomitant use of NSAIDs with gastroprotecitve agents such as PPI, misoprostol or antihistamines. Selective COX-2 inhibitors can also be good alternatives in some cases. Due to their irreversible binding to H^+/K^+ ATPase and its inhibition, which is the common final step in gastric acid secretion, PPIs are the most effective protective agent and very popular in practice (26).

On the other hand, standard doses of antihistamines are not as effective in prevention of PUD (27). Misoprostol found no significant place in prevention of PUD due to its side effects and its use during pregnancy is forbidden (4).

1.2.3. H2 receptor antagonists

H2 receptor blockers or H2 receptor antagonists (H2RAs) such as ranitidine, cimetidine, nizatidine and famotidine play a significant role in the treatment of gastrointestinal diseases (28). However, proton pump inhibitors (PPIs) are a valuable substitute for H2RA (29). H2RAs are used to supress gastric acid secretion in disorders such as:gastric or duodenal ulcers, heartburn or indigestion, gastric hypersecretion, they are also used for stress ulcer prophylaxis, gastritis, gastrointestinal hemorrhage esophagitis and urticaria (28).

The effectiveness of H2RAs strongly depends on the intensity of the dosing regimen, the duration of therapy and severity of gastric disease. They can affect the suppression of gastric acid release by approximately 70%, which makes them less potent than PPIs. Also, H2RA inhibit basal gastric secretion, so their effectiveness has a key role in suppressing nocturnal acid secretion. Nocturnal acidity levels affect the healing of duodenal ulcers, so evening dosing of H2RAs antagonists is an appropriate therapy in most cases (28). H2Ras are well tolerated, with side effects of fatigue, diarrhea, muscle aches, and drowsiness, headache and constipation . Less common side effects involve those with a strong effect on the central nervous system (CNS) such as: headache, confusion, slurred speech, and hallucinations. Elderly patients are more prone to such adverse events, especially after intravenous administration. Cimetidine is an exception among H2Ras; due to its high affinity for liver oxidase it may interact with other drugs. It also has some serious adverse events. Therefore, its usage has been significantly

reduced (30). Cimetidine is known as a competitive inhibitor of the hepatic oxidase enzyme, which leads to a clinically important interactions with drugs metabolized by the same enzymes. By inhibiting CYP, cimetidine may cause increased plasma concentrations of propranolol, β-adrenoceptors, phenobarbital, warfarin, theophylline, various benzodiazepines, estradiol, tricyclic antidepressants, phenytoin, calcium channel blockers and lidocaine. Cimetidine may be the cause of gynecomastia and impotence in men and galactorrhea in women. In addition, long-term usage of higher doses reduces testosterone binding to the androgen receptor and inhibt estradiol hydroxylation by CYP (e.g., CYP1A2, CYP2C9, and CYP2D6) (28).

1.3. Herbal treatment for peptic ulcer disease

Phytotherapy is the study of the use of herbal extracts for the purpose of healing and health promotion and as such is present from the ancient history. Today, we witness a growing interest in alternative therapies, including phytotherapy (31, 32). A part of that can be explained by tendency to avoid adverse effects of conventional drugs. Therefore medicinal herbs represent a new avenue in medical treatments. In the context of treating PUD, certain herbal extracts have showed some exciting results and can serve as complimentary therapy (33).

Some of the adverse effects of drugs commonly used for the treatment of PUD such as anticholinergics, antacids, antibiotics, bismuth and antihistamines are impotence, arrhythmias, bone marrow suppression, hypersensitivity, etc. (34, 35). Therefore, investigations into new pharmacologically active agents by screening various plant extracts have led to discovery of safe and effective gastroprotective agents. In particular, herb extracts with antioxidative effect are considered to be a significant reservoir of options for the tratment of PUD (34).

Medicinal plants achieve their healing properties by producing different renewable secondary metabolites, called phytochemical ingredients. Those phytochemicals were shown to serve as a mechanism of protection against various pathogens (36).

The rise in the number of resistant pathogens has had an impact on pharmaceutical companies into changing their strategy in the development of antibiotics and to exploring the options of developing new drugs from medicinal herbs (37). Nevertheless, synthetic antibiotics remain dominant to date.

1.3.1. Garlic (*Allium sativum*)

Allium sativum belongs to the *liliaceae* family, and is commonly known as "garlic". The chemical ingredients of this plant are found in the acrid volatile oil. Phytochemicals that cause a strong garlic flavor are formed when plant cells are damaged by crushing, chopping or chewing, causing the breakdown of various sulfur - containing compounds stored in the cell fluid. Consequently, the released compounds are responsible for the strong hot taste and smell of garlic (38).

Health benefit of garlic has been well documented through the history. It is considered to be a significant source of phytochemicals with antioxidant activity. These include organosulfur compounds of garlic (involving S-allyl-Lcysteine (SAC) sulfoxides and δ-glutamyl-S-allyl-L-cysteins), flavonoids and phenolic compounds. The powerful antioxidant action of aqueous garlic extract is based on scavenging reactive oxygen species (ROS) ability cellular antioxidant enzymes such as superoxide dismutase (SOD), glutathione peroxidase and catalase (39). Aqueous garlic extract (GE) can inhibit lipid peroxidation and activation of oxidative transcription factors. These mechanisms may explain the use of garlic in the treatment of PUD (40).

Given that raw garlic is easily convertable into bio-inactive form, various types of GE with different composition of bioactive ingredients have been identified. Indeed, their biological efficacy has been confirmed in a number of studies. Nowadays, commercially available garlic preprations such as essential oil, powder or GE have been shown to have beneficial and health promoting effect (41). The main effects of GE are reduction of lipoprotein oxidation, lowering blood glucose and antioxidant effect by scavenging ROS and induction antioxidant enzymes. GE were also shown to have antitumor activity by promoting programmed cell death and inducing cell cycle arrest (42). With regards to the beneficial effects of GE that may contribute to PUD prevention and ulcer healing, it was observed that GE reduces gastric mucosa inflammation caused by *H. pylori* infection (43). Allyl-methyl plus methyl-allyl thiosulfinate and allicin from acetonic GE have bacteriostatic effect on *H. pylor*i (44, 45). In addition, there is some evidence of gastric adenocarcinoma risk reduction (46).

1.4. AGS cell line

Human gastric adenocarcinoma epithelial cell line (AGS) was derived from a tissue sample of human gastric adenocarcinoma (Barranco et al.,1983). This is well - differentiated cell line with all the characteristics of gastric epithelial cells such as the ability of secreting mucus and true gastric epithelial cell morphology. Despite the fact that AGS cells belong to the human adenocarcinoma cell line, they are well differentiated and are therefore used in gastroprotection studies (47, 48). Unlike the primary cultures, AGS can be successfully maintained long term by subculturing. For example, AGS has been used for investigation of the role of gastrin receptors in cell growth stimulation (49) and in investigation of mechanisms by which *H. pylori* invades gastric epithelium (50, 51). In humans, gastric epithelial cells are exposed to the oxidative ambience caused by the intake of reactive species formed and the oxidative reactions during the process of food digestion (52). AGS are also exposed to food-derived polyphenols that have not been previously metabolized by the liver (53). Because of these reasons, these cells can be use as a unique model in which different cytoprotective mechanisms mediated by polyphenols could be active (47, 54).

The investigations of gastroprotective effect of various compounds, which are oftentimes performed on animal models, have included new cell culture technologies, that reduced the use of laboratory animals. For instance, Zheng et al. used the human AGS cell line to explore the cell - protective effects of antiulcer ingredients. Their results demonstrated significant correlation with previous studies on primary rat gastric cell line that was used to determine the effects of antiulcer and cytotoxic agents on the stomach mucosa. However, the primary rat gastric cell line can not be sustained or maintained for a long period because of the potential for overgrowth of contaminating cells, such as fibroblasts. Moreover, the presence of contaminating cells has potential to disrupt the investigation which aims at observing biochemical changes significant for mucus-secreting epithelial cells (55). Finally, AGS cell line models allow the evaluation of specific agents on the healing of a pre-existent gastric epithlial damage. The proliferative capacity of gastric epithelial cells is important for the renewal and repair of the gastric epithelium, before and after the injury (56).

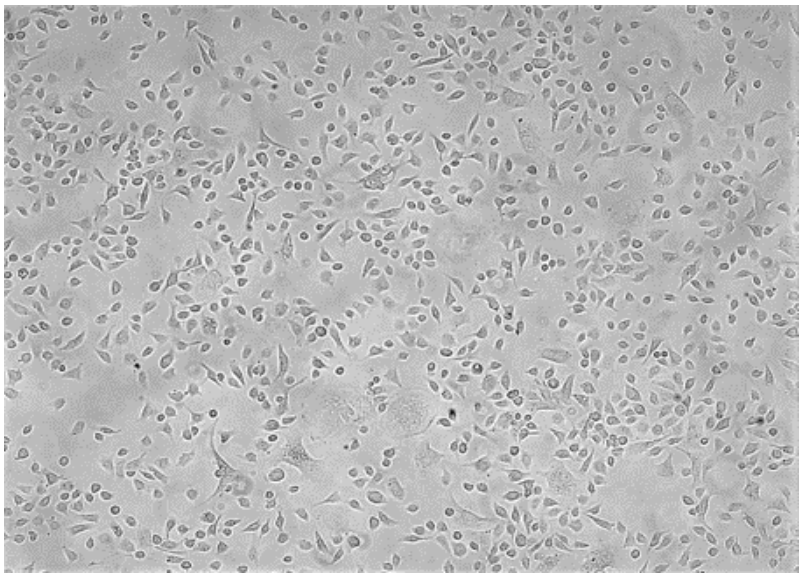

Figure 1.1. Dark field micrographs of live-AGS cell line. The figure was made by the author.

1.5. Sodium taurocholate

Sodium taurocholate (NaT) is the bile salt composed of taurine and cholic acid. Bile has detergent-like toxicity owing to the heterogeneous mixture of bile salts, cholesterol bilirubin enzymes and phospholipids. NaT functions as a antimicrobial agent, solubilising membrane lipids, including the dissociation of membrane proteins (57). Bile acid reflux in the stomach has been implicated in the development of PUD and gastritis, and Na^+ / taurocholate instilled into the stomach cause serious biochemical mucosal damage (58). The gastric mucosa is constantly exposed to high concentrations of acid and the proteolytic enzyme, pepsin. Therefore, reflux of bile acids, ethanol consumption and intake of NSAIDs may lead to serious damage (59). In addition, it has been shown that acidified NaT introduced into the stomach of rats causes marked H^+ back diffusion and extensive gastric mucosal lesions (56). Therefore, the use of AGS a cell culture model of PUD can definitively prove that using the bile salts, such as NaT can lead to development of gastric lesions. Also, the use of AGS, the cell model of PUD can be used to investigate different mechanisms involved in the development of PUD.

1.6. Markers of cell damage and oxidative stress

1.6.1. Antioxidant enzymes

Improving the antioxidant capacity of gastric epithelial cells may provide stronger protection against oxidative damage and consequent ulceration (60). Studies of the expression of enzymes such as SOD can elucidate their role in assessing the effects of NaT and antioxidants on gastric epithelial cells in PUD.

SODs are crucial enzymatic antioxidants that rapidly catalyze the dismutation of superoxide, and promote its removal. Also, SOD catalytically convert superoxide radicals in hydrogen peroxide and thus protect cells from free radical damage (60). It has been shown that there is decreased SOD expression in cells loaded with free radicals, while expression was increased in those treated with antioxidants. Therefore, SOD expression is used as an indicator of the effectiveness of the antioxidant effect (61).

The Thioredoxin (TRX) system is one of the major antioxidant systems in mammalian cells, and Thioredoxin (TRX-1) as small redox-active protein has significant redox-regulating function and antioxidant activity. Also, TRX-1 participates in regulation of several cell functions, such as, proliferation, metabolism, cytoprotection, differentiation and apoptosis. In PUD, TRX-1 has important role in the host defence mechanism against the ROS-related gastric mucosal injury including *H. pylori* infection. These proteins function inhibit or activate apoptotic signalling molecules such as apoptosis signal-regulating kinase 1 and Ras or transcription factors like the Nuclear Factor Pathway (NF-κB). However, it remains unclear, whether Trx-1 is hampers the mechanism of NaT-induced gastric mucosal injury (62) (63).

1.6.2. Cell Proliferation Assay

Proliferating cell nuclear antigen (PCNA) is 36,000 molecular weight multifunctional protein with singificant role essential for replication and repair of DNA. The fact that synthesis of PCNA begins during the late phase of S and G2 phases of the cell cycle, makes it a suitable assay for cell proliferation (64), and for determination of mitotic potential of the cells with GE by histochemical staining against PCNA.

1.6.3. Intracellular GSH level

Intracellular level of reduced glutathione (GSH) is an important protective factor of gastric mucosa from ethanol-induced damage both *in vivo* and *in vitro*, and is the major thiol antioxidant of the cell. The oxidized form of glutathione is glutathione disulfide (GSSG). Glutathione is the major solubile antioxidant and is present in high concentrations in the cytosol, nucleus and mitochondria. GSH in the nucleus maintains the reduction of some proteins required for DNA repair and expression. The main protective properties of GSH against oxidative stress are based to the action of GSH as a co-factor of certain enzymes that stop oxidative stress (e.g. glutathione peroxidase GPx) and its participation in the transport of amino acids across the membrane; it also purifies hydroxyl radicals, detoxifies hydrogen peroxide and lipid peroxides *via* the catalytic reaction of glutathione peroxidase.Therefore, GSH concentration can be used as a reliable marker of oxidative stress in cells (65).

1.6.4. Cyclooxygenase

Another key gastroprotective mechanism involves prostaglandins (PG) biosynthesized from arachidonic acid by cyclooxygenase (COX), with prostaglandins E1 and E2. PGs play a role in the synthesis of mucus and bicarbonate and in stimulating gastric defense factors such as blood flow in the mucosa, maintenance of the integrity of the mucosa, proliferation of epithelial cells and suppression of inflammatory cells infiltration. PG have been shown to protect the gastrointestinal mucosa from necrotizing agents, stress, and NSAIDs (66).

1.6.5. Genetic factors

The changes in the ATP-binding cassette, sub-family G, member 2 (ABCG2) gene could be one of the possible genetic factors adding to the development of PUD. It encodes protein named ABCG2 which is a half-transporter belonging to the ABC transporters superfamily. ABCG2 protein utilises energy from adenosine triphosphate (ATP) hydrolysis to transport substrates and has a protective role in elimination of xenobiotics from cells into the extracellular space. ABCG2 has been found in the apical membrane of cells in the brain, ovaries, prostate, testes, placenta and gastrointestinal tract, which confirms the assumption of its protective role by aggravating the passage of xenobiotics through the cell membranes. It was observed that the more intense the infection of PUD, the higher the level of ABCG2 expression (67). It is not

known, whether GE impact ABCG2 expression in AGS cells, the cell culture model of PUD, therefore this point is worth investigating in AGS cells.

Nuclear Factor Kappa B Subunit 2 (NFKB2) is a protein coding gene for the transcription factor complex , NF-κB. It is found in various cell types and has a central role as endpoint of numerous signal transduction initiated by stimuli associated to many biological processes such as; tumorigenesis, cell growth, inflammation, and apoptosis. Five members have been identified in mammals: NF-κB1/p105, NF-κB2/p100, c-Rel, RelA, and RelB. In a normal cellular pathway, NF-κB activation is regulated by its core element known as IκB kinase (IKK), that phosphorylates IκBα and IκBβ at the sites that mediate their ubiquitination and degradation. The relative expression level of NFKB2 is decreased in gastric cancer. In contrast, NFKB2 expression is increased in PUD which might suggest inhibition of , NF-κB pathway during carcinogenesis (68, 69).

1.6.6. Cell Cytoskeleton protiens

The cell cytoskeleton is involving in numeros biological processes, such as motility, adhesion and intracellular transport. Changes in cytoskeletal components consisting of actin filaments, intermediates filaments and microtubules lead to the development of several diseases, such as cancer, liver and ulcus disease. Consequently, the changes in the structure of the cytoskeleton may cause anomalies in the cells and consequently lead to invasiveness (70). Cell cytoskeleton protein F-actin has a significant role in cell motility and force generation (71, 72) Membrane proteins main function involves numerous signaling proteins, channel proteins or cytokine receptors, hence changes in the F-actin redistribution are an indirect measure of a cells ability to act on external stimuli.

In our model of PUD, this refers to the ability of AGS cells following external stimulation by NaT, LPZ, and GE. Furthermore, since the actin fibers are anchored internally to the cell membrane, a significant and permanent disruption of the membrane structure will lead to a disruption in the structure, distribution and amount of F-actin. Moreover, to the best of our knowledge, the effect of NaT on cell cytoskeleton have not been thoroughly investigated yet.

1.7. Cell membrane phospholipids and cholesterol

Plasma membrane, an outer edge of the each mammalian cell, is composed of amphipathic lipids and proteins with the primary role in protection from the external environment. Lipids are the main components of the mammalian cell membranes with cholesterol being the molecule of highest importance for cell membrane structure. Cholesterol (CH) accounts for approximately 30% of all lipids within the cell membrane (73, 74). This essential molecule is composed of the four fused hydrocarbon rings with small hydroxyl group and an eight-carbon branched aliphatic tail. CH is synthesized in the series of enzymatic processes within endoplasmatic reticulum and then transferred through the Golgi to the cell membrane (75). Almost 90% of free CH, not esterified CH, resides within plasma membrane. The structure of CH molecule determines the rigidity of the cell membrane thus different density of CH within membrane results in different stages of rigidity. This uneven distribution of CH within membranes in various cells results in CH-rich domains termed lipid rafts (LR) (76). LR influences several biological processes such as transmembrane receptor signaling and virus entrance. Beside its key role in membrane maintenance, CH is crucial in synthesis of hormones, bile acids and is a precursor for vitamin D (77). It is well established that the cell membrane in mammalians cells is composed of two monolayers, differing in lipid composition. The outer layer has abundant phosphatidylcholine and sphingomyelin while cytoplasmic inner layer usually contains more phosphatidylethanolamine and phosphatidylserine (78). This four major phospholipids (PH) account for more than half of the lipids within mammalian plasma. The PH consists of hydrophilic head and hydrophobic tail. They exhibit multiple roles such as forming the permeability barrier of the cell membrane, providing precursors for signaling processes and participating in signal transduction (76, 79).

As a novel approach to exam the mechanism of medicinal effect of garlic and its compounds is to use AGS cells as the model of PUD. Our aim is to investigate at the molecular and immunohistochemical level on how GE reverses changes in the cell membrane composition.

2. HYPOTHESIS

Based on previous studies on the effect of NaT on cell cultures and the effect of garlic extracts on oxidative damage, we will test the the following hypotheses using AGS, a cell culture model of PUD.

1. NaT added in a cell culture model of ulcer disease causes oxidative stress and increasing its concentration reduces cell viability.
2. Pretreatment with GE attenuates the gastric lession through the induction of mucus synthesis and the expression of SOD, TRX1, ABCG2 and NFKB2 in a cell culture model of ulcer disease.
3. Pretreatment with GE increase the production of PGE2 and suppress depletion of cellular reduced GSH in a cell culture model of ulcer disease.
4. Pretreatment of AGS cells with GE reverses changes in the cell membrane composition and cytoskeletal protein levels induced by NaT exposure.

3. RESEARCH OBJECTIVES

Based on the hypothesis, the following specific objectives were set:

1. To establish cell culture model of PUD on AGS and measure the toxic effect of NaT.
2. To measure the effect of varying concentrations of GE and exposure required for survival in cultured AGS cells.
3. To determine mitotic potential of the cells before and after treatment with GE by PCNA staining.
4. To measure the amount of cellular reduced GSH in AGS cells before and after treatment with GE.
5. Perform immunoassay to compare the amount of PGE2 in cultured AGS cells before and after treatment with GE.
6. To determine the expression level of SOD, TRX1, ABCG2 and NFKB2 in cultured AGS cells before and after treatment with GE.
7. To investigate the correlation of gene expression with cell survival parameters and the amount of secreted PGE2 and GSH in the cultured AGS.
8. To characterize the effect of LPZ, GE and NaT on F-actin distribution in AGS cells.
9. To examine the morphological changes in cell membrane structure caused by the addition of NaT, LPZ and GE by using CL and PH specific stains.

4. MATERIALS AND METHODS

4.1. Study design

The study was structured as a randomized controlled trial.

4.2. Materials

Human epithelial gastric cell line AGS (ATCC CRL-1739) was used in all experimental procedures. The AGS cell line was derived from fragments of a tumor resected from a patient with no prior therapy. Cells were sub-cultivated in 10 cm dishes in Roswell Park Memorial Institute Medium (RPMI-1640), (Sigma-Aldrich Co, USA) containing 2 mM L-glutamine supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1% antibiotic/antimycotic solution (Thermo Fisher Scientific Inc., Waltham, MA, USA). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 (v/v) in air. For sodium taurocholate toxicity studies, cells were maintained in RPMI-1640 without FBS.

Sodium taurocholate was purchased from a commercial supplier in powder form (Sodium oxalate, Sigma-Aldrich, St. Louis, MO, USA). In order to get 100 mM solution 2.685 mg of sodium taurocholate was dissolved in 5 mL of phosphate saline buffer (PBS) (Sigma-Aldrich, Saint Louis, MO, SAD) which was then sterilized by filtration.

Garlic extracts was purchased from a commercial supplier in the form of an oil solution (Sigma-Aldrich, St. Louis, MI, USA). In its commercial form it is insoluble in water and thus insoluble in RPMI-1640 medium. Garlic oil stock was prepared in 6% BSA in a 1:6 garlic oil to Bovine Serum Albumin (BSA) ratio and solubilized by homogenizing with an ultrasonic homogeniser (Bandelin Sonoplus 2070) for 15 seconds.

Lansoprazole was purchased from a commercial supplier in the powder form (≥98% powder (TLC); Sigma Aldrich, St.Louis, MO, USA). In order to be used for application in cell cultures, a stock solution was prepared. Lansoprazole stock was prepared in 96% ethanol and

homogenised with ultrasonic homogeniser (Bandelin Sonoplus 2070) for 15 seconds. Working solution was prepared by diluting LPZ stock solution 1:9 with RPMI-1640.

4.3. Methods

4.3.1. Cell culture

AGS are mucus-secreting epithelial cells presenting numerous characteristics of well differentiated gastric cells, including morphology, microvilli, and mucus production and have been used as a model for testing of gastroprotective drug potentials (47). Cells were cultured in 10 cm. Culture dishes in Roswell Park Memorial Institute medium (RPMI-1640), (Sigma-Aldrich, St. Louis, MO, USA) containing 2 mM L-glutamine supplemented with 10% fetal bovine serum (FBS), (Thermo Fisher Scientific Inc., Waltham, MA, USA) and 1% antibiotic/antimycotic solution (Thermo Fisher Scientific Inc., Wal-tham, MA, USA). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2 (v/v) in air. Cells were passaged every 3 – 4 days to maintain 75% confluency.

The culturing protocol was following:

1. 9 ml of RPMI-1640 medium with FBS (growth medium) was transferred to a 15 ml Falcon tube.
2. The cryotube with frozen AGS cells was thawed by partially immersing it for 2 minutes in the water bath heated up to 37 °C.
3. The thawed cell suspension was transferred by sterile pipette into a RPMI-1640 medium with FBS in the Falcon tube prepared in step 1.
4. The cell suspension was centrifuged at room temperature (RT) at 130g for 5 minutes.
5. The supernatant was discarded, and cells are resuspended in 5 ml of RPMI-1640 medium with FBS.
6. Cells were counted using trypan blue exclusion and Neubauer Hemocytometer.
7. The final cell count/ml for culture was determined, and 10 ml of cell suspension was transferred to 10 cm^2 culture dish.
8. The next day, the RPMI- 1640 medium was replaced with the 10 ml of a fresh medium.
9. Cells were grown to 85% confluency.

10. After reaching the sufficiently confluency, the growth medium was removed, and cells were trypsinized with 5 ml of preheated trypsin solution for 5 minutes and lightly shaken until cells visibly detached from the culture dish.
11. RPMI-1640 growth medium (5 mL) was added to trypsin/cell suspension to stop the trypsin activity. The solution was transferred to a 15 ml Falcon tube and centrifuged at room temperature at 130 g for 5 minutes.
12. The supernatant was removed, and cells were resuspended in 10 ml of fresh RPMI-1640 growth medium.
13. Cells were counted using trypan blue exclusion and Neubauer Hemocytometer.
14. The final concentration of $4x10^5$ cells/ml was prepared, and 10 ml of cell suspension was transferred to each 100 mm culture dish.
15. Cells were grown to 90% confluency and then harvested for further experiments.
For purposes of maintaining viable cells every 3 - 4 days cell were trypsinised and cultured into new dish. This coincide with total of 75% confluency prior trypsinization.

4.3.2. Examination of sodium taurocholate toxicity in AGS cell line

To determine NaT- induced damage, AGS cells were sub-cultivated in RPMI 1640 cell culture medium supplemented with 10% fetal bovine serum (FBS), and 1% antibiotic/antimycotic solution. To establish NaT toxicity, cells were maintained in RPMI 1640 without FBS. The antiproliferative and cytotoxic effect was determined by a colorimetric MTT [3-(4, 5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay (Sigma Aldrich, St. Louis, MO, USA) according to the manufacturer's protocol.

MTT test procedure to determine the numbers of viable cells following NT treatment is described below:
1. Cells were sown in 96-well. At least 3 biological replicas per treatment concentration or time were used.
2. The growth medium was replaced with mediums containing NaT in varying concentrations NaT (2, 4, 8 and 10 mM), and incubated for for 30 minutes, 1, 4, 12 and 24 hours in triplicates, respectively.
4. 10 µl of MTT stock solution was pipetted into each well, resulting in 0.5 mg/ml of tetrazolium bromide concentration.

5. Plates were placed in an incubator for 4 hours to produce formazan crystals.

6. After incubation, 100 µl of MTT solvent was added into each well with MTT stock solution, and formazan crystals were dissolved by repeated pipetting 10 times.

7. 96-well plates were read on the iMark microplate reader (Bio-rad, Hercules, California, United States) at 450 nm.

8. The final result was calculated as $OD_{450\ nm}$ percentage value against the untreated group.

4.3.3. Measurement of garlic extracts activity to the epithelial damage caused by sodium taurocholate

4 mM NaT; 10 µM LPZ; 100, 150, 250 and 350 µg/ml concentrations of GE were used in the experiments.

Cells were plated at a density of $4x10^5$ cells/ml in 6-well plates and were grown for 24 hours. The cell subgroups for determination of gastroprotective effect of GE were designed as follows; group A, AGS cells grown in RPMI-1640 medium as a negative control; group B, AGS cells treated only with lansoprazole as a positive control; group C, AGS cells treated with NaT only; group D, AGS cells pre-treated with LPZ and exposed to Nat; groups E-H, cells pre-treated with increasing concentrations of GE and exposed to NaT. Cells in these groups were pre-treated with LPZ and GE for 24, 48 and 72 hours and subsequently exposed to NaT for 1 h. Subsequently, cells were trypsinized to determine cell viability using trypan blue exclusion and Neubauer Hemocytometer counting. Results were expressed as a percentage relative to negative controls of at least three independent experiments.

4.3.4. Measurement of GSH concentration

To determine the level of free radicals accumulation, the concentration of glutathione (GSH) was measured by ELISA. Cells were plated at a density of $4x10^5$ cells/mL in 6-well plates and were grown for 24 hours. The cell subgroups for determination of gastroprotective effect of GE were as follows; AGS cells grown in RPMI-1640 medium; AGS cells treated only with LPZ; AGS cells treated with 4 NaT only; cells pre-treated with LPZ and exposed to Nat groups E-H, cells pre-treated with increasing concentrations of GE and exposed to NaT. Cells

in these groups were pre-treated with LPZ and GE for 24, 48 and 72 hours and subsequently exposed to NaT for 1 h.

GSH concentration was determined using a commercially available kit (Glutathione Assay Kit, Sigma-Aldrich, Saint Louis, MO, USA). The cells were centrifuged 600 x g to obtain cell suspension at the bottom of the tube. The supernatant was discarded, 5% 5-sulfosalicylic acid was added to the cell pellet and vortexed. The suspended cells were frozen and thawed twice and then centrifuged at 10000 x g for 10 minutes. The supernatant was removed and used to measure GSH concentration. The reaction to measure the GSH concentration was placed in a 96-well polystyrene plate. 10 µl of sample and 150 µl of working solution (working solution was prepared according to the manufacturer's instructions using the solution included in the assay) were added to the well, incubated for 5 minutes and 50 µl of NADPH solution was added with a multichannel pipette.
The response was measured using an iMarkTM Microplate Absorbance Reader at 405 nm. Results were expressed as micromoles per milliliter.

4.3.5. Measurement of PGE2 concentration

To determine PGE2 concentration, the day after the AGS cells became confluent, eight subgroups were denoted as follows: untreated cells, cells treated with with LPZ only, cells treated with NaT only, cells pre-treated with LPZ and subsequently exposed to NaT, and cells pre-treated with four different concentrations of GE and subsequently exposed to NaT. On the second day, cells were exposed to the LPZ and GE in four different concentrations for 24, 48 and 72 hours. To induce oxidative stress in cells, NaT was added in appropriate wells according previously described experiments.

The PGE2 content was determined by an enzyme immunoassay kit (Elabscience, Houston, Texas, USA) as described by manufacturer. Briefly, after incubation, cells were washed with pre-cooled PBS and dissociated by trypsin. Cells were collected into the tubes and centrifuged for 5 minutes at 1000 x g. Cell pellets were washed 3 times with pre-cooled PBS. The freeze-thaw process was repeated for several times, and centrifuged for 10 minutes at 1500xg at 4°C. The cell fragments were removed, and supernatant was collected to carry out the assay. To measure the GSH levels cell supernatants were placed in a 96-well polystyrene plate. 50 µl of standard working solution (working solution was prepared according to the

manufacturer's instructions using the solution included in the assay) was added to the well, incubated for 45 minutes at 37°C. The solution was aspirated from each well, and 350 µl of wash buffer was added to each well (this step was repeated 3x). After that, 100 µl of HRP Conjugate working solution was added and incubated for 30 minutes at 37°C. The solution was aspirated from each well, and the wash process was repeated for five times. 90 µl of Substrate reagent was added to each well, covered with a plate sealer and incubated for 15 minutes at 37°C. In the last step 50 µl of Stop Solution was added to each well.

The response was measured using an iMarkTM Microplate Absorbance Reader at 450 nm. The values were calculated according to the manufacturer's instructions. Results were expressed in picograms per milliliter per well.

4.3.6. Total RNA isolation and RT-PCR analysis.

On the first day of experiment, cells were plated at a density of $4x10^5$ cells/ml of growth medium in 6-well plates. Five subgroups were denoted as follows: untreated cells, cells treated with LPZ only, cells treated with NaT only, cells pre-treated with LPZ and subsequently exposed to NaT, and cells pre-treated with the highest concentration of GE (350µl) and subsequently exposed to NaT. On the second day, cells in the appropriate wells were exposed to the LPZ and the highest concentration of GE for 24 hour. On the third day, to induce oxidative stress in cells NaT was added in appropriate wells according previously described experiments. The medium was removed from all wells, washed with PBS, the cells were trypsinized with 500 µl of trypsin which was then deactivated with 500 µl of medium. The cells thus obtained were stored in RNAlater solution (RNAlater® Sigma-Aldrich, Co. LLC.) at -20°C until total RNA was isolated.

Total RNA was isolated using a commercial kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Cells were added to 350 µl of high guanidine thiocyanate buffer and vortexed briefly to lyse and homogenize. Then 350 µl of 70% ethanol was added. Samples were transferred to a centrifuge column and centrifuged at 10,000 rpm for 15 seconds to bind RNA to the column membrane. The column was washed three times to remove any impurities from the membrane. After that, the centrifuge column was placed in a new centrifuge tube and 40 µl of distilled water was added to the column and centrifuged at 10000 rpm for 1 minute to dissolve the RNA in water. RNA pellet was stored in ethanol at -20°C.

The concentration of RNA was determined from isolated total RNA samples and the synthesis of the first cDNA strand was started using a commercial kit (PrimeScript First Strand cDNA Synthesis Kit, Takara Bio, Inc.) according to the manufacturer's instructions. After incubation for 5 minutes at 65°C and cooling on ice, incubation was performed for 10 minutes at 30°C and at 42°C for 45 minutes. The enzyme was then inactivated at 95°C for 5 minutes. The resulting cDNA was stored at -20 ° C until preparation for the PCR reaction.

First strand cDNA was synthesized by manufacturer's protocol (PrimeScript First StrandcDNASynthesis Kit, Takara Bio, Otshu-Shi, Japan). The synthesized cDNA was amplified using specific primer sequences as follows:

- β actin (sense 5'-GCACCACACCTTCTACAATG-3', antisense 5'-TGCTTGCTGATCCACATCTG-3');
- SOD (sense5'-ATGGTGGCCTTCTTGTTCTGC-3', antisense 5'-GTGCTGTGGGTGCGGCACACC-3');
- NFKB2 (sense 5'-CCATGACAGCAAATCTCC-3', antisense 5'-TAAACTTCATCTCCACCC-3');
- ABCG2 (sense 5'-ATGTCAACTCCTCCTTCTAC-3 'antisense5'-AATGATCTGAGCTATAGAGGC-3');
- TRX1 (sense 5-GGGGTTGCGTTAGTGGATTTTTG-3' antisense 5'-GACGACTTCGCCATCTTTTGTGA-3').

PCR conditions were: for β actin denaturation at 94°C for 3 min, annealing at 56.7°C for 45 s, elongation at 72°C for 1 min in 30 cycles; for SOD denaturation at 94°C for 3 min, annealing at 59°C for 45 s, elongation at 72°C for 1 min in 30 cycles; for NFKB2 denaturation at 94°C for 3 min, annealing at 50°C for 45 s, elongation at 72°C for 1 min in 30 cycles ; for ABCG2 denaturation at 94°C for 3 min, annealing at 54°C for 45 s, elongation at 72°C for 1 min in 30 cycles; for TRX1 denaturation at 94°C for 3 min, annealing at 50°C for 45 s, elongation at 72°C for 1 min in 30 cycles ; for ABCG2 denaturation at 94°C for 3 min, annealing at 54°C for 45 s, elongation at 72°C The PCR products were run on 0.8% agarose gel, stained with SYBR Safe DNA Gel Stain (Thermo Fischer Scientific,Waltham, MA, USA), visualized and semi quantified by ImageJ software using QuantIF ImageJ macro (80).

Results were expressed as a percentage relative to negative controls. All experiments were done in triplicate.

4.3.7. Preparation of coverslips

The washing protocol was following: 1 part of 30 % hydrogen peroxide (Kemika, Zagreb, Croatia) was mixed with 9 parts of concentrated (96 %) sulfuric acid (Kemika, Zagreb, Croatia).

1. In 300 mL wide throat Erlenmeyer flask, 100 coverslips were placed (20 × 20mm square), and a mixture of sulfuric acid and hydrogen peroxide was poured on glass slides.
2. Gentle swirl motion was used to cover all coverslips with the solution, which was repeated at least 3 times during the following 30 minutes.
3. After 30 minutes, under the fume hood, acid was poured from the flask into a 5 L-bucket of distilled water to safely dilute it. 200 ml of fresh distilled water was added to the flask with coverslips to dilute leftover acid.
4. Coverslips were rinsed with water, which was then safely discarded into chemical waste, or by dilution with large amounts of tap water.
5. 200 ml of distilled water was poured into a flask and placed on an orbital shaker (100 rpm) for 1 hour.
6. After 1 hour, water was discarded from the flask, and fresh 200 ml of distilled water was added. The washing cycle was repeated another 5 times (in total, 6 hours of washing).
7. After the last wash, distilled water was discarded, and the coverslips were dried by adding 100 ml of ACS or HPLC grade methanol, the flask was swirled for 5 minutes. Methanol was then discarded into chemical waste.
8. Aluminum foil was placed over the flask opening, and coverslips were dry sterilized at 270°C for 5 hours.
9. After sterilisation in each well of 6 well plate one coverslip was added with sterile forceps
Coverslips had to be treated with adhesive molecule to ensure firm cell attachemnt. For this poly-D-Lysine was used (Sigma Aldrich, St. Louis, MO, USA).
10. Poly-D-Lysine stock was prepared by dissolving 5 mg of poly-D-lysine powder in 5 ml of sterile deionised water in final concentration od 1 mg/ml
11. To prepare working solution, stock was diluted 1:200 with sterile deionised water, and 1.25 ml of working solution was added to each well.
12. Poly-D-lysine was incubated for 1 hour in cell incubator and was removed afterwards
13. Coverslips were left to dry overnight in sterile laminar under UV-C light

4.3.8. Visualization of the F-actin cytoskelet with Rhodamine Phalloidin stain

To determine the organization and structure/function relationships of a filamentous structures in a cell model of PUD, the AGS cells were grown on glass cover-slips inside a 6 well plate grown to 80% confluency in RPMI-1640 plus 10% FBS. After 24 hours, cells were tretead as follows: untreated cells (grown in RPMI-1640 medium), cells treated with LPZ only, cells treated with NaT only, cells pretreated with LPZ and subsequently exposed to NaT and cells pretreated with four different concentrations of GE and subsequently exposed to NaT. On the third day, according to the above described protocol, cells were exposed to 4mM NaT for 1 hour. AGS cell morphology (F-actin cytoskeleton protein) were visualized by Rhodamine Phalloidin Reagent (Abcam Inc., Cambridge, UK) according to the manufacturer's instructions. Briefly, cell culture medium was aspirated carefully to avoid dislodging of any cells from the plate. Cells were washed once in PBS and fixed in 2% formaldehyde in PBS at room temperature for 10-30 minutes. 0.1% Triton X-100 (Haihang Industry Co.,Ltd, USA) in PBS was added into the fixed cells for 3-5 minutes followed by 2-3 times washes with PBS. Conjugate working solution; 100 µl of 1X Phalloidin was added to each well of fixed cells. Cells were incubated in dark, at room temperature for 60 minutes. Nuclei were counterstained using 4′,6-diamidino-2-phenylindole (DAPI) (1 µg/ml in methanol). Subsequently, cells were rinsed 2-3 times with PBS. The cells were analyzed using an AxioSkop 2 MOT microscope (Car Zeiss, Göttingen, Germany). Cell images were obtained by using a 40 × dry immersion objective ajdusted against stain negative control with Olympus DP70 camera (American laboratory trading, Inc., East Lime, CT, USA) and without postacquisition enhancement of images. Total and immunopositive nuclei were counted in ImageJ software using QuantIF ImageJ macro (80).

4.3.9. Proliferating cell nuclear antigen staining

The cells were grown on glass cover-slips inside a 6 well plate After the treatment, the RPMI-1640 medium was removed from each well of the 6 well plate and 2 mL of 2% formaldehyde was added. Glass slides were incubated in formaldehyde for 30 minutes at 4°C. After incubation, formaldehyde was removed, and 3 ml of 1X PBS was pipetted in each well. Fixed cells were stored at 4 °C until further use.

1st day of experiment:

0.5% Triton X-100 and 0.5 % NP-40 protein detergent (Thermo Fisher Scientific Inc., Waltham, MA, USA) in 1X PBS was added into the fixed cells and incubated for 2h at 4°C. After incubation, the stock of Triton X-100 and NP-40 solution was removed and the solution of primary antibodies dissolved in blocking solution in 1:250 dilution was added in each plate, and incubated overnight at 4°C.

Blocking solution: 2.5% horse serum, (Sigma Aldrich, St. Louis, MO, USA); 2% goat serum, (Sigma Aldrich, St. Louis, MO, USA); 1x BSA (Sigma Aldrich, St. Louis, MO, USA).

2nd day of experiment:

After incubation of the primary antibodies cells were washed three times for 10 min with precooled 1X PBS. After washing, secondary antibodies were prepared in blocking solution (2.5% horse serum; 2% goat serum; 1X BSA) in 1:500 dilution and added in each plate and incubated for 2 h at room temperature. After incubation with secondary antibodies the wash process with 1X PBS was repeated three times. Following washing cells were incubated in a 1:1000 diluted fluorophore-labeled streptavidin (BD Biosciences, USA) for 1 h at room temperature in the dark. After incubation of streptavidin, glass slides were washed for 10 minutes with 2 ml of 1 μM of Hoechst prepared in 1X PBS. The last three washes were done for 10 min in 1X PBS.

The glass slides were imaged on the Axioskop 2 MOT microscope with mounted Olympus D70 camera controlled through computer program DP Manager 1.2.1.107 and DP Controller 1.2.1.108 .

4.3.10. *In situ* localization of cholesterol and phospholipids cell membrane

To determine the localization of cholesterol and phospholipids, the cells were grown on glass cover-slips inside a 6 well plate grown to 85% confluency in RPMI-1640 plus 10% FBS. The day after the cells become confluent, eight subgroups were denoted as follows: untreated cells, cells treated with with LPZ only, cells treated with NaT only, cells pre-treated with LPZ and subsequently exposed to NaT, and cells pre-treated with four different concentrations of GE and subsequently exposed to NaT. On the second day, cells were exposed to the LPZ and GE in four different concentrations for 24, 48 and 72 hours. To induce oxidative stress in cells, the NaT was added in appropriate wells according previously described experiments.

Cholesterol-23-(dipyrromethene boron difluoride)-24-norcholesterol (Cayman chemical company, Michigan, USA) and Sulforhodamine (Santa Cruz Biotechnology, Inc., USA) was prepared in RPMI-1640 medium without FBS in a 1:10000 ratio. The dye was applied on live and treated cells followed by incubation for 30 min. Afterwards, the dye solution was removed and the cells were fixed with 2% formaldehyde and placed for 30 min at 4°C. After that, formaldehyde was discarded and cells were rinsed for 10 min with 2 ml of 1 μM of Hoechst (Sigma Aldrich, St. Louis, MO, USA) prepared in 1X PBS.

The cells were imaged using an AxioSkop 2 MOT microscope (Car Zeiss, Göttingen, Germany) equipped with fluorescence and Zeiss filter sets 15 and 01. Cell images were obtained by using a 40 × dry immersion objective ajdusted against stain negative control with Olympus DP70 camera (American laboratory trading, Inc., East Lime, CT, USA) and without postacquisition. Quantification of images was performed in image J-FIJI.

After incubation of streptavidin, glass slides were washed for 10 minutes with 2 ml of 1 μm of Hoechst prepared in 1X PBS. The last three washes were done for 10 min in 1X PBS. The glass slides were imaged on the Axioskop 2 MOT microscope with mounted Olympus D70 camera controlled through computer program DP Manager 1.2.1.107 and DP Controller 1.2.1.108 .

4.3.11. Statistical analysis

The statistical program Statistica 12 (TIBCO, Palo Alto, CA USA) was used for statistical analyses. For statistical significance data was analyzed with One way and Two way ANOVA post hoc Tukey HSD. Data eligability for analysis with ANOVA was determined with Shapiro Wiilk test for normality of distribution and with Bartlett's F test for homoscedasticity on samples. P values of *$p<0.05$, **$p<0.01$ and ***$p<0.001$ were considered statistically significant. A sample in minimal biological triplicate was used for each statistical analysis.

5. RESULTS

5.1. Establishment of the cell culture model of peptic ulcer disease and assessment of the toxic effect of NaT

The toxicity of the NaT on the AGS cell viability were assessed by MTT assay after treatment with four different doses of NaT; 2, 4, 8 and 10 mM, at different time periods: 30 minutes, 1, 4, 12 and 24 hours, respectively. Cell survival was determined, and compared to untreated control (Figure 5.1.).

Cell viability was higher than 50% after 30 min exposure to 2, 4, 8 and 10 Mm NaT. However, significant reduction after 4 hours exposure to 2 mM NaT showed 48% of cell viability, while after 12 hours showed 39% and after 24 h viability was 24%, shown in Table 5.1. After 24 hours exposure to 10 mM NaT showed 6.85% of cell viability. Significant differences can be observed between the lowest and highest concentrations of NaT. Hence, as exposure time increased, cell viability decreased significantly. However, the treatment with 4 mM NaT for 60 minutes had a pronounced effect; cell viability was 50% compared to untreated control as shown in Figure 5.1. According to the MTT results, the concentration and length of exposure to NaT were selected.

Reduction of cell viability by 50% required 4 mM NaT after exposure for 1hour. This concentration and exposure time was used in all subsequent experiments on AGS model of PUD. Each experiment was repeated at least three times to ensure consistency of the results.

Post hoc analysis showed a time-dependent effect and NaT concentration-dependent effect on cell viability. Hence, cell viability after exposure to varying NaT concentrations and varying time periods in AGS cell line has shown significant results (Table 5.2.). Statistical significance in cell survival was observed between almost all NaT concentrations and the time of cell exposure to NaT. However, cell viability of untreated controls compared to varying NaT concentrations was statistically significant, while groups of untreated controls in different time periods did not show significant difference in their cell viability Also, there was no siginificant difference between 4 and 12 hours treatment of exposure to 8 mM NaT compared to 10 mM NaT in the same exposure periods (Table 5.2.).

Nevertheless, the 4 mM concentration of NaT selected to establish model of ulcer showed a statistically significant difference compared to the remaining three concentrations at different exposure periods.

Table 5.1. Viability of AGS cells after exposure to varying NaT concentrations and varying time periods determined by MTT assay expressed as a percentage relative to untreated control.

Duration of treatment	RPMI-1640 medium	NaT 2mM	NaT 4mM	NaT 8 mM	NaT 10 mM	F values; (degrees of freedom)			p-values		
	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Time period	Treatment	Time period x treatment	Time period	Treatment	Time period x treatment
30 min	100.0; 0.78	92.54; 0.60	81.97; 0.34	66.47; 0.45	58.53; 0.72	11840 (4,125)	32370 (4,125)	884.4 (16,125)	<0.001	<0.001	<0.001
1 h	100.0; 0.32	66.15; 3.63	50.03; 1.68	30.08; 0.27	15.63; 0.26						
4 h	100.0; 0.34	48.78; 0.99	30.0; 0.48	15.32; 0.57	13.85; 0.37						
12 h	100.0; 0.59	39.65; 0.58	28.17; 0.86	13.16; 0.49	11.67; 0.42						
24 h	100.0; 0.48	24.92; 0.46	16.31; 0.64	7.81; 0.44	6.85; 0.43						

Two way Anova; P values of ***$p < 0.001$ were considered statistically significant. SD = standard deviation; The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; SD = standard deviation.

Table 5.2. Post hoc Tukey test of two-way ANOVA results for cell viability after exposure to varying NaT concentrations and varying time periods in AGS cell line.

Survivability rate measured by MTT test				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
30 min	RPMI-1640 medium	30 min	NaT 2 mM	<0.001
30 min	RPMI-1640 medium	30 min	NaT 4 mM	<0.001
30 min	RPMI-1640 medium	30 min	NaT 8 mM	<0.001
30 min	RPMI-1640 medium	30 min	NaT 10 mM	<0.001
30 min	NaT 2 mM	30 min	NaT 4 mM	<0.001
30 min	NaT 2 mM	30 min	NaT 8 mM	<0.001
30 min	NaT 2 mM	30 min	NaT 10 mM	<0.001
30 min	NaT 2 mM	1h	NaT 2 mM	<0.001
30 min	NaT 2 mM	4h	NaT 2 mM	<0.001
30 min	NaT 2 mM	12h	NaT 2 mM	<0.001
30 min	NaT 2 mM	24h	NaT 2 mM	<0.001
30 min	NaT 4 mM	30 min	NaT 8 mM	<0.001
30 min	NaT 4 mM	30 min	NaT 10 mM	<0.001
30 min	NaT 4 mM	1h	NaT 4 mM	<0.001
30 min	NaT 4 mM	4h	NaT 4 mM	<0.001
30 min	NaT 4 mM	12h	NaT 4 mM	<0.001
30 min	NaT 4 mM	24h	NaT 4 mM	<0.001
30 min	NaT 8 mM	30 min	NaT 10 mM	<0.001
30 min	NaT 8 mM	1h	NaT 8 mM	<0.001
30 min	NaT 8 mM	4h	NaT 8 mM	<0.001
30 min	NaT 8 mM	12h	NaT 8 mM	<0.001
30 min	NaT 8 mM	24h	NaT 8 mM	<0.001
30 min	NaT 10 mM	1h	NaT 10 mM	<0.001
30 min	NaT 10 mM	4h	NaT 10 mM	<0.001
30 min	NaT 10 mM	12h	NaT 10 mM	<0.001
30 min	NaT 10 mM	24h	NaT 10 mM	<0.001
1h	RPMI-1640 medium	1h	NaT 2 mM	<0.001
1h	RPMI-1640 medium	1h	NaT 4 mM	<0.001
1h	RPMI-1640 medium	1h	NaT 8 mM	<0.001
1h	RPMI-1640 medium	1h	NaT 10 mM	<0.001
1h	NaT 2 mM	1h	NaT 4 mM	<0.001
1h	NaT 2 mM	1h	NaT 8 mM	<0.001
1h	NaT 2 mM	1h	NaT 10 mM	<0.001
1h	NaT 2 mM	4h	NaT 2 mM	<0.001
1h	NaT 2 mM	12h	NaT 2 mM	<0.001
1h	NaT 2 mM	24h	NaT 2 mM	<0.001
1h	NaT 4 mM	1h	NaT 8 mM	<0.001
1h	NaT 4 mM	1h	NaT 10 mM	<0.001
1h	NaT 4 mM	4h	NaT 4 mM	<0.001
1h	NaT 4 mM	12h	NaT 4 mM	<0.001
1h	NaT 4 mM	24h	NaT 4 mM	<0.001
1h	NaT 8 mM	1h	NaT 10 mM	<0.001
1h	NaT 8 mM	4h	NaT 8 mM	<0.001
1h	NaT 8 mM	12h	NaT 8 mM	<0.001
1h	NaT 8 mM	24h	NaT 8 mM	<0.001
1h	NaT 10 mM	12h	NaT 10 mM	<0.001

Survivability rate measured by MTT test				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
1h	NaT 10 mM	24h	NaT 10 mM	<0.001
4 hours	RPMI-1640 medium	4h	NaT 2 mM	<0.001
4 hours	RPMI-1640 medium	4h	NaT 4 mM	<0.001
4 hours	RPMI-1640 medium	4h	NaT 8 mM	<0.001
4 hours	RPMI-1640 medium	4h	NaT 10 mM	<0.001
4 hours	NaT 2 mM	4h	NaT 4 mM	<0.001
4 hours	NaT 2 mM	4h	NaT 8 mM	<0.001
4 hours	NaT 2 mM	4h	NaT 10 mM	<0.001
4 hours	NaT 2 mM	12h	NaT 2 mM	<0.001
4 hours	NaT 2 mM	24h	NaT 2 mM	<0.001
4 hours	NaT 4 mM	4h	NaT 8 mM	<0.001
4 hours	NaT 4 mM	4h	NaT 10 mM	<0.001
4 hours	NaT 4 mM	24h	NaT 4 mM	<0.001
4 hours	NaT 8 mM	24h	NaT 8 mM	<0.001
4 hours	NaT 10 mM	24h	NaT 10 mM	<0.001
12 hours	RPMI-1640 medium	12h	NaT 2 mM	<0.001
12 hours	RPMI-1640 medium	12h	NaT 4 mM	<0.001
12 hours	RPMI-1640 medium	12h	NaT 8 mM	<0.001
12 hours	RPMI-1640 medium	12h	NaT 10 mM	<0.001
12 hours	NaT 2 mM	12h	NaT 4 mM	<0.001
12 hours	NaT 2 mM	12h	NaT 8 mM	<0.001
12 hours	NaT 2 mM	12h	NaT 10 mM	<0.001
12 hours	NaT 2 mM	24h	NaT 2 mM	<0.001
12 hours	NaT 4 mM	12h	NaT 8 mM	<0.001
12 hours	NaT 4 mM	12h	NaT 10 mM	<0.001
12 hours	NaT 4 mM	24h	NaT 4 mM	<0.001
12 hours	NaT 8 mM	24h	NaT 8 mM	<0.001
12 hours	NaT 10 mM	24h	NaT 10 mM	<0.001
24 hours	RPMI-1640 medium	24h	NaT 2 mM	<0.001
24 hours	RPMI-1640 medium	24h	NaT 4 mM	<0.001
24 hours	RPMI-1640 medium	24h	NaT 8 mM	<0.001
24 hours	RPMI-1640 medium	24h	NaT 10 mM	<0.001
24 hours	NaT 2 mM	24h	NaT 4 mM	<0.001
24 hours	NaT 2 mM	24h	NaT 8 mM	<0.001
24 hours	NaT 2 mM	24h	NaT 10 mM	<0.001
24 hours	NaT 4 mM	24h	NaT 8 mM	<0.001
24 hours	NaT 4 mM	24h	NaT 10 mM	<0.001

P values of ***$p < 0.001$ were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate.

5.2. Gastroprotective effect of garlic extracts in a cell culture model of peptic ulcer disease

To determine effects of GE on cell survival, cells were pretreated with GE in four different concentrations for 24, 48 and 72 hours, as shown in Table 5.3. Different concentrations of GE had a significant effect on AGS cell survival. Treatment resulted a dose-dependent increase in the number of cells alive; higher concentrations of GE resulted higher survival rate. Hence, the number of apoptotic cells decreased by the increasing concentrations of GE and increasing exposure time of cells to garlic pretreatment. This effect can be observed at 24, 48 or 72 hours after pretreatment with GE and subsequent damaged with NaT, shown in Table 5.3.

In AGS cells, pretreatment wih GE at concentrations 100, 150, 250 and 350 μg/ml after 24, 48 and 72 hours showed statistically significant higher survival compared to NaT - alone treated cells, while there was no significant difference compared to untreated cells. Further, no statistically significant difference was observed between 150, 250 and 350 μg/ml concentrations of GE and cells treated with LPZ/NaT in any period of time, while concentration of GE at 100 μg/ml compared to LPZ/NaT was statistically significant after 48 hours (Table 5.3).

Post hoc analysis showed a time-dependent effect on cell viability; effect at longer periods of cell exposure to garlic pretreatment. Consequntly, the more cells undergo the process and apoptosis was decreased after NaT damage (Table 5.4). On the other hand, after 24, 48 and 72 hours there was no significant difference between cells pretreated with LPZ and subsequently with NaT and with cells pretreated with highest concentration of GE subsequently exposed to NaT. (Table 5.4.). Also, after 24, 48 and 72 hours significant difference was observed between 100, 150 and 250 μg/ml of GE subsequently exposed to NaT and untreated control. However, the highest concentration of GE after 72 hours did not show statistically significant cell viability compared to untreated control (Table 5.4.).

Table 5.3. Cell viability of AGS cell line after exposure to varying treatments and varying time periods determined by cell counting expressed as a percentage relative to untreated control.

Duration of treatment	RPMI-1640 medium	LPZ 10µm	NaT 4 mM	LPZ/NaT	GE 100µg/ ml/NaT	GE 150µg/ ml/NaT	GE 250µg/ ml/ NaT	GE 350µg/ ml/ NaT	F values; (degrees of freedom)			p-values		
	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Time period	Treatment	Time period x treatment	Time period	Treatment	Time period x treatment
24h	100.0; 1.02	99.30; 1.08	72.82; 6.22	82.81; 2.33	81.80; 4.59	87.21; 4.48	87.87; 2.89	91.93; 2.26	5.27 (2,48)	128.6 (7,48)	4.25 (14,48)	<0.01	<0.001	<0.001
48h	100.0; 0.04	98.27; 1.66	59.37; 4.01	92.38; 4.77	79.71; 2.52	84.10; 3.03	87.04; 0.80	89.09; 1.45						
72h	100.0; 0.04	97.38; 0.90	67.72; 2.69	91.08; 1.99	84.17; 1.63	87.37; 1.24	89.97; 1.17	92.17; 1.81						

Two way Anova; P values of ***p < 0.001and **p < 0.01 were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts; SD = standard deviation.

Table 5.4. Post hoc Tukey test of two-way ANOVA results for cell viability after exposure to varying treatments and varying time periods in AGS cell line.

Survivability rate measured by cell counting.				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
24h	RPMI-1640 medium	24h	NaT	<0.001
24h	RPMI-1640 medium	24h	LPZ/NaT	<0.001
24h	RPMI-1640 medium	24h	GE 100µg/m/NaT	<0.001
24h	RPMI-1640 medium	24h	GE 150µg/ml/NaT	<0.001
24h	RPMI-1640 medium	24h	GE 250µg/ml/NaT	<0.001
24h	LPZ	24h	NaT	<0.001
24h	LPZ	24h	LPZ/NaT	<0.001
24h	LPZ	24h	GE 100µg/ml/NaT	<0.001
24h	LPZ	24h	GE 150µg/ml/NaT	<0.001
24h	LPZ	24h	GE 250µg/ml/NaT	<0.001
24h	NaT	24h	LPZ/NaT	<0.001
24h	NaT	24h	GE 100µg/ml/NaT	<0.001
24h	NaT	24h	GE 150µg/ml/NaT	<0.001
24h	NaT	24h	GE 250µg/ml/NaT	<0.001
24h	NaT	24h	GE 350µg/ml/NaT	<0.001
24h	NaT	48h	NaT	<0.001
24h	LPZ/NaT	24h	GE 350µg/ml/NaT	<0.05
24h	LPZ/NaT	48h	LPZ/NaT	<0.01
24h	LPZ/NaT	72h	LPZ/NaT	<0.05
24h	GE 100µg/ml/NaT	24h	GE 350µg/ml/NaT	<0.01
48h	RPMI-1640 medium	48h	NaT	<0.001
48h	RPMI-1640 medium	48h	GE 100µg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 150µg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 250µg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 350µg/ml/NaT	<0.001
48h	LPZ	48h	NaT	<0.001
48h	LPZ	48h	GE 100µg/ml/NaT	<0.001
48h	LPZ	48h	GE 150µg/ml/NaT	<0.001
48h	LPZ	48h	GE 250µg/ml/NaT	<0.01
48h	LPZ	48h	GE 350µg/ml/NaT	<0.05
48h	NaT	48h	LPZ/NaT	<0.001
48h	NaT	48h	GE 100µg/ml/NaT	<0.001
48h	NaT	48h	GE 150µg/ml/NaT	<0.001
48h	NaT	48h	GE 250µg/ml/NaT	<0.001
48h	NaT	48h	GE 350µg/ml/NaT	<0.001
48h	LPZ/NaT	48h	GE 100µg/ml/NaT	<0.001
48h	GE 100µg/ml/NaT	48h	GE 350µg/ml/NaT	<0.05
72h	RPMI-1640 medium	72h	NaT	<0.001
72h	RPMI-1640 medium	72h	LPZ/NaT	<0.05
72h	RPMI-1640 medium	72h	GE 100µg/ml/NaT	<0.001
72h	RPMI-1640 medium	72h	GE 150µg/ml/NaT	<0.001
72h	RPMI-1640 medium	72h	GE 250µg/ml/NaT	<0.01
72h	LPZ	72h	NaT	<0.001
72h	LPZ	72h	GE 100µg/ml/NaT	<0.001
72h	LPZ	72h	GE 150µg/ml/NaT	<0.01
72h	LPZ	72h	LPZ/NaT	<0.001

Survivability rate measured by cell counting.				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
72h	NaT	72h	GE 100µg/ml/NaT	<0.001
72h	NaT	72h	GE 150µg/ml/NaT	<0.001
72h	NaT	72h	GE 250µg/ml/NaT	<0.001
72h	NaT	72h	GE 350µg/ml/NaT	<0.001

P values of *p < 0.05, **p < 0.01 and ***p < 0.001 were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts.

5.3. Determination of PCNA in a cell culture model of peptic ulcer disease

In AGS model, the treatment caused a statistically significant difference depending on both, the time period and combination of the time period and treatment (Table 5.5.).

After 24 hours, results showed significantly increased mitotic potential in cells pretreated with 150, 250 and 350 μg/ml subsequent damaged with NaT compared to untreated cells. Also, significantly increased mitotic potential was observed in cells pretreated with GE and cells damaged with NaT only. However, the lowest concentration of GE did not show significant difference compared to untreated control. Also, the results showed that mitotic potential of the cells was significantly decreased after 24 hours when cells were pretreated with the lowest concentration of GE and significantly increased in the remaining three groups of GE damaged with NaT (Figure 5.1.). However, after 48 hours cells pretreated with 150, 250 and 350 μg/ml of GE showed significant decrease in mitotic potential compared to 24 hours pretreatment with 150, 250 and 350 μg/ml of GE damaged with NaT (Figure 5.1 and Figure 5.2.). After 72 hours, mitotic potential in cells pretreated with GE and exposed to NaT started to increase again (Table 5.5. and Figure 5.3.).

After 48 hours statistically significant difference was observed between untreated controls compared to cells pretreated with LPZ only and cells pretreated with 100, 150, 250 and 350 μg/ml of GE damaged with NaT. Moreover, mitotic potential of cells pretreated with GE was decreased compared to the cells treated with NaT only (Figure 5.2.).

However, after 72 hours cells treated with NaT only showed decreased mitotic potential compared to the cells pretreated with LPZ only, and cells pretreated with 150 and 250 μg/ml of GE damaged with NaT (Table 5.6. and Figure 5.3.). Also, after 72 hours, treatment with LPZ only showed significant difference compared to the cells damaged with NaT only. Cells pretreated with GE subsequently damaged with NaT showed increased mitotic potential compared to the cells damaged with NaT only. However, there was no significant difference between cells pretreated with LPZ only and cells pretreated with LPZ and subsequent damaged with NaT (Table 5.6. and Figure 5.3.).

Post hoc analyses showed that 150, 250 and 350 μg/ml concentrations of GE caused three to four times higher PCNA proliferation relative to the lowest concentration of GE. Also, the untreated cells did not have a significant effect on cell proliferation as garlic extracts had.

Table 5.5. Percentage of proliferating cell nuclear antigen positive nuclei after exposure to varying treatments and varying time periods in AGS cell line.

Duration of treatment	RPMI-1640 medium	LPZ 10 μM	NaT 4 mM	LPZ/NaT	GE 100μg/ml/NaT	GE 150μg/ml/NaT	GE 250μg/ml/NaT	GE 350μg/ml/NaT	F values; (degrees of freedom)			p-values		
	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Time period	Treatment	Time period x treatment	Time period	Treatment	Time period x treatment
24h	8.68; 3.59	22.14; 10.89	13.70; 3.20	20.28; 2.45	6.41; 3.85	23.25; 8.62	23.78; 5.57	21.55; 5.08	11.41 (2,118)	5.31 (7,118)	7.92 (14,118)	<0.001	<0.001	<0.001
48h	23.56; 6.02	9.74; 6.44	16.88; 8.04	13.04; 2.65	9.86; 6.56	8.46; 4.82	9.57; 2.44	10.44; 6.02						
72h	4.21; 4.68	18.40; 4.66	4.52; 2.91	14.26; 9.03	11.91; 6.06	16.64; 8.27	28.52; 7.38	12.47; 3.89						

Two way Anova; P values of ***p < 0.001were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts; SD = standard deviation.

Table 5.6. Post hoc Tukey test of two-way ANOVA results for proliferating cell nuclear antigen after exposure to varying treatments and varying time periods in AGS cell line.

PCNA				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
24h	RPMI-1640 medium	24h	GE 150μg/ml/NaT	<0.05
24h	RPMI-1640 medium	24h	GE 250μg/ml/NaT	<0.05
24h	RPMI-1640 medium	48h	RPMI-1640 medium	<0.05
24h	LPZ	24h	GE 100μg/ml/NaT	<0.01
24h	GE 100μg/ml/NaT	24h	GE 150μg/ml/NaT	<0.01
24h	GE 100μg/ml/NaT	24h	GE 250μg/ml/NaT	<0.01
24h	GE 100μg/ml/NaT	24h	GE 350μg/ml/NaT	<0.05
24h	GE 150μg/ml/NaT	48h	GE 150μg/ml/NaT	<0.01
48h	RPMI-1640 medium	48h	LPZ	<0.05
48h	RPMI-1640 medium	48h	GE 100μg/ml/NaT	<0.05
48h	RPMI-1640 medium	48h	GE 150μg/ml/NaT	<0.01
48h	RPMI-1640 medium	48h	GE 250μg/ml/NaT	<0.05
48h	RPMI-1640 medium	72h	RPMI-1640 medium	<0.001
48h	GE 250μg/ml/NaT	72h	GE 250μg/ml/NaT	<0.001
72h	RPMI-1640 medium	72h	LPZ	<0.01
72h	RPMI-1640 medium	72h	GE 150μg/ml/NaT	<0.05
72h	RPMI-1640 medium	72h	GE 250μg/ml/NaT	<0.001
72h	LPZ	72h	NaT	<0.05
72h	NaT	72h	GE 250μg/ml/NaT	<0.001
72h	LPZ/NaT	72h	GE 250μg/ml/NaT	<0.05
72h	GE 100μg/ml/NaT	72h	GE 250μg/ml/NaT	<0.01
72h	GE 250μg/ml/NaT	72h	GE 350μg/ml/NaT	<0.01

P values of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were considered statistically significant. The data shown are representative of at least three independent experiments. NaT=sodium taurocholate; LPZ=lansoprazole; GE=garlic extracts.

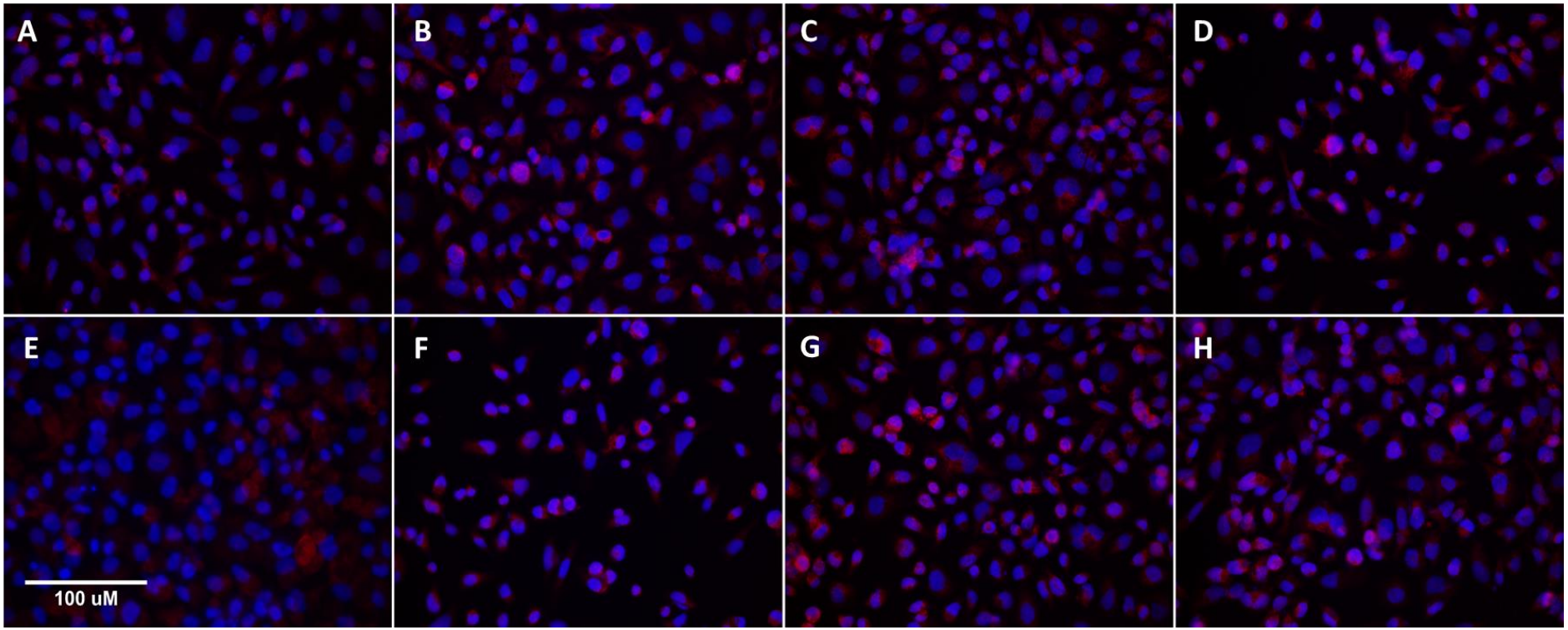

Figure 5.1. Visualization of the proliferating cell nuclear antigen positive nuclei with Streptavidine Cy5PE labbeled antibodies in AGS cells pretreated with LPZ and GE for 24 hours, and treated with NaT for 1hour. The figure was made by the author.

Blue - nuclei stained with DAPI, red-signal of positive nuclei. A - RPMI 1640 (untreated control), B - lansoprazole (LPZ 10μm), C - sodium taurocholate (NaT/4mM), D - sodium taurocholate (NaT 4mM) and lansoprazole (LPZ 10μM), E - sodium taurocholate (NaT 4mM) and garlic extracts (GE 100μg/ml), F - sodium taurocholate (NaT 4mM) and garlic extracts (GE 150 μg/ml), G - sodium taurocholate (4mM) and garlic extracts (GE 250μg/ml), H - sodium taurocholate (4mM) and garlic extracts (GE 350μg/ml). Size bar represents 100 μm.

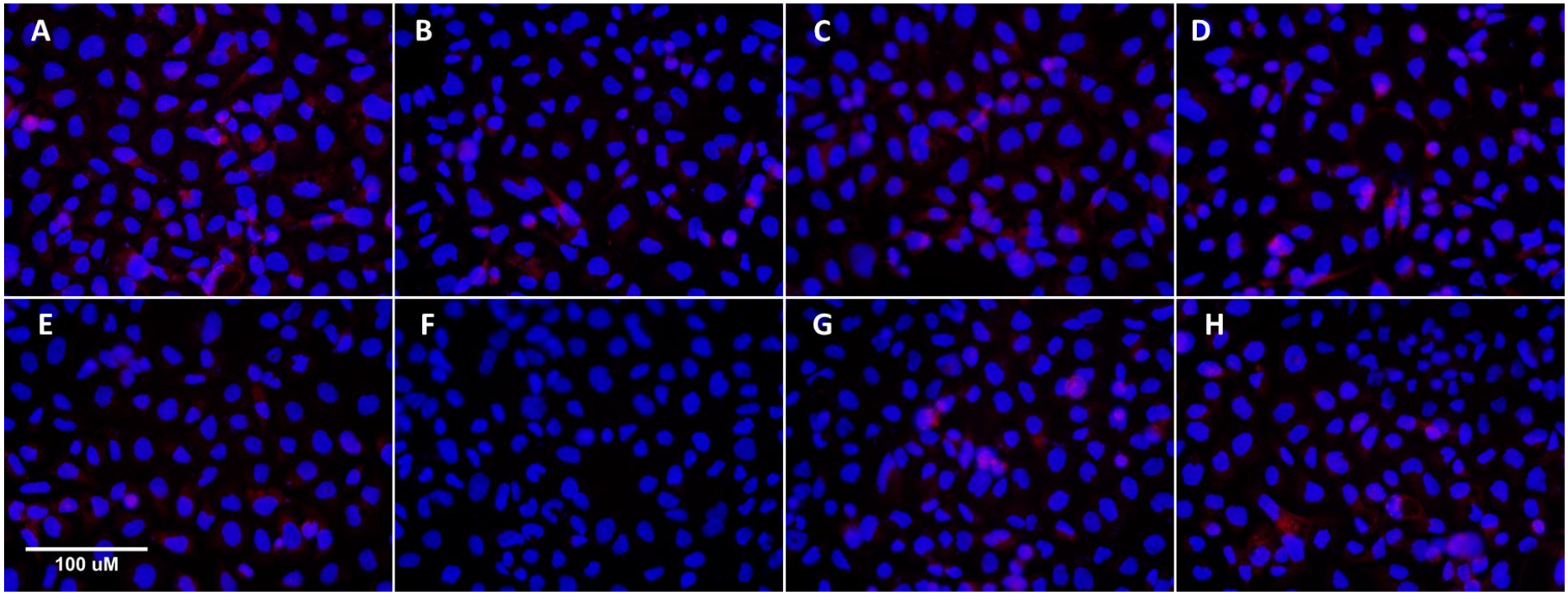

Figure 5.2. Visualization of the proliferating cell nuclear antigen positive nuclei with Streptavidine Cy5PE labbeled antibodies in AGS cells pretreated with LPZ and GE for 48 hours, and treated with NaT for 1hour. The figure was made by the author.

Blue - nuclei stained with DAPI, red-signal of positive nuclei. A - RPMI 1640 (untreated control), B - lansoprazole (LPZ 10µm), C - sodium taurocholate (NaT/4mM), D - sodium taurocholate (NaT 4mM) and lansoprazole (LPZ 10µM), E - sodium taurocholate (NaT 4mM) and garlic extracts (GE 100µg/ml), F - sodium taurocholate (NaT 4mM) and garlic extracts (GE 150 µg/ml), G - sodium taurocholate (4mM) and garlic extracts (GE 250µg/ml), H - sodium taurocholate (4mM) and garlic extracts (GE 350µg/ml). Size bar represents 100 µm.

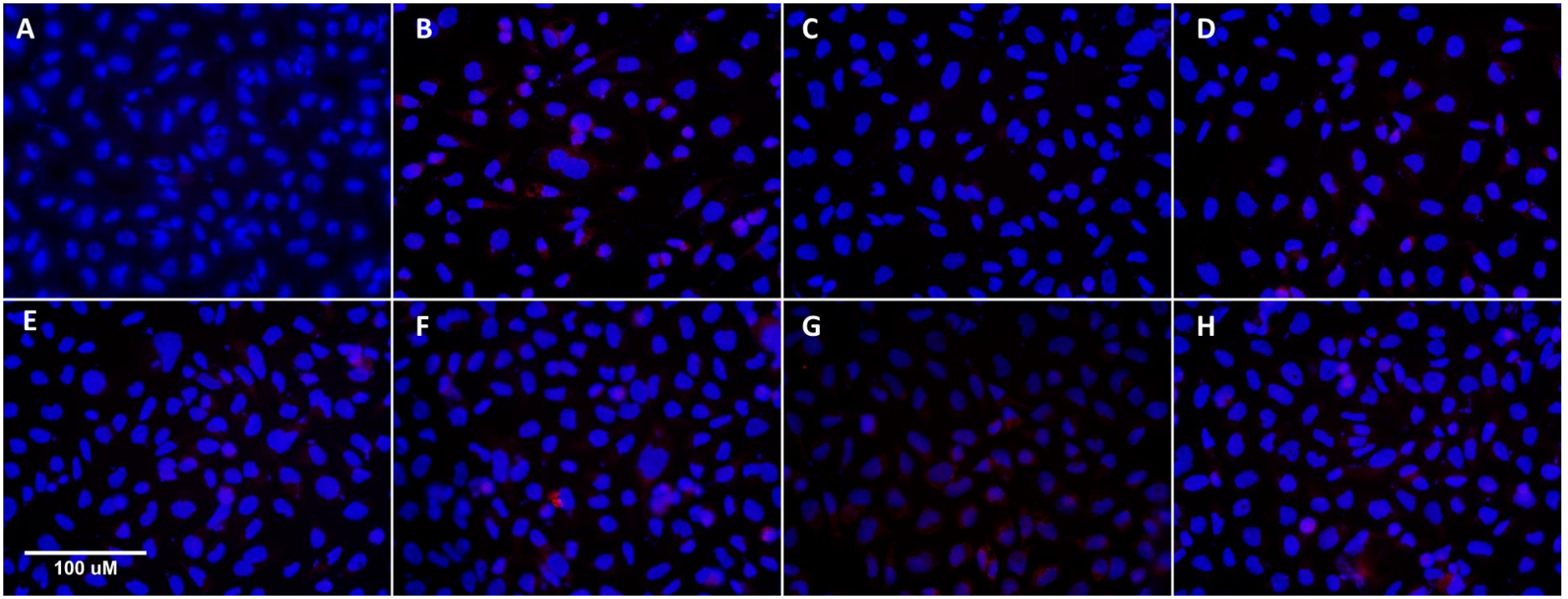

Figure 5.3. Visualization of the proliferating cell nuclear antigen positive nuclei with Streptavidine Cy5PE labbeled antibodies in AGS cells pretreated with LPZ and GE for 72 hours, and treated with NaT for 1hour. The figure was made by the author.

Blue - nuclei stained with DAPI, red-signal of positive nuclei. A - RPMI 1640 (untreated control), B - lansoprazole (LPZ 10µm), C - sodium taurocholate (NaT/4mM), D - sodium taurocholate (NaT 4mM) and lansoprazole (LPZ 10µM), E - sodium taurocholate (NaT 4mM) and garlic extracts (GE 100µg/ml), F - sodium taurocholate (NaT 4mM) and garlic extracts (GE 150 µg/ml), G - sodium taurocholate (4mM) and garlic extracts (GE 250µg/ml), H - sodium taurocholate (4mM) and garlic extracts (GE 350µg/ml). Size bar represents 100 µm.

5.4. Measurement of GSH concentration in a cell culture model of peptic ulcer disease

To evaluate cellular redox tone, GSH levels were measured in above mentioned subgroups. Untreated cells were used as a control.

In AGS model, the treatment caused a statistically significant difference depending on both, the time period and combination of the time period and treatment. (Table 5.7.).

After 24, 48 and 78 hours, treatment with NaT only showed significant decrease of GSH levels compared to untreated control and LPZ treatment only. After 24 hours, cells treated with NaT only showed significant decrease of GSH levels compared to LPZ/NaT treatment, shown in Table 5.7. However, after 48 and 72 hours, GSH concentrations started to decrease in LPZ/NaT treatment. However, pretreatment with the highest concentration of GE for 24, 48 and 72 hours showed significant recovery of GSH levels compared to the cells treated with NaT only. Although, the highest levels of GSH were recorded after 24 hours, while after 48 hours the levels of GSH were lower, but still with higher levels compared to GSH levels in cells treated with NaT.

Pretreatment with GE after 72 hours subsequent damaged with NaT showed significant decrease of GSH, although at highest concentration of GE, levels of GSH were higher compared to cells treated with NaT only (Table 5.7.).

Moreover, there was significant difference in GSH levels between cells pretreated with LPZ and subsequent damaged with NaT and cells treated with LPZ only. Additionally, LPZ as positive control showed higher levels of GSH compared to cells pretreted with GE and subsequnt with NaT (Table 5.7.).

After 24, 48 and 72 hours untreated control showed increased levels of GSH concentrations compared to cells pretreated with varying concentrations of GE for 24, 48 and 72 hours subsequent damaged with NaT for 1 hour (Table 5.7.).

The post-HOC analysis found significant differences between the treated sample groups with GE exposed to NaT in all three time periods. Also, after 24, 48 and 72 hours, it was found significant difference between cells pretreated with GE and subequent damaged with NaT at and cells treated with LPZ and NaT only . However, the post-HOC analysis found no significant

differences between the treated sample groups with 100, 150 and 250 GE μg/ml of GE for 72 hours, while it was present between groups of samples treated with GE at 350 μg/ml. Statistically significant difference was not found between LPZ treatments only in all three time periods. Also, there was no observed significant difference between untreated groups and LPZ treated only cells in all three time periods (Table 5.8.).

However, statistically significant results were observed between LPZ/NaT and LPZ in all three time periods. The same was observed between LPZ/NaT and cells damaged with NaT only, shown in Table 5.8.

Table 5.7. Effects of varying treatments and varying time periods on the levels of GSH in AGS cell line determined by GSH measurements by spectrophotometry at 415 nm. Data are expressed as a percentage relative to untreated control.

Duration of treatment	RPMI-1640 medium	LPZ 10μM	NaT 4mM	LPZ/NaT	GE 100μg/ml/NaT	GE 150μg/ml/NaT	GE 250μg/ml/NaT	GE 350μg/ml/NaT	F values; (degrees of freedom)			p-values		
	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Time period	Treatment	Time period x treatment	Time period	Treatment	Time period x treatment
24h	100.0; 2.37	96.30; 3.95	39.01; 0.52	60.40; 1.84	48.25; 2.37	51.95; 3.43	59.87; 2.90	72.01; 3.43	173 (2,48)	1056 (7,48)	28.07 (14,48)	<0.001	<0.001	<0.001
48h	100.0; 3.26	102.1; 2.71	54.86; 1.63	36.65; 1.90	39.09; 2.17	43.71; 0.81	46.16; 0.54	56.76; 1.90						
72h	100.0; 1.08	94.32; 2.97	44.83; 1.62	35.37; 2.43	38.07; 1.35	39.42; 1.08	41.04; 1.08	52.67; 0.27						

P values of ***$p < 0.001$and **$p < 0.01$ were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts; SD = standard deviation.

Table 5.8. Post hoc Tukey test of two-way ANOVA for results of GSH levels after exposure to varying treatments and varying time periods in AGS cell line.

GSH				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
24h	RPMI-1640 medium	24h	NaT	<0.001
24h	RPMI-1640 medium	24h	LPZ/NaT	<0.001
24h	RPMI-1640 medium	24h	GE 100μg/ml/NaT	<0.001
24h	RPMI-1640 medium	24h	GE 150μg/ml/NaT	<0.001
24h	RPMI-1640 medium	24h	GE 250μg/ml/NaT	<0.001
24h	RPMI-1640 medium	24h	GE 350μg/ml/NaT	<0.001
24h	LPZ	24h	NaT	<0.001
24h	LPZ	24h	LPZ/NaT	<0.001
24h	LPZ	24h	GE 100μg/ml/NaT	<0.001
24h	LPZ	24h	GE 150μg/ml/NaT	<0.001
24h	LPZ	24h	GE 250μg/ml/NaT	<0.001
24h	LPZ	24h	GE 350μg/ml/NaT	<0.001
24h	NaT	24h	LPZ/NaT	<0.001
24h	NaT	24h	GE 100μg/ml/NaT	<0.01
24h	NaT	24h	GE 150μg/ml/NaT	<0.001
24h	NaT	24h	GE 250μg/ml/NaT	<0.001
24h	NaT	24h	GE 350μg/ml/NaT	<0.001
24h	NaT	48h	NaT	<0.001
24h	LPZ/NaT	24h	GE 100μg/ml/NaT	<0.001
24h	LPZ/NaT	24h	GE 150μg/ml/NaT	<0.01
24h	LPZ/NaT	24h	GE 350μg/ml/NaT	<0.001
24h	LPZ/NaT	48h	LPZ/NaT	<0.001
24h	LPZ/NaT	72h	LPZ/NaT	<0.001
24h	GE 100μg/ml/NaT	24h	GE 250μg/ml/NaT	<0.001
24h	GE 100μg/ml/NaT	24h	GE 350μg/ml/NaT	<0.001
24h	GE 100μg/ml/NaT	48h	GE 100μg/ml/NaT	<0.001
24h	GE 100μg/ml/NaT	72h	GE 100μg/ml/NaT	<0.001
24h	GE 150μg/ml/NaT	24h	GE 250μg/ml/NaT	<0.05
24h	GE 150μg/ml/NaT	24h	GE 350μg/ml/NaT	<0.001
24h	GE 150μg/ml/NaT	48h	GE 150μg/ml/NaT	<0.001
24h	GE 150μg/ml/NaT	72h	GE 150μg/ml/NaT	<0.001
24h	GE 250μg/ml/NaT	24h	GE 350μg/ml/NaT	<0.001
24h	GE 250μg/ml/NaT	48h	GE 250μg/ml/NaT	<0.001
24h	GE 250μg/ml/NaT	72h	GE 250μg/ml/NaT	<0.001
24h	GE 350μg/ml/NaT	48h	GE 350μg/ml/NaT	<0.001
24h	GE 350μg/ml/NaT	72h	GE 350μg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	NaT	<0.001
48h	RPMI-1640 medium	48h	LPZ/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 100μg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 150μg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 250μg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 350μg/ml/NaT	<0.001
48h	LPZ	48h	NaT	<0.001
48h	LPZ	48h	LPZ/NaT	<0.001
48h	LPZ	48h	GE 100μg/ml/NaT	<0.001
48h	LPZ	48h	GE 150μg/ml/NaT	<0.001
48h	LPZ	48h	GE 250μg/ml/NaT	<0.001
48h	LPZ	48h	GE 350μg/ml/NaT	<0.001

GSH				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
48h	LPZ	72h	LPZ	<0.05
48h	NaT	48h	LPZ/NaT	<0.001
48h	NaT	48h	GE 100µg/ml/NaT	<0.001
48h	NaT	48h	GE 150µg/ml/NaT	<0.001
48h	NaT	48h	GE 250µg/ml/NaT	<0.01
48h	NaT	72h	NaT	<0.001
48h	LPZ/NaT	48h	GE 250µg/ml/NaT	<0.001
48h	LPZ/NaT	48h	GE 350µg/ml/NaT	<0.001
48h	GE 100µg/ml/NaT	48h	GE 350µg/ml/NaT	<0.001
48h	GE 150µg/ml/NaT	48h	GE 350µg/ml/NaT	<0.001
48h	GE 250µg/ml/Na	48h	GE 350µg/ml/NaT	<0.001
72h	RPMI-1640 medium	72h	NaT	<0.001
72h	RPMI-1640 medium	72h	LPZ/NaT	<0.001
72h	RPMI-1640 medium	72h	GE 100µg/ml/N	<0.001
72h	RPMI-1640 medium	72h	GE 150µg/ml/NaT	<0.001
72h	RPMI-1640 medium	72h	GE 250µg/ml/NaT	<0.001
72h	RPMI-1640 medium	72h	GE 350µg/ml/NaT	<0.001
72h	LPZ	72h	NaT	<0.001
72h	LPZ	72h	LPZ/NaT	<0.001
72h	LPZ	72h	GE 100µg/ml/NaT	<0.001
72h	LPZ	72h	GE 150µg/ml/NaT	<0.001
72h	LPZ	72h	GE 250µg/ml/NaT	<0.001
72h	LPZ	72h	GE 350µg/ml/NaT	<0.001
72h	NaT	72h	LPZ/NaT	<0.01
72h	LPZ/NaT	72h	GE 350µg/ml/NaT	<0.001
72h	GE 100µg/ml/NaT	72h	GE 350µg/ml/NaT	<0.001
72h	GE 150µg/ml/NaT	72h	GE 350µg/ml/NaT	<0.001
72h	GE 250µg/ml/NaT	72h	GE 350µg/ml/NaT	<0.001

P values of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts.

5.5. Measurement of PGE2 concentration in a cell culture model of peptic ulcer disease

To evaluate the role of PG in AGS model of ulcer disease PGE2 concentrations as powerful stimulus of gastric mucus were measured.

In AGS model, the treatment caused a statistically significant difference depending on both, the time period and combination of the time period and treatment (Table 5.9.).

After 24, 48 and 72 hours pretreatment with GE at concentrations 100, 150, 250 and 350 μg/ml showed higher stimulation of PGE2 synthesis compared to untreated cells and cells treated with LPZ only. The GE at 350 μg/ml presented a strong effect in AGS cell line increasing the levels of PGE2 compared with NaT solely. After 24 hours there was no significant difference in PGE2 synthesis between GE at 100 μg/ml and cells treated only with NaT. However, after 48 and 72 hours treatment with GE at 100 μg/ml showed increased PGE2 synthesis compared to cells treated with NaT only (Table 5.9).

24 hour NaT treatment resulted in significant increase of PGE2 levels in AGS cell line compared to untreated control and LPZ only. In contrast, 48 and 72 hours NaT treatment showed significant decrease in PGE2 levels. Significant difference between the NaT treated cells and untreated cells can be detected at all three time periods. Pretreatment with LPZ for 24, 48 and 72 hours showed significant increase of PGE2 levels in cells compared to untreated control and treatment with LPZ only. However, PGE2 levels were significant increased in LPZ/NaT treatment compared to NaT only (Table 5.9.).

The post-HOC analysis showed significant differences in PGE2 levels between the treated cells compared to untreated cells. However, The post-HOC analysis found no significant differences between the treated sample groups for all three time periods. Statistically significant difference was found between the lowest and highest concentrations of GE in all three time periods, however, there was no significant difference between the groups with highest concentrations of GE in all three time periods (Table 5.10.).

There was no significant difference in PGE2 stimulation between NaT treated groups in all three time periods, while significant difference was found between groups treated with LPZ/NaT and higher concentrations of GE in all three time periods (Table 5.10.).

Finally, no significant difference was found between untreated cells and cells treated with LPZ only in all three time periods. There was also no observed significant difference between untreated groups and LPZ only treated cells in all three time periods shown in Table 5.10.

Table 5.9. Effects of varying treatments and varying time periods on the levels of PGE2 in AGS cell line determined by PGE2 measurements by spectrophotometry at 450 nm. Data are expressed as a percentage relative to untreated control.

Duration of treatment	RPMI-1640 medium	LPZ 10µM	NaT 4mM	LPZ/NaT	GE 100µg/ml /NaT	GE 150µg/ml /NaT	GE 250µg/ml /NaT	GE 350µg/ml /NaT	F values; (degrees of freedom)			p-values		
	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Time period	Treatment	Time period x treatment	Time period	Treatment	Time period x treatment
24h	100.0; 4.76	102.83; 4.50	123.57; 4.12	177.68; 1.28	125.76; 3.47	166.09; 16.49	182.32; 8.24	189.28; 5.66	28.44 (2,48)	457.9 (7,48)	10.87 (14,48)	<0.001	<0.001	<0.001
48h	100; 1.28	102.42; 1.71	115.13; 2.42	143.55; 0.85	146.84; 2.14	155.69: 2.71	178.69; 2.85	190.25; 1.85						
72h	100.0; 1.30	107.70; 2.61	117.58; 0.58	145.77; 0.87	145.48; 1.16	154.92; 3.34	178.75; 2.76	190.67; 2.47						

Two way Anova; P values of ***p < 0.001 were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts; SD = standard deviation.

Table 5.10. Post hoc Tukey test of two-way ANOVA for results of PGE2 levels after exposure to varying treatments and varying time periods in AGS cell line presenting significant results.

PGE2				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
24h	RPMI-1640 medium	24h	NaT	<0.001
24h	RPMI-1640 medium	24h	LPZ/NaT	<0.001
24h	RPMI-1640 medium	24h	GE 100μg/ml/NaT	<0.001
24h	RPMI-1640 medium	24h	GE 150μg/ml/NaT	<0.001
24h	RPMI-1640 medium	24h	GE 250μg/ml/NaT	<0.001
24h	RPMI-1640 medium	24h	GE 350μg/ml/NaT	<0.001
24h	LPZ	24h	NaT	<0.001
24h	LPZ	24h	LPZ/NaT	<0.001
24h	LPZ	24h	GE 100μg/ml/NaT	<0.001
24h	LPZ	24h	GE 150μg/ml/NaT	<0.001
24h	LPZ	24h	GE 250μg/ml/NaT	<0.001
24h	LPZ	24h	GE 350μg/ml/NaT	<0.001
24h	NaT	24h	LPZ/NaT	<0.001
24h	NaT	24h	GE 150μg/ml/Nat	<0.001
24h	NaT	24h	GE 250μg/ml/Nat	<0.001
24h	NaT	24h	GE 350μg/ml/Nat	<0.001
24h	LPZ/NaT	24h	GE 100μg/ml/NaT	<0.001
24h	LPZ/NaT	48h	LPZ/NaT	<0.001
24h	LPZ/NaT	72h	LPZ/NaT	<0.001
24h	GE 100μg/ml/NaT	24h	GE 150μg/ml/NaT	<0.001
24h	GE 100μg/ml/NaT	24h	GE 250μg/ml/NaT	<0.001
24h	GE 100μg/ml/NaT	24h	GE 350μg/ml/NaT	<0.001
24h	GE 100μg/ml/NaT	48h	GE 100μg/ml/NaT	<0.01
24h	GE 150μg/ml/NaT	24h	GE 250μg/ml/NaT	<0.05
24h	GE 150μg/ml/NaT	24h	GE 350μg/ml/NaT	<0.001
24h	GE 150μg/ml/NaT	72h	GE 150μg/ml/NaT	<0.01
48h	RPMI-1640 medium	48h	NaT	<0.05
48h	RPMI-1640 medium	48h	LPZ/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 100μg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 150μg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 250μg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 350μg/ml/NaT	<0.001
48h	LPZ	48h	LPZ/NaT	<0.001
48h	LPZ	48h	GE 100μg/ml/NaT	<0.001
48h	LPZ	48h	GE 150μg/ml/NaT	<0.001
48h	LPZ	48h	GE 250μg/ml/NaT	<0.001
48h	LPZ	48h	GE 350μg/ml/NaT	<0.001
48h	NaT	48h	LPZ/NaT	<0.001
48h	NaT	48h	GE 100μg/ml/NaT	<0.001
48h	NaT	48h	GE 150μg/ml/NaT	<0.001

PGE2				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
48h	NaT	48h	GE 250µg/ml/NaT	<0.001
48h	NaT	48h	GE 350µg/ml/NaT	<0.001
48h	LPZ/NaT	48h	GE 250µg/ml/NaT	<0.001
48h	LPZ/NaT	48h	GE 350µg/ml/NaT	<0.001
48h	GE 100µg/ml/NaT	48h	GE 250µg/ml/NaT	<0.001
48h	GE 100µg/ml/NaT	48h	GE 350µg/ml/NaT	<0.001
48h	GE 150µg/ml/NaT	48h	GE 250µg/ml/NaT	<0.001
48h	GE 150µg/ml/NaT	48h	GE 350µg/ml/NaT	<0.001
72h	RPMI-1640 medium	72h	NaT	<0.05
72h	RPMI-1640 medium	72h	LPZ/NaT	<0.001
72h	RPMI-1640 medium	72h	GE 100µg/ml/NaT	<0.001
72h	RPMI-1640 medium	72h	GE 150µg/ml/NaT	<0.001
72h	RPMI-1640 medium	72h	GE 250µg/ml/NaT	<0.001
72h	RPMI-1640 medium	72h	GE 350µg/ml/NaT	<0.001
72h	LPZ	72h	LPZ/NaT	<0.001
72h	LPZ	72h	GE 100µg/ml/NaT	<0.001
72h	LPZ	72h	GE 150µg/ml/NaT	<0.001
72h	LPZ	72h	GE 250µg/ml/NaT	<0.001
72h	LPZ	72h	GE 350µg/ml/NaT	<0.001
72h	NaT	72h	LPZ/NaT	<0.001
72h	NaT	72h	GE 100µg/ml/NaT	<0.001
72h	NaT	72h	GE 150µg/ml/NaT	<0.001
72h	NaT	72h	GE 250µg/ml/NaT	<0.001
72h	NaT	72h	GE 350µg/ml/NaT	<0.001
72h	LPZ/NaT	72h	GE 250µg/ml/NaT	<0.001
72h	LPZ/NaT	72h	GE 350µg/ml/NaT	<0.001
72h	GE 100µg/ml/NaT	72h	GE 250µg/ml/NaT	<0.001
72h	GE 100µg/ml/NaT	72h	GE 350µg/ml/NaT	<0.001
72h	GE 150µg/ml/NaT	72h	GE 250µg/ml/NaT	<0.001
72h	GE 150µg/ml/NaT	72h	GE 350µg/ml/NaT	<0.001

P values of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts.

5.6. Distribution of the F-actin cytoskeleton in a cell culture model of peptic ulcer disease

Deformations and distribution of the F-actin cytoskeleton are involved in the epithelial cells damage. To assess the effect of ulcerogenic agent on epithelial gastric cells, distribution of F-actin was determined compared to untreated control.

Treatment with NaT only did not show significant difference compared to untreated cells, however after 48 hours, cells treated with NaT only for 1 h showed the greatest distribution of F actin. Ater 24 pretreatment with LPZ and cells treated with LPZ and subsequent with NaT caused a significant decrease in the total amount of actin within the cell (Figure 5.4. and Figure 5.5). However, after 48 hours it was observed increased amount of F-actin shown in Table 5.11. and Figure 5.5. Interestingly, after 24 hours pretreatment with the highest concentration of garlic (350 μg/ml) damaged with NaT had the highest distribution of F actin compared to the remaining three concentrations of garlic, while after 72 hours it showed a significant decrease, and the lowest distribution of F-actin was observed. Pretreatment with GE exposed to NaT in the highest concentration at 350 μg/ml showed statistically significant reduction in distribution of F-actin compared to untreated control (Table 5.11., Figure 5.5. and Figure 5.6.).

The post-HOC analysis showed significant differences in F-actin distribution between the treated cells compared to untreated cells. However, The post-HOC analysis found no significant differences between the treated sample groups in all three time periods. Statistically significant difference was found between the lowest and highest concentrations of GE in all three time periods (Table 5.12.). Also, significant difference was observed in F-actin distribution between NaT treated groups, groups treated with LPZ only and cells pretreated with LPZ and subsequent with NaT in all three time periods. Significant difference was not found between groups treated with the highest concentration of GE in all three time periods, however difference was observed in other groups of GE after 48 and 72 hours compared to pretreatment after 24 hours (Table 5.12.). After 48 and 72 hours significant difference in F-actin distribution between all GE groups damaged subsequently with NaT and cells damaged with NaT only was seen. Moreover, after 72 hours the same was observed between all groups of GE damaged with NaT and cells treated with LPZ only. Statistically significant difference was observed between LPZ treatments in all three time periods, shown in Table 5.12.

Table 5.11. Effects of varying concentrations of varying treatments and varying time periods on the levels of F-actin distribution in AGS cell line determined by F-actin quantification. Data are expressed as a percentage relative to untreated control.

Duration of treatment	RPMI-1640 medium	LPZ 10µM	NaT 4mM	LPZ/NaT	GE 100µg/ml/NaT	GE 150µg/ml/NaT	GE 250µg/ml/NaT	GE 350µg/ml/NaT	F values; (degrees of freedom)			p-values		
	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Time period	Treatment	Time period x treatment	Time period	Treatment	Time period x treatment
24h	100.0; 9.49	56.83; 8.77	92.10; 5.33	58.21; 6.21	79.06; 12.57	84.63; 7.70	66.36; 11.27	102.89; 11.38	196.40 (2,96)	18.46 (7,96)	13.30 (14,96)	<0.001	<0.001	<0.001
48h	100.0; 23.48	131.52; 43.68	309.84; 58.03	183.86; 14.17	149.87; 10.57	190.99; 24.71	160.68; 25.06	141.98; 24.58						
72h	100.0; 21.88	101.41; 23.41	107.59; 19.03	77.99; 11.26	72.93; 7.59	65.69; 9.99	62.56; 11.03	58.79; 4.97						

Two way Anova; P values of ***p < 0.001 were considered statistically significant. . The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts; SD = standard deviation.

Table 5.12. Post hoc Tukey test of two-way ANOVA for results of F-actin distribution after exposure to varying treatments and varying time periods in AGS cell line presenting significant results.

F-Actin				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
24h	RPMI-1640 medium	72h	RPMI-1640 medium	<0.001
24h	LPZ	48h	LPZ	<0.001
24h	LPZ	72h	LPZ	<0.001
24h	NaT	48h	NaT	<0.001
24h	NaT	72h	NaT	<0.001
24h	LPZ/NaT	48h	LPZ	<0.001
24h	LPZ/NaT	72h	LPZ	<0.001
24h	GE 100µg/ml/NaT	48h	GE 100µg/ml/NaT	<0.001
24h	GE 100µg/ml/NaT	72h	GE 100µg/ml/NaT	<0.01
24h	GE 150µg/ml/NaT	48h	GE 150µg/ml/NaT	<0.001
24h	GE 250µg/ml/NaT	48h	GE 250µg/ml/NaT	<0.001
24h	GE 250µg/ml/NaT	72h	GE 250µg/ml/NaT	<0.05
48h	RPMI-1640 medium	48h	NaT	<0.001
48h	RPMI-1640 medium	48h	LPZ/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 150µg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 250µg/ml/NaT	<0.05
48h	RPMI-1640 medium	72h	RPMI-1640 medium	<0.001
48h	LPZ	48h	NaT	<0.001
48h	LPZ	48h	GE 150µg/ml/NaT	<0.05
48h	NaT	48h	LPZ/NaT	<0.001
48h	NaT	48h	GE 100µg/ml/NaT	<0.001
48h	NaT	48h	GE 150µg/ml/NaT	<0.001
48h	NaT	48h	GE 250µg/ml/NaT	<0.001
48h	NaT	48h	GE 350µg/ml/NaT	<0.001
48h	NaT	72h	NaT	<0.001
48h	GE 150µg/ml/NaT	72h	GE 150µg/ml/NaT	<0.01
72h	RPMI-1640 medium	72h	GE 150µg/ml/NaT	<0.05
72h	RPMI-1640 medium	72h	GE 250µg/ml/NaT	<0.01
72h	RPMI-1640 medium	72h	GE 350µg/ml/NaT	<0.01
72h	LPZ	72h	GE 150µg/ml/NaT	<0.05
72h	LPZ	72h	GE 250µg/ml/NaT	<0.01
72h	LPZ	72h	GE 350µg/ml/NaT	<0.001
72h	NaT	72h	GE 100µg/ml/NaT	<0.05
72h	NaT	72h	GE 150µg/ml/NaT	<0.001
72h	NaT	72h	GE 250µg/ml/NaT	<0.001
72h	NaT	72h	GE 350µg/ml/NaT	<0.001

P values of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts.

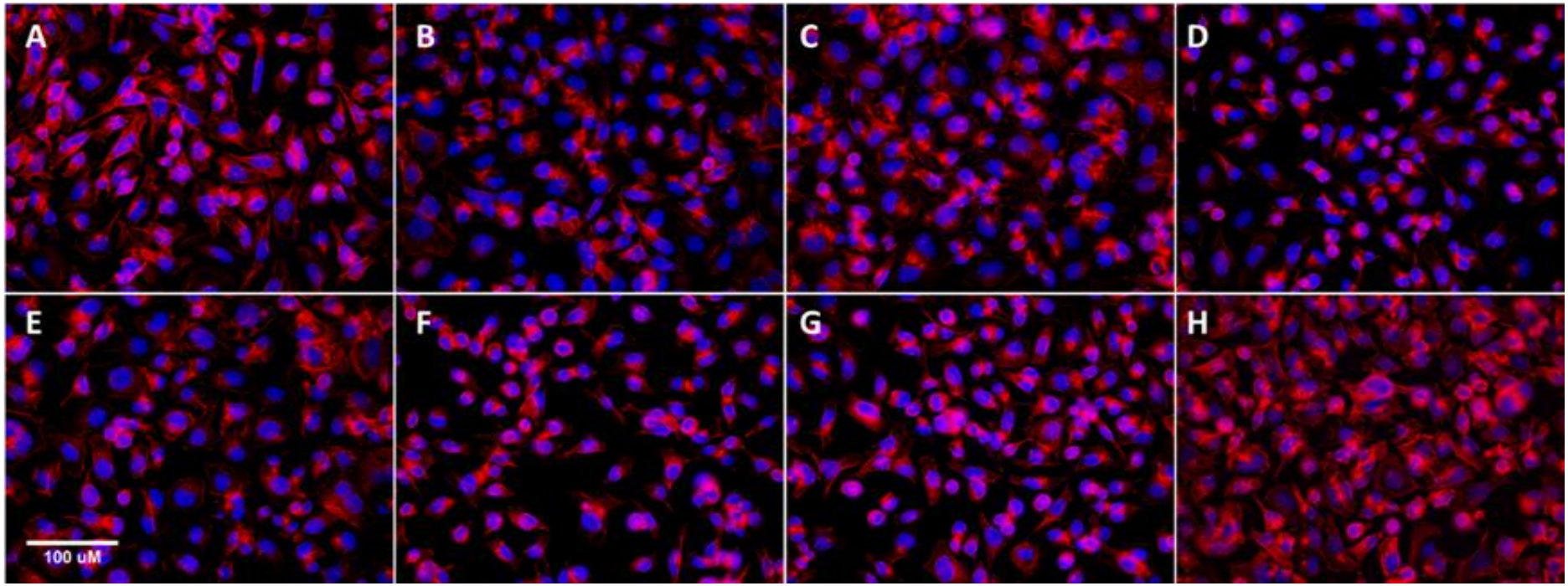

Figure 5.4. Visualization of the F-actin cytoskelet with Rhodamine Phalloidin stain in AGS cells pretreated with LPZ and GE for 24 hours, and treated with NaT for 1hour. The figure was made by the author.

Blue - nuclei stained with DAPI, red - F actin distribution. A - RPMI 1640 (untreated control), B - lansoprazole (LPZ 10µm), C - sodium taurocholate (NaT/4mM), D - sodium taurocholate (NaT 4mM) and lansoprazole (LPZ 10µM), E - sodium taurocholate (NaT 4mM) and garlic extracts (GE 100µg/ml), F - sodium taurocholate (NaT 4mM) and garlic extracts (GE 150 µg/ml), G - sodium taurocholate (4mM) and garlic extracts (GE 250µg/ml), H - sodium taurocholate (4mM) and garlic extracts (GE 350µg/ml). Size bar represents 100 µm.

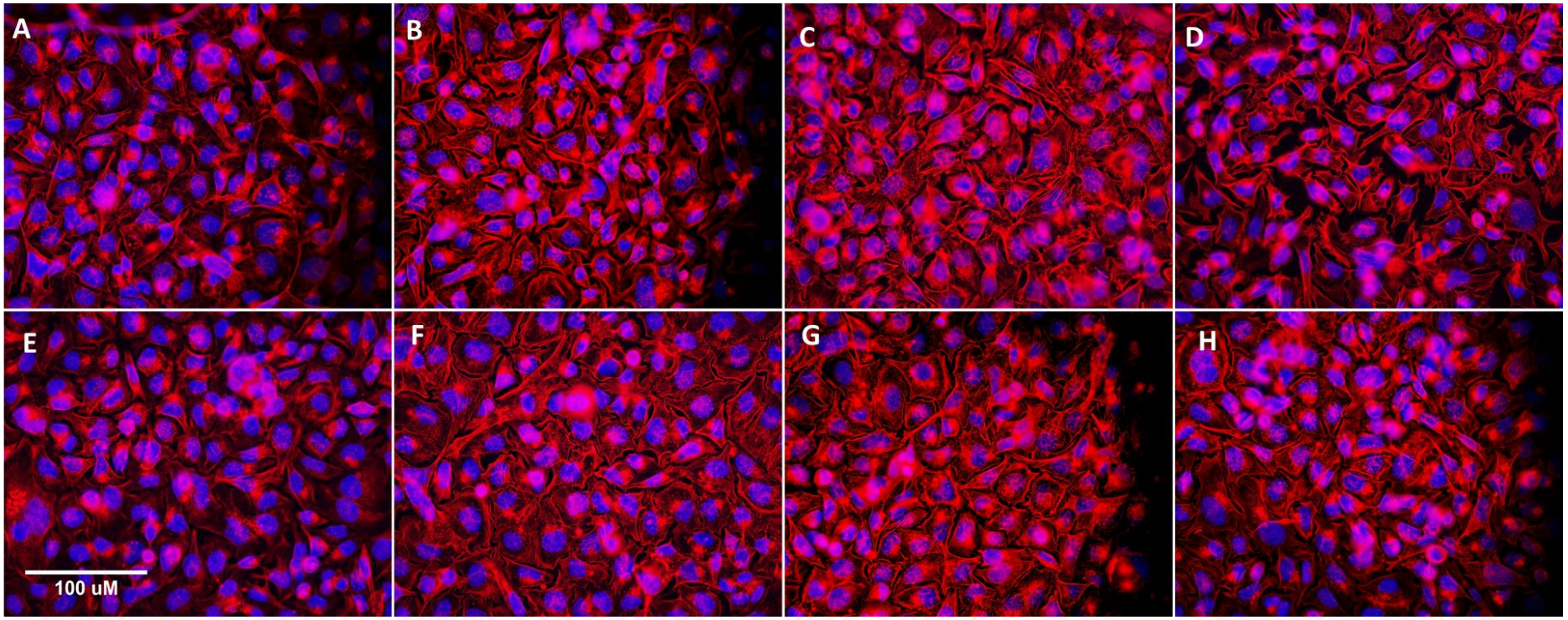

Figure 5.5. Visualization of the F-actin cytoskelet with Rhodamine Phalloidin stain in AGS cells pretreated with LPZ and GE for 48 hours, and treated with NaT for 1hour. The figure was made by the author.

Blue - nuclei stained with DAPI, red - F actin distribution. A - RPMI 1640 (untreated control), B - lansoprazole (LPZ 10µm), C - sodium taurocholate (NaT/4mM), D - sodium taurocholate (NaT 4mM) and lansoprazole (LPZ 10µM), E - sodium taurocholate (NaT 4mM) and garlic extracts (GE 100µg/ml), F - sodium taurocholate (NaT 4mM) and garlic extracts (GE 150 µg/ml), G - sodium taurocholate (4mM) and garlic extracts (GE 250µg/ml), H - sodium taurocholate (4mM) and garlic extracts (GE 350µg/ml). Size bar represents 100 µm.

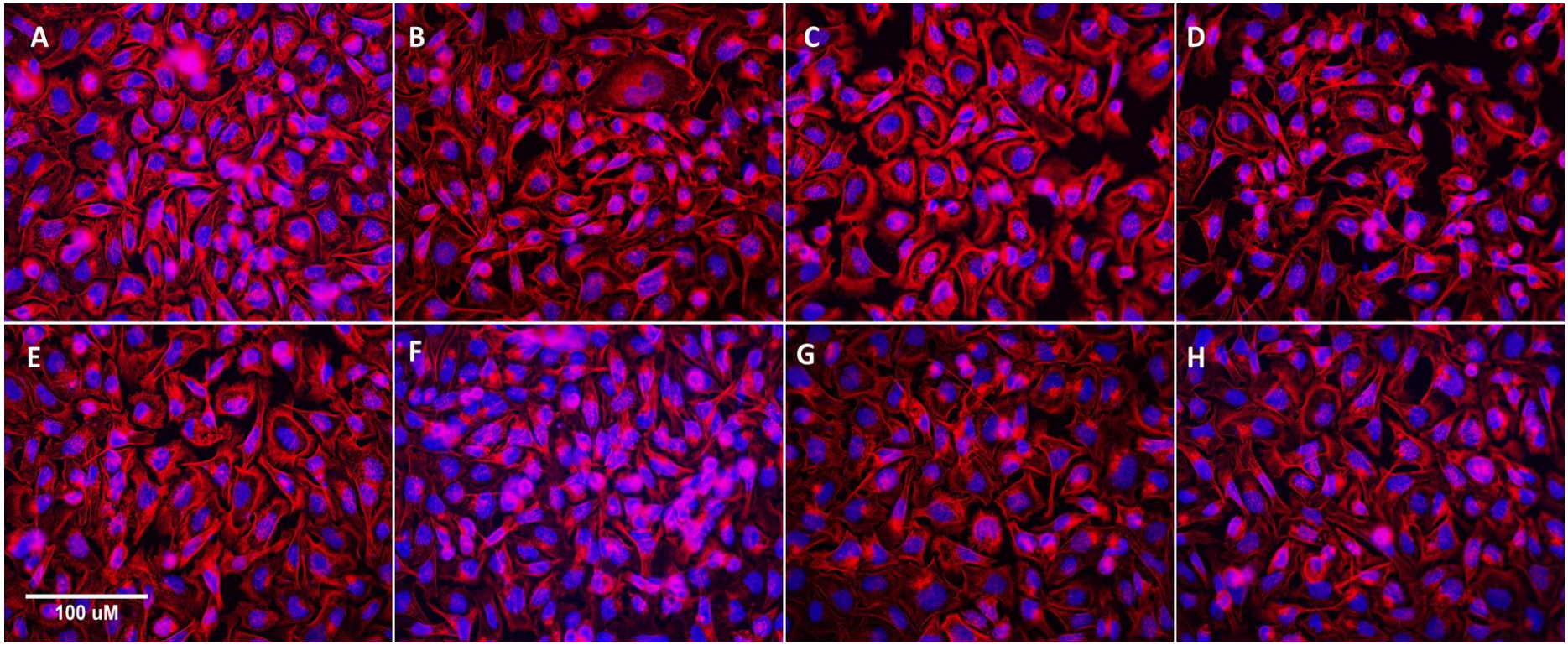

Figure 5.6. Visualization of the F-actin cytoskelet with Rhodamine Phalloidin stain in AGS cells pretreated with LPZ and GE for 72 hours, and treated with NaT for 1hour. The figure was made by the author.

Blue - nuclei stained with DAPI, red - F actin distribution. A - RPMI 1640 (untreated control), B - lansoprazole (LPZ 10µm), C - sodium taurocholate (NaT/4mM), D - sodium taurocholate (NaT 4mM) and lansoprazole (LPZ 10µM), E - sodium taurocholate (NaT 4mM) and garlic extracts (GE 100µg/ml), F - sodium taurocholate (NaT 4mM) and garlic extracts (GE 150 µg/ml), G - sodium taurocholate (4mM) and garlic extracts (GE 250µg/ml), H - sodium taurocholate (4mM) and garlic extracts (GE 350µg/ml). Size bar represents 100 µm.

5.7. Expression of SOD, ABCG2, NFKB2 and TRX1 in a cell culture model of peptic ulcer disease

There were five subgroups denoted as follows: untreated cells, cells treated with LPZ only, cells treated with NaT only, cells pre-treated with LPZ and subsequently exposed to NaT, and cells pre-treated with the highest concentration of GE (350µl) and subsequently exposed to NaT. On the first day of experiment, cells were plated at a density of $4x10^5$ cells/ mL of growth medium in 6-well plates. On the second day, cells in the appropriate wells were exposed to the LPZ and the highest concentration of GE for 24 hour. On the third day, to induce oxidative stress in cells NaT was added in appropriate wells according previously described experiments.

Since SOD plays a key role against oxidant generation, hence our experiment was to analysis of the SOD regulated gene expression in AGS cell culture model of PUD. As expected, SOD expression was lower in cells treated with NaT only compared to untreated control, shown in Table 5.13 and Figure 5.7. On the other hand, post hoc Tukey test showed that SOD expression was higher in cells pretreated with the highest concentration on GE and then exposed to NaT compared to the cells treated with NaT solely, shown in Table 5.14. Nevertheless, no significant difference between the groups was observed. In contrast, cells treated with LPZ had demonstrated higher levels of SOD expression compared to cells treated with NaT only (Figure 5.7.). Moreover, there was significant difference between cells pretreated with LPZ/NaT compared to cells treated with NaT only (Table 5.14.).

To determine possible genetic factors contributing to the development of PUD changes in ABCG2 gene expression was investigated. ABCG2 expression was decreased in cells pretreated with the highest concentration of GE followed by exposure to NaT, and cells pretreated with LPZ solely, compared to untreated control (Table 5.13 and Figure 5.8.). AGS cells treated only with NaT had lower expression of ABCG2 compared to untreated cells as shown in Table 5.13 and Table 5.15 and Figure 5.8. There were no other significant differences between the groups.

NF-κB encoded by NFKB2 gene is activated by inflammatory factors such as IL-8, and infection during the development of peptic ulcer. To determine the possible genetic factors important for the development of PUD relative expression level of NFKB2 gene was studied.

There was strong expression of NFKB2 in cells treated with NaT solely compared to untreated control, as shown in Table 5.13 and Figure 5.9. Further, post hoc Tukey analysis showed that expression of NFKB2 at highest concentration of GE exposed to NaT was significantly lower compared to cells treated with NaT only (Table 5.16.). In addition, there was strong expression of NFKB2 in cells treated with NaT only compared to the cells pretreated with LPZ and subsequently exposed to NaT and cells treated with LPZ only. In contrast, it was no significant difference between cells pretreated with highest concentration of GE subsequent exposed to NaT and cells pretreated with LPZ and subsequent exposed to NaT (Table 5.16.).

Since nuclear and cytosolic form of TRX, known as TRX1 is involved in scavenging ROS and regulating redox-sensitive transcription factors in mammalian cells, our experiment on analyzed TRX1 gene expression in AGS cell culture model of PUD. As expected, TRX1 expression was lowest in cells treated with NaT only compared to untreated control (Figure 5.10.). Furthermore, cells treated with LPZ solely demonstrated higher levels of TRX1 expression compared to the untreated control. Cells pretreated with GE and subsequently damaged with NaT showed significant difference of TRX1 expression compared to untreated cells, while there was no significant difference in TRX1 expression between cells pretreated with LPZ/NaT and untreated control (Table 5.13. and Figure 5.10).

Post hoc Tukey test in cells treated with GE, LPZ, LPZ/NaT showed significant increase of TRX1 expression compared to the cells treated with NaT only (Table 5.17.).

Table 5.13. Effects of 24 h treatment with LPZ and GE damaged with NaT for 1h on the expression of SOD, ABCG2, NFKB2 and TRX1 in AGS cell line. The gene expression analysis was done by RT-PCR. Data are expressed as a percentage relative to untreated control.

Treatment	RPMI- 1640 medium	LPZ 10µM	NaT 4mM	LPZ/ NaT	GE 350µg/ml/Nat	One way Anova	
Gene	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	F values; (degrees of freedom)	p-values
SOD	100.00 30.92	125.41 5.75	44.77 2.98	107.42 12.12	66.39 9.07	13 (4,14)	<0.001
ABCG2	100.0; 4.88	48.18; 0.45	60.35; 11.93	69.53; 23.08	54.27; 19.82	5.68 (4,14)	<0.5
NFKB2	100.0; 46.34	168.77; 77.41	317.37; 42.39	109.66; 17.44	124.39; 12.17	11.61 (4,14)	<0.001
TRX1	100.0; 0.19	111.51; 7.41	46.19; 1.27	99.64; 3.27	80.46; 3.37	125.9 (4,14)	<0.001

One way Anova; P values of *p < 0.5 and ***p < 0.001 were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts; SD = standard deviation.

Table 5.14. Post hoc Tukey test of one way ANOVA results for SOD gene expression 24 hours after treatment with LPZ and GE damaged with NaT for 1 h in AGS cell culture model presenting significant results.

Expression of SOD gene after 24 hours from treatment		
The first set of variables (A)	The second set of variables (B)	Post hoc Tukey HSD p value A vs B
RPMI-1640 medium	NaT	<0.05
LPZ	NaT	<0.001
LPZ	GE 350μg/ml/NaT	<0.01
NaT	LPZ/NaT	<0.01

P values of *p < 0.05, **p < 0.01 and ***p < 0.001 were considered statistically significant. The data shown are representative of at least three independent experiments. NaT=sodium taurocholate; LPZ=lansoprazole; GE=garlic extracts.

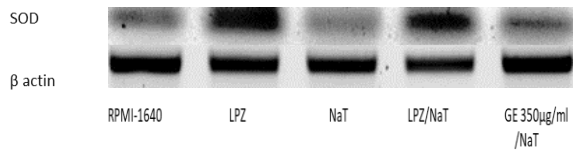

Figure 5.7. Representative figure of gel analysis of SOD expression compared to β actin. The data shown are representative of three independent experiments. The gene expression analysis was done by RT-PCR and obtained results were semi-quantified by ImageJ software using. The data shown are representative of three independent experiments. Superoxide dismutase (SOD).

Table 5.15. Post hoc Tukey test of one way ANOVA results for ABCG2 gene expression 24 hours 24 hours after treatment with LPZ and GE damaged with NaT for 1 h in AGS cell culture model presenting significant results.

Expression of ABCG2 gene after 24 hours from treatment		
The first set of variables (A)	The second set of variables (B)	Post hoc Tukey HSD p value A vs B
RPMI-1640 medium	LPZ	<0.05
RPMI-1640 medium	GE 350µg/ml/NaT	<0.05

P values of *p < 0.05 were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts.

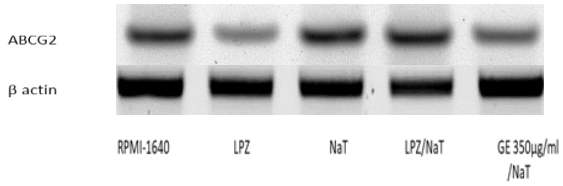

Figure 5.8. Representative figure of gel analysis of ABCG2 expression compared to β actin. The data shown are representative of three independent experiments. The gene expression analysis was done by RT-PCR and obtained results were semi-quantified by ImageJ software using. ATP-binding cassette sub-family G, member 2 (ABCG2).

Table 5.16. Post hoc Tukey test of one way ANOVA results for NFKB2 gene expression 24 hours 24 hours after treatment with LPZ and GE damaged with NaT for 1 h in AGS cell culture model presenting significant results.

Expression of NFKB2 gene after 24 hours from treatment		
The first set of variables (A)	The second set of variables (B)	Post hoc Tukey HSD p value A vs B
RPMI-1640 medium	NaT	<0.001
LPZ	NaT	<0.05
NaT	LPZ/NaT	<0.001
NaT	GE 350μg/ml /NaT	<0.01

P values of *p < 0.05, *p < 0.01, *p < 0.001 were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts.

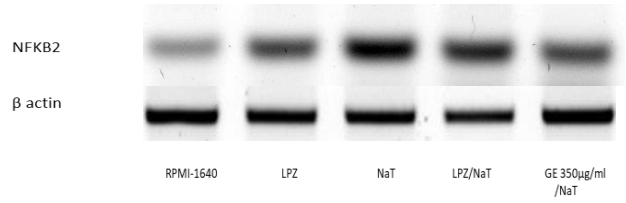

Figure 5.9. Representative figures of gel analysis of NFKB2 expression compared to β actin expression. The values are represented as means ±SD. The data shown are representative of three independent experiments. The gene expression analysis was done by RT-PCR and obtained results were semi-quantified by ImageJ software using. The data shown are representative of three independent experiments. Nuclear Factor Kappa B Subunit 2 (NFKB2).

Table 5.17. Post hoc Tukey test of one way ANOVA results for TRX1 gene expression 24 hours after 24 hours after treatment with LPZ and GE damaged with NaT for 1 h GE in AGS cell culture model presenting significant results.

Expression of TRX1 gene after 24 hours from treatment		
The first set of variables (A)	The second set of variables (B)	Post hoc Tukey HSD p value A vs B
RPMI-1640 medium	NaT	<0.001
RPMI-1640 medium	LPZ	<0.05
RPMI-1640 medium	GE 350μg/ml /NaT	<0.001
LPZ	NaT	<0.001
LPZ	LPZ/NaT	<0.001
LPZ	GE 350μg/ml /NaT	<0.001
NaT	LPZ/NaT	<0.001
NaT	GE 350μg/ml /NaT	<0.001
LPZ/NaT	GE 350μg/ml /NaT	<0.001

P values of *p < 0.05 and ***p <0.001 were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts.

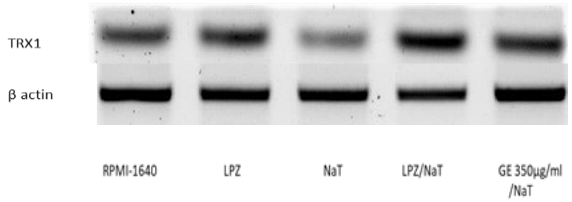

Figure 5.10. Representative figures of gel analysis of TRX1 expression compared to β actin expression. The values are represented as means ±SD. The data shown are representative of three independent experiments. The gene expression analysis was done by RT-PCR and obtained results were semi-quantified by ImageJ software using. The data shown are representative of three independent experiments. Thioredoxin 1 (TRX1).

5.8. Cholesterol and phospholipids in a cell culture model of ulcer disease

5.8.1. Cholesterol staining

In the AGS model, NaT treatment caused a statistically significant difference depending on both, the time period and combination of the time period and treatment (Table 5.18.).

After 24, 48 and 72 hours, significant differences were seen between cells pretreated with GE at concentrations (100, 150, 250 and 350 μg/ml) followed by damage with NaT and untreated cells. Pretreatment with LPZ only for 72 hours, and LPZ/NaT showed significant difference compared to untreated control. Moreover, after 72 hours pretreatment with LPZ only and LPZ/NaT showed increased integrated density values of cholesterols compared to the same treatments after 24 and 48 hours. Additionally, cells pretreated with GE at concentrations (100, 150, 250 and 350 μg/ml) and subsequent damaged with NaT also showed increased integrated density values of cholesterols compared to the same treatments after 24 and 48 hours (Figures 5.11., 5.12. and 5.13.). After 72 hours, pretreatment with LPZ subsequent damaged with NaT showed significant difference of density values of cholesterol compared to the cells pretreated with GE at concentrations (100, 150, 250 and 350 μg/ml) subsequent damaged with NaT (Table 5.18.).

Post hoc analysis showed that integrated density values of cholesterols in cells pretreated with GE concentrations (100, 150, 250 and 350 μg/ml) were significantly different between 24 and 48 hours. Also, after 48 hours, GE concentrations (100, 150, 250 and 350 μg/ml) damaged with NaT showed significantly difference compared to untreated cells. However after 72 hours, pretreatment with GE did not show significant difference compared to untreated control (Table 5.19.).

In contrast, after 72 hours statistically significant difference was observed between cells treated with LPZ only compared to cells pretreated with NaT only and cells pretreated with GE (100, 150, 250 and 350 μg/ml) and subsequent damaged with NaT. Moreover, after 72 hours cells pretreated with GE at concentrations (250 and 350 μg/ml) damaged with NaT showed significant difference compared to the cells treated with NaT only (Table 5.19.).

Table 5.18. Effects of varying concentrations of varying treatments and varying time periods on the integrated density value of cholesterol in AGS cells. Data are presented as integrated density values per cell. Data are expressed as a percentage relative to untreated control.

Duration of treatment	RPMI-1640 medium	LPZ 10μM	NaT 4mM	LPZ/NaT	GE 100μg/ml/NaT	GE 150μg/ml/NaT	GE 250μg/ml/NaT	GE 350μg/ml/NaT	F values; (degrees of freedom)			p-values		
	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Time period	Treatment	Time period x treatment	Time period	Treatment	Time period x treatment
24h	100.0; 33.44	57.97; 9.55	51.59; 5.16	71.84; 8.41	63.94; 9.81	80.06; 9.10	71.90; 12.83	68.97; 6.19	68.81 (2,96)	18.45 (7,96)	10.82 (14,96)	<0.001	<0.001	<0.001
48h	100.0; 38.75	63.08; 8.83	31.46; 5.44	73.0; 20.88	34.23; 6.22	36.41; 5.34	44.75; 4.96	42.53; 5.51						
72h	100.0; 50.31	263.02; 25.26	68.76; 40.02	244.08; 41.26	87.04; 16.72	122.07; 33.21	160.77; 5.90	157.18; 16.88						

Two way Anova; P values of ***p < 0.001 were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts; SD = standard deviation.

Table 5.19. Post hoc Tukey test of two-way ANOVA for results of cholesterol staining after exposure to varying treatments and varying time periods in AGS cell line presenting significant results.

Cholesterol				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
24h	RPMI-1640 medium	24h	LPZ	<0.001
24h	RPMI-1640 medium	24h	NaT	<0.001
24h	RPMI-1640 medium	24h	GE 100µg/ml/NaT	<0.01
24h	RPMI-1640 medium	72h	RPMI-1640 medium	<0.001
24h	LPZ	72h	LPZ	<0.001
24h	LPZ/NaT	72h	LPZ/NaT	<0.05
24h	GE 100µg/ml/NaT	48h	GE 100µg/ml/NaT	<0.01
24h	GE 150µg/ml/NaT	48h	GE 150µg/ml/NaT	<0.001
24h	GE 250µg/ml/NaT	48h	GE 250µg/ml/NaT	<0.01
24h	GE 350µg/ml/NaT	48h	GE 350µg/ml/NaT	<0.01
48h	RPMI-1640 medium	48h	NaT	<0.001
48h	RPMI-1640 medium	48h	GE 100µg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 150µg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 250µg/ml/NaT	<0.01
48h	RPMI-1640 medium	48h	GE 350µg/ml/NaT	<0.01
48h	LPZ	72h	LPZ	<0.001
48h	LPZ/NaT	72h	LPZ/NaT	<0.001
48h	GE 250µg/ml/NaT	72h	GE 250µg/ml/NaT	<0.01
48h	GE 350µg/ml/NaT	72h	GE 350µg/ml/NaT	<0.01
72h	RPMI-1640 medium	72h	LPZ	<0.001
72h	RPMI-1640 medium	72h	LPZ/NaT	<0.001
72h	LPZ	72h	NaT	<0.001
72h	LPZ	72h	GE100µg/ml/NaT	<0.001
72h	LPZ	72h	GE 150µg/ml/NaT	<0.001
72h	LPZ	72h	GE 250µg/ml/NaT	<0.001
72h	LPZ	72h	GE 350µg/ml/NaT	<0.001
72h	NaT	72h	LPZ/NaT	<0.001
72h	NaT	72h	GE 250µg/ml/NaT	<0.001
72h	NaT	72h	GE 350µg/ml/NaT	<0.001
72h	LPZ/NaT	72h	GE 100µg/ml/NaT	<0.001
72h	LPZ/NaT	72h	GE 150µg/ml/NaT	<0.001
72h	LPZ/NaT	72h	GE 250µg/ml/NaT	<0.05
72h	LPZ/NaT	72h	GE 350µg/ml/NaT	<0.01

P values of *p < 0.05, **p < 0.01 and ***p < 0.001 were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts.

5.8.2. Phospholipids staining

In AGS model, the treatment caused a statistically significant difference depending on both, the time period and combination of the time period and treatment (Table 5.20.).

After 24 hours significant differences were noticed between cells pretreated with the lowest concentration of GE (100μg/ml) subsequent damaged with NaT and remaining three concentrations of GE, while after 72 hours GE at concentration (250μg/ml) showed increased percentage of integrated density values of phospholipids compared to the remaining three concentrations of GE, shown in Table 5.20.

After 24 hours, cells treated with NaT only showed decreased percentage of integrated density compared to cells treated with NaT after 48 and 72 hours. Also, after 24, 48 and 72 hours it was observed significant difference between cells treated with NaT only and untreated cells (Table 5.20., and Figures 5.11., 5.12. and 5.13.).

After 72 hours pretreatment with LPZ only and LPZ/NaT showed significant difference compared to those after 24 and 48 hours (Table 5.20.).

Post hoc analysis showed that integrated density values of phospolipids in cells pretreated with GE concentrations (100, 150, 250 and 350 μg/ml) damaged with NaT were significantly different between 24 and 48 hours. Also, there was no significant difference between GE and untreated controls after 24 hours, while after 48 hours, GE concentrations (100, 150, 250 and 350 μg/ml) damaged with NaT showed significantly difference compared to untreated cells, shown in Table 5.21.

In contrast, after 72 hours statistically significant difference was observed between cells treated with LPZ only compared to cells treated with NaT only and cells pretreated with GE (100, 150, 250and 350 μg/ml) and subsequent damaged with NaT. Moreover, after 72 hours cells pretreated with GE at concentrations (250 and 350 μg/ml) showed significant difference compared to the cells pretreated with NaT only. Finally, after 72 hours pretreatment with the highest concentration of GE (350 μg/ml) damaged with NaT showed the same effect as the lowest effect of GE (100 μg/ml) after 24 hours, shown in Table 5.21 and Figure 5.13.

Table 5.20. Effects of varying concentrations of varying treatments and varying time periods on the integrated density value of phospholipids in AGS cells. Data are presented as integrated density values per cell. Data are expressed as a percentage relative to untreated control.

Duration of treatment	RPMI-1640 medium	LPZ 10µM	NaT 4mM	LPZ/NaT	GE 100µg/ml/NaT	GE 150µg/ml/NaT	GE 250µg/ml/NaT	GE 350µg/ml/NaT	F values; (degrees of freedom)			p-values		
	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Time period	Treatment	Time period x treatment	Time period	Treatment	Time period x treatment
24h	100.0; 25.39	18.12; 0.69	2.75; 0.99	2.60; 0.82	73.94; 48.14	5.50; 1.86	10.02; 3.17	9.01; 3.62	68.81 (2,96)	18.45 (7,96)	10.82 (14,96)	<0.001	<0.001	<0.001
48h	100.0; 32.79	36.59; 4.77	18.54; 3.58	24.63; 4.56	15.27; 2.89	21.97; 6.46	15.93; 2.17	19.79; 3.03						
72h	100.0; 48.59	208.12; 32.76	21.43; 14.40	210.36; 57.57	21.51; 4.51	23.20; 6.81	114.16; 15.96	76.35; 12.36						

Two way Anova; P values of ***p < 0.001 were considered statistically significant. . The data shown are representative of at least three independent experiments. NaT=sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts; SD = standard deviation.

Table 5.21. Post hoc Tukey test of two-way ANOVA for results of phospholipids staining after exposure to varying treatments and varying time periods in AGS cell line presenting significant results.

Phospholipids				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
24h	RPMI-1640 medium	24h	LPZ	<0.001
24h	RPMI-1640 medium	24h	NaT	<0.001
24h	RPMI-1640 medium	24h	GE 100µg/ml/NaT	<0.001
24h	RPMI-1640 medium	72h	RPMI-1640 medium	<0.001
24h	LPZ	72h	LPZ	<0.001
24h	LPZ/NaT	72h	LPZ/NaT	<0.05
24h	GE 100µg/ml/NaT	48h	GE 100µg/ml/NaT	<0.01
24h	GE 150µg/ml/NaT	48h	GE 150µg/ml/NaT	<0.001
24h	GE 250µg/ml/NaT	48h	GE 250µg/ml/NaT	<0.001
24h	GE 350µg/ml/NaT	48h	GE 350µg/ml/NaT	<0.01
48h	RPMI-1640 medium	48h	NaT	<0.001
48h	RPMI-1640 medium	48h	GE 100µg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 150µg/ml/NaT	<0.001
48h	RPMI-1640 medium	48h	GE 250µg/ml/NaT	<0.01
48h	RPMI-1640 medium	48h	GE 350µg/ml/NaT	<0.01
48h	LPZ	72h	LPZ	<0.001
48h	LPZ/NaT	72h	LPZ/NaT	<0.001
48h	GE 250µg/ml/NaT	72h	GE 250µg/ml/NaT	<0.01
48h	GE 350µg/ml/NaT	72h	GE 350µg/ml/NaT	<0.01
72h	RPMI-1640 medium	72h	LPZ	<0.001
72h	RPMI-1640 medium	72h	LPZ/NaT	<0.001
72h	LPZ	72h	NaT	<0.001
72h	LPZ	72h	GE 100µg/ml/NaT	<0.001
72h	LPZ	72h	GE 150µg/ml/NaT	<0.001
72h	LPZ	72h	GE 250µg/ml/NaT	<0.001
72h	LPZ	72h	GE 350µg/ml/NaT	<0.001
72h	NaT	72h	LPZ/NaT	<0.001
72h	NaT	72h	GE 250µg/ml/NaT	<0.01
72h	NaT	72h	GE 350µg/ml/NaT	<0.001
72h	LPZ/NaT	72h	GE 100µg/ml/NaT	<0.001
72h	LPZ/NaT	72h	GE 150µg/ml/NaT	<0.001
72h	LPZ/NaT	72h	GE 250µg/ml/NaT	<0.05
72h	LPZ/NaT	72h	GE 350µg/ml/NaT	<0.01

P values of *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts.

5.8.3. Colocalization of cholesterol and phospholipids

In AGS model, the treatment caused a statistically significant difference depending on both, the time period and combination of the time period and treatment (Table 5.22.).

After 24 h, treatment with LPZ only caused a significant increase in colocalization and redistribution of membrane compartments, compared to the untreated control, cells treated with NaT only, and combination of LPZ/NaT treatment. Also, cells treated with LPZ alone after 24 hours showed higher percentage of colocalization compared to other groups except those treated with the lowest concentration of GE (100 μg/ml) damaged with NaT. However, after 48 and 72 hours the colocalization of the lowest concentration of GE was decreased. After 48 hours it was shown that cells treated with LPZ only had decreased percentage of colocalization compared to cells treated with the same treatment after 24 hours. After 72 hours, colocalization of cells treated with LPZ only was similar to the colocalization of LPZ only after 48 hours. However, after 72 hours, combination of LPZ/NaT and NaT only resulted in increased colocalization compared to untreated control. Interestingly, after 24 hours, lowest conce ntration of GE damaged with NaT showed significant increase of phospholipids and cholesterol dissaray compared to untreated control and remainig three concentrations of GE damaged with NaT, while after 72 hours that concentration was significantly decreased and similir to untreated control. On the other hand, after 72 hours GE concentration at (250 μg/ml) showed significant increase of colocalization compared to untreated control and reamining three concentrations of GE (Table 5.22. and Figures 5.11., 5.12., and 5.13.).

Post hoc analysis showed that there was no significant difference between GE groups and untreated control after 24 hours, however, cells pretreated with LPZ only showed significant difference compared to untreated cells after 24 hours. Moreover, after 24 hours treatment with LPZ only showed significant difference compared to (150, 250 and 350 μg/ml) of GE. The lowest concentration of GE showed significant difference during the varying time periods. After 72 hours, treatment with LPZ/NaT showed significant difference compared to cells pretreated with different concentrations of GE extracts and subsequent damaged with NaT (Table 5.23.).

Additionally, after 72 hours, it was observed significant difference in colocalization between different groups of GE pretreatments subsequently damaged with NaT (Figure 5.13.).

Table 5.22. Effects of varying concentrations of varying treatments and varying time periods on the colocalization of cholesterol and phospholipids in AGS cells. Data are presented as integrated density values per cell. Data are expressed as a percentage relative to untreated control.

Duration of treatment	RPMI-1640 medium	LPZ 10µM	NaT 4mM	LPZ/NaT	GE 100µg/ml/NaT	GE 150µg/ml/NaT	GE 250µg/ml/NaT	GE 350µg/ml/NaT	F values; (degrees of freedom)			p-values		
	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Mean; SD	Time period	Treatment	Time period x treatment	Time period	Treatment	Time period x treatment
24h	14.21; 4.20	25.24; 5.17	14.51; 2.69	6.06; 5.14	21.97; 4.35	13.09; 1.85	12.80; 1.74	14.04; 5.72	11.65 (2,108)	8.82 (7,108)	11.06 (14,108)	<0.001	<0.001	<0.001
48h	12.91; 2.50	14.42; 3.55	6.29; 0.27	12.33; 3.89	7.80; 2.75	9.85; 4.31	7.84; 1.37	11.47; 6.10						
72h	8.49; 4.94	16.21; 9.01	14.74; 6.95	28.15; 3.14	8.88; 2.32	6.99; 2.49	21.36; 2.93	8.15; 5.22						

Two way Anova; P values of ***p < 0.001 were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts; SD = standard deviation.

Table 5.23. Post hoc Tukey test of two-way ANOVA for results of cholesterol and phospholipids colocalization after exposure to varying treatments and varying time periods in AGS cell line presenting significant results.

Colocalization of phospholipids and cholesterol				
The first set of variables (A)		The second set of variables (B)		Post hoc Tukey HSD p-value A vs B
Time period	Treatment	Time period	Treatment	
24h	RPMI-1640 medium	24h	LPZ	<0.001
24h	LPZ	24h	LPZ/NaT	<0.001
24h	LPZ	24h	GE 150μg/ml/NaT	<0.01
24h	LPZ	24h	GE 250μg/ml/NaT	<0.01
24h	LPZ	24h	GE 350μg/ml/NaT	<0.05
24h	LPZ	48h	LPZ	<0.05
24h	LPZ/NaT	24h	GE 100μg/ml/NaT	<0.001
24h	LPZ/NaT	72h	LPZ/NaT	<0.001
24h	GE 100μg/ml/NaT	48h	GE 100μg/ml/NaT	<0.001
24h	GE 100μg/ml/NaT	72h	GE 100μg/ml/NaT	<0.001
48h	LPZ/NaT	72h	LPZ/NaT	<0.001
48h	GE 250μg/ml/NaT	72h	GE 250μg/ml/NaT	<0.001
72h	RPMI-1640 medium	72h	LPZ/NaT	<0.001
72h	RPMI-1640 medium	72h	GE 250μg/ml/NaT	<0.05
72h	LPZ	72h	LPZ/NaT	<0.01
72h	NaT	72h	LPZ/NaT	<0.001
72h	LPZ/NaT	72h	GE 100μg/ml/NaT	<0.001
72h	LPZ/NaT	72h	GE 150μg/ml/NaT	<0.001
72h	LPZ/NaT	72h	GE 350μg/ml/NaT	<0.001
72h	GE 100μg/ml/NaT	72h	GE 250μg/ml/NaT	<0.001
72h	GE 150μg/ml/NaT	72h	GE 250μg/ml/NaT	<0.001
72h	GE 250μg/ml/NaT	72h	GE 350μg/ml/NaT	<0.001

P values of $*p < 0.05$, $**p < 0.01$ and $***p < 0.001$ were considered statistically significant. The data shown are representative of at least three independent experiments. NaT = sodium taurocholate; LPZ = lansoprazole; GE = garlic extracts.

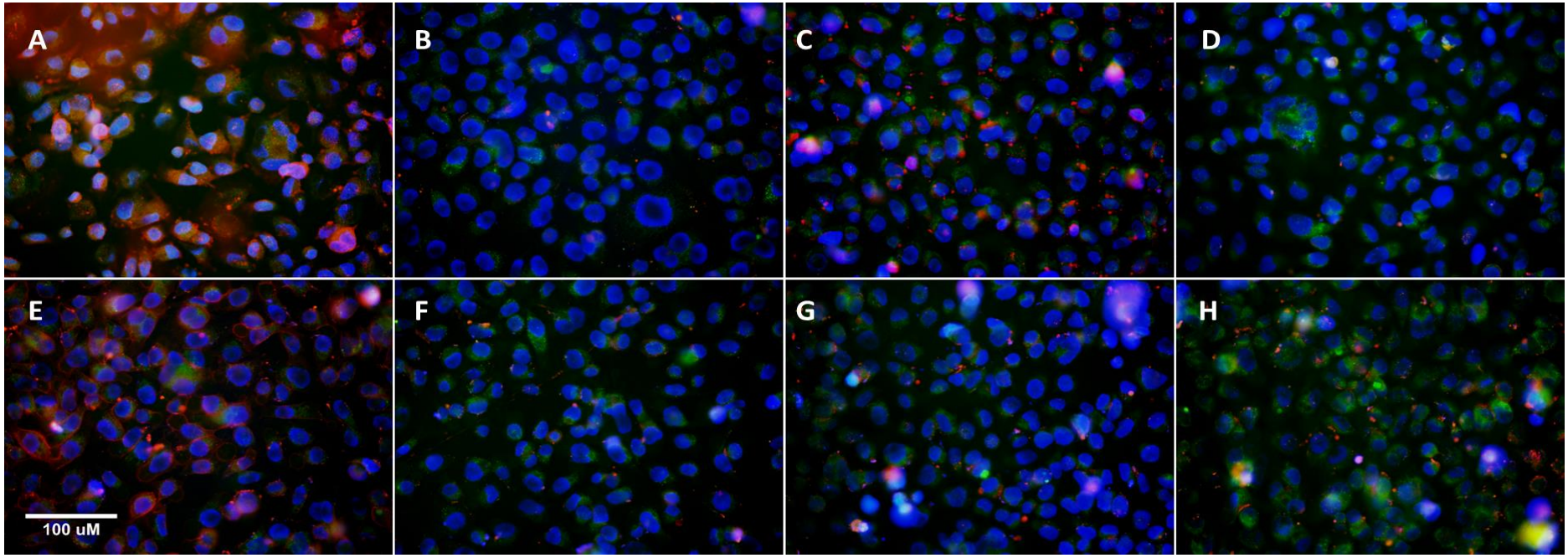

Figure 5.11.Visualization of the cholesterol and phospholipids in AGS cells pretreated with LPZ and GE for 24 hours, and treated with NaT for 1hour. The figure was made by the author.

Blue - nuclei stained with DAPI, red - phospholipids, green - cholesterol, orange - colocalization . A - RPMI 1640 (untreated control), B - lansoprazole (LPZ 10µm), C - sodium taurocholate (NaT/4mM), D - sodium taurocholate (NaT 4mM) and lansoprazole (LPZ 10µM), E - sodium taurocholate (NaT 4mM) and garlic extracts (GE 100µg/ml), F - sodium taurocholate (NaT 4mM) and garlic extracts (GE 150 µg/ml), G - sodium taurocholate (4mM) and garlic extracts (GE 250µg/ml), H - sodium taurocholate (4mM) and garlic extracts (GE 350µg/ml). Size bar represents 100 µm.

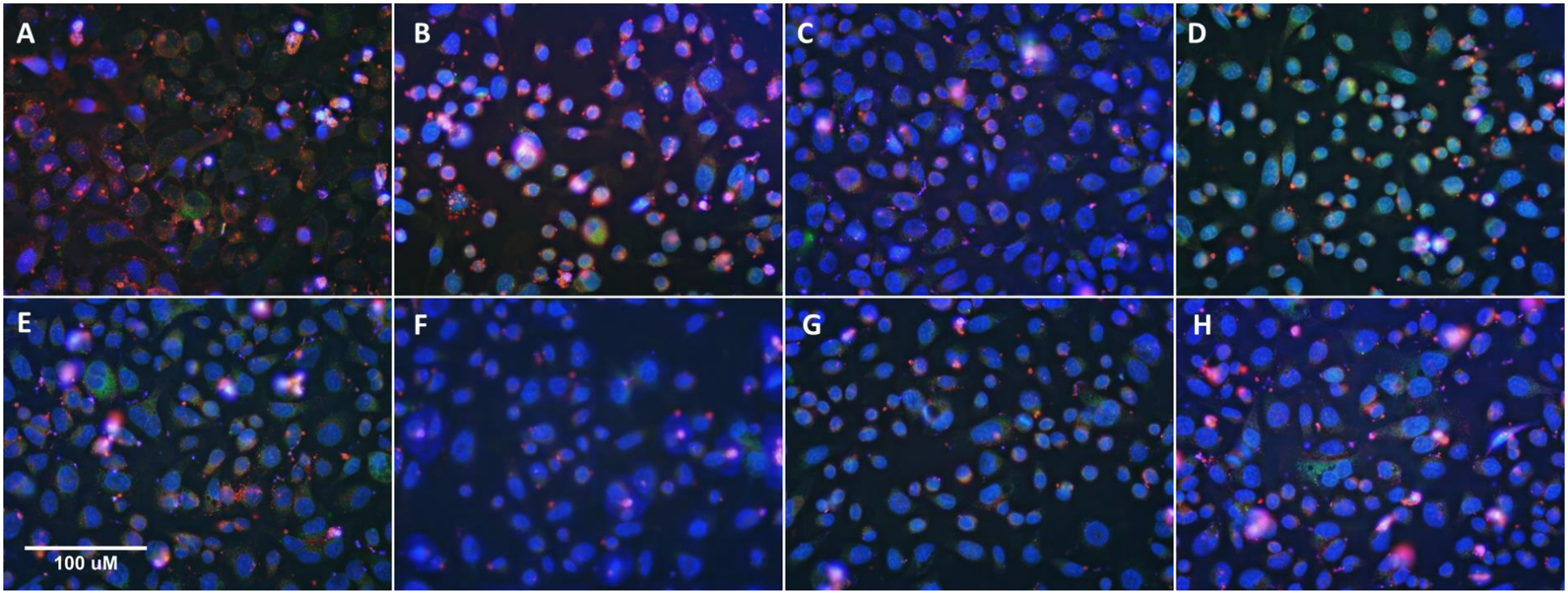

Figure 5.12. Visualization of the cholesterol and phospholipids in AGS cells pretreated with LPZ and GE for 48 hours, and treated with NaT for 1hour. The figure was made by the author.

Blue - nuclei stained with DAPI, red - phospholipids, green - cholesterol, orange - colocalization . A - RPMI 1640 (untreated control), B - lansoprazole (LPZ 10µm), C - sodium taurocholate (NaT/4mM), D - sodium taurocholate (NaT 4mM) and lansoprazole (LPZ 10µM), E - sodium taurocholate (NaT 4mM) and garlic extracts (GE 100µg/ml), F - sodium taurocholate (NaT 4mM) and garlic extracts (GE 150 µg/ml), G - sodium taurocholate (4mM) and garlic extracts (GE 250µg/ml), H - sodium taurocholate (4mM) and garlic extracts (GE 350µg/ml). Size bar represents 100 µm.

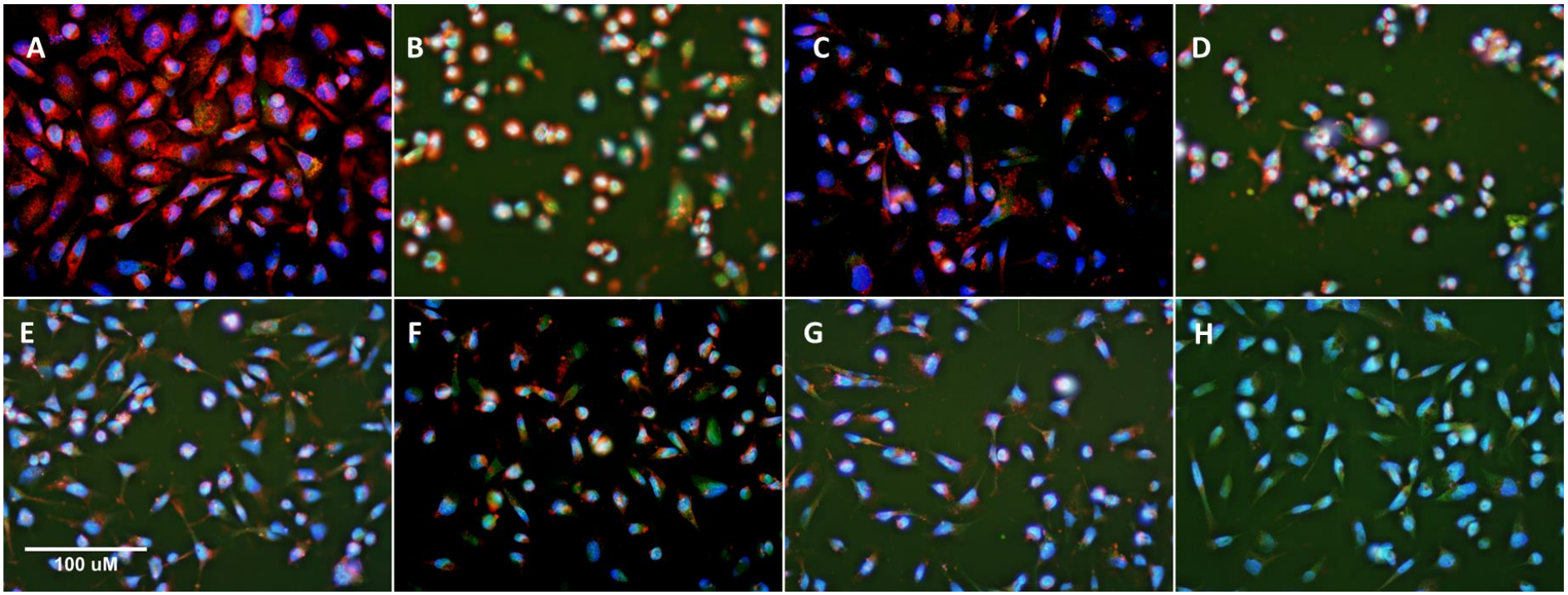

Figure 5.13. Visualization of the cholesterol and phospholipids in AGS cells pretreated with LPZ and GE for 72 hours, and treated with NaT for 1hour. The figure was made by the author.

Blue - nuclei stained with DAPI, red - phospholipids, green - cholesterol, orange - colocalization . A - RPMI 1640 (untreated control), B - lansoprazole (LPZ 10µm), C - sodium taurocholate (NaT/4mM), D - sodium taurocholate (NaT 4mM) and lansoprazole (LPZ 10µM), E - sodium taurocholate (NaT 4mM) and garlic extracts (GE 100µg/ml), F - sodium taurocholate (NaT 4mM) and garlic extracts (GE 150 µg/ml), G - sodium taurocholate (4mM) and garlic extracts (GE 250µg/ml), H - sodium taurocholate (4mM) and garlic extracts (GE 350µg/ml). Size bar represents 100 µm.

6. DISCUSSION

PUD is a chronic multifactorial disease, affecting up to 10% of the world's population (4, 33). Conventional treatments of PUD, such as H2 receptor antagonists and PPIs, have demonstrated adverse effects, relapses, and various drug interactions. In contrast, it has been found that medicinal plants and their chemical crude extracts are useful in the prevention and treatment of several diseases. Hence, in the past few years, interest in alternative therapies and the usage of herbal products has grown, especially for those produced from medicinal plants (31). GE have shown promise in the treatment of several disease, and have been proposed as an appropriate preventive treatment for PUD, because of their antioxidant activity (34, 35). Despite other positive effects of garlic and its extracts, the potential effect on the prevention of PUD has not yet been investigated.

6.1. Sodium taurocholate induced epithelial damage as a model of peptic ulcer disease

The gastroprotective effect of different ingredients against the bile-induced injury on the gastric mucosa (48, 56). Reflux of bile salts from the duodenum into the stomach was investigated had long been thought to be one of the etiology of gastric ulcer. The reflux mechanism was thought to be caused by mucosal damage, and acid hyposcretion has often been observed. Mechanism of acid hyposecretion is considered to be due to hydrogen ion back diffusion, replaced by a sodium ion moving in the opposite direction to the stomach lumen (81). The effect of instillation of NaT on stimulated stomachs of patients with duodenal ulcer was investigated. It was reported that instillation of NaT into the stomach led to reductions in pentagastrin-stimulated volume, acid and sodium outputs. These observations are not consistent with the back-diffusion theory, but suggest direct inhibition of parietal cells gastric secretion with NaT (82). However, the effect of NaT in gastric epithelial cells is not fully understood.

Hoshino at al., reported that all gastric irritants tested such as bile salts, ethanol, NSAIDs, hydrogen peroxide, etc., induced apoptosis through the pathway in which mitochondrial dysfunction plays significant role (83), while Theoduloz at al., suggested that pathogenesis of NaT-induced gastric lesions occurs directly and indirectly through oxygen-

derived free radicals (56). Determining the survival rate of experimental models in a dose-dependent and time-dependent manner is important to properly monitor the cells response to NaT toxicity effect and provides a preliminary measure of how to proceed further with the experiments. In our study, the toxicity of the NaT on the AGS cell viability were assessed by MTT assay after treatment with four different doses of NaT; 2, 4, 8 and 10 mM, at different time periods: 30 minutes, 1, 4, 12 and 24 hours, respectively.

We showed that treatment with 4mM NaT for 60 minutes caused a reduction of 50.03% in cell viability compared to untreated controls (Table 5.1.). This concentration and exposure time was used in all subsequent experiments on AGS model of PUD. In contrast to our study, Theoduloz at al., used 8 mM NaT for 30 min to cause mucosal injury on AGS and MRC-5 fibroblasts (56). In our study, this concentration and time of exposure produced 60% cell survival.

In our study, the toxic effect of NaT after 4, 12, and 24 hours caused a significant decline in cell survival even at the lowest concentration. For instance, 2 mM NaT caused 48% cell survival after 4 hours, 4 mM, 30%, 8 mM 15%, while 10 mM NaT resulted in 13% of cell survival. Moreover, cells exposed to NaT for 12 and 24 hours, showed significant decreased in viability; after 24 hours, 10 mM NaT caused 6% of cell viability. MTT assay is a widely used method for determination of cell viability, however, it requires a well-calibrated experimental setup with minimal variation among experiments. Also, MTT assay relies on mitochondria to metabolise yellow tetrazolium bromide to purple formazan, while in apoptosis, mitochondria undergo fragmentation, which caused reduction of dye formation (84). Hence, these limitations of the MTT method may be the reasons for the variability of cell survival in different experiments.

6.2. Gastroprotective effect of garlic extracts in a cell culture model of peptic ulcer disease

Examination of various plant extracts has led to the discovery of new pharmacological ingredients with effective gastroprotective activity. For instance, antioxidant properties of GE were used as the main reason for the treatment of PUD (34). In the last few years, a number of *in vivo* studies have shown the antiproliferative and antioxidant activity effects of several compounds derived from GE. These include flavonoids, flavanols, polyphenols, and sulfur-

containing compounds, known as main compounds of its bioactivity, which play significant role in scavenging free radicals (4).

However, the potential gastroprotective effect of GE in *in vitro* studies specially on the gastric human cell line has not yet been fully elucidated. Our studies here demonstrated AGS cells, pretreatment with GE at concentrations 100, 150, 250 and 350 μg/ml after 24, 48 or 72 hours showed statistically significant higher survival compared to NaT-alone treated cells, while there was no significant difference compared to untreated cells. These results indicate that cytoprotective compounds of garlic have significant role in protection of AGS cells against NaT-induced damage binding bile salts, consequently forming a barrier to avoid the mucous membranes injury. Our results can be compared with previous investigation which demonstrated antioxidant dose dependent effect in reducing oxidative stress (65). Further, no statistically significant difference was observed between 150, 250 and 350 μg/ml concentrations of GE and cells treated with LPZ/NaT in any period of time, while concentration of GE at 100 μg/ml compared to LPZ/NaT was statistically significant after 48 hours. Furthermore, no difference was present in the highest concentration of GE compared to LPZ and untreated control which indicates on synergistic effect between GE and LPZ.

Suzukiy at al., demonstrated that LPZ enhances the proliferation and migration of mouse gastric mucosal cell line (GSM06) during gastric mucosal wound repair (85). GE were used in the treatment of numerous disorders such as in maintenance of body electrolytes, hypertension, and also as an antifungal, antibacterial, and antiviral (86). Also, Adeniyi et al. showed that *H. Pylori* was inhibited by a concentration of GE at a dose of 6 mg/ml. Similar to our study, the aim of this study was also to focus on assessing the positive therapeutic effect of garlic pretreatment in ulcus disease, and to examine the potential mechanisms of action of garlic and its antioxidant effect (87). Other studies have shown that GE (45) or garlic powder tablets (88) maintain *in vitro* activity against *H. pylori* and ulcus disease (89). In addition, it has been shown that plants with antioxidant capacity as the main mechanism are used as a herbal container for the treatment of ulcer disease. Moreover, medicinal herbs, including garlic, have achieved their medicinal properties thanks to their ability to produce renewable secondary metabolites, also known as phytochemical ingredients (4). Numerous studies have shown that the crucial role of garlic has been observed in the antioxidant effect by removing ROS, reduces the induction of antioxidants in serum glucose enzyme and inhibits lipoprotein oxidation. Garlic extracts were

shown to have antitumorigenic effect by promoting apoptosis and inducing cell cycle arrest (46).

The results of our study showed that pretreatment with GE for 24 hours leads to an increase in PCNA levels, these levels were similar to the pre-treatment with LPZ. Our results has twofold significance, the first indicating that cell division has been activated (which could be demonstrated by further experimentation with the addition of 5-bromo-2′-deoxyuridine) while the second reason for the increase in PCNA levels suggests that cells began to repair DNA damage due to damage to the NaT. Garlic most likely does not cause cell death but encourages cells to replicate DNA which is an extremely beneficial effect especially since gastric damage caused by gastritis can be repaired more quickly. However, after 48 hours, there was a decline in PCNA levels, and re-growth of PCNA after 72 hours. It is still unclear why the decline occurred after 48 hours andthere was re-recovery after 72 hours, therefore, further study of cell exposure to longer pretreatments could elucidate whether the growth of PCNA levels corresponds to shorter or longer pretreatments with GE.

Finally, from these results, we can conclude that GE is a potent synergist of LPZ and can promote ulcer healing and prevent recurrence.

6.3. Immunoassay analysis of GSH and PGE2 in a cell culture model of peptic ulcer disease

Previous *in vitro* and *in vivo* studies have shown that intracellular GSH protects gastric mucosal cells against ethanol-induced damage (3, 90). In several studies plant terpenes and their derivatives have been shown to have gastroprotective activity and produced a significant rise of GSH concentration in AGS cells (91, 92). Rodriguez at al., results were confirmed by our current study in which the concentration of GSH increased, after the cells were pretreated with GE and subsequently damaged by NaT. Our data on GSH levels clearly showed that NaT damage increased oxidative stress, while pretreatment with GE resulted in a beneficial effect on reducing oxidative stress. After 24, 48 and 78 hours, treatment with NaT alone showed significant decrease of GSH levels compared to untreated control and cells treated with LPZ. Moreover, pretreatment with GE for 24, 48 and 72 hours showed significant recovery of GSH levels compared to the cells damaged with NaT. Interestingly, the highest levels of GSH were

recorded after 24 hours., while after 48 and 72 hours the levels of GSH were lower, but still greater than GSH levels in cells treated with NaT. Our study was confirmed by a previous study that showed that GSH values were also highest after 24-hour antioxidant pretreatment (65). The mechanism of gastroprotective effects of the GE on gastric damage induced by NaT may be related to its anti-inflammatory actions and its antioxidant properties, which increases GSH activities (93).

Our results confirmed the strong role of garlic as a natural antioxidant against oxidative damage. In a study performed by Stagos et al., quercetin, a well known plant polyphenol found in GE significantly increased GSH level and inhibited the reduction of GSH both *in vivo* and *in vitro* (94). Therefore, GSH activity of GE provide another evidence to support usage of GE in prevention of PUD. Furthermore, the role of GE in previous *in vivo* and in *vivo* studies has shown preservation of glutathione reductase and peroxidase levels, where glutathione reductase was involved in the conversion of oxidized glutathione to glutathione (95, 96).

PGE2 is a powerful inhibitor of gastric acid secretion and stimulus of gastric mucus and bicarbonate synthesis. In order to determine the cytoprotective effect of PGE2 on gastric mucosal cells, in study from Hoshino at al., studied the effect of PGE2 on gastric induced apoptosis in guinea pig gastric mucosal cells. This research demonstrated that PGE2 inhibited the apoptosis caused by numeros irritants, such as ethanol, hydrochloric acid and hydrogen peroxide (83). PGE2-mediated gastroprotective role of *J. sambac* had also been investigated by Miller. The results of this research showed that the gastroprotective effect of *J. sambac* is mediated by PGE2. Moreover, measurement of PGE2 levels demonstrated that its biosynthesis was significantly increased by the *J. sambac* compound (97). PGs have shown crucial role in mucosal defense; they stimulate bicarbonate and mucus secretion, increase epithelial cell resistance to cytotoxin-induced injury, and maintain mucosa blood flow. PGs maintain the synthesis and secretion of gastric mucus, which is also one of the additional proofs of their gastroprotective role (98).

In our study, NaT as gastric irritant did not show significant effect on increased secretion of PGE2 compared to LPZ or untreated control, while in combination with LPZ secretion of PG was increased. This would suggest that our model of PUD is a very complex inflammatory process involving different mediators of inflammation, however damage caused with NaT solely did not show the most significant role in PGE2 synthesis. However, in previous study on

rat models, single dose of LPZ did not affect the synthesis of PG (99). Also, omeprazole failed to affect PG production from gastric mucosal cells (100).

De Olinda et al., reported that *Magonia glabrata* protected against gastric lesions caused by indomethacin and ethanol by increasing production of PG (101). In our study, pretreatment with GE at concentrations 100, 150, 250 and 350 μg/ml showed higher stimulation of PGE2 synthesis compared to untreated cells and cells treated with LPZ only. However, stimulation of PGE2 synthesis was greatest after 24 hours, while after 48 and 72 hours a significant decrease in PGE2 concentrations. Also, the highest concentration of GE (350 μg/ml) presented a strong effect in AGS cell line increasing the levels of PGE2 compared to the cells damaged with NaT. Hence, results of our study are in agreement with study of Theoduloz at al., that showed how derivates of medicinal plant Jatropha isabelii affect increases of PGE2 synthesis after damage with bile salt (56). It is known that garlic contains fatty acids responsible for increasing level of PG which modulates inflammation. Several studies have showed that flavonoids (abundant in garlic) increased the gastric PGE2 level in an ethanol and acetic-induced gastric model in mice (102, 103). These studies suggest a significant role of medicinal plants on increasing PG production. Therefore, more studies are needed to elucidate the mechanisms by which garlic stimulates PG synthesis.

6.4. The role of SOD, ABCG2, NFKB2 and TRX1 as markers in oxidative and cellular damage caused by sodium taurocholate in peptic ulcer disease

SOD as antioxidant enzyme specialized for physiological defense strategies plays significant role against ROS and free radicals (104, 105). It has been shown that SOD expression in cells loaded with free radicals is decreased, while expression was increased in those treated with antioxidants (61). In our study, treatment with GE resulted in increased SOD expression. Therefore, SOD expression could be used as an indicator of the effectiveness of the antioxidant effect, and could play a significant role in gastroprotective effect. Previous studies support this hypothesis; Wu at al., showed that water extract of *Acrostichum aureum* increased SOD in ethanol-induced gastric injury model in rats (106). Study from Tahir at al., showed that ethanolic extract of *Hyssopus officinalis L*. significantly increased the mucus barrier and SOD, while decreased the ulcer indices (107). In our study, SOD expression was significantly reduced after the onset of NaT-induced ulcer, and this reduction was prevented by prior treatment with

GE. Therefore, in our study decreased SOD expression in NaT-damaged gastric epithelial cells may be due to increased production of ROS leading to decreased activity of this enzyme (98). The significant inhibition of PUD in cells pretreated with GE was comparable with LPZ which is a standard drug used for curing PUD. These results indicated the participation of GE as antioxidants in the gatroprotective effect of GE in PUD.

Protein product of the ABCG2 gene has been found in various gastrointestinal tissues (67). Previous research reported that *H. pylori* in PUD could increase the ABCG2 gene expression (108). Some studies have shown that *H. pylori* infection is associated with the intensity of ABCG2 expression in the development of peptic ulcer. Expression was significantly more common in higher intensity of *H. pylori* infection compared to low intesity infection (67). In contrast, in our model of PUD was observed downregulation of ABCG2 expression in cells treated with NaT. Morever, cells pretreated with the GE and subsequently exposed to NaT, and cells pretreated with LPZ were shown decrease expression of ABCG2. Untreated cells had higher expression of ABCG2. Our results indicate that the differences in the modulatory effects of NaT and GE on ABCG2 gene expression could be related to the different experimental paradigms used. Our conclusion is supporeted by previous researches that showed the level of ABCG2 expression differed among numerous studies and cases (67). Because, ABCG2 protein is not found in the epithelium of the stomach, Diestra at al., reported that ABCG2 gene expression could be derive from the capillaries endothelial cells, where expression of the protein was demonstrated (109). Hence, it could be speculated that expression of ABCG2 gene in model of PUD could be under an influence of NaT. Considering this and the previous findings, the decreased expression of ABCG2 in the model of ulcer disease caused by NaT damage remains unanswered. On the other hand, changes in the ABCG2 gene such as single nucletiode polymophism (SNPs) or mRNA gene expression, may affect gene function or its level. This can lead to loss its protective functions and thus increases the risk of developing an ulcer. However, it would be interesting to investigate the changes in the ABCG2 gene, as some studies have shown that this could be one of the convincing genetic factors contributing to the development of PUD (110, 111).

NF-κB plays significant role in relief complications in chronic diseases and stress conditions. The sulfur compounds present in aged garlic extracts are known as key regulators of the inflammatory response, acting by reducing NF-κB activation, consequently preventing the production of proinflammatory cytokines such as IL-1 and IL-6. Several studies have shown

that is activated by inflammatory factors such as IL-8 and *H.pylori* during development of PUD (92, 112). Significant number of chemical compounds in herbal plants, such as flavonoids, phenolic compounds, coumarins, alkaloids, essential oils etc., showed anti-inflammatory activity by inhibiting molecular meta-inflammatory mediators in inflammatory responses (113, 114). In a recent study from Jeong at al., *Populus deltoides* leaf extract which has strong anti-inflammatory effect significantly inhibited the levels of NF-κB activation (115). In this study, the IκBα phosphorylation and inhibition of p65, along with nuclear translocation by leaf extracs of *P. deltoides*, showed that the their inflammatory response is connected with NF-κB signaling. Geng et al. demonstrated that Jurkat T cells treated with S-allylcysteine (SAC), a garlic extract, were able to prevent the NF-κB activation, while Schäfer at al., reported the ability of the garlic components SAC, allicin and diallyldisulfide to inhibit activation of NF-κB (116, 117). The modulating effects of garlic components in human blood have been shown to be associated with inhibition of NF-κB activity (118). In contrast, NFkB is crucial in modulating the expression of proinflammatory genes, such as COX-2 (119). Increased NF-κB activity leads to several inflammatory diseases, such as atherosclerosis and arthritis (120). In agreement, our data showed that GE inhibited NF-κB activation. Obviously, GE have strong influence on NF-κB regulatory pathway under stress conditons. Therefore, we can conclude that inhibition of NFKB2 gene expression could belong to the major inhibitory pathway of COX-2 and PGE2, after treatment with GE. Zebrowska at al., showed that the relative expression level of NFKB2 is decreased in gastric cancer as opposed to PUD (69). In our current study, there was a significant upregulation of NFKB2 expression in cells treated with NaT compared to other subgroups, indicating that NaT increases cellular damage and could have a significant role in development of PUD. Taken together, in our further research, it is crucial to elucidate which of the mediator inhibitors and regulators of inflammatory reactions are mediated by NF-κB the modulation signaling pathway in AGS model of PUD.

Aerobic organisms have a developed TRX system of antioxidant proteins that contributes to maintaining a reducing environment in cells. Also, TRX1 as a small multifunctional redox protein exists widely in organism, induced by numerous types of stresses and protects cells from several stresses caused by harmful substances, bacterium or virus (121). Both TRX isoforms, TRX1 and TRX2, have also been shown to participate in different cellular functions and lead to the activation of various molecular mechanisms including redox balance, cell proliferation and DNA replication (122). Previous studies showed that TRX-1 expression

in transgenic mice decreased focal ischemic brain damage, and thioacetamide or lipopolysaccharide-induced hepatitis (123, 124). Kawasaki at al., demonstrated that TRX1 suppresses gastritis induced by *H. Pylori* (125). However, it is not clear whether TRX-1 is involved in the host defense mechanism against PUD caused by bacteria or bile acid and bile salt. Our results could suggest a positive response, as we observed strong TRX1 expression in the cells pretreated with GE and LPZ.

Finally, in our study gene expression was determined after 24 hours because we were interested in the initial response of the cells to the short-term effect of GE and NaT, because in that time period there was significant effect on GSH and PGE2 synthesis. However, membrane stability and cellular antioxidant response are more informative after 48 and 72 hours. This is primarily because the cells try to defend themselves long term against membrane disruption by several mechanisms that require a complete analysis of a transcriptome that is beyond the scope of the dissertation. Therefore, it would be interesting precisely predict and elucidate the mechanisms of NaT toxicity and the gastroprotective effect of GE in further investigations by microarray analysis of gene expression in AGS cells at three time periods (24, 48 and 72 hours).

6.5. F-actin distribution as in a cell culture model of peptic ulcer disease

Actin cytoskeleton is a dynamic, fibrous network which is regulated with cordinated action of actin-binding proteins. Prevoius studies had shown that deformations of the actin cytoskeleton are involved in the epithelial cell damage induced by the taurocholate, ethanol and acetylsalicylic acid. However, ROS seems to underlie ethanol, but not acetylsalicylic acid or taurocholate, induced cytoskeletal disruption (126). Quite unexpectedly, in our study damage with NaT did not cause reduction of F-actin distribution, moreover, after 72 hours cells treated with NaT for 1 h showed the highest amount of F-actin. After 72 hours, the same results were observed in the cells treated with LPZ only, however, after 24 hours the amount of F-actin was decreased. Hence, we can only assume that the reduced distribution of F actin, after 24 hours with the addition of LPZ is the result of actin filaments reorganization. On the other side, the treatment with NaT as oxidative stress on F actin structure is less pronounced. Our data are comparable with previous study on rat gastric mucosal cells which had shown that exposure to 1-5 mmol/L taurocholate did not induce any changes in the actin bundles, while actin were moderately damaged by 10 mmol/L of taurocholate (126).

Gruhlke at al., had shown in their study that after treatment with 25-100 μM allicin for 10 minutes, the cells become amorphous. At the same time, actin filaments were lost. Furthermore, the nucleus was poorly visible in the treatment with allicin, by staining cells with DAPI (127). The reason for this is probable due to the oxidation of S-thioalylations of actin and actin-binding proteins that regulate the composition and disassembly of actin filaments. Although cytoskeletal proteins are among the most abundant S-thioallylated proteins, the mechanism of actin cytoskeletal disorders remains unclear. In our study after 24 hours pretreatment with the highest concentration of GE damaged with NaT had the highest distribution of F actin compared to the remaining three concentrations of garlic, while after 72 it showed a significant decrease, and the lowest distribution of F-actin was observed. However, some research has shown that there are parallels with the reversible depolymerization; polymerization of actin filaments using glutathionylation; deglutathylation, respectively (128, 129). Dumontet at al., had shown that treatment with low doses of 0.5 μM allicin for 30 minutes had no effect on actin cytoskeleton, leading to the conclusion that allicin inhibits cell division by interfering with spindle formation during mitosis. In human T-cells exposure to higher concentrations of 25 μM allicin for 1 hour caused inhibition of actin polymerization (130).

Recently study had shown that the cytoplasmic branches of the actin cytoskeleton are lost in allicin-treated cells. This indicate that GE has strong benefical effect on the actin cytoskeleton (127). Therefore, the results from our study support the protective role of GE in AGS cells damaged with NaT. Notwithstanding, proteins of cytoskeleton are most abundantly S-thioallylated proteins, but the mechanism of actin cytoskeleton disruption has to be further investigated. With these results we could conclude that short-term pretreatment with the highest concentration of GE achieved significant gastroprotective role, while exposure of cells with that concentration for 72 hours caused an oxidative effect. Finally, GE can cause apoptosis or necrosis depending on the dose and duration of treatment, however biocompatible doses affect signaling cascades and cellular metabolism.

6.6. Morphological changes of the cell membrane in the cell culture model of peptic ulcer disease

Cell membrane is continuously changing interface of the cell with everything that surrounds it and its composition is tightly regulated, and ratios of PL and CH are important for

proper functioning. LR are important part of the cell membrane and it serves as anchoring spot for transmembrane receptors such as insulin receptor and it will accommodate glycolipids that are important in cell to cell communications (76). NaT has detergent like properties, and because of that, high concentrations of bile salts have been used in numerous studies as membrane lysis agents (131, 132). Hence, NaT had expected effects on the cell membrane washing out both CH and PL parts of the membrane. In particular, after 24 hours, cells damaged with NaT showed decreased integrated density of PL compared to cells treated with NaT after 48 and 72 hours. NaT also had an effect on washing out CH in all three time periods, but still less than in PL. After 24 and 48 hours our study has shown unexpected effect when colocalization of CH and PL stains were examined with no significant change in redistribution. This is usually not the case when detergents are applied on the cell membranes because it will cause a partial extraction of PL part of the membrane leaving behind detergent resistant and CH rich microdomains or it will cause total disarray within the cell membrane (133-135). However, after 72 h, exposure to 4 mM NaT for 1 h led to expected significant change in redistribution of CH and PL. Hence, our study coincides with study from Powell and his colleagues which shown that the signaling effects of bile acids and salts can correlate with hydrophobicity and membrane affinity in HCT116 cells (136). Therefore, bile salts may interfere with plasma membrane organization and affect cellular signaling. Although there is still no evidence for membrane-mediated effects of bile salts on cell surface signaling components.

Our studies also demonstrated that cells pretreated with the highest GE concentration and subsequently damaged with NaT in all three time periods did not show significant redistribution of PL and CH, indicating a beneficial effect of garlic in our model. LPZ showed increased redistribution in all three time periods, however, it was the most pronounced after 24 and 72 hours. What is interesting in our study is that LPZ had similar extracting effects on the cell membranes as NaT did, resulting significant increase in mixing of cholesterol and phospholipids thus causing disarray. Fako at al., investigated the effect of LPZ on fatty acid synthase in live cells by determining lipid synthesis. The result of their research showed that LPZ inhibits cellular fatty acids synthase activity in the cells (137). This effects with short-term exposure were not previously attributed to LPZ and it can stem from it affecting de novo synthesis of the fatty acids and shifting cholesterol metabolism which was previously described in prolonged exposure to the LPZ (138-140).

Various reactions associated with phytochemicals such as garlic occur in the membrane lipid bilayers or in the environment of membrane lipids. For this reason, the interaction with the lipids which make biomembrane is mentioned as one of the crucial mechanisms resulting from the different effect of phytochemicals (141). Tsuchiya et al., found that methanol extracts from the wild plant *Evodiopanax innovans* lead to a decrease in the fluidity of biomimetic CH and PL membranes (142). Rendu at al., demonstrated that ajoene an antiplatelet compound derived from garlic reduced the microviscosity of lipid membranes by acting on the inner side of the lipid bilayers but not affecting the external hydrophilic components (143). The use of garlic and the effect of its extracts as a new approach to the medicinal treatment have been relatively studied due to their interaction with lipids and the effect on changing membrane fluidity (142). For instance, in the tumor cells, diallyl sulfide (DAS) a component of garlic caused rigidifying of biomembranes interacting with its inherent lipids. In fact, membrane rigidification causes prevention lipid peroxidation by interfering with free radicals diffusion in membrane lipid bilayers and reduces it the effectiveness of their reaction (144). Other studies have shown that allyl derivatives modify membrane fluidity by interacting with the membrane lipids, which would pharmacologically mean that it may be one of the possible mechanism in the healing effect of garlic (145, 146). The cellular and structural specificity of membrane rigidity is related to the membrane lipid composition of target cells and the hydrophobicity of different extracts of garlic (147). In our study, treatments with GE could not save the membrane PL and CL completely but the trend of preservation of CH and PL and its rations within the membrane was observed despite being washed out with NaT. Hence, this is a good marker for further *in vivo* investigations on gastric damage rat model.

7. CONCLUSIONS

Based on this study results, the following conclusions can be drawn:

- The toxicity of the NaT on the AGS cell viability showed that reduction of cell viability by 50% required 4 mM NaT after exposure for 1 hour.
- Pretreatment wih GE at concentrations 100, 150, 250 and 350 μg/ml for 24, 48 and 72 hours showed statistically significant higher survival compared to NaT-alone treated cells.
- Pretreatment with the highest concentration of GE for 72 hours subsequently damaged with NaT showed the highest cell viability.
- Staining cells for PCNA indicated that mitotic potential of cells pretreated with 150, 250 and 350 μg/ml of GE for 24 and 72 hours exposed to NaT was increased, while in cells pretreated with 150, 250 and 350 μg/ml of GE for 48 exposed to NaT mitotic potential was decreased.
- Treatment with NaT resulted in a decrease in GSH concentrations, while pretreatment with GE exposed to NaT led to recovery and an increase in GSH concentrations.
- Pretreatment with GE for 24 hours resulted in the highest concentrations of GSH, while after 48 and 72 hours GSH concentrations started to decrease. Also, the most significant recovery of GSH was achieved with the highest concentration of GE.
- Pretreatment with different concentrations of GE for 24, 48 and 72 hours increased PGE2 concentrations, while exposure of cells to NaT showed decreased synthesis of PGE2.
- Treatment with NaT showed decreased expression of SOD, while pretreatment with GE exposed to NaT showed increased expression. The strongest expression of SOD was observed in the pretreatment with LPZ and LPZ/NaT.
- Treatment with NaT, and pretreatment with LPZ only, LPZ and GE subsequently exposed to NaT caused decreased expression of ABCG2.
- Treatment with NaT showed increased expression of NFKB2, while pretreatment with LPZ only, LPZ and GE subsequently exposed to NaT showed significantly decreased expression of NFKB2 compared to the cells treated with NaT solely, but increased compared to untreated control.

- Treatment with NaT showed decreased expression of TRX1 compared to untreated control, while pretreatment with LPZ and GE subsequently exposed to NaT caused increased expression compared to the treatment with NaT only, but lower than untreated control and treatment with LPZ only.
- Positive correlation of pretreatment with the highest concentration of GE damaged with NaT and cell survival, synthesis of GSH and PGE2 was observed.
- Positive correlation of SOD, NFKB2 and TRX 1 with LPZ and GE pretreatment were seen, while ABCG2 expression was not changed.
- After 48 hours F-actin distribution was significantly pronounced in cells treated with NaT, cells treated with LPZ only and cells pretreated with LPZ exposed to NaT.
- After 24 hours, pretreatment with the highest concentration of GE damaged with NaT led to the significantly pronounced distribution of F-actin, while after 72 hours distribution was less pronounced.
- After 24, 48 and 72 hours, cells damaged with NaT significantly decreased integrated density values of CL and PL, while colocalization of CL and PL stains were examined with no significant changes in redistribution.
- Pretreatment with LPZ for 24 and 48 hours significantly decreased integrated density values of CL and PL, with significant changes in redistribution, while pretreatment for 72 hours significantly increased density values of PL and CL, causing significant increase in mixing PH and CL dissaray.
- Pretretments with GE exposed to NaT did not save the PL and CH completely, but the trend of preservation of CH and PL and its rations within the membrane was observed despite being washed out with NaT.

8. SUMMARY

Objectives: PUD is a chronic disease affecting up to 10% of the world's population. A cellular model of PUD can be established in AGS by NaT. The aim of the study was to explore effects of GE pretreatment and LPZ addition in the cell culture model of PUD by examining oxidative stress, F-actin distribution and determine morphological changes in cell membrane structure.

Study design: Cells were plated at a density of $4x10^5$ cells/mL in 6-well plates and were grown for 24 hours. Cells were pretreated with LPZ and GE for 24, 48 and 72 hours and subsequently exposed to NaT for 1 h.

Material and methods: The AGS cell line was used as a model of PUD. The establishment of the NaT model was determined by the MTT test. Evaluation was done by determination of mitotic potential of the cells by by staining against (PCNA); GSH and PGE2 concentrations by ELISA; AGS proliferation by cell counting; expression of SOD, ABCG2, NFKB2 and TRX1 by RT PCR; F-actin cytoskeleton visualization by semi-quantification of Rhodamine Phalloidin stain; morphological changes in cell membrane structure by using CL and PH specific stains.

Results: Our results showed significant reduction of cell damage after NaT incubation when the AGS cells were pretreated with LPZ ($p<0.001$) and increasing concentrations of GE ($p<0.001$). Pretreatment with different concentrations of GE increased PGE2 and suppressed depletion of GSH ($p<0.001$). Positive correlation of SOD, NFKB2 ($p<0.01$) and TRX 1 ($p<0.001$) with LPZ and GE pretreatment were seen, while ABCG2 expression was not changed. ($p<0.001$). Pretreatment of the cells with GE reverses changes in the cell membrane composition and cytoskeletal protein levels induced by NaT exposure ($p<0.001$).

Conclusion: GE pretreatment had gastroprotective effect in the cell model of PUD. Further experiments are needed to fully elucidate the mechanism of the protective role of GE in PUD.

Keywords: peptic ulcer disease, sodium taurocholate, garlic extracts, lansoprazole, AGS cell line

9. SAŽETAK

Ciljevi: Peptična ulkusna bolest (PUB) je kronična bolest koja pogađa do 10% svjetske populacije. Stanični model PUB-a može se uspostaviti u staničnom modelu ljudskih gastričnih epitelnih stanica (AGS) djelovanjem žučne soli; natrijevog taurokolata (NaT). Cilj istraživanja bio je istražiti učinke predtretmana ekstrakata češnjaka (EČ) i lanzoprazola (LPZ) u staničnom modelu ulkusne bolesti ispitivanjem oksidativnog stresa, distribucije F-aktina i određivanjem morfoloških promjena u strukturi stanične membrane.

Ustroj studije: Stanice su nasađene u koncentraciji $4x10^5$ stanica/mL te su potom inkubirane 24 sata. Stanice su potom tretirane LPZ i EČ 24, 48 i 72 sata i zatim izložene NaT tijekom 1 sata.

Materijal i metode: AGS stanična linija korištena je kao model PUB. Uspostava NaT modela određena je MTT testom. Procjena je učinjena određivanjem mitotičkog potencijala stanica bojenjem proliferirajućeg staničnog jezgrenog antigena (PCNA); koncentracije glutationa (GSH) i prostaglandina E2 (PGE2) pomoću ELISA; učinka EČ na stanično preživljenje brojanjem stanica Neubauerovim hemocitometrom; ekspresije SOD, ABCG2, NFKB2 i TRX1 pomoću RT PCR-a; Vizualizacijom citoskeleta F-aktina polukvantificiranjem Rhodamine Phalloidin boje; morfoloških promjena u strukturi stanične membrane bojanjem kolesterola i fosfolipida specifičnim bojama.

Rezultati: Rezultati naše studije pokazali su značajno smanjenje oštećenja stanica nakon inkubacije NAT-om, kada su AGS stanice prethodno tretirane LPZ-om ($p < 0,001$) i povećanjem koncentracija EČ ($p < 0,001$). Predtretman različitim koncentracijama EČ povećao je PGE2 i potisnuo iscrpljivanje GSH ($p < 0,001$). Uočena je pozitivna korelacija SOD, NFKB2 ($p < 0,01$) i TRX 1 ($p < 0,001$) s LPZ i predtretmanom EČ, dok ekspresija ABCG2 nije promijenjena. ($p < 0,001$). Predtretman stanica s EČ uzrokovao je promjene u sastavu stanične membrane i razine proteina citoskeleta izazvane izloženošću stanica NaT-u ($p < 0,001$).

Zaključak: Predtretman EČ imao je gastroprotektivni učinak u staničnom modelu PUB-i. Potrebni su daljnji eksperimenti kako bi se u potpunosti razjasnio mehanizam zaštitne uloge EČ u PUB-i.

Ključne riječi: Peptična ulkusna bolest, natrijev taurokolat, ekstrakti češnjaka, lanzoprazol, AGS stanična linija

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19. **Kuna, Lucija**; Jakab, Jelena; Omanović, Tea; Raguž-Lučić, Nikola; Smolić, Martina; Včev Aleksandar Pharmaceutical residues in the aquatic environment. Water for all, Osijek, Croatia, 2017.

20. Omanović, Tea; Kizivat, Tomislav; Raguž-Lučić, Nikola; Ninčević, Vjera; Jakab, Jelena; **Kuna, Lucija**; Smolić, Robert; Bilić-Čurčić, Ines; Včev, Aleksandar; Smolić, Martina. Evaluation of molecular mechanism differences in in vitro models of drug induced and fatty-acids induced NAFLD. The 1st International Conference on Fatty Liver, Seville, Spain, 2017. str.

21. Jakab J, **Kuna L**. Erasmus week & workshop, Prag (13.-17. ožujka 2017). Medicine and tourism.

22. Bojanic K, Kizivat T, **Kuna L**, Wagner J, Smolic R, Bilic Curcic I, Kralik K, Smolic M, Tucak A, Vcev A: Patients compliance correlates to pharmacogenomics of drugs used in the treatment of early stage breast cancer. IUAES Congress, 4-9 May 2016, Dubrovnik, Croatia.

23. **Kuna L**, Jakab J. An alternative to red bull. Summer school on stress, Osijek (13. - 17. lipnja 2016.)

24. Bojanic K, Kizivat T, **Kuna L**, Wagner J, Smolic R, Bozic I, Mrso M, Raguz-Lucic N, Vcev A, Smolic M: Pharmacogenomics of anastrazole could predict interpatient variability in the intensity of adverse effects. Ninth ISABS Conference on Forensic and Anthropologic Genetics and Mayo Clinic Lectures in Individualized Medicine, Bol, Croatia, June 2015.

Scientific projects:

1. 2021. - „Uloga interleukina 33 i kalpain proteaze 1 u razvoju akutnog respiratornog distres sindroma u bolesnika s pneumonijom uzrokovanom SARS-CoV-2 virusom" (MEFOS-IP-2020) IP project of the Faculty of Medicine, University J. J. Strossmayer in Osijek. - Project member. Project manager: assoc. prof. Robert Smolić.

2. 2020. - 2021. „Biljezi lipogeneze i mitohondrijske disfunkcije u procjeni amiodaronom izazvanog jetrenog oštećenja" (MEFOS-IP14-2020). IP project of the Faculty of Medicine, University J. J. Strossmayer in Osijek. - Project member. Project manager: assoc. prof. Robert Smolić.

3. 2019. - 2020. „Procjena izražaja SOST i DKK1 proteina u gingivalnoj krevikularnoj tekućini kod parodontalnih pacijenata na kroničnoj terapiji statinima" (FDMZ-IP7-2019), IP project of the Faculty of Dental Medicine and Health, University J. J. Strossmayer in Osijek. - Project member. Project manager: prof. Martina Smolić.

4. 2019. - 2020. „Osobitosti signalnih mehanizama pomoću kojih diallyl disulfid (DADS) smanjuje epitelno oštećenje u AGS i RGM-1 staničnim modelima ulkusne bolesti" (MEFOS-6-2019). IP project of the Faculty of Medicine, University J. J. Strossmayer in Osijek. - Project member. Project manager: assoc. prof. Robert Smolić.

5. 2018. - 2019. „Djelovanje antiksidanasa i anliza gena uključenih u oksidativni stres kod epitelnog oštećenja izazvanog natrijevim turokolatom u staničnom modelu ulkusne bolesti" (VIF-2018-FDMZ-03). VIF project of the Faculty of Dental Medicine and Health, University J. J. Strossmayer in Osijek. - Project manager.

6. 2016. - 2017. „Djelovanje spojeva ekstrakta češnjaka i đumbira na epitelno oštećenje izazvano natrijevim turokolatom u staničnom modelu ulkusne bolesti" (VIF2016-MEFOS-2)**.** VIF project of the Faculty of Medicine, University J. J. Strossmayer in Osijek. - Project member. Project manager: prof. Aleksandar Včev.

International mobility as a guest scientist or student:

Erasmus study exchange, Third Faculty of Medicine, Department for The Study of Obesity and Diabetes, Charles University Prague; June, 2018.

Summer School of Molecular Medicine, University Hospital of Jena, Germany, August 14 - September 14, 2017.

Awards:

2020. - Award for the best scientific article published in 2019 in the category of young scientist; Faculty of Dental Medicine and Health Osijek, Faculty of Dental Medicine and Health Osijek, Osijek, December, 2020.

Kuna, Lucija; Jakab, Jelena; Smolić, Robert; Raguž-Lučić, Nikola; Včev, Aleksandar; Smolić, Martina. Peptic Ulcer Disease: A Brief Review of Conventional Therapy and Herbal Treatment Options. // J. Clin. Med. 2019, 8(2), 179.

Courses:

- „Writing a Journal Article…and Getting it published“, 4-6th September, 2019, Bern, Switzerland
- Workshop for mentors, December 7, 2016, Osijek, Croatia
- Course on Laboratory Animal Science –LabAnim,17-26th November 2016, Department for Animal Physiology, Faculty of Science, Zagreb, Croatia
- qPCR basics and best practices for effective planning, realization and data analysis. Mannheim, Germany, 27th October 2016
- Laboratory immunotechniques in molecular biology research", University of Pecs Medical School, Pecs, Hungary (14-18 December 2015)

Membership in scientific and professional societies:

- Croatian Society of Personalized medicine
- Croatian Society of Human Genetics
- Croatian Pharmacological Society
- Croatian Biological Society (Societas biologorum croatica)
- Organizational committee of 10th ISABS Conference on Forensic and Anthropologic Genetics Mayo Clinic Lectures Individualized Medicine, 19-24 June 2017, Dubrovnik, Croatia

Foreign language:

English C1 level