A THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Antiulcer Activity, Toxicity, and Possible Mechanisms of Action of *Opuntia ficus-indica* Fruit-Derived Materials

백년초(*Opuntia ficus-indica*) 열매 유래 물질의 항궤양 효과, 독성 및 작용 기전

By

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Major in

Interdisciplinary Program in Agricultural Biotechnology

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Abstract

Gastritis is a common disease among Korean adults who take mainly very salty and spicy foods. Usually, symptoms include epigastric pain, nausea, vomiting, abdominal pain, indigestion, and bloating. Pathophysiology of gastritis is due to a lack of equilibrium between the gastric aggressive factor (acid, pepsin, and *Helicobacter pylori*) and the mucosal defense factor (gastric mucus, bicarbonate secretion, prostaglandins, and innate resistance of the mucosal cells). There are several types of medicines used to treat a gastric ulcer. However, these treatments have side effects. There is a pressing need to develop a new gastritis treatment with fewer side effects. Plants are regarded to represent a reservoir of potential therapeutics and therefore the efforts to search for novel compounds from medicinal plants have been continued.

Opuntia ficus-indica (Cactaceae) has been used in traditional medicine of many countries. It is widely cultivated in Jeju Island, Korea, for use in the manufacture of health foods. The aim of this study was to develop *O. ficus-indica* fruits extract as an antiulcer botanical drug. The antiulcer activity of OF-80E (80% ethanol extract of *O. ficus-indica* fruits) was assessed using the ethanol-, non-steroidal anti-inflammatory drugs (indomethacin, aspirin, and diclofenac)-, and stress-induced gastritis rat models. The results were compared with those of commercially available drugs, Stillen® tablet and Mucosta® tablet. In addition, the acute toxicity, sub-chronic toxicity, genotoxicity, and safety pharmacology studies of OF-80E were analyzed under Organization for Economic Cooperation and Development guideline and Good Laboratory Practice regulations for human safe consumption. Finally, the possible mechanism underlying the antiulcer actions of OF-80E in gastritis models were elucidated using biochemical and molecular analyses.

Inhibition of aspirin-induced cytotoxicity in AGS cells assay-guided fractionation of the *O. ficus-indica* fruits led to the identification of two active compounds through spectroscopic analyses, including electron ionization mass spectrometry and nuclear magnetic resonance spectroscopy. The two antiulcer constituents were the flavonoids aromadendrin and narcissin. Based on the IC_{50} values, the flavone, aromadendrin (<0.5 μM), and the flavonol, narcissin (<0.5 μM), were more effective in inhibition of aspirin-induced cytotoxicity in AGS cells than other flavones, naringenin (5.9 μM), eriodictyol (>10 μM) and taxifolin (1.1 μM), and flavonols, kaempferol, quercetin, and isokaempfride (>10 μM).

OF-80E was more effective than commercially available drugs to protect gastric mucosal damage against aggressive factors. In ethanol-, non-steroidal anti-inflammatory drugs-, and stress-induced gastritis rat models, OF-80E inhibited gastric hemorrhagic lesions and histological tissue damage effectively comparing than commercially available drugs.

In a single dose oral toxicity study, the approximate lethal dose of OF-80E in both male and female of Sprague Dawley (SD) rats was higher than 10000 mg/kg. In a 13-week repeated oral toxicity study, the no observed adverse effect level of OF-80E was 2000 mg/kg/day for both sexes of SD rats. In a 4-week repeated oral toxicity study, the maximum tolerance dose of OF-80E was 1500 mg/kg/day for both sexes of beagle dogs. In *Salmonella typhimurium* and *Escherichia coli* reverse mutation studies, OF-80E did not cause mutation. In a chromosome aberration test, OF-80E did not cause chromosomal aberration in Chinese hamster lung cells. In micronucleus assay, OF-80E did not induce micronuclei in the mammalian bone marrow cells. Single oral administration of OF-80E to rodent at below 5000 mg/kg did not affect the central nervous system of ICR mice and did not induce adverse effects on the respiratory system of SD rats. OF-80E did not effect on the human ether-a-go-go related gene channel up to the concentration of 500 μg/mL, indicating that the effect of OF-80E on cardiovascular system was to be low.

OF-80E increased glutathione reduced by aspirin in AGS cells and decreased by indomethacin in rats. OF-80E increased prostaglandin E_2 levels reduced by aspirin in AGS cells. Decreased adherent mucus was synthesized and stimulated, by OF-80E pretreatment in indomethacin-induced gastritis rats. OF-80E inhibited myeloperoxidase activity in indomethacin-induced rat gastric mucosal and reduced tumor necrosis factor-α in stress-induced rat gastric mucosal.

Key world: gastritis, *Opuntia ficus-indica* fruit, gastro-protective effect, oral toxicity study, genotoxicity, safety pharmacology

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Contents

List of Abbreviations

ANOVA	analysis of variance
ATCC	American type culture collection
B(a)P	benzo(a)pyrene
b.i.d.	bis in die
BW	body weight
Ca^{2+}	calcium ion
CBC	complete blood cell
CCK	cholecystokinin
CHL	Chinese hamster lung
CHO	Chinese hamster ovary
Cl^{-}	chloride ion
CNS	central nervous system
CPA	cyclophosphamide monohydrate
CMC-Na	carboxyl methyl cellulose sodium salt
COX	cyclooxygenase
DMSO	dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
ELISA	enzyme-linked immunosorbent assay
EMS	ethyl methane sulfonate
FBS	fetal bovine serum
FDA	Food and Drug Administration

GBD	Global Burden of Disease
GI	gastrointestinal
GSH	glutathione
GLP	good laboratory practice
hERG	human ether-a-go-go-related gene
HPLC	high-performance liquid chromatography
HPMC	hydroxy propyl methyl cellulose
IACUC	Institutional Animal Care and Use Committee
ICH	International Council for Harmonization
K^+	potassium ion
KP	Korean pharmacopoeia
MC	methyl cellulose
MEM	minimum essential medium
MFDS	Ministry of Food and Drug Safety
MNPCE	micronucleated polychromatic erythrocyte
MPLC	medium pressure liquid chromatography
MPO	myeloperoxidase
MTD	maximum tolerance dose
MTT	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide
Na^+	sodium ion
NCE	normochromatic erythrocyte
NMR	nuclear magnetic resonance
NOAEL	no observed adverse effect level

NSAID	non-steroidal anti-inflammatory drug
OD	optical density
OECD	Organization for Economic Cooperation and Development
PBS	phosphate-buffered saline
P-CAB	potassium-competitive acid blocker
PCE	polychromatic erythrocyte
PGE_2	prostaglandin E_2
p.o.	post oral
PPI	proton pump inhibitor
QSAR	quantitative structure-activity relationship
RBC	red blood cell
RCC	relative cell count
SD	Sprague Dawley
std	standard deviation
SEM	standard error of the mean
SPSS	Statistical Package for the Social Sciences
SPF	specific pathogen-free
t.i.d.	ter in die
TLC	thin-layer chromatography
TNF-α	tumor necrosis factor-alpha
US	United States
WBC	white blood cell
WIRS	water immersion restraint stress

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I. Introduction

Gastritis is a common disease among Korean adults who take mainly very salty and spicy foods. Usually, symptoms such as nausea, vomiting, abdominal pain, indigestion, and bloating appear, but gastritis may be found during health without any symptoms. Dyspepsia, heartburn, non-cardiac chest pain, abdominal pain, chronic diarrhea, and constipation are common symptoms experienced by individuals without histopathological explanation. Common gastrointestinal (GI) diseases such as peptic ulcer, chronic gastritis, gastroesophageal reflux disease, duodenogastric reflux, and irritable bowel syndrome are clinical symptom-based diagnoses with significant overlap and symptom variability over time. It is a recurrent chronic illness that affects approximately 10% of the world's population [1] and is thought to be caused by a lack of equilibrium between the gastric aggressive factor (acid, pepsin, and *Helicobacter pylori*) and the mucosal defense factor (gastric mucus, bicarbonate secretion, prostaglandins, and innate resistance of the mucosal cells) [2].

Currently, therapeutics like antacids, anticholinergics, proton pump inhibitors, and H_2-receptor antagonists are used to treat gastric ulcers [3]. However, these drugs have drawbacks with onset of hypersensitivity, gynecomastia, impotence, arrhythmia, and hematopoietic changes with continuous use [4]. Therefore, there is a pressing need to develop new improved antiulcer agents with fewer side effects. Plants are regarded to represent a reservoir of potential therapeutics and the efforts to search for novel compounds from medicinal plants have been continued.

Historically, botanicals were an intrinsic part of the United States (US) Food and Drug Administration (FDA). However, since the 1940s, most US drugs have been single 'active' molecule ingredients, or 'New Chemical Entities'. When a natural product is the source material

for a new drug, the process most favored for pharmaceutical development has been identification, isolation, purification, and whenever feasible, synthesis of the active drug substance [5]. The FDA has been advising sponsors on how to develop botanicals as drugs. By the time the guidance was made public, the agency had received hundreds of inquiries and filings on botanicals. Within the past two decade, two botanical new drugs have been approved by the FDA. Veregen® (sinecatechins; ointment, 15%; Medigene, Germany) is proprietary extract of green tea (*Camellia sinesis* Kuntze) for the topical treatment of genial and perianal warts [6]. Fulyzaq® (Crofelemer; 125 mg tablet; Salix Pharmaceuticals, USA) is proprietary extract of the blood-red latex of the South American croton tree (*Croton lechleri* Müll. Arg.) [7]. Both drugs were shown to meet the same legal criteria for safety and efficacy and are manufactured to the same quality standards as other prescription drugs in the US. In Korea, seven botanical new drugs have been launched and approximately twenty botanical drugs have been studied.

Opuntia ficus-indica (L.) Miller (Cactaceae) grows in all the semiarid counties throughout the world and is especially cultivated in the Mediterranean region and Central America. Both its fruits and cladodes have been used in traditional medicine of many countries. In addition, there have been extensive studies on the biological activities of this plant [8]. *O. ficus-indica* has been presumed to be introduced as an ornament plant to Korea. Since then, it has been naturalized as *O. ficus-indica* and is widely cultivated in Jeju Island, Korea, for use in the manufacture of health foods, such as tea, jam, and juice. The dried powder of the fruits and stems of the cactus significantly inhibited HCl-ethanol, aspirin, and indomethacin-induced gastric lesions in rats without any change in gastric juice secretion [9]. Its bioactive chemical constituents include several flavonoids that have been isolated from this plant species [10].

The aim of this study was to assess whether OF-80E, 80% ethanol extract of *O. ficus-indica* fruits, had antiulcer activity in comparison with commercially available drugs, Stillen® tablet and

Mucosta® tablet, using various *in vivo* and *in vitro* models. In addition, toxicity studies to predict toxicity of OF-80E in clinical trials were conducted. Finally, the possible mechanism underlying the antiulcer actions of the isolated constituents was elucidated using biochemical and molecular analyses.

II. Literature reviews

1. Gastritis (Gastric ulcer)

Gastric acid is a highly acidic fluid produced by parietal cells in the stomach that is involved in the breakdown of food as well as the elimination of any swallowed microorganisms. Under normal conditions, the stomach produces a mucosal barrier that protects the organ from the aggressions of gastric acid and digestive enzymes. When the mucosal barrier breaks down, an ulcer may develop [11].

A peptic ulcer is an area of mucosal damage, typically 3 mm or greater in size, that may affect the stomach (gastric ulcer), proximal duodenum (duodenal ulcer) [12] or both . Gastric ulcer typically develops in the angulus of the lesser curvature, although they may be found anywhere from the pylorus to the cardia. Duodenal ulcer generally develops in the bulb, where gastric contents pass into the small intestine [13].

An analysis by the National Institute of Diabetes and Digestive and Kidney Disease has estimated that the total cost of peptic ulcer disease in the US in the year 2004 was approximately 3.1 billion USD. Direct costs, including hospital services, outpatient endoscopy, physician services, prescription and OTC drugs, nursing home health care, were calculated at 2.6 billion USD, while indirect costs, including supplementary meal, transportation for going to hospital, were approximately 520 million USD [14]. Regarding drug therapy, approximately 26.5 billion USD was spent worldwide on proton pump inhibitor (PPI), among the most widely used antiulcer drugs, in 2008 [15].

According to the Global Burden of Disease (GBD) study, there were almost 50400000 (range 48835000 to 52173000) incident cases of peptic ulcer disease worldwide in 2013. The age-standardized incidence rate was 810.7 per 100000 (range 786.1 to 838.9) [1]. Dutch researchers

have concluded that the incidence of gastric ulcers in that country remained stable from 1996-2005, while the incidence of duodenal ulcers decreased steadily over the same period [16]. The prevalence of gastric ulcer among patients undergoing GI endoscopy in the primary care setting in Havana, Cuba, was 6.2% [17].

In a study of asymptomatic individuals undergoing route health check-ups in Taiwan, 4.7% were found to have gastric ulcers, 3.9% had duodenal ulcers, and 0.9% had both [18]. In a population-based study of 3600 randomly selected inhabitants of Shanghai, China, 17.2% had endoscopically confirmed peptic ulcer; and one-third of these were gastric ulcer. The majority of the cases detected were asymptomatic [19].

In India, the frequency of gastric ulcer decreased slightly, from 2.9 to 2.7%, between 1988 and 2008; more significant decreases were seen in the rate of duodenal ulcers during the same period [20]. According to the results of a population-based study in Ardabil, Iran, the prevalence of endoscopically confirmed gastric ulcer in the country in 3.3% [21].

Although gastric ulcers can occur at any age [22], they are most common between 25~64 years of age [23]. The incidence of gastric ulcer increases with age because of the likelihood of prolonged use of non-steroidal anti-inflammatory drugs (NSAIDs), a high prevalence of *H. pylori* infection in person older than 50 years as well as age-related cellular changes in the gastric mucosa [24].

1.1. Pathophysiology and risk factors

Until recently, the development of ulcer disease was attributed to overproduction of acid and mucosal barrier destruction as a results of stress, smoking, or dietary factors [12]. In 1982, Australian physicians Robin and Barry Marshall identified the potential link between peptic ulcers and *H. pylori*, although the medical community did not support this opinion until later in the 20th

century [25, 26]. Ulcers develop from a complex relationship of acid levels, the presence of *H. pylori* and/or extended use of NSAID including aspirin and ibuprofen [12]. Ulceration in the absence of either *H. pylori* infection or a history of NSAID use is extremely rare, but may be associated with neoplasia, acid hypersecretory states (i.e., Zollinger-Ellison syndrome), Crohn's disease of the stomach or duodenum, viral infection with herpes simplex virus or cytomegalovirus, or radiation damage [13]. Use of selective serotonin reuptake inhibitor or warfarin, especially in combination with aspirin, has also been associated with gastric ulceration [27, 28]. Smoking slows the healing rate of existing ulcers and increases the risk of ulcer recurrence [23], but is not itself a proven cause of ulcers. Emotional stress may be a contributing factor, but is seldom the only cause [13]. Genetic factors also appear to contribute: patients with a family history of peptic ulcer area at an increased risk [29]. Increasing age and male gender are also associated with a greater risk of gastric ulcer [24].

1.1.1. *Helicobacter pylori*

Helicobacter pylori is spiral-shaped bacterium that penetrates into and adheres to the protective mucous layer of the stomach [13, 25]. During the preliminary stage of infection, the organism invades and begins to multiply close to surface epithelial cells, leading to epithelial mucin depletion, cellular exfoliation, and compensatory regeneration. The acute response is mediated by the release of bacterial lipopolysaccharide, activation of mast cells and release of proinflammatory cytokines, as well as alterations in gastric acid production. Despite these changes, this phase is generally subclinical and asymptomatic and often resolves spontaneously. In patients whose immune systems fail to clear the organism, however, infection and resulting gastritis may progress from the acute to chronic stage, which is mediated by a T helper cell-directed immune response [13, 30] and a chronic inflammatory response [31]. The long-term

health effects of *H. pylori* infection include chronic gastritis, gastric and duodenal ulcer, distal stomach cancer and mucosa-associated lymphoid tissue lymphoma, as well as several extra gastric diseases of diverse body systems [32, 33]. Due to its proven association with gastric cancer, the pathogen has been classified as a group I human carcinogen by the International Agency for Research on Cancer.

Helicobacter pylori infection is typically acquired in childhood (before age 10) and may persist throughout the person's life [34]. Acquisition of *H. pylori* depends greatly on conditions of sanitation and hygiene; prevalence of infection ranges from approximately 10% in Western countries to nearly 100% in developing nations [32]. It is interesting to note that while more than 50% of the global population in chronically infected with *H. pylori* only 5~10% of individuals infected with the microorganism actually go on to develop peptic ulcer disease [13]. Humans are the only known host of *H. pylori*. Transmission among humans is typically by the oral-fecal route in less developed countries, while it is more common by the oral-to-oral route in developed countries [32]. Studies have shown that *H. pylori* remains viable in water for prolonged periods, indicating that the pathogen may be transmitted in drinking water biofilms [35]. Another possible route of transmission is exposure to an *H. pylori*-infected individual with gastroenteritis (i.e., through contact with vomitus, saliva, or diarrheal stools) [36].

Varying clinical results from *H. pylori* infection are believed due, in part, to differences in virulence among strains. Approximately 60~80% of all *H. pylori* express an antigenic protein known as *Cag*A, the product of the *cag*A gene, which resides in the *cag* pathogenicity island (*cag*PAI) [37]. The *cag*PAI is a complex of virulent genes and a coding region for the type IV phosphorylated secretion system. Through this system, many virulent gene products or proteins are phosphorylated into the host cells. People infected with *H. pylori* strains containing the *cag*PAI demonstrate increased levels gastric mucosal inflammation and epithelial cell damage compared

to people infected with non-*cag*A strains, and thus are at increased risk for the development of peptic ulcer disease and distal gastric cancer [38]. The *vac*A-positive genotype of *H. pylori* is also associated with increased morbidity [13]. Other factors determining the nature (acute or chronic) and outcome of *H. pylori* infection include the interaction of the pathogen with the mucosal barrier and resulting immunopathogenic response, pattern of histological gastritis induced, alterations in the homeostasis of gastric hormones and acid secretion, environmental risk factors and host genetic factors, among others [13].

1.1.2. Nonsteroidal anti-inflammatory drugs

NSAIDs such as aspirin are a widely used class of anti-inflammatory drugs that inhibit inflammatory prostaglandin production. Unfortunately, such as inhibitory action has deleterious effects in areas relying on prostaglandin production, including gastric mucosal protection and renal blood flow. NSAIDs also damage the GI tract via other mechanisms including effects on neutrophil function [39]. Altering gastric mucosal blood flow in a non-prostaglandin-dependent manner; direct irritant effects including the concept of ion trapping and interference with growth factors and ulcer healing mechanisms.

The mechanism of action of NSAIDs is accomplished by blocking the enzyme cyclooxygenase (COX), which is responsible for the conversion of arachidonic acid to prostaglandins. Prostaglandins liberated from arachidonic acid by cyclooxygenase are short-lived substances that act as local hormones (autocoids) important in normal physiology and pathologic conditions. Prostaglandins promote mucus production and enhance bicarbonate production, two important characteristics of the barrier, and improve blood flow to the GI wall, further adding to the resistant abilities of the gastric mucosa. Prostaglandin E_2 (PGE_2) is the principal eicosanoid in inflammatory conditions. Along with their benefits, side effects involving the GI system have

emerged that have limited the usefulness of NSAIDs [40].

While the direct contact of these medicines with the stomach can indeed cause damage, the main way, that they cause ulcers is by suppressing gastric prostaglandin synthesis [13], leading to easier damage to the stomach lining caused by acid and bile. Normally, the stomach has three defenses against digestive juices: mucus that coats the stomach lining and shields it from stomach acid, the chemical bicarbonate that neutralizes stomach acid, and blood circulation to the stomach lining that acids in cell renewal and repair. NSAIDs hinder all these protective mechanisms and with the stomach's defenses down, digestive juices can damage the sensitive stomach lining and cause ulcers. For this reason, "safety-coating" of preparations of aspirin and similar products dose little to decrease the long-term ulcer risk from these medicines. Acid suppression is currently the primary method of preventing and managing NSAID-associated ulcer disease [13]. NSAID-induced ulcers usually heal once the person stops taking the medication.

Two isoforms of cyclooxygenase have been identified. COX-1 has gastro-protective effect, whereas COX-2 produces proinflammatory mediators. Classic NSAIDs (e.g., ibuprofen) inhibit COX-1 more than COX-2, whereas the newer class of NSAIDs (e.g., celecoxib, rofecoxib) preferentially inhibits COX-2, and are associated with a somewhat lower rate of gastric ulcers, albeit with an increased risk of adverse cardiovascular effects [13].

1.2. Morbidity and mortality

Complications of gastric ulcer include upper GI bleeding [28] and perforation and gastric outlet obstruction (ulcer penetration into surrounding tissues) [23]. Gastrointestinal bleeding can cause anemia and may be fatal, although improved awareness and treatment in recent years has led to a reduced incidence of mortality from bleeding [27]. According to a US healthcare survey, hospitalizations for peptic ulcer disease decreased by nearly 30% (37.2% decrease for duodenal

ulcers and 19.6% decrease for gastric ulcer) between 1993 and 2006. Inpatient mortality rates for peptic ulcer disease decrease from 3.8 to 2.7% in the same period; perforation was the most important cause of ulcer-related mortality [41]. Hospitalizations for bleeding peptic ulcer also decreased in Sweden, from a rate 63.9 per 100000 populations in 1987 to 35.3 per 100000 in 2005. However, age-standardized 30-days mortality rate increased during the same period, from 5.3 to 6.2% [42].

According to the World Health Organization's 2015 GBD study, there were 267500 (range 249400 to 290000) deaths caused by peptic ulcer disease worldwide in 2015, giving an age-standardized mortality rate of 4.1 per 100000 [43]. According to GBD 2013, an estimated 40612 (range 31643 to 46710) people in China alone died as a result of peptic ulcer disease in 2013, giving an age-standardized death rate of 3.23 per 100000 [44].

An association between long-term infection with *H. pylori* and the development of gastric cancer has been confirmed. Gastric ulcer disease and gastric cancer have etiologic factors in common. A likely cause of both is atrophic gastritis induced by *H. pylori*. By contrast, there appear to be factors associated with duodenal ulcer disease that protect against gastric cancer [38]. History of gastric ulcer, especially gastric ulcer of relatively recent diagnosis, has also been linked to an increased risk of pancreatic cancer, according to a prospective cohort study involving more than 50000 male health professionals [45].

1.3. Diagnosis

Diagnosis of gastric ulcer is guided by the presence of certain symptom including epigastric pain; pain beginning 2~5 h after eating or on an empty stomach; and nocturnal pain causing the patient to awaken and that is relieved by food intake, antacids, or antisecretory agents [23]. An upper GI series showing a gastric ulcer, or an esophagogastroduodenoscopy and biopsy showing

a gastric ulcer is often diagnostic. The investigation of choice is an upper GI endoscopy, taking particular notice of the lesser curve and pre-pyloric regions. A peptic ulcer is diagnosed upon observation of a mucosal break at least 5 mm in diameter and covered in fibrin. Smaller breaks are classified as erosions. Gastric ulcers typically develop in the angulus of the lesser curvature, although they may be found anywhere from the pylorus to the cardia. Duodenal ulcers generally develop in the bulb, where gastric contents pass into the small intestine [13].

Several invasive and noninvasive methods may be used to diagnose *H. pylori* infection [46]. Serological tests that measure specific *H. pylori* immunoglobulin G antibodies can determine if a person has been infected. The sensitivity and specificity of these assay range from 80 to 95%, depending on the assay used. Another diagnostic method is the urea breath test. In this test, the patient is given ^{13}C-labeled urea drink [13]. *H. pylori* metabolizes the urea rapidly, and the labeled carbon is absorbed. This labeled carbon can then be measured as CO_2 in the patient's expired breath to determine whether *H. pylori* is present. The sensitivity and specificity of the breath test ranges from 94 to 98%. The stool antigen test, while being less convenient, is also highly accurate [23, 46].

Endoscopic examination is indicated in patients presenting with alarm symptoms (GI bleeding, inexplicable weight loss, or anemia) and in all patients over 50 years presenting with a newly developed ulcer [46]. During endoscopy, biopsy specimens should be obtained from the antral and body on fundus mucosa so that diagnosis of *H. pylori* can be made by urease or histological testing. The biopsy urease test, a colorimetric test based on the ability of *H. pylori* to produce urease, provides rapid testing at the time of biopsy. Histological identification of organisms is considered the gold standard of diagnosis tests [13]. Polymerase chain reaction has been found useful as a method of detecting *H. pylori* in biofilms, particularly drinking water biofilms, which have been identified as a possible method of waterborne transmission of pathogen

[35].

1.4. Prevention

Since the source of *H. pylori* is not yet known, recommendations for avoiding infection have not been made. In general, it is always wise for persons to wash hands thoroughly, to eat food that has been properly prepared, and to drink water from a safe, clean source. It is not known how *H. Pylori* is transmitted or why some patients become symptomatic while others do not. The bacteria are most likely spread from person to person through fecal-oral or oral-oral routes. Possible environmental reservoirs include contaminated water sources. Patients requiring ongoing treatment with NSAIDs for pain or cardiovascular risk reduction should receive prophylactic antiulcer therapy. PPIs have been shown more effective than histamine H2 antagonists for purposes of NSAID-induced ulcer prevention [47].

2. Therapeutic agents of gastritis

The goals of ulcer therapy include eliminating pain, promoting ulcer healing, and preventing the development of new lesions [12, 29]. The choice of treatment depends on the suspected etiology of the ulcer. Pharmacological approaches to ulcer eradication address eliminating the *H. pylori* bacteria using one or more antibiotics plus an acid-suppressing drug or bismuth component [46, 48]. Therapy for *H. pylori* infection consists of 10~14 days of one or two effective antibiotics, such as amoxicillin, tetracycline (not to be used for children under 12 years of age), metronidazole or clarithromycin, plus either ranitidine bismuth citrate, bismuth subsalicylate or a PPI [46]. Acid suppression by an H2 blocker or PPI in conjunction with the antibiotics helps alleviate ulcer-related symptoms (i.e., abdominal pain, nausea), heal gastric mucosal inflammation and prevent ulcer recurrence, and may enhance efficacy of the antibiotics

against *H. pylori* at the gastric mucosal surface [47].

Antibiotic resistance and patient noncompliance are the two major reasons for treatment failure [48]. Eradication rates for combination regimens range from 90~96%, depending on the regimen used and length of treatment [48]. Lifestyle changes may improve the treatment of ulcer, such as avoidance of alcohol, smoking, NSAIDs, illicit drugs, and caffeine [23]. Drugs marketed for the treatment of gastric ulcer are summarized in the Table 1.

Table 1. Drugs marketed for the treatment of gastric ulcer

Drug name	Organization	Year & country of first launch
Histamine H2 antagonists		
Cimetidine (Tagamet)	GlaxoSmithKline	1977
Ranitidine hydrochloride (Znatac)	GlaxoSmithKline	1981
Famotidine (Famodil)	Astellas	1985 (Japan)
Roxatidine acetate hydrochloride (Altat)	Aska Pharmaceuticals / Takeda	1986 (Japan)
Nizatidine (Axid)	Lilly	1987 (UK)
Ranitidine bismuth citrate (Pyloid)	GlaxoSmithKline	1995 (UK)
Lafutidine (Stogar)	Taiho	2000 (Japan)
Proton pump inhibitors		
Omeprazole (Losec)	AstraZeneca	1988 (Sweden)
Lansoprazole (Prevacid)	Takeda	1992 (France)
Pantoprazole sodium (Protonix)	Takeda	1994 (Germany)
Rabeprazole sodium (Aciphex)	Eisai	1997 (Japan)
Esomeprazole magnesium (Nexium)	AstraZeneca	2000 (Sweden)
Ilaprazole (Aldenon)	Livzon	2008 (China)
Esomeprazole sodium (Nexium I.V.)	AstraZeneca	2009 (Sweden)
Potassium-competitive acid blockers		
Revaprazan hydrochloride (Revanex)	Yuhan	2007 (Korea)
Vonoprazan fumarate (Takecab)	Takeda	2015 (Japan)
Tegoprazna (K-cap)	CJ HealthCare	2019 (Korea)

Drug name	Organization	Year & country of first launch
Cytoprotectives		
Sucralfate (Carafate)	Chugai	1970
Sofalcone (Solon)	Taisho	1984 (Japan)
Sulglicotide (Gliptide)	Sirton Pharmaceuticals	1985 (Italy)
Teprenone (Selbex)	Eisai	1985 (Japan)
Plaunotol (Kelnac)	Daiichi Sankyo	1986 (Japan)
Troxipide (Aplace)	Kyorin	1986 (Japan)
Midoriamin (Midoriamine)	Mitsubishi Tanabe Pharma	1987 (Japan)
Zinc acexamate (Copinal)	Vinas	1988 (Spain)
Irsogladine maleate (Gaslon)	Nippon Shinyaku	1989 (Japan)
Rebamipide (Mucosta)	Otsuka	1990 (Japan)
Ecabet sodium (Gastrom)	Mitsubishi Tanabe Pharma	1993 (Japan)
Polaprezinc (Promac)	Zeria	1994 (Japan)
Dosmalfate (Diotul)	FAES	2000 (Spain)
Antimuscarinic agents		
Methscopolamine bromide (Pamine)	Pfizer	1947
Propantheline bromide (Pro-Banthine)	Shire	1953 (US)
Mepenzolate bromide (Cantil)	Sanofi	1958
Glycopyrronium bromide (Robinul)	Pfizer	1962 (US)
Pirenzepine hydrochloride (Gastrozepin)	Boehringer Ingelheim	1977
Atropine sulfate (Nulev)	Schwarz Pharma	2001 (US)
CCKB/gastrin antagonists		
Proglumide (Milid)	Rottapharma	1967 (Italy)
Prostaglandins		
Misoprostol (Cytotec)	Pfizer	1985 (Switzerland)
Bismuth		
Bismuth subsalicylate	Procter & Gamble	1928
Antacids		
Almagate (Almex)	Almirall	1984 (Spain)

Drug name	Organization	Year & country of first launch
Antibiotics		
Metronidazole (Flagyl)	Pfizer	1960
Amoxicillin trihydrate (Amolin)	GlaxoSmithKlein	1972
Clarithromycin (Klaricid)	Abbott	1990 (Ireland, Italy)

2.1. Histamine H2 antagonists

Histamine H2 receptor antagonists decrease hydrochloric acid production by competing with histamine for receptor sites on gastric parietal cells. H2 blockers with five-membered rings (cimetidine, ranitidine, and famotidine) and an H2 blocker with a six-membered ring (roxatidine) are effective at inhibiting nocturnal acid secretion, but less effective at inhibiting meal-stimulated acid secretion. The rapid loss of acid suppression activity by H2 receptor antagonists may be attributed to tolerance [48]. They are useful in treating NSAID-induced gastric or duodenal ulcers as well as for keeping ulcers in remission when used as maintenance therapy, although their efficacy may be compromised in patients continuing on NSAID therapy [13].

2.2. Proton pump inhibitors

PPIs exhibit more potent and longer-lasting inhibition of gastric acid secretion as compared with histamine H2 receptor antagonists. This is primarily due to differences in their mechanism of action. PPIs block histamine-, gastrin-, and acetylcholine-mediated sources of acid production and inhibit gastric secretion at the final common pathway of the H^+/K^+ adenosine triphosphatase (ATPase) proton pump [48, 49]. Histamine H2 receptor antagonists, on the other hand, only block receptor sites mediated by histamine. In contrast to H2 antagonists, tolerance has not been observed in patients receiving PPIs.

Oral PPIs such as omeprazole, rabeprazole, pantoprazole, and the intravenous PPI

esomeprazole sodium act via selective inhibition of H^+/K^+ adenosine triphosphatase in the secretory canaliculus of the stimulated parietal cell. All PPIs are prodrugs, active only after deprotonation and in the presence of active acid secretion, for which they should be taken shortly before a meal for optimum efficacy [47, 48, 50]. They inhibit more than 90% of 24-h acid secretion, as compared to 65% with H2 receptor antagonists. They covalently bind acid-secreting enzyme by permanently inactivating the enzyme. Restoration of acid secretion requires synthesizing new pumps, which have a half-life of 18 h. Their duration of action thus depends on the rate of *de novo* proton pump regeneration. PPIs also have some anti-*H. pylori* activity [47]. However, they have a short half-life (approximately 90 min) and thus are not as effective as H2 antagonists in suppressing nocturnal acid output [49]. They also have a slow onset of action, with most PPIs reaching maximal effect only after several days of administration [47]. Improved metabolism has been achieved using single-isomer versions of some PPIs, exemplified by esomeprazole, and with delayed-delivery systems [47]. The intravenous PPI esomeprazole is especially effective for the prevention of recurrent peptic ulcer bleeding after endoscopic therapy [51].

PPIs are generally considered to be safe and are among the most widely prescribed medications worldwide. In the last decade, however, as a result of their ever-increasing use, PPIs have been found to cause a range of side effects and complications, leading to growing concerns about their long-term use [52]. A growing body of evidence shows that prolonged use of PPIs can result in structural changes in the gastric mucosa, most notably the development of parietal cell hyperplasia and hypertrophy. In older adults, treatment with PPIs has been linked in observational studies to increased risk of community-acquired pneumonia and enteric infections; however, other studies have failed to replicate the pneumonia-related findings [15, 52]. PPIs may impede the correct absorption of certain dietary nutrients. Suppression of secretory function by PPIs results

in decreased absorption of calcium in the intestine, thereby moderately increasing bone fracture risk in long-term PPI users [53-55]. Based on this evidence, in May 2010, the US FDA issued a warning to physicians regarding a possible increase in the risk of bone fractures (specifically vertebral, wrist, and hip) in patients treated for long periods or with high doses of PPIs. This risk has not been observed in patients treated with histamine H2 antagonists. Nonetheless, an extensive review of these and other putative side effects of PPIs has concluded that their benefits of their appropriate use far outweigh the associated potential risks [52].

To overcome the shortcomings of existing PPIs, a newer-generation class of compounds known as potassium-competitive acid blockers (P-CABs) has been developed and evaluated in clinical trials. P-CABs inhibit H^+/K^+-ATPase in a potassium-competitive and reversible manner, with higher pKa values and improved stability at low pH. These properties endow the P-CABs with improved pharmacokinetic properties, as manifested by a less variable onset of action, reduced acid liability, prolonged efficacy over the 24-h period and more consistent efficacy across a wide range of patient profiles [48, 50, 56]. In late December 2014, the Japanese Ministry of Health, Labour and Welfare approved vonoprazan fumarate, the first member of the P-CAB class, for the treatment of acid-related diseases. Vonoprazan (Takecab) was launched in Japan in early 2015. In July 2018, the Korea Ministry of Food and Drug Safety (MFDS) approved tegoprazan, the member of the P-CAB class, for the treatment of acid-related disease. Tegoprazan (K-CAB) was launched in Korea in March 2019.

2.3. Antacids

Aluminum- (Amphojel, Basaljel) magnesium- (Riopin, Mylanta), calcium-, or carbonate-based antacids help to reduce the acid load within the gastric lumen. They act by reducing the amount of acid delivered to the duodenum, raising the gastric pH to at least 3.0 and reducing the

conversion of pepsinogen into pepsin, thereby decreasing pepsin activity. Antacids are not used as first-line therapy but are useful in supplementing other methods during the initial days of treatment. Their principle side effects include altered bowel motility (diarrhea and/or constipation) and duodenogastric reflux [29].

2.4. Prostaglandin derivatives

Endogenous prostaglandins, especially PGE_2, play a pivotal role in protecting the gastric mucosa and in promoting the healing of gastric ulcers. NSAIDs deplete endogenous prostaglandins, contributing to the development of gastric ulcers. Prostaglandin derivatives such as misoprostol are gastric protecting agents that exhibit a multifactorial mechanism of action. They form a viscous adhesive substance that protects the GI lining against pepsin, peptic acid, and bile salts. These cytoprotective agents are used for short-term management of NSAID-induced gastric ulcers and may also be used to prevent gastric ulcers [57-59].

2.5. Antimuscarinic agents

Antimuscarinic agents (pirenzepine, atropine) inhibit gastric secretion by exerting anticholinergic activity on the parietal cells. Antimuscarinics that are used for GI smooth muscle spasm include the tertiary amines atropine sulphate and dicycloverine hydrochloride (dicyclomine hydrochloride) and the quaternary ammonium compounds propantheline bromide and hyoscine butylbromide. The quaternary ammonium compounds are less lipid-soluble than atropine and may be less likely to cross the blood-brain barrier; they are also less well absorbed. Although central nervous system (CNS)-like side effects such as confusion are thereby reduced, peripheral nervous system side effects are common with quaternary ammonium compounds. Nonselective antimuscarinics (e.g., belladonna alkaloids) are outmoded treatments, any clinical virtues being

outweighed by atropinic side effects [constipation, transient bradycardia (followed by tachycardia, palpitations, and arrhythmias), reduced bronchial secretions, urinary urgency and retention, dilatation of the pupils with loss of accommodation, photophobia, dry mouth, flushing and dryness of the skin].

2.6. Anti-*Helicobacter pylori* therapy

Eradication of *H. pylori* improves the healing of gastric and duodenal ulcers and reduces the risk of recurrence or rebleeding [60] and, when therapy is initiated within 6 months of diagnosis, decreases the risk of hospitalization for major bleeding events [61].

Triple therapy incorporating clarithromycin, amoxicillin, and a PPI is generally considered first-line therapy for eradication of *H. pylori*, although metronidazole may be used in place of amoxicillin when resistance to the latter is a concern [12, 62]. Bismuth- and non-bismuth-based quadruple therapy regimens are also used in the first-line setting. Levofloxacin-containing triple therapy (PPI, levofloxacin, and amoxicillin) may be used as rescue therapy [12]. Although treatment duration has traditionally been 10~14 days, shorter treatment regimens are being evaluated in clinical trials in order to improve compliance, reduce costs, and circumvent potential adverse events [60].

As a component of drug combination therapy, clarithromycin effectively treats duodenal ulcer or gastric ulcer associated with *H. pylori* infection. Clarithromycin inhibits bacterial growth, possibly by blocking dissociation of peptide t-RNA from ribosomes, arresting RNA-dependent protein synthesis. Amoxicillin effectively treats duodenal ulcer or gastric ulcer associated with *H. pylori* infection. Amoxicillin interferes with synthesis of cell wall mucopeptides during active multiplication, resulting in bactericidal activity against susceptible bacteria. Metronidazole effectively treats gastric ulcer associated with *H. pylori* infection. Metronidazole is active against

various anaerobic bacteria and protozoa and appears to be absorbed into cells. Intermediate-metabolized compounds formed, bind DNA, and inhibit protein synthesis, causing cell death.

Antibiotic resistance is a significant problem impairing the efficacy of anti-*H. pylori* agents [12]. Resistance to nitroimidazoles (e.g., metronidazole), for example, is encountered in approximately 35% of *H. pylori* strains in industrialized countries, while in developing countries nearly all strains are nitroimidazole-resistant. Macrolide resistance is somewhat lower, affecting approximately 15% and 25~50% of *H. pylori* strains in westernized and developing countries, respectively. Clarithromycin resistance is increasingly common and is the main reason for failure to cure *H. pylori* infection with existing treatment regimens [12, 63]. Resistance to penicillin was rare until the late 20th century but is now detected with increasing frequency. Studies of resistance mechanisms in *H. pylori* have demonstrated that the pathogen easily develops antibiotic resistance *de novo*, although horizontal gene transfer via natural transformation cannot be excluded as another possible mechanism of resistance acquisition [46].

2.6.1. Combination therapy

Current standards for combination therapy have been proposed based on the Maastrich two Consensus Report, the National Institute for Clinical Excellence, and the European Helicobacter Study Group. According to the suggested model, first-line combination therapy for a patient with confirmed *H. pylori* consists of amoxicillin [1000 mg bis in die (b.i.d.)] plus clarithromycin (500 mg b.i.d.) and a PPI (20~40 mg b.i.d.). As an alternative, metronidazole (500 mg b.i.d.) can be substituted for amoxicillin in the suggested regimen. In either case, treatment should be continued for 7~14 days. If patient does not respond (failure rates as high as 20~50% have been reported for these regimens in clinical practice [64] second-line quadruple drug therapy should be initiated, incorporating metronidazole [500 mg ter in die (t.i.d.)], tetracycline (500 mg, four times daily),

bismuth (120 mg, four times daily) and a PPI (20~40 mg b.i.d.), continuing for 7~10 days [46]. In a randomized, open-label, noninferiority phase III trial, quadruple therapy (bismuth subcitrate potassium/metronidazole/tetracycline plus omeprazole) for seven days yielded eradication rates of 80%, as compared to 55% for standard therapy (omeprazole/amoxicillin/clarithromycin), with similar safety and adverse event profiles. Given the improved efficacy of the quadruple drug regimen together with the rising prevalence of drug-resistant *H. pylori*, it has been recommended that quadruple drug therapy be considered for first-line treatment [64, 65]. Because of putative toxicity concerns, however, bismuth is not available in all countries [13].

In Japan, where eradication rates with triple therapy have been decreasing and are currently around 70%, the incorporation of rabeprazole as the PPI component in a triple therapy regimen has been evaluated in a large-scale nationwide study. In 3162 patients with *H. pylori*-positive gastric/duodenal ulcer, an 80% eradication rate was obtained with triple therapy incorporating rabeprazole (10 mg), amoxicillin (750 mg), and clarithromycin (200 or 400 mg) administered twice daily for seven days. The combination was deemed safe and effective in this population [66].

In Italy and other countries where standard triple therapy has suffered a significant decline in cure rates, sequential therapy has emerged as a highly effective treatment option. According to this regimen, an initial five-day course of therapy with a PPI plus amoxicillin is followed by five more days of PPI plus clarithromycin and tinidazole. Eradication rates of 90% or higher have been obtained using sequential therapy [63, 67].

2.6.2. Anti-*Helicobacter pylori* vaccines

Although the success rate of antibacterial therapy is high (approximately 90%), vaccination represents an attractive alternative approach for a variety of reasons, especially in the developing

world. Advantages of vaccination over drug therapy include ease of administration, circumventing problems of adherence and preventing reinfection, as well as the potential to treat even asymptomatic infections [32].

Several attempts have been made to develop an anti-*H. pylori* vaccine, but to date none have progressed beyond the earliest stages of clinical testing. The earliest vaccines tested were subunit vaccines; although these proved promising in animal studies, efficacy in humans was much lower and adjuvant-related side effects were encountered [32]. One potential approach is the development of vaccines directed to one or more so-called virulence factors. *H. pylori* has several virulence factors, and some of these are believed to be potentially useful for incorporation in a vaccine. A few early studies using different vaccine formulations have demonstrated the ability of vaccines to reduce *H. pylori* load in infected humans. The mechanism by which protection exerted is unknown, although nonclassical immune factors appear to be involved. In addition to mechanistic studies, further studies are required to determine the most effective antigen(s), route of administration, appropriate small- and large-animal models, and adjuvants for formulation in an eventual anti-*H. pylori* vaccine [32, 68-70].

2.7. CCKB antagonists

The peptide hormone and neurotransmitter cholecystokinin (CCK) is widely distributed throughout the GI tract and CNS, where it is involved in the regulation of various biological functions. The action of CCK is mediated by two distinct receptor subtypes: CCKA, which are highly selective for cholecystokinin, and CCKB, which are selective for both CCK and gastrin [47]. CCKA receptors are found in peripheral tissues such as gallbladder, pancreas, and ileum, as well as in discrete brain areas. CCKB receptors are present throughout the brain and are also found in the stomach. The biological roles of the peripheral CCKA receptors are well

characterized and include gallbladder contraction, enzyme secretion, and gut motility. Peripheral CCKB receptors principally mediate the stimulation of gastric acid secretion and regulate gastric mucosal hypertrophia. CCK and gastrin antagonists have been pursued for the treatment of gastric acid secretion disorders, including gastric ulcer.

3. Study of new drug from plant extracts to treat gastritis

3.1. Plant extracts with antigastritis activity

Among the studied plant extracts, those belonging to the Asteraceae, followed by the Combretaceae and Fabaceae families, were the most frequently studied and were reported to have promising wound healing, antioxidant, anti-inflammatory, cytoprotective, gastric secretion inhibition, mucus production improvement, HSP70 up-regulation, *Bax* protein down-regulation, antisecretory, and anti-*H. pylori* effects. Table 2 is briefly described the studied plant extracts with antiulcerogenic activity, its corresponding modes of action and studied models.

Table 2. Plant extracts with gastro-protective activity

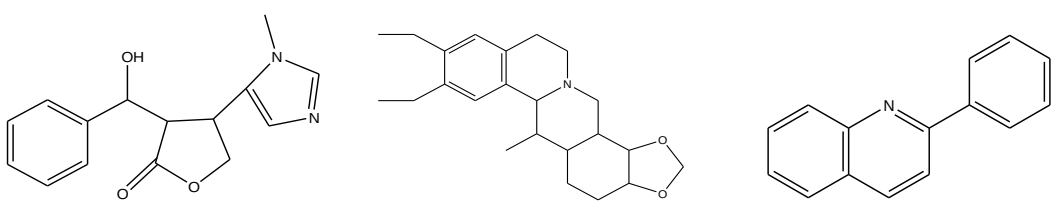
Family	Scientific name	Mechanism of gastro-protection	Reference
Acanthacea	*Barleria lupulina*	Anti-inflammatory, wound healing, antisecretory	[71]
Acanthacea	*Eremomastax speciosa*	Wound healing, antisecretory, mucus production, antioxidant, cytoprotective	[72]
Anacardiaceae	*Anacardium occidentale*	Wound healing, antioxidant, anti-inflammatory, cytoprotective	[73]
Anacardiaceae	*Anacardium humile*	Wound healing, mucus production, anti-inflammatory, cytoprotective	[74]
Apiaceae	*Centella asiatica*	Wound healing, mucus production, antioxidant, anti-inflammatory	[75]
Asteraceae	*Artemisia asiatica*	Wound healing, antioxidant, anti-inflammatory, mucus production, gastro-protective	[76]
Asteraceae	*Baccharis dracunculifolia*	Wound healing, antioxidant, mucus production	[77]
Asteraceae	*Baccharis trimera*	Wound healing, antisecretory, antioxidant	[78]
Asteraceae	*Hieracium gymnocephalum*	Wound healing, anti-inflammatory	[79]
Asteraceae	*Tanacetum larvatum*	Wound healing, anti-inflammatory, antioxidant	[80]
Asteraceae	*Vernonia condensate*	Wound healing, inhibition of gastric secretion, antioxidant, mucus production, cytoprotective	[81]
Asteraceae	*Solidago chilensis*	Wound healing, antioxidant, antisecretory, mucus production	[82]
Bignoniaceae	*Kigelia Africana*	Wound healing, antioxidant	[83]
Boraginaceae	*Cordia dichotoma*	Wound healing, antioxidant, anti-inflammatory	[84]
Boraginaceae	*Cordia verbenacea*	Wound healing, antioxidant, cytoprotective	[85]
Caesalpinieae	*Caesalpinia sappan*	Wound healing, antioxidant, cytoprotective, anti-inflammatory	[86]
Caesalpinieae	*Archidendron jiringa*	Wound healing, mucus production, antioxidant	[87]
Caesalpinieae	*Alhagi maurorum*	Antioxidant, antiapoptotic	[88]
Calophyllaceae	*Mammea Americana*	Wound healing, antisecretory	[89]
Capparaceae	*Capparis zeylanica*	Wound healing	[90]
Celastraceae	*Maytenus robusta*	Wound healing, mucus production, antioxidant, anti-inflammatory, cytoprotective, gastro-protective, antisecretory	[91]

Family	Scientific name	Mechanism of gastro-protection	Reference
Cibotiaceae	*Cibotium barometz*	Wound healing, antioxidant, HSP70 up-regulation, *Bax* protein down-regulation, mucus production	[92]
Combretaceae	*Terminalia arjuna*	Wound healing, anti-*H. pylori*, antisecretory	[93]
Combretaceae	*Terminalia belerica*	Wound healing, antisecretory, mucus production	[94]
Combretaceae	*Terminalia catappa*	Wound healing, anti-*H. pylori*, mucus production, anti-inflammatory, cytoprotective	[95]
Combretaceae	*Terminalia chebula*	Wound healing, antisecretory, cytoprotective	[96]
Combretaceae	*Terminalia coriacea*	Wound healing, antisecretory, mucus production, antioxidant	[97]
Combretaceae	*Terminalia fagifolia*	Wound healing, antisecretory, antioxidant	[98]
Crassulaceae	*Bryophyllum pinnatum*	Wound healing, anti-inflammatory	[99]
Cucurbitaceae	*Momordica cymbalaria*	Wound healing, antisecretory	[100]
Cucurbitaceae	*Mukia maderaspatana*	Wound healing, antioxidant, anti-inflammatory, mucus production	[101]
Cyperaceae	*Cyperus rotundus*	Wound healing, antioxidant activity, anti-inflammatory	[102]
Fabaceae	*Cassia sieberiana*	Wound healing, cytoprotective, antioxidant, anti-inflammatory	[103]
Fabaceae	*Parkia speciosa*	Wound healing, mucus production, antioxidant, anti-inflammatory, HSP70 up-regulation, *Bax* protein down-regulation	[104]
Fabaceae	*Tamarindus indica*	Wound healing, antisecretory	[105]
Hypericaceae	*Cratoxylum arborescens*	Wound healing, anti-*H. pylori*, antisecretory, mucus production, antioxidant, antiapoptotic, anti-inflammatory, cytoprotective	[106]
Lamiaceae	*Calamintha officinalis*	Wound healing, antioxidant	[107]
Lamiaceae	*Hyptis suaveolens*	Wound healing, cytoprotective, anti-inflammatory	[108]
Lamiaceae	*Tectona grandis*	Wound healing, inhibition of gastric secretion	[109]
Meliaceae	*Spondias mombin*	Wound healing, antioxidant, anti-inflammatory, inhibition of gastric secretion	[110]
Meliaceae	*Toona ciliate*	Wound healing, antisecretory, cytoprotective	[111]
Moraceae	*Ficus religiosa*	Wound healing, inhibition of gastric secretion	[112]

Family	Scientific name	Mechanism of gastro-protection	Reference
Moringaceae	*Moringa oleifera*	Wound healing, serotonin release, antisecretory, cytoprotective, anti-inflammatory	[113]
Myristicaceae	*Myristica malabarica*	Wound healing, anti-inflammatory, angiogenesis, cytoprotective	[114]
Papaveraceae	*Argemone Mexicana*	Wound healing	[115]
Piperaceae	*Piper betle*	Wound healing, antioxidant, mucus production, anti-inflammatory	[116]
Rhamnaceae	*Scutia buxifolia*	Wound healing, antioxidant	[117]
Rhamnaceae	*Ziziphus jujuba*	Anti-*H. pylori*, mucus production, antioxidant	[118]
Rubiaceae	*Morinda citrifolia*	Wound healing, antisecretory, antioxidant, anti-inflammatory	[119]
Salvadoraceae	*Salvadora indica*	Wound healing, cytoprotective	[120]
Santalaceae	*Osyris quadripartita*	Wound healing	[121]
Talinaceae	*Talinum portulacifolium*	Wound healing, antisecretory	[122]
Urticaceae	*Cecropia glaziovii*	Wound healing, antisecretory	[123]
Zingiberaceae	*Aframomum pruinosum*	Wound healing, anti-*H. pylori*, mucus production, anti-inflammatory, cytoprotective	[124]

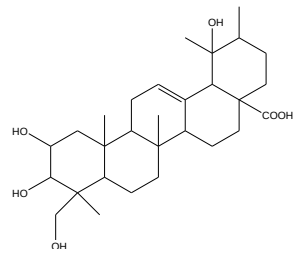
3.2. Phytochemicals with antigastritis activity

Biological activity of plants is mainly related to the presence of plant secondary metabolites, which have a specific function and role. A wide pool of phytochemicals including tannins, flavonoids, alkaloids, terpenoids, and phenolic glycosides have been reported to be responsible for the observed gastro-protective and antiulcerogenic properties of the various plants used in gastritis management. This suggests plants and their bioactive phytochemicals as upcoming viable sources of antiulcer agents. Furthermore, the therapeutic benefits of plant extracts may be attributed both to a single component or even to the combined action of a mixture of phytoconstituents. Figure 1 shows the most common and widely used plant secondary metabolites with antiulcer activity, which majorly includes alkaloids, flavonoids, phenolic acids, and essential oils. Numerous plant secondary metabolites have been reported to display antiulcer activity through different mechanisms of action in many experimental models of ulcers that are induced by ethanol, acetic acid, NSAIDs, stress, *H. pylori*, and so on. Indeed, plant secondary metabolites exert antiulcer activity through different mechanism; predominantly via antioxidant, anti-inflammatory, antimicrobial, antisecretory, anticholinergic, and cytoprotective effects. The phytochemical-related modes of action are summarized in Table 3.

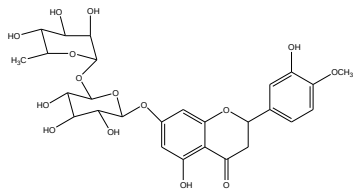


Epiisopilturine Cavidine 2-Phenylquinoline

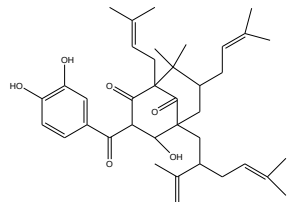
Figure 1. Structures of phytochemicals evaluated as antigastritis agents.



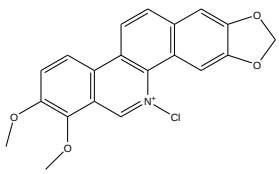
23-Hydroxytormentic acid 28-*O*-glucoside



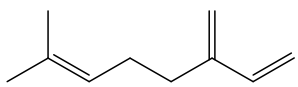
Hesperidin



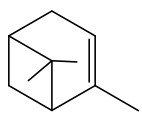
Garcinol



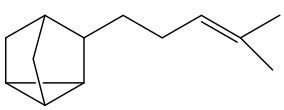
Chelerythrine



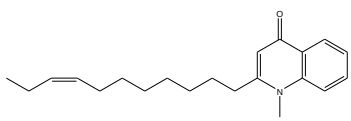
β-Myrcene



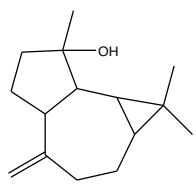
α-Pinene



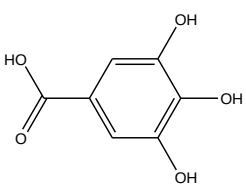
α-Santalene



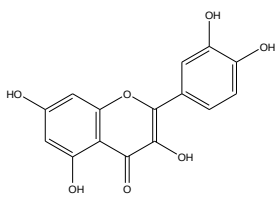
1-methyl-2-[(z)-8-tridecenyl]-4-(1*H*)-quinolone



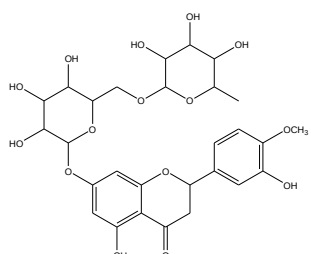
Spathulenol



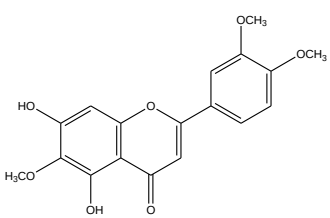
Gallic acid



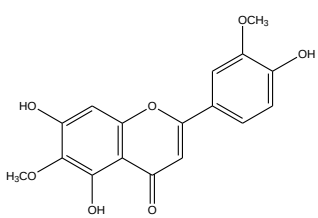
Quercetin



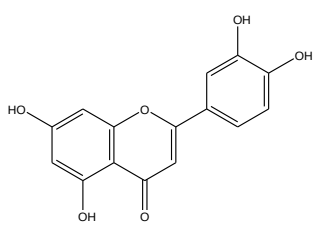
Hesperidin



Eupatilin



Jaceosidin



Luteolin

Figure 1. (continued).

Table 3. Antigastritis activities of phytochemicals

Phytochemical	Plant source	Mode of action	Reference
Alkaloids			
1-methly-2-[(z)-8-tridecenyl]-4-(1*H*)-quinolone	*Evodia rutaecarpa*	Highly selective activity against *H. pylori*	[125]
2-Phenylquinoline	*Galipea longiflora*	SOD and GST activity normalization, increased GSH and reduced LPO and TNF-α levels in gastric mucosa	[126]
Cavidine	*Corydalis impatiens*	Increased mucosa GSH, SOD and PGE_2 levels, decreased IL-6 and TNF-α levels	[127]
Chelerythrine	Papaveraceae and Rutaceae family	Reduced myeloperoxidase activity, IL-6 and TNF-α levels and inhibited NO	[128]
Epiisopiloturine	*Pilocarpus microphyllus*	Reduced pro-inflammatory cytokines, oxidative stress and increased gastric mucosal blood flow	[129]
Terpenoids			
23-hydroxytormentic acid 28-*O*-glucoside	*Rubus coreanus*	Increased SOD and GPx activity	[130]
α-Pinene	*Pistacia atlantica*	Antibacterial activity on metronidazole-resistant *H. pylori*	[131]
α-Santalene	*Gallesia integrifolia*	Gastroprotective and curative effects, due to antioxidant, anti-inflammatory, antisecretory, mucogenic and nitrergic and activity	[132]
β-Myrcene	*Citrus aurantium*	Decreased gastric and duodenal lesions, SOD activity, increased gastric mucus production, mucosal MDA levels and GPx and GR activity	[133]
Phenolic			
Gallic acid	Widespread in plant kingdom	Inhibited gastric acid secretion or through antioxidant action	[134]

Phytochemical	Plant source	Mode of action	Reference
Flavonoids			
Eupatilin	*Artemisia asiatica*	Gastroprotective activity through antioxidant and anti-inflammatory action	[76]
Garcinol	*Garcinia indica*	Suppressed superoxide anion, hydroxyl, and methyl radicals	[135]
Hesperidin	*Citrus sinensis*	Increased GSH and mucin levels, prevented oxidative cell injury	[136]
Jaceosidin	*A. asiatica*	Gastroprotective activity through antioxidant and anti-inflammatory action	[76]
Luteolin	*A. asiatica*	Gastroprotective activity through antioxidant and anti-inflammatory action	[76]
Quercetin	Widespread in plant kingdom	Antioxidant, antisecretory, anti-inflammatory and mucosa regeneration	[137]

3.3. Herbal medicines tested in clinical trials

Herbal medicines have been used to treated gastric ulcer for millennia. Several controlled studies have demonstrated that herbal medicines are effective in treating human gastric ulcer (Table 4). Eighty five percent of patients with gastric ulcer showed improvement after orally given a herbal mixture three times daily for 6 weeks [138]. Similarly, oral herbal mixtures two or three times daily for 2 months induced a >90% improvement in patients with gastric ulcer [139, 140]. The efficacy of herbal medicines in treating gastric ulcer is comparable to that of famotidine, a histamine H2 receptor antagonist. Herbal medicine is comparable or superior to cimetidine in treating either gastric or duodenal ulcers [141-144]. It has been reported that oral herbal medicines for 4 weeks achieved superior efficacy to cimetidine in treating gastric and duodenal ulcers, as well as gastritis [143]. Stillen® tablet (60 mg of *A. asiatica* 95% ethanol extract per tablet, t.i.d.)

was compared the efficacy and the safety with cetraxate (600 mg, a mucosal protective agent) in 512 patients with erosive gastritis [145]. The efficacy evaluation related to endoscopic cure rate was found to be significant in Stillen® tablet (52%) in comparison with cetraxate group (35%). Both Stillen® tablet and cetraxate produced no treatment-associated adverse events.

Moreover, combination of herbal medicine and ranitidine exhibited a synergistic effect in treating gastric ulcer [146]. Herbal medicine effectively cures gastric ulcer and prevent its recurrence. For example, oral herbal tablets induced a 62.4% cure rate while the recurrence rate was 17.7% after 1-year follow-up [147]. In contrast, treatment with ranitidine only achieved a 50.7% cure rate, and the recurrence rate was 54.1%. Likewise, oral combination of omeprazole and herbal medicine for 4 weeks significantly reduced gastric ulcer recurrence rate (25%) compared with omeprazole alone (57.1%) after 6 months follow-up [148].

Taken together, these results demonstrated that herbal medicines alone are effective in treating gastric ulcer and preventing recurrence. Combination of herbal medicines and conventional regimens exhibits a synergistic effect in the management of gastric ulcer. Although all mixtures that are listed in Table 4 are effective for gastric ulcer, herbal medicines should be given according to each patient's internal conditions as defined by the theory of traditional herbal medicine to gain an optimal benefit.

Table 4. Efficacy and safety of herbal medicines for gastric ulcer in humans

Herbal extract	No. patients (M/F)[a]	Treatment day	Efficacy		Adverse effects	Reference
			Herbal extract	Positive control		
Rhizoma Coptidis, Radix Sanguisorbae, radix paeoniae alba, rhizoma bletilla, Chickens Gizzard membrane	60 (41/19)	42	Cure[b] rate: 20% Effective[c] rate: 85%	Cure rate: 20% Effective rate: 72%	None	[138]
Radix Astragali, *Radix Aucklandiae*, *Fructus Aurantii*, *Immaturus*, *Cortex Magnoliae Officinalis*, Chickens Gizzard membrane, *radix notoginseng*, *radix paeoniae alba*, *Radix*, *Scutellariae*, *Radix Glycyrrhizae*	50 (35/25)	60	Cure rate: 72% Effective rate: 96%	ND[d]	Vomiting in one case	[139]
Radix Astragali, radix codonopsis, poria, Rhizoma Atractylodis Macrocephalae, dried orange peel*, Radix Glycyrrhiza*	84 (43/41)	60	Effective rate: 93%	ND	ND	[140]
Radix Bupleuri, Radix Codonopsis, radix paeoniae alba, rhizoma corydalis, rhizoma bletilla, margarita, indigo naturalis, radix glycyrrhizae	26 (15/11)	28	Effective rate: 92%	Effective rate: 92%	Temporary diarrhea at beginning	[149]
Margarita*, borax, Rhizoma Coptidis, rhizoma bletilla, indigo naturalis, amber*	90 (ND)	30	Cure rate: 89% Effective rate: 97%	Cure rate: 83% Effective rate: 90%	None	[141]
R*hizoma curculiginis, Herba Epimedii, Radix Astragali, rhizoma bletilla, poria, Fructus Amomi, Radix Glycyrrhizae Preparata*	62 (44/18)	30	Cure rate: 82% Effective rate: 98%	Cure rate: 81% Effective rate: 93%	5 cases dry mouth; 7 cases constipation	[142]
Radix Codonopsis, Herba Taraxaci, Radix Salviae miltiorrhizae, Rhizoma Atractylodes alba, Radix Glycyrrhizae	30 (22/8)	56	Cure rate: 50% Effective rate: 87%	Cure rate: 40% Effective rate: 70%	ND	[143]
Ramulus Cinnamomi, Radix Paeoniae Alba, Radix, Glycyrrhizae Preparata, Rhizoma Zingiberis Recens, Fructus Jujubae, Sacchaium Granorum, Radix Cynanchi Paniculati	80 (58/22)	28	Cure rate: 45% Effective rate: 94%	Cure rate: 10% Effective rate: 74%	ND	[144]

Herbal extract	No. patients (M/F)[a]	Treatment day	Efficacy		Adverse effects	Reference
			Herbal extract	Positive control		
Radix Astragali, Taraxacum mongolicum Hand, tokyo violet herb, Bulbus Lilii, Radix Linderae, Radix Salvia miltiorrhiza, radix paeoniae alba, Radix Glycyrrhizae	12 (ND)	28	Cure rate: 100%	Cure rate: 63%	ND	[150]
Artemisia asiatica (Stillen® tablet)	520 (ND)	14	Cure rate: 52%	Cure rate: 51%	None	[145]

[a] M: male; F: female.
[b] Cure: clinical symptoms disappeared.
[c] Effective: clinical symptoms improved.
[d] ND: not determined.

4. *Opuntia ficus-indica* (L.) Miller

Figure 2. *O. ficus-indica* plant and its fruits.

Opuntia ficus-indica, commonly called prickly pear or nopal cactus, belongs to the dicotyledonous angiosperm Cactaceae family (Figure 2), a family that includes approximately 1500 species of cactus. This plant species is a tropical and subtropical plant. It can grow in arid and semi-arid climates with a geographical distribution encompassing Mexico, Latin America, South Africa, and Mediterranean countries [151]. Most of regions are concentrated in Mexico (from which it is native) and the Americas. This plant species has spread to some African and Asian regions. In Korea, it has been cultivated Jeju island.

Opuntia ficus-indica is employed in health, nutrition, and cosmetics in the form of tea, jam, juice, and oil extracted from seeds. It is used as herbal remedy for diverse health problems in different countries. For instance, in the sub-Saharan traditional medicine pharmacopeia, *O. ficus-indica* flowers and fruits are given as antiulcer or antidiarrheal agents; flowers are administered as an oral antihemorrhoid medication, and cladode sap as a treatment for whooping cough. On the other hand, indigenous populations consume substantial amounts of either fresh or dry fruits as food. In these populations, *O. ficus-indica* cladodes, fruits, and flowers are featured for their interesting contents of antioxidants, pectin polysaccharides, and fibers.

Opuntia ficus-indica fruits and stems have been traditionally used in folk medicine in several countries for several medicinal purpose [152]. However, many researchers have focused

their investigations for studying genus *Opuntia* in order to discover the properties of plant that could form the basis of their use in the prevention and cure of chronic disease. Therefore, clinical pharmacological interests in the efficacy and safety of the phytochemicals present in genus *Opuntia* have grown during recent years due to the realization that many people try to self-medicate with this plant species.

4.1. Nutritional contents and bioactive constituents of *Opuntia ficus-indica*

The main component of *O. ficus-indica* cladodes is water (80~95%), followed by small amounts of carbohydrates (3~7%), fiber (1~2%), and protein (0.5~1%); other compounds are only partly known and have not been quantitatively determined [153]. *O. ficus-indica* also represent phytochemicals, such as phenolic acids and flavonoids [154].

Cactus dietary fiber is composed of several chemical components that are resistant to digestive enzymes such as cellulose, hemicelluloses, pectin, lignin, gums, etc. [155]. The benefits associated with fiber content are well known, especially for the prevention of illnesses such as diabetes, treatment of GI disorders, illnesses associated with low dietary fiber intake, reduction of glucose value in the blood, antihyperlipidemic, and antihypercholesterolemic effects [8].

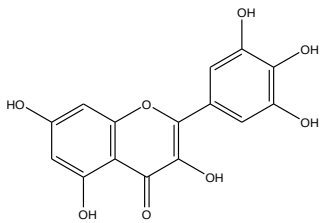
Opuntia ficus-indica was found to have various classes of bioactive compounds (Figure 3). It has been established that the amount of phytochemicals varies between genus *Opuntia*; for example, fruits contain taurine (7.7~11.2 mg/100 g fresh fruit) for Sicilian cultivars but at a lower concentration than that reported for American and African cultivars [156]. The content of total phenolic and polyphenolic compounds (free and conjugated) is in concentrations of 80~90 mg/100 g dried weight, which include aromadendrin, taxifolin, dihydroquercetin, isorhamnetin, vitexin, kaempferol, quercetin, betalanins, betacyanins, rutin, isorhamnetin and derivatives like myricetin, orientin, and some derivatives of pyrone [157]. The profiles of phenolic and non-

phenolic components reported to be present in *O. ficus-indica* are presented in Table 5, as described by Ahuja *et al.* [158].

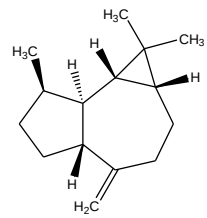
Table 5. Phenolic and non-phenolic compounds in *O. ficus-indica* plant tissues

Tissue	Major compounds
Flower	Gallic acid Quercetin 3-*O*-rutinoside Quercetin 7-*O*-rutinoside Kaempferol 3-*O*-rutinoside Quercetin 3-*O*-glucoside Isorhamnetin 3-*O*-robinobioside Isorhamnetin 3-*O*-galactoside Isorhamnetin 3-*O*-rutinoside Isorhamnetin 3-*O*-glucoside
	Isorhamnetin 7-*O*-rutinoside Isorhamnetin 3-*O*-rhamnosyl 7-*O*-rutinoside Kaempferol 3-*O*-arabinoside
	Other compounds Terpene (limonene, linalool, germacrene D, aromadendrene, squalene) Ester and alkaloid (ethyl linoleate) Sterol Carboxylic acid (linoleic, palmitic, octanoic, butanedioic, pentanedioic) Phenolic acid: hydroxycinnamic acid derivative (5-hydroxyferulic acid-rhamnosidehexoside, caffeoyl methoxycinnamoyl quinic acid, 1,4-diferuloyl syringic acid, 4-*p*-coumaroyl caffeic acid, 1,5-dicaffeoyl ferulic acid; 1,4-syringicferuloyl 4-coumaroyl caffeic acid)
Whole fruit / pulp	Flavonoid Myricetin Quercetin Isorhamnetin Kaempferol Luteolin Isorhamnetin glycoside Kaempferol glucoside Catechin
	Phyllocactin Protocatechuic acid
	4-hydroxybenzoic acid Ferulic acid Vanillic acid (also isovanillic acid) *Trans*-coumaric and *trans*-cinnamic acid Syringic, fukic, piscidic, and eucomic acid
	Betalains Taxifolin Orientin Vitexin

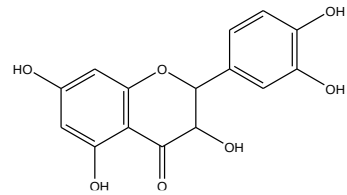
Tissue	Major compounds
	Betaxanthin Pigment Portulacaxanthin I, portulacaxanthin III, muscaaurin, indicaxanthin, (*S*)-serine-betaxanthin, (*S*)-valine-betaxanthin, (*S*)-isoleucine-betaxanthin, (*S*)-phenylalaine-betaxanthin, vulgaxanthin I, vulgaxanthin II, miraxanthin II, betanin, iso-betanin
	Volatile organic compound (VOC) 2-decanynoic acid, γ-terpinene, linalool, α-farnesene etc.
	Other compounds Carotenoid [Lutein-5,6-epoxide, (all-*E*)-β-criptoxanthin, (all-*E*)-α-carotene, (all-*E*)-bcarotene, (9*Z*)-β-carotene, lycopene] Ascorbic acid and tocopherol Amino acid (taurine, cystine) Biothiol (glutathione)
Seed	Phenolic acid Feruloyl-sucrose isomer 1 Feruloyl-sucrose isomer 2 Sinapoyl-diglucoside Flavonoid Tannin
	Fatty acid (linoleic, oleic, palmitic, stearic, vaccenic); sterols, vitamin E
Peel / skin	Total phenolic acid Flavonoids – kaempferol, quercetin, isorhamnetin, isorhamnetin glucoside
	17-decarboxybetanin, betanin
	Xanthophyll
	Fatty acid; mineral (Fe, Mn, Mg, Ca, Zn); glucose, fructose
	Phospholipid and glycolipid
	Phytoestrogen
Cladode	Phenolic acid – gallic acid, coumaric acid, 3,4-dihydroxybenzoic, 4-hydroxybenzoic, ferulic, salicylic, vanillic syringic, synaptic acid, protocatechuic acid etc.
	Flavonoid – isorhamnetin, quercetin, kaempferol, isoquercetin, nicotiflorin, rutin, narcissin
	Alkanes (heptadecane) Ascorbic acid β-carotene, Lutein
	Pectic polysaccharide Lignan
Stem	Flavonoid Glycoside, coumarin
	Terpenoid Triterpene, labdane-type diterpene
	Tannin Polysaccharide Fatty acid (azelaic acid, palmitelaidic acid)



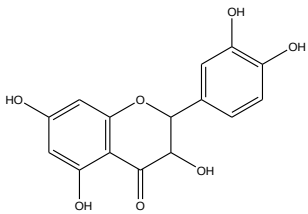
Myricetin



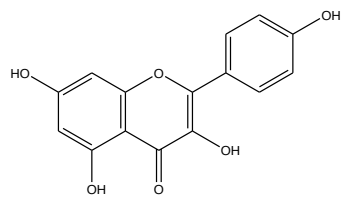
Aromadendrene



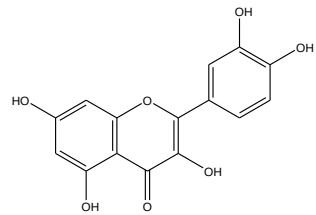
Taxifolin



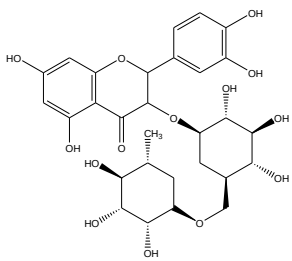
Dihydroquercetin



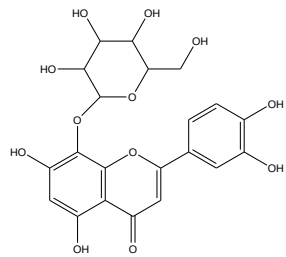
Kaemferol



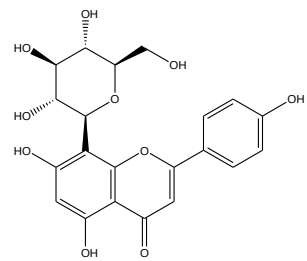
Quercetin



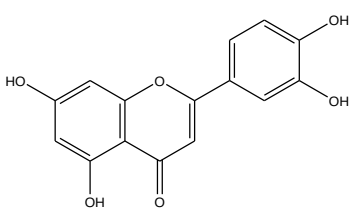
Rutin



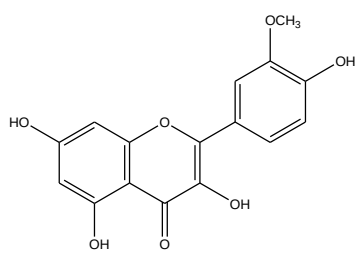
Orientin



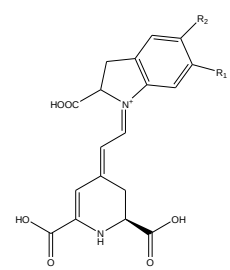
Vitexin



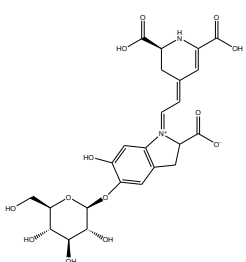
Luteolin



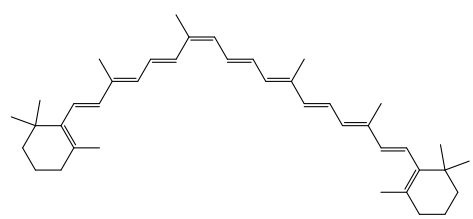
Isorhamnetin



Betacyanin



Betanin



β-caroten

Figure 3. Structures of important phytochemicals in *O. ficus-indica*.

4.2. Biological activities of *Opuntia ficus-indica*

Opuntia species contain a wide array of phenolic and non-phenolic constituents which singly or synergistically exert biological activities. Biological activities and source of *O. ficus-indica* and *in vivo* and *in vitro* models are summarized in Table 6, as described by El-Mostafa *et al.* [159].

Table 6. Major biological activities of *O. ficus-indica* in different experimental models

Biological activity	Source	*In vivo* and *in vitro* model
Hypolipidemic and hypocholesterolemic	Cladode powder	Rat
	Cladode	Mouse
	Seed powder and oil	Rat
Antidiabetic	Capsule: cladode and peel extract	Human
	Cactus powder in capsule	Human
	Aqueous extract of the cladode and fruit	Rat
	Cladode and fruit skin extract (capsule)	Human
Hypoglycemic	Polysaccharide extracted from cladode	Rat
	Extract powder racket after drying	Rat
Anti-inflammatory	Indicaxanthin from fruit	Human intestinal epithelial cell line (Caco-2 cells) stimulated by cytokine IL-1β
	Lyophilized extract of cladode	Human chondrocyte cultures stimulated with IL-1β
	Indicaxanthin from fruit	Rat Pleurisy
	Methanol extract of stem (active substance: β-sitosterol)	Mouse
	Methanol extract of fruit (Betalain, indicaxanthin)	*In vitro* study of human myeloperoxidase
Anti-inflammatory and antioxidant	Butanol and methanol extract of fruit	*In vivo* study in gerbil, i*n vitro* studies in cultured mouse cortical cell
Antioxidant	Betalain a pigment purified from fresh	Endothelial cells human

Biological activity	Source	*In vivo* and *in vitro* model
	pulp	umbilical vein
	Betanin from fruit extract	Human RBC
	Ethanol extract of stem	Mouse splenocyte
	Flavonoids of whole fruit	Rat
	Glycoprotein extracted from *O. ficus-indica*	Mouse
	Fruit	Human
Antimicrobial	Methanol extract of cladode	Bacteria: *Campylobacter jejuni*
	Methanol, ethanol, and aqueous extract of cladode	Bacteria: *Vibrio cholerae*
	Hexane extract from flower	Bacteria: *Staphylococcus aureus* *Pseudomonas aeruginosa*
	Aqueous and alcohol extract of cladode	Bacteria: *Proteus mirabilis*

4.2.1. Flower

Opuntia flowers come in various colors but colors progression during flowering begins from white through yellow to red/orange/pink/peach/cream. Flowers are auxiliary, large, bisexual, and possess tepals arrange spirally [160]. The *O. ficus-indica* flowers are considered as by-products and are usually cast-off after fruit separation. Compared to the maceration method of extraction, the Soxhlet method was more efficacious and demonstrated the presence of thermostable polyphenols which contributed significantly to the antioxidant activity of *O. ficus-indica* flowers. Similarly, the green and economic processing method of accelerated solvent extraction gave improved yield/expression of flavonoids and phenols than the maceration method [161].

The presence of flavonoids in extracts has also been associated with anti-inflammatory, antiatherosclerotic, and antiangiogenic bioactivities [161-163]. The *Opuntia* flower methanol and mucilage extracts have shown complete wound healing capacities on day thirteen of treatment

[164]. The plant flowers are rich in natural bioactive polyphenolic components which could find use in food industry, as well as in pharmaceutical and cosmeceutical preparation. These functions give them added value and reduce their environmental impact as waste materials [161].

The antioxidative and cardioprotective activities of flavonoids arise from the ability to inhibit lipid peroxidation, chelate redox-active metals, and inhibit processes involving reactive oxygen species. They do this by electron transfer, activating antioxidant enzymes, and inhibiting oxidases [158]. Anti-inflammatory activity is brought about by the inhibition of inflammatory pathway and enzyme involved, and induction of nitric oxide synthase and COX-2 enzymes [165]. The induction of cell apoptosis by active compounds is related to anticancer activity where proapoptotic protein expression is elevated. Chromatin condensation, mitochondria-transmembrane-de-polarization protein decrease, and cellular shrinkage in cancer cells are some of the reported anticancer mechanisms of action [166]. Phytosterols act by reducing intestinal absorption of cholesterols. They act also able to elevate the levels of antioxidant enzymes and induce cell death [158].

4.2.2. Fruit/pulp

Opuntia fruits are the succulent, ellipsoid, and edible part of the plant. They are usually about 7 cm long but can have a wide range of colors which include green, white reddish, purple, and yellow [160]. Betalains contributes to fruit color and biological activity of fruit pulp. Biological activity in betalain compounds have been linked to intrinsic structural features such as those attributed to the betalamic acid core of betalain structures which appear to play a modified role in enhancing or reducing biological activity [167]. The number of position of hydroxy groups and glycosylation in these pigmented compounds determine their antioxidative activities, as is the case with flavonoids phenolic constituents in *Opuntia* fruits [167].

Opuntia fruit/pulp extracts possess antidiabetic, cardioprotective, neuroprotective, anti-inflammatory, and hepatoprotective properties [158]. Betanin was reported to reduce chronic myeloid leukemia K562 cell spread, and cell death was inhibited. The ethyl acetate extract of the fruits containing flavonoids, *trans*-taxifolin, and dihydrokaempferol also suppressed HeLa cervical carcinoma cell proliferation, while normal human BJ fibroblasts were unaffected, which suggests potential application as intervention for human cervical carcinoma management [168].

The antioxidant activity of *Opuntia* fruits is reported to be comparable with that of red oranges and grapes [169]. Antioxidant activity have been reported for purified *Opuntia* fruit polysaccharides [170]. Antioxidant activity and vitamin C content varied according to different fruit samples. Purple fruit of *O. ficus-indica* usually showed the highest antiradical ability [171]. In general, the biological capacities and nutraceutical benefits of *Opuntia* fruit species may be due to the synergistic effects of betalains, flavonoids, and other biologically active constituents present. The highlighted applications suggest that *Opuntia* fruit/extracts have potential for industrial application in the food, agricultural, and nutraceutical industries [158].

4.2.3. Seed

Opuntia seeds refer to the small hard part of the *Opuntia* fruit which consists of the embryo and its food source, protected within a seed testa/coat. It is mature plant ovule which may be sown and capable of germination and growth into a whole new plant [160]. Clinical evidence has shown that polyunsaturated fatty acids can help in managing stroke and coronary heart disease symptoms. Interestingly, *Opuntia* seed oil extracted without the use of solvent showed significant reduction in alloxan-induced death *in vivo* [172]. It has been reported that DPPH (2,2-diphenyl-1-picrylhydrazyl) scavenging activity was reported to contribute to this effect. The seed oil achieved 86% inhibition compared to vitamin C which gave 97% DPPH inhibition after 90 min of mice

treatment [172]. This reduction capacity can be attributed to vitamin K1, tocopherols, β-carotene, phenolic compounds, and unsaturated fatty acids in the seed extract [173, 174].

Significant differences in antioxidant activity have been recorded for ground seeds compared to whole *Opuntia* seeds which was attributed to their high total phenol composition. Seeds are found in abundance could be explored for their phenolic and non-phenolic compound compositions, as well as for their biological activities which are related to the aromaticity of phenol compound structure, number of hydroxy group, and nature of other chemical substituents present [158].

4.2.4. Peel/skin

Opuntia feel makes up 60% of the entire fruit. Peels are the rinds of the *Opuntia* fruit which are often discarded after separation from the fruit and are regarded as a by-product [173]. As food ingredients, fruit peels can be considered a source of fiber for prebiotic preparations and antioxidant [175]. Non-digestible oligosaccharides from plant by-products with prebiotic potential can undergo fermentative processes involving lactic acid-producing bacteria and bifidobacteria to produce new compounds, with enhanced health benefits [175].

The antioxidant peel constituents include oleuro, pyrogallol, benzoic, 3-OH tyrosol, ellagic, chorogenic, protocatechuic acid, epicatechin, and gallic acid. Peel extracts used in hamster diet supplementation increased excretion of cholesterol and lowered liver cholesterol levels [176]. Mechanism of extracts hypocholesterolemic activities include inhibition of hepatic HMG-CoA activity, interference with cholesterol absorption in the intestine and bile acid trafficking and antioxidant activity [176]. Flavonoid glycosides dominate the flavonoid profile of the fruit peels [177]. Other bioactive constituents include phytoestrogens and terpene alcohols [175]. The fruit peel extract have also exhibited anticancer activity in Ehrlich ascites carcinoma cells [178].

The physicochemical properties of peel fibers can be exploited in the food industry to improve food product parameters such as shelf-life, sensory attributes, staling, and viscosity. Peels are cheap and readily available, and these factors enhance the potential for commercialization. They may also be applied as fat or sugar substitutes to stabilize oxidative processes in foods, and oil and water retention capacity enhancers [158].

4.2.5. Cladode

Cladodes are the spiny or spineless paddle-like, oblong (up to 70~80 cm), thick and succulent part of the *Opuntia* plant species with varying withs. They possess a waxy, water repellent epidermis and are capable of photosynthesis and asexual reproduction. Small bristles (glochidia) may be present with barbs in the areoles [160]. Cladode extracts of *O. ficus-indica* have been shown to lower cholesterol levels and have antiulcer and anti-inflammatory activities [179]. Nopal extract containing quercetin, isorhamnetin, kaempferol, and vitamin C have also shown antioxidant activity [180] and hepatoprotective activity [181]. Antidiabetic and antioxidant activities of cladode extracts have been attributed to significant amounts of polysaccharides, as well as lignans, flavonoids, and phenolic acids in the cladode extracts [182]. The cladode extracts rich in polysaccharides and polyphenols were also able to decrease the hydroxyl radical-induced oxidation of linoleic acid and DNA and may be used as surfactant and natural antioxidants in food and pharmaceutical industries [183]. Mucilage, pectin, and total pectic polysaccharide fractions of *O. ficus-indica* cladodes were characterized and showed antioxidant activity [184]. The radical scavenging abilities of cladode extract may be linked to presence of phenolic antioxidant and vitamin contents [185]. Cladodes of the *Opuntia* cultivars have further demonstrated useful anticarcinogenic selenium-enriched chemotherapeutic properties and could serve as food providing advanced dietary pharmacology to help fight human disease [171].

III. Materials and Methods

1. Preparation of test materials

1.1. Instrumental analysis

^{1}H and ^{13}C-nuclear magnetic resonance (NMR) spectra were recorded in DMSO-d_6 and methanol-d_4 on Avance 600 spectrometer (Bruker, Karlsruhe, Germany) at 400 and 300 MHz and 100 and 75 MHz, respectively, using tetramethylsilane as an internal standard, and chemical shifts are given in δ (ppm). UV spectra were obtained in methanol on a UV-2101 spectrophotometer (Shimadzu, Kyoto, Japan) and mass spectra were obtained via liquid chromatographic introduction into a Finnigan LCQ advantage Max ion trap mass spectrometer, equipped with a Finnigan Surveyor Modular HPLC system (Thermo Fisher Scientific, Waltham, MA, USA). Ascentis Express C-18 column (4.6 × 150 mm, 2.7 μm, Supelco, Bellefonte, PA, USA) was used. Diaion HP20 (250~850 μm, Mitsubishi, Tokyo, Japan) and Sephadex LH-20 (25~100 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden) were used for column chromatography. Pre-coated silica gel plates (silica gel 60 F_{254}, 0.25 and 0.5 thicknesses, Merck, Darmstadt, Germany) were used for analytical thin-layer chromatography (TLC). An Agilent 1200 high-performance liquid chromatography (Agilent, Santa Clara, CA, USA) and an Isolera™ Prime medium pressure liquid chromatography (Biotage, Uppsala, Sweden) were used to isolate the active principles. Biotage® SNAP KP-C18-HS (12 g, C_{18}, 100 Å, 30 μm) column, Agilent ZORBAX Eclipse XDB column (9.4 × 250 mm, 5 μm) and Capcell Pak UG120 column (4.6 × 250 mm, 5 μm, Shiseido, Tokyo, Japan) were used.

1.2. Chemicals and reagents

The two commercially available antigastritis agents used in these studies were Mucosta® tablet and Stillen® tablet. they were purchased from Wonjin Pharm (Seoul, Korea). Ethanol, HCl, aspirin, indomethacin, and diclofenac sodium were purchased from Sigma-Aldrich (St. Louis, MO, USA). Betanin was purchased from TCI (Tokyo, Japan). Narigenin, eriodictyol, taxifolin, kaemperol, quercetin, isokaempfride, and 3-*O*-methyl quercetin were obtained from Sigma-Aldrich. All of the other chemicals and reagents used in this study were of reagent-grade quality and are available commercially.

1.3. Preparation of *O. ficus-indica* fruit ethanol extracts

The freeze-dried purple powders of *O. ficus-indica* fruit were purchased from west agricultural technology center in Jeju. Each powder (100 g) of *O. ficus-indica* fruits were extracted with 1 L ethanol [0 (water), 10, 20, 30, 40, 50, 60, 70, 80, 90, and 95% by volume] twice at room temperature 1 day and filtered. The combined filtrate was concentrated by rotary evaporation at 40ºC and was dried using freeze dryer (Eyela, Tokyo, Japan) (Table 7).

Table 7. Ethanol extraction of *O. ficus-indica* fruits

Ethanol (%)	Yield[a]
0 (Water)	48.5
10	48.9
20	51.6
30	51.6
40	52.8
50	51.9

60	48.8
70	50.2
80	42.5
90	39.3
95	22.2

[a] (dry weight of extracts / dry weight of *O. ficus-indica* fruit) × 100

1.4. Bioassay-guided fractionation and isolation of O. *ficus-indica* fruits

Freeze-dried powders (4 kg) of *O. ficus-indica* fruits were extracted with ethanol (10 L) twice at room temperature for 1 day and filtered. The combined filtrate was concentrated by rotary evaporation at 40ºC to yield 111.6 g of a dark purple soft extract. The extract (20 g) was sequentially partitioned into hexane-soluble (4.9 g), chloroform-soluble (4.9 g), butanol-soluble (13.4 g), and water-soluble (88.9 g) portions for subsequent bioassays. This fractionation procedure was repeated five times. The organic solvent-soluble fractions were concentrated at 40ºC, and water-soluble fraction was concentrated at 50ºC. To isolate the active constituents, each *O. ficus-indica* fruit-derived fractions (0.4~100 μg/mL) was tested in aspirin induced cytotoxicity assay as described by Graziani *et al*. [186].

The butanol-soluble fraction was the most biologically active fraction and was subjected to HP-20 diaion column chromatography through elution with a three gradient of water and methanol [100:0 (2 L), 50:50 (2 L), and 0:100 (2 L) by volume] to yield 3 fractions (each of which reached 2 L) (Figure 4). Column fractions were monitored by TLC on silica gel plates developed with a chloroform and methanol (9:1 by volume) mobile phase. Fraction B-3 was separated by medium-pressure liquid chromatography (MPLC) with UV detector at 254 nm and SNAP column cartridge through elution with a 50% methanol at a flow rate of 10 mL/min to give seven fractions

(each of which reached 50~120 mL). Fraction B-3-4 was subjected to Sephadex LH-20 column chromatography with methanol to provide five fractions (each of which had a volume of 200 mL). Compound **1** was obtained from B-3-4-4 by recrystallization. Another active fraction B-3-5 was subjected to Sephadex LH-20 column chromatography with methanol to afford three fractions (each of which reached 200 mL). Fraction B-3-5-2 was separated by preparative high-performance liquid chromatography (HPLC) with a mobile phase of acetonitrile and water at a flow rate of 5 mL/min. Finally, active compound **2** was isolated at a retention time of 18.834 min (Figure 5).

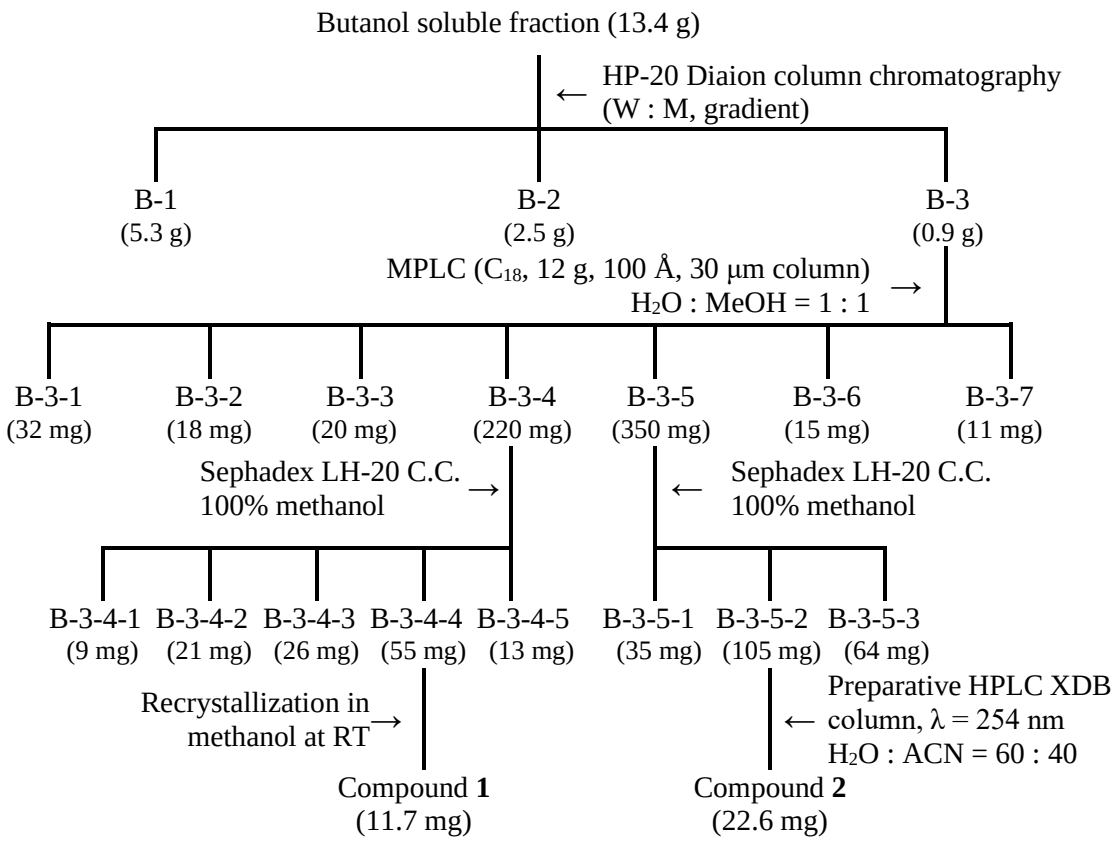


Figure 4. Procedure to isolate the active constituents from of *O. ficus-indica* fruits.

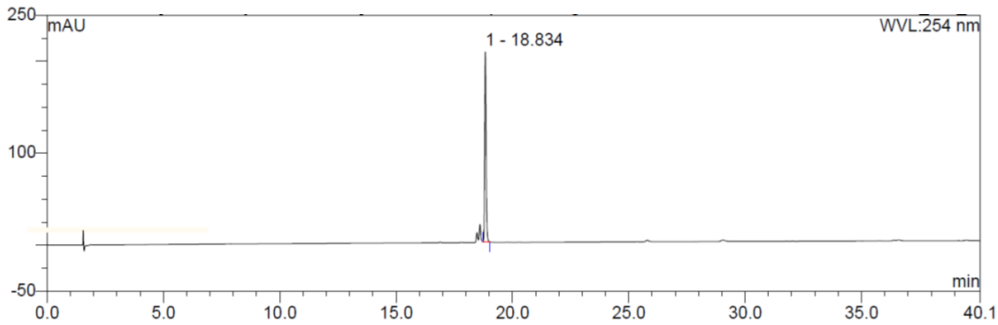


Figure 5. HPLC chromatogram of compound 2.

1.5. High-performance liquid chromatography with diode array detector and electrospray ionization mass spectrometry chemical analysis

HPLC-DAD-ESIMS analysis, a Finnigan LCQ advantage Max ion trap mass spectrometer, equipped with a Finnigan Surveyor Modular HPLC system was used. The injection sample was prepared by filtering through nylon membrane filters (0.2 μm). Chromatographic separation was achieved on an Ascentis Express C18 column. The mobile phase consisted 0.1% formic acid (v/v) as an aqueous mobile phase (A) and acetonitrile as an organic mobile phase (B) at a flow rate of 0.3 mL/min, with gradient elution as follows: 0 min, 20% (v/v) B; 25 min, 80% B; 25.1 min, 20% B; 30 min, 20% B. The injection volume was 10 μL of the sample solution that were used for the HPLC analysis. ESI was used as the ionization source for the MS in negative mode. The MS parameters were as follow: source voltage 4.8 kV, capillary voltage 3.0 V, the scanned mass range was 100~1000 mass unit, nebulizing gas was pure nitrogen with flow rate of 8 mL/min and block and capillary temperature 275 ºC. For the UV spectra were recorded on the DAD detector at λ = 190~600 nm and the cell and column temperature were set at 30ºC.

1.6. High-performance liquid chromatography analysis of narcissin and aromadendrin

HPLC analysis of narcissin and aromadendrin in *O. ficus-indica* fruit ethanol extracts were performed using Agilent Alliance 1200 HPLC System. Sample (10 μL) were applied to a capcellpack UG120 column. The mobile phase was 0.1% formic acid (v/v) as an aqueous mobile phase (A) and 0.1% formic acid (v/v) acetonitrile as an organic mobile phase (B). The isocratic condition was a follow: 0~30 min, 18% B. The flow rate was set at 1.0 mL/min and UV absorbance was monitored at 353 and 290 nm. Maximum UV absorbance of narcissin and aromadendrin were 353 and 290 nm, respectively. Narcissin and aromadendrin were analyzed simultaneously in same condition. Chromatographic data were collected and analyzed using and an Agilent ChemStation chromatography software (version B.04.03). In analysis of narcissin, the calibration curve was plotted from 5 to 100 μg/mL with a correlation coefficient greater than 0.999. Precision values were determined as the coefficient of variations (each, n = 3). The precision ranged from 0.02 to 0.20%, with accuracy ranging from 97.8 to 101.3%. In analysis of aromadendrin, the calibration curve was plotted from 5 to 100 μg/mL with a correlation coefficient greater than 0.999. Precision values were determined as the coefficient of variations (each, n = 3). The precision was ranged from 0.01 to 0.10%, with an accuracy ranging from 98.7 to 103.5%. The results obtained were reproducible and satisfied the Korea MFDS criterion (MFDS, 2003).

1.7. High-performance liquid chromatography analysis of betanin

HPLC analysis of betanin in *O. ficus-indica* fruit ethanol extracts were performed using Agilent Alliance 1200 HPLC System. Sample (10 μL) were applied to a capcellpack UG120 column. The mobile phase was 0.1% formic acid (v/v) with 5% (v/v) acetonitrile as an aqueous

mobile phase (A) and 0.1% formic acid (v/v) 95% (v/v) acetonitrile as an organic mobile phase (B). The gradient condition was a follow: 0−25 min, 0−50% B. The flow rate was set at 1.0 mL/min and UV absorbance was monitored at 535 nm. Chromatographic data were collected and analyzed using an Agilent ChemStation chromatography software. The calibration curve was plotted from 5 to 100 μg/mL with a correlation coefficient greater than 0.999. Precision values were determined as the coefficient of variations (each, n = 3). The precision ranged from 0.5 to 5.0%, with accuracy ranging from 97.0 to 102.3%. The results obtained were reproducible and satisfied the MFDS criterion (MFDS, 2003).

1.8. Mass production of OF-80E (*O. ficus-indica* fruits 80% ethanol extract)

Of all tested ethanol extracts, 80% ethanol extract was most effective. The code name of *O. ficus-indica* fruits 80% ethanol extract was named OF-80E. OF-80E (25 kg) was manufactured by bGMP (bulk good manufacturing practice) plant (Bioland, Cheongju, Chungbuk, Korea). Freeze-dried powders (100 kg) of *O. ficus-indica* fruits were extracted by extract solvent that were consist of 800 L ethanol [Korean pharmacopoeia (KP) grade] and 200 L water (KP grade) for 4 h stirred. Filtrate was filtered by filter (1 μm) pressor. Sequentially, 400 L ethanol (KP grade) and 100 L water (KP grade) were added to residue for 2 h stirred. Filtrate was filtered by filter (1 μm) pressor. The combined filtrate was concentrated at below 40ºC and was vacuum dried. Finally, dark purple dried powder (25 kg) was obtained.

2. Evaluation of gastro-protective activity in an *in vitro* model

2.1. Gastric AGS cell cultures

Human epithelial gastric cell line, AGS (ATCC CRL-1739) cells, was purchased from the

America type culture collection (ATCC, Manassa, VA, USA). The AGS cells were monolayers in the Roswell Park Memorial Institute medium (Sigma-Aldrich) with 10% heat inactivated fetal bovine serum (FBS) (Gibco, Waltham, MA, USA), 100 IU/mL penicillin (Sigma-Aldrich), and 100 µg/mL streptomycin (Sigma-Aldrich) in humidified incubator (Thermo Fisher Scientific, Waltham, MA, USA) with 5% CO_2 in 37ºC.

2.2. Ethanol-induced cytotoxicity in AGS cells

The protective effect of the OF-80E and betanin against ethanol-induced damage in AGS cells was assessed using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromine] assay according to the method described by Yeo *et al*. [187]. The AGS cells were seeded in 96-well plates with a density of 1×10^4 cells/well. After 24 h, the cells were treated with medium containing the OF-80E at concentrations of 1, 10, 100, 200, and 500 µg/mL or betanin at concentrations of 1, 2, 5, 10, and 50 µM. The control group was treated with serum free medium. After 1 h, the medium was added with 9% ethanol for 30 min. The cell viability was determined using MTT assay. The MTT reagents were dissolved in phosphate-buffered saline (PBS) and added to each well at a final concentration of 0.5 mg/mL. The cells were then incubated for 1 h. The supernatant was removed, and 100 µL of dimethyl sulfoxide (DMSO) was added to each well to dissolve the formazan salts formed. Absorbance (optical density, OD) was measured at 570 nm. The percentage of cell viability was determined using the following equation:

cell viability (%) = (OD control – OD sample) / OD control × 100

2.3. Aspirin-induced cytotoxicity in AGS cells

The protective effect of the *O. ficus-indica* fruit ethanol extracts, solvent partition isolated fractions, OF-80E, betanin, and flavonoid compounds against aspirin-induced apoptosis in AGS

cells was assessed using the MTT assay according to the method described by Graziani *et al*. [186]. The AGS cells were seeded in 96-well plates with a density of 1×10^4 cells/well. After 24 h, the cells were treated with medium containing the ethanol extracts at concentrations of 0.4, 1.2, 3.7, 11.1, 33.3, and 100 μg/mL or the solvent partition isolated fractions at concentrations of 0.04, 0.12, 0.37, 1.11, 3.33, and 10 μg/mL or the OF-80E at concentrations of 1, 10, 100, 200, and 500 μg/mL or betanin at concentrations of 1, 2, 5, 10, and 50 μM and flavonoid compounds at concentrations of 0.5, 1, 5, and 10 μM. The control group was treated with serum free medium. After 1 h, the medium was added with aspirin (4 mM) for 24 h. The cell viability was determined as described above.

2.4. Determination of reduced glutathione level in AGS cells

In order to evaluate the concentration of glutathione (GSH), AGS cells were seeded in 24 well plates with a density 1×10^5 cells/well plate for 24 h. The cells were then treated with medium containing the OF-80E at a concentration of 100 μg/mL or betanin at a concentration of 10 μM. The control group was treated with serum free medium. After 1h, the medium was added with aspirin (4 mM) for 24 h. Cells were washed with ice-cold PBS, harvested by scraping into PBS, and homogenized in 300 μL of extraction buffer [0.1% Triton X-100 and 0.6% sulfosalicylic acid in KPE (0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5)]. GSH in the acid-soluble supernatant was analyzed with an enzyme assay using microplate reader (Molecular Devices, San Jose, CA, USA) [188]. GSH content was expressed a nmole/mg protein, determined by the Bradford assay kit (Bio-Rad Laboratory, Hercules, CA, USA).

2.5. Determination prostaglandin E_2 level in AGS cells

In order to evaluate the concentration of PGE_2, AGS cells were seeded in 24 well plates

with a density 1×10^5 cells/well plate for 24 h. After 24 h, The cells were treated with medium containing the OF-80E at a concentration of 100 μg/mL or betanin at a concentration of 10 μM. The control group was treated with serum free medium. After 1 h, the medium was added with aspirin (4 mM) for 24 h. PGE_2 levels were determined in the medium using an enzyme-linked immunosorbent assay (ELISA) kit (Cayman, Ann Arbor, MI, USA) according to the manufacture's protocol.

2.6. Data analysis

All data were presented as means ± standard errors of mean (SEM). Statistical analyses were performed with one-way analysis of variance (ANOVA), followed by Dunnett's *post hoc* test. *P* values < 0.05 were considered statistically significant. All statistical tests were carried out using the computer program Graph Pad Prism 7 (GraphPad Software, San Diego, CA, USA).

3. Evaluation of gastro-protective activity in an *in vivo* model

3.1. Animals

The animals used in this study were male Sprague Dawley (SD) rats (7 weeks and 25~30 weeks old) (Orient, Gapyeong, Gyeonggi, Korea). In all experiments, 7 weeks old rats were used except diclofenac-induced gastritis. Rats were allowed to acclimate in polycarbonate cages (W235 × L380 × H175 mm) with 5 animals per cage on aspen chip bedding for a week to the controlled environment (temperature, 20~23ºC; 12 h light cycle from 9:00 to 21:00; food, Agribrand Purina Korea, and water *ad libitum*). After acclimation 250~300 g rats were used for the experiments. The procedures used for the care and euthanasia of animals complies with the Guide for Care and Use of Laboratory Animals published by US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and was approved by International Animal Care and

Use Committee (IACUC) of Gyeonggi Bio Center (12-03-0046, 12-04-0002, 12-05-0021, 12-05-0039, 12-06-0032, 12-07-0034, and 12-08-0009).

3.2. Ethanol-induced gastritis rats

The experiment was carried out according to the method of Mizui and Doteuchi [189]. OF-80E, Stillen® tablet and Mucosta® tablet were dissolved in 1% hydroxy propyl methyl cellulose (HPMC) before use. After 24 h of fasting, the rats (8 weeks old, n = 10/group) were pre-treated orally with OF-80E [50, 100, 150, and 200 mg/kg body weight (BW)], Stillen® tablet (60 mg/kg BW as *A. asiatica* ethanol extract), Mucosta® tablet (100 mg/kg BW as rebamipide), and vehicle (1% HPMC). 1 h after tested sample treatments, the rats were received 5 mL/kg acidified ethanol [150 mM HCl in 60% ethanol; post oral (p.o.)]. 1 h later, the rats had been euthanized in a CO_2 chamber, the stomachs were removed and then the area of the lesions was measured.

3.3. Indomethacin-induced gastritis rats

The experiment was carried out according to the method of Urushidani *et al*. [190] with slight modifications. After 24 h of fasting, the rats (8 weeks old, n = 10/group) were pre-treated orally with OF-80E, Stillen® tablet, Mucosta® tablet, and vehicle, as described above. 1 h after tested sample treatments, the rats received indomethacin in 0.5% carboxyl methyl cellulose sodium salt (CMC-Na) (100 mg/kg; p.o.) to induce acute gastric ulcer. 6 h later, the stomachs of the rats were removed and then the area of the lesions was measured, as described above.

3.4. Aspirin-induced gastritis rats

The experiment was carried out according to the method of Naito *et al*. [191] with slight modifications. After 24 h of fasting, the rats (8 weeks old, n = 7/group) were pre-treated with

orally OF-80E, Stillen® tablet, Mucosta® tablet, and vehicle, as described above. Thirty minutes after, rats were anesthetized by intramuscular injection of 15 mg/kg tiletamine/zolazepam (Zoletil50®; Virbac Lab., Carros, France) and 9 mg/kg xylazine (Rompun®; Bayer, Leverkusen, Germany). If the tail was moved (a sign of awakening) during the operation, an additional 5 mg/kg tiletamine/zolazepam and 3 mg/kg xylazine were injected by intramuscular. The abdomen was opened below the xiphoid process, and the pyloric end of the stomach was ligated. The stomach was placed back carefully, and the abdomen was sutured. After 30 min of tested sample treatments, the rats received aspirin in 0.5% CMC-Na (200 mg/kg, p.o.) to induce acute gastric ulcer. 6 h later, the stomachs of the rats were removed and then the area of the lesions was measured, as described above.

3.5. Stress-induced gastritis rats

Water immersion restraint stress (WIRS) is widely accepted for studying stress ulcers. The experiment was carried out according to the method of An *et al*. [192] with slight modifications. After 24 h of fasting, the rats (8 weeks old, n = 7/group) were pre-treated orally with OF-80E, Stillen® tablet, Mucosta® tablet, and vehicle, as described above. Thirty minutes after tested sample treatments, the rats were placed in individual restraint cages and vertically immersed in a water bath (20ºC) to the level of the xyphoid process for 6 h. The stomachs of the rats were removed and then the area of the lesions was measured, as described above.

3.6. Diclofenac-induced gastritis rats

As people age, the gastro cells damage caused by NSAIDs intensified, so old rats (20~25 weeks old) were used in this test. The experiment was carried out according to the method of Matsui *et al*. [193] with slight modifications. Daily for 14 days, the rats (n = 7/group) were pre-

treated orally with OF-80E, Stillen® tablet, Mucosta® tablet, and vehicle, as described above. 1 h after tested sample treatments, the rats received diclofenac sodium 0.5% CMC-Na (50 mg/kg; p.o.) to induce gastric ulcer. After treated 14 day, the stomachs of rats were removed and then the area of the lesions was measured, as described above.

3.7. Determination of gastric lesion index

The removed rat stomachs were immediately fixed in 2% formalin for 5 min. The stomachs were collected and opened along the greater curvature, washed in saline, fixed between Petri dishes, and photographed (Sony Cyber-Shot Dsc-h2, Tokyo, Japan) at 72 dpi resolution (2816 × 2112 pixels). Hemorrhagic or ulcerative lesions were measured, and the lesion area was compared to that of the total area of each stomach through computerized planimetry using the Image J program (National Institutes of Health, Bethesda, MD, USA). The area of the damage in each rat was summed, and the total was used as the lesion index. The lesion inhibition ratio of each group was calculated using the following equation: lesion inhibition (%) = [lesion area of vehicle control group (mm^2) – lesion area of test group (mm^2)] / [lesion area of vehicle control group (mm^2)] ×100

3.8. Gastric adherent mucus assay

The level of mucus bound to the gastric epithelial surface was determined according to the method of Kang *et al*. [194]. The glandular portion of the stomach was excised and immersed for 2 h in 0.1% alcian blue (Sigma-Aldrich) in sucrose solution. The unbound dye was then removed by two subsequent washing, and the mucus-bound dye was eluted by immersing the stomach in 30% docusate sodium salt solution for 2 h. After centrifugation of the eluent, the optimal density of the alcian blue solution was read at 620 nm and calculated using the calibration curve. The

results were expressed as the concentration (μg/g tissue) of alcian blue adhering to the gastric mucosal surface.

3.9. Measurement of mucosal myeloperoxidase and tumor necrosis factor-alpha

The scraped mucosa was homogenized, and the membrane-bound myeloperoxidase (MPO) was solubilized. The homogenate was sonicated, thawed, and centrifuged. The supernatant was used to determine a gastric mucosal MPO concentration using ELISA kit (HK 105-02 rat MPO kit; Hycult Biotech, Noord-Brabant, Netherlands) according to the manufacturer's instructions. The concentration of tumor necrosis factor-alpha (TNF-α) in the supernatant was measured using an ELISA kit specific for rat TNF-α (Quantikine® immunosaasy kit; R&D Systems, Minneapolis, MN, USA).

3.10. Measurement of reduced glutathione level of mucosa

The scraped mucosa was washed with ice-cold PBS, harvested by scraping into PBS, and homogenized in extraction buffer [0.1% Triton X-100 and 0.6% sulfosalicylic acid in KPE (0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5)]. GSH in the acid-soluble supernatant was analyzed with an enzyme assay using a microplate reader (Molecular Devices) [188]. GSH content was expressed as nmole/mg protein, using by the Bradford assay kit (Bio-Rad Laboratory).

3.11. Measurement of histological index of gastric tissue

The histological index of gastric tissue was determined according to the method of Rosers [195]. The stomach tissues of different groups were fixed with 10% buffered formalin, dehydrated, cleared with xylene, and infiltrated (at 60ºC) and embedded with paraffin wax. Paraffin blocks

were cut at 5 μm and affixed to slides and then stained. Sections were stained with hematoxylin and eosin for general histological method. The assessment criteria of histological score was according to the method described by Dokmeci *et al*. [196] : 0, normal mucosa; 1, epithelial cell damaged; 2, glandular disruption, vasocongestion or edema in the upper mucosa; 3, mucosal disruption, vasocongestion or edema in the mid-lower mucosa; and 4, extensive mucosal disruption involving the full thickness of the mucosa. The overall sum value of the damage score of each of the fields was taken as the histology index for the section.

3.12. Data analysis

All data were presented as means ± SEMs. Statistical analyses were performed with one-way ANOVA, followed by Dunnett's *post hoc* test ($P < 0.05$). All statistical tests were carried out using the computer program Graph Pad Prism 7.

4. Toxicity studies of OF-80E

4.1. Single dose oral toxicity study in Sprague Dawley rats

4.1.1. Animals

Specific pathogen-free (SPF) SD rats were obtained from Koatech (Namyangju, Gyeonggi, Korea). Rats were allowed to acclimate in stainless steel cages (W215 × L355 × H200 mm) with 5 animals per cage on aspen chip bedding for a week to the controlled environment (temperature, 23 ± 2ºC; humidity, 55 ± 15%; ventilation of 10~20 air change/h, 150~300 lux luminous intensity; and 12 h light cycle from 9:00 to 21:00) in the experimental animal facility at Preclinical Research Center, Chemon (Yongin, Gyeonggi, Korea). Throughout the study period, the temperature and humidity of animal room were measured every hour with a computer-based automatic sensor, and

the environmental conditions such as ventilation frequency and luminous intensity were monitored on a regular basis. The room was maintained at a room temperature of 21.4~22.7ºC, and a relative humidity of 55.6~60.1%. Animals were offered irradiation-sterilized pellet diet (Teklad certified irradiation global 18% protein rodent diet, 2918C, Harlan Laboratories, Indianapolis, IN, USA) *ad libitum*. Underground water disinfected by ultraviolet sterilizer and ultrafiltration were given via water bottle *ad libitum*. After acclimation 250~300 g rats were used for the experiments. The rats were allowed to acclimate the environment condition described above for 1 week prior to study initiation. After acclimation 214~229 g (male) and 149~161 g (female) rats were used for the experiments. The present study was approved by IACUC of Preclinical Research Center, Chemon (Serial no., 12-R103). This study was conducted under guideline of Organization for Economic Cooperation and Development (OECD) [197] as well as good laboratory practice (GLP) regulations for nonclinical laboratory studies of Korea MFDS [198].

4.1.2. Experimental design

Healthy male and female SD rats (7 weeks old) were randomly assigned to four group (5/sex/group). Vehicle [1% methyl cellulose (MC)] or graded dose of OF-80E (2500, 5000, and 10000 mg/kg of BW) were administered to rats by oral gavage once at dose of 20 mL/kg of BW. The rats were observed for mortality and clinical signs every hour for 6 h after dosing during the first 24 h and then once daily for a total of 14 days. BWs were recorded on days 0, 1, 7, and 14 after the treatment. On day 14 after administration, all animals were etherized with the CO_2 gas and complete post-mortem examinations were performed on all vital organs.

4.1.3. Data analysis

The data of BWs were expressed as means ± stds (standard deviations) and were subjected to a Student's *t*-test to compare the treatment group with the vehicle control group. The significance level was judged at a probability value of $P < 0.05$. The commercial statistics program, Statistical Package for the Social Sciences (SPSS) software (ver. 10.1K) (SPSS, Chicago, IL, USA), was used for analyses.

4.2. Thirteen-week repeated-dose oral toxicity study with a four-week recovery in Sprague Dawley rats

4.2.1. Experimental design

The present study was approved by IACUC of Preclinical Research Center, Chemon (Serial no., 12-R519). Healthy male and female SD rats were randomly assigned to four groups (10/sex/group). Vehicle and highest dose groups were assigned more animal (5/sex/group) that were for recovery study. Vehicle (1% MC) or graded dose of OF-80E (500, 1000, and 2000 mg/kg of BW) were administered to rats by oral gavage once daily for 13 weeks at dose of 10 mL/kg of BW. During the administration and observation periods, animals were observed daily for clinical signs including mortality, and if any, the type, date, and severity of signs were recorded individually. Animals with severe symptoms were isolated to prevent cannibalism, and if dead animal found, they were weighed immediately and processed in the same way as scheduled necropsy. Body weight, food and water consumption were recorded weekly throughout the study.

4.2.2. Urine collection and blood sampling

In the last week (main group, 13 weeks administration; recovery group, 4 weeks recovery after 13 weeks administration) of observation, 5 animals/sex/group of the main and recovery

group were housed in a metabolic cage (W255 × D255 × H475 mm, Jeungdo, Seoul, Korea) for urine collection. The urinalysis was performed on about 1 mL of urine sample freshly collected for 3~4 h and the volume of urine collected for 24 h was used to measure the total volume of urine. Animal were fasted overnight (with water available) prior to the blood sampling for clinical pathology. The samples were taken from the posterior vena cava of all animals of scheduled necropsy under deep isoflurane (Ifran liquid, Hana Pharm.) anesthesia.

4.2.3. Urinalysis

About 0.3 mL of urine was soaked into a test strip (Multistix 10SG, Siemens, Munich, Germany) and was analyzed by a Siemens CliniTek Advantus automatic analyzer to measure following parameters. The urine color was observed in the animal room and the results were entered into the automatic analyzer.; urine color, bilirubin, pH, nitrite, clarity, ketone body, protein, occult blood, glucose, specific gravity, and urobilinogen. The urine that had been subjected to the urine color test was centrifuged (1500 rpm, 425 RCF, MF300, Hanil Scientific, Gimpo, Gyeonggi, Korea) for 5 min. The sediments were stained with Stenheimer-Marbin method and following items were counted with a microscope.; leukocytes [white bold cell (WBC)], epithelial cells, erythrocyte [red blood cell (RBC)], and cast.

4.2.4. Hematological test

Approximately 1 mL blood was collected in a complete blood cell (CBC) bottle (Vacutainer 3 mL, Becton Dickinson, Franklin Lakes, NJ, USA) that contained anticoagulant EDTA-2K and the following parameters were detected with a Siemens ADVIA 2120 coulter counter.; RBC, red cell distribution width, neutrophil, haematocrit, hemoglobin distribution width, lymphocyte, hemoglobin concentration, mean platelet volume, monocyte, mean corpuscular volume, platelet,

eosinophil, mean cell hemoglobin, basophil, WBC, mean cell hemoglobin concentration, large unstained cells, and reticulocytes. At necropsy, approximately 1.8 mL blood was dispensed into microtube containing 0.2 mL of 3.2% sodium citrate. Plasma was then obtained from centrifugation and the following parameters were measured with a coagulation time analyzer. (ACL 100, Instrumentation Laboratory, Bedford, MA, USA); activated partial thromboplastin time and prothrombin time.

4.2.5. Clinical biochemistry test

About 2 mL of blood was dispensed into a 5 mL vacutainer tube (Insepack, Sekisui, Tokyo, Japan) with a clot activator. The blood was left at room temperature for 15~20 min for clotting and then centrifuged at 3000 rpm for 10 min using Hanil Scientific 1902 RCF, Combi-514R centrifuge. The following parameters were measured by a AU680 serum biochemistry analyzer (Beckman Coulter, Brea, CA, USA).; aspartate aminotransferase, total cholesterol, creatinine, alanine aminotransferase, triglyceride, inorganic phosphorus, alkaline phosphatase, total protein, creatine phosphokinase, total bilirubin, albumin, albumin/globulin ratio, glucose, blood urea nitrogen, calcium ion (Ca^{2+}), sodium ion (Na^{+}), potassium ion (K^{+}), and chloride ion (Cl^{-}).

4.2.6. Histopathology

After blood sampling for clinical pathology, the animals were sacrificed by exsanguination from the vena cava and aorta. The body surface, subcutis, head, and all internal organs of thoracic and abdominal cavities were examined, and recorded necropsy's opinion. The following organs of all animals were weighed (paired organs were weighed separately) using a BP221S electronic balance (Sartorius AG, Göttingen, Germany), and the organ weights were converted to relative organ weights based on the organ-to-fasted BW ratio.; ovary, testis, lung, spleen, kidney, uterus,

epididymis, brain, liver, adrenal gland, prostate gland, heart, pituitary gland, and thymus.

The organs/tissues listed below were removed from each animal and preserved in 10% neutral buffered formalin; brain, pituitary gland, lungs, heart, thymus, spleen, adrenal gland, kidney, liver, seminal vesicles, ovary[#], uterus, vagina, skin, stomach, duodenum, jejunum, ileum, cecum, colon, rectum, eye with optic nerve[#], harderian gland[#], thyroid gland[#], tongue, trachea, esophagus, pancreas, mesenteric lymph node, gross lesions, thoracic spinal cord, skeletal muscle, peripheral nerve, femorotibial joint, urinary bladder, testis[#], epididymis[#], prostate gland, parathyroid gland[#], salivary gland[#], aorta, mammary gland, born marrow sternum, mandibular lymph node[#] ([#] Paired organs were removed separately and fixed). Eyes and testes were preserved in Davidson's fixative and Bouin's fixative, respectively.

Tissue slide were prepared from all fixed tissues of vehicle control and test substance of high- dose group, and single-substance related group, and organs with gross findings of low- and middle-dose groups. Tissue slides were also additionally prepared from skeletal muscle and peripheral nerve in low- and middle-dose groups because these organs were appeared to be affected by the test substance at the high dose. The histopathological findings were processed by the Pristima® (Xybion Medical Systems, Princeton, NJ, USA) program (version 6.1.0 build 31). Standardized System for Nomenclature and Diagnostic Criteria-Guides for Toxicology Pathology of the Society of Toxicology [199], International Harmonization of Nomenclature and Diagnostic Criteria were also utilized [200].

4.2.7. Data analysis

Parametric multiple or nonparametric multiple comparison procedures were used for inter-group comparison. The data were statistically analyzed with the program SPSS software (ver. 10.1K). The data of BWs, food and water consumptions, urinalysis, urine volume, hematological

and serum biochemistry tests, and organ weights were subjected to one-way ANOVA test for the significance which was considered at a probability value of $P < 0.05$.

4.3. Four-week repeated-dose oral toxicity study in beagle dogs

4.3.1. Animals

This study was used pure-bred beagle dogs supplied by Woojung BSC (Suwon, Gyeonggi, Korea). The animals were housed under controlled conditions (temperature, 23 ± 3ºC; humidity, 55 ± 15%; ventilation of 10~20 air change/h; 150~300 lux luminous intensity; and 12 h light cycle from 9:00 to 21:00) in the experimental animal facility at Preclinical Research Center, Chemon. Throughout the study period, the temperature and relative humidity of animal room were measured every hour with a computer-based automatic sensor, and the environmental conditions such as ventilation frequency and luminous intensity were monitored on a regular basis. The room was maintained at a room temperature of 20.7~22.9ºC, and a relative humidity of 57.8~64.1%.

Each dog was offered a daily ration of 300 g of solid food supplied by Cargill Agri Purina (Seongnam, Gyeonggi, Korea). Water was disinfected by ultraviolet sterilizer and ultrafiltration and made available *ad libitum* using an automatic water supplier. Examination of water was performed by the authorized Gyeonggi Institute of Health & Environment, and water quality satisfied the standards for the drinking water. The animals were individually housed in a stainless-steel cage (W895 × L795 × H765 mm) with the mesh bottom throughout the whole housing period.

Total 10 beagles (5 males and 5 females) were used after they were assessed for general health and parasite infections 2 weeks prior to study start. The dogs were 6~7 months of age and weighed 6.5~7.7 kg (male)/6.2~7.2 kg (female) at the start of treatment. Animals were macroscopically examined and individually weighed upon receipt, and then acclimated under laboratory conditions for 15 days. General observations were made once a day and only healthy

animals were used for this study after blood examinations. The present study was approved by IACUC of Preclinical Research Center, Chemon (Serial no., 12-D030).

4.3.2. Experimental design

Healthy male and female beagle dogs were randomly assigned to five group (1/sex/group). Control (gelatin capsule only) or graded dose of OF-80E (300, 600, 1000, and 1500 mg/kg of BW) were administered to dogs by oral gavage once at dose using gelatin capsule. Every animal was observed at least once a day for clinical sign including mortality and if there is any sign, the data and severity of the symptom were recorded individually. The first day of administration was designated as day 1. All animals were weighted on day 1 (before administration), and from then on once a week and on the day of necropsy. The BW for the necropsy was measured after fasting overnight. Food consumption was measured on the first day of administration, from then once a week during study period. Weighed food was given to each cage, and the remaining quantity on next day was subtracted to calculate the mean daily consumption. Eyes were macroscopically observed for all animals before administration and within a week before scheduled necropsy.

4.3.3. Urine collection and blood sampling

All animals were examined before administration and within a week before scheduled necropsy on urine collecting plate for urine collection. Approximately 0.3 mL of fresh urine was collected and used for urinalysis. All animals were fasted overnight (for 16~24 h, water available) before blood sampling. Blood samples were taken from a cephalic vein of all animals before administration and on the day scheduled necropsy.

4.3.4. Urinalysis

About 0.3 mL of urine was soaked into a Siemens Multistix 10SG test strip and was analyzed by a Siemens CliniTek Advantus automatic analyzer to measure following parameters. The color of urine was observed with naked eye and the results were input into the automatic analyzer.; urine color, clarity, glucose, bilirubin, ketone body, specific gravity, pH, protein, urobilinogen, nitrile, occult blood, and leucocyte.

4.3.5. Hematological test

Approximately 1 mL of blood was put in a Becton Dickinson CBC bottle (Vacutainer 3 mL) that contained EDTA-2K, an anticoagulant. Then, the following parameters were detected with a Siemens ADVIA 2120 coulter counter.; RBC, haematocrit, hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red cell distribution width, hemoglobin distribution width, mean platelet volume, platelet, white blood cell, neutrophil, lymphocyte, monocyte, eosinophil, basophil, and large unstained cells.

4.3.6. Clinical biochemistry test

About 2 mL of blood was added into a 5 mL Sekisui Insepack vacutainer tube containing clot activator. The blood was left at room temperature for 15~20 min for clotting and then centrifuged at 3000 rpm for 10 min, as described earlier. The following parameters were measured by the serum biochemistry analyzer.; aspartate aminotransferase, alanine aminotransferase, creatine phosphokinase, total bilirubin, glucose, gamma glutamyl transferase, total cholesterol, triglyceride, total protein, albumin, albumin/globulin ratio, blood urea nitrogen, creatinine, inorganic phosphorus, Ca^{2+}, K^{+}, Na^{+}, and Cl^{-}.

4.3.7. Histopathology

After blood sampling from cephalic vein for blood tests, all animals were anesthetized by pentobarbital sodium (Hanlim Pharm., Yongin, Gyeonggi, Korea), and euthanized by exsanguinations from the axillary artery and vein. The external appearance, subcutis, head, and all the internal organs in the abdominal and thoracic cavities were observed grossly and the findings were recorded. The following organs were weighed (paired organs were weighed separately) with a Sartorius BP3100S electronic balance, and the organ-to-fasted BW ratio (relative organ weights) was calculated.; ovary, uterus, adrenal gland, pituitary gland, thymus, prostate gland, testis, epididymis, spleen, kidney, heart, lung, brain, liver with gall bladder, and thyroid gland with parathyroid gland. Statistical analysis was not be performed as number of animals was insufficient for analysis.

4.4. Bacterial reverse mutation test

4.4.1. Test strains and materials preparation

Salmonella typhimurium TA100 and TA1535 and *Escherichia coli* WP2 *uvr*A were used for the detection of base-pair substitution type mutation. and *S. typhimurium* TA98 and TA1537 were used for the detection of frame-shift type mutation. The above tester strains were obtained from Molecular Toxicology (Boone, NC, USA). The viable cell counts of five tester strains were 0.57~1.85×10^9 CFU/mL in *S. typhimurium* bacterial strains and 2.44×10^9 CFU/mL in *E. coli*. To evaluate the toxicity and solubility (precipitation) of OF-80E, a pilot experiment was performed with all bacterial strains. OF-80E was dissolved in DMSO (Sigma-Aldrich). The positive control substances were dissolved in either sterile distilled water (Daihan pharm., Seoul, Korea) or DMSO; sodium azide was dissolved in distilled water, 2-aminoanthracene, acridine mutagen ICR-191, 4-nitroquinoline-*N*-oxide and benzo(a)pyrene [B(a)P] were dissolved in DMSO. All

substances were purchased from Sigma-Aldrich. The positive control substances listed above have been chosen among the ones widely used in the bacteria reverse mutation assays or are listed in the OECD guideline TG471 [201]. Rat liver microsomal enzyme (S9) prepared male SD rats induced with aroclor 1254, was obtained from Molecular Toxicology. S9 mixture was prepared by mixing S9 with fresh cofactor solution (Wako, Osaka, Japan). The 1 mL of S9 mixture contained 950 μL of 8 μM of $MgCl_2 \cdot 6H_2O$, of 33 μM of KCl, of 5 μM of glucose-6-phosphate, of 4 μM of NADPH, of 4 μM of NADH, of 100 μM of sodium phosphate buffer (pH 7.4) and ,50 μL of S9.

4.4.2. Experimental procedures

The direct plate incorporation method was used to make plates. For the plating assay, the followings were added to each sterile tube containing 2.0 mL of top agar held at 45°C in a heating block: 0.1 mL of OF-80E solution, 0.5 mL of S9 mixture (or sodium phosphate buffer, pH 7.4), and 0.1 mL of bacterial culture. The contents were vortexed, poured onto minimal glucose agar plate and evenly distributed. Negative control plates were treated with 0.1 mL of vehicle instead of the test substance, and positive control plates were treated with each positive control substance by the method described above. After the treatment was done and the top agar was hardened, all plates were turned over and incubated at 37°C for 48 h, then number of revertant colonies per plate were counted. A preliminary range-finding study was done to select top dose of the main study. The preliminary study was performed in the presence (+S9) and absence (−S9) of exogeneous metabolic activation system and used one plate for each dose. Based on the test results, the dose levels of main study were set at 15, 50, 150, 500, 1500, and 5000 μg/plate for all bacteria strains. Corresponding negative and positive controls were also utilized. Five tester strains and triplicate plates were used for each dose. When counting the colonies, each plate was checked for

the formation of background lawn, contamination, and other abnormality. The plates for sterility check were examined for any visible colonies due to contaminations. It was considered as positive if there is a reproducible increase in the mean number of revertant colonies/plate at one or more dose levels in at least one strain with or without metabolic activation system. Dose-responsiveness of number of colonies and increase factor against negative control were also considered. Growth inhibition was determined by background lawn turned into thinner or significant decrease in colony number against the negative control. There is no criterion for 'significant decrease', so it was determined by 50% decrease in number of colonies against the negative control.

4.5. Chromosome aberration test in CHL cells

4.5.1. Test system

Chinese hamster lung (CHL) cells (ATCC CRL-1935) were obtained from ATCC. This cell line has been demonstrated to be sensitive to the clastogenic activity of a variety of chemical agent. The cells were grown in reconstituted minimum essential medium, which was supplemented with 2200 mg sodium bicarbonate, 292 mg L-glutamine, antibiotics (penicillin and streptomycin), and 100 mL of FBS (Gibco) per liter. Cultures were incubated in humidified incubator at 37 ± 1°C in an atmosphere of 5% CO_2. 6×10^4 cells per flask (culture surface 25 cm^2) (Falcon, Boston, MA, USA) were seeded into 5 mL of medium and incubated for 3 days before the chemical treatment. The 1 mL of S9 mixture contained 700 μL of 8 μM of $MgCl_2 \cdot 6H_2O$, of 33 μM of KCl, of 5 μM of glucose-6-phosphate, of 4 μM of NADPH, of 4 μM of NADH, of 100 μM of sodium phosphate buffer (pH 7.4), and 300 μL of S9. OF-80E was dissolved in DMSO. B(a)P and ethyl methane sulfonate (EMS) (Sigma-Aldrich) were used as positive control substances in the presence and absence of metabolic activation system, respectively (OECD guideline TG 473). B(a)P was dissolved in DMSO and EMS was diluted with the culture medium

just before the treatment.

4.5.2. Experimental procedure

Dose range were selected based on the results of a range-finding test conducted on the test substance, using the same treatment regimen, in both the presence and absence of metabolic activation system with one culture per dose. Cells were treated with 8 levels (5 to 5000 mg/mL), and the treatments were repeated in the narrower ranges. The condition of the treatment mixtures was observed with unaided eyes at the start and end of the treatment. Precipitation was defined as settlement of fine substances of the test substance at the end of treatment. Cells were removed from flasks and counted about 24 h after the start of the treatment. With the cell counts, relative cell count (RCC) was calculated as follows and used as an indicator of current cytotoxicity:

RCC = (cell count of treatment flask/cell count of control flask) × 100 (%)

Doses of the treatment series were set as shown as Table 8.

Table 8. Doses of the treatment series of chromosome aberration test

Treatment series	Metabolic activation	Treatment time, recovery time (h)	Dose of test substance (μg/mL)	Positive control, dose (μg/mL)
1	+	6, 18	1250, 2500, 5000	B(a)P, 20
2	−	6, 18	1250, 2500, 5000	EMS, 800
3	−	24, 0	1250, 2500, 5000	EMS, 600

Before the treatment, the old medium was replaced with 2.2 mL (series 1) and 4.5 mL (series 2 and 3) of fresh medium per flask. The prepared test substance was added to each flask at least 1 h after the medium change. Negative control cultures were treated with the vehicle only. The treatment schedules and compositions of treatment mixtures are shown as Table 9.

Table 9. Treatment schedules and compositions of chromosome aberration test

Treatment series	Medium + Test substance	S9 mixture	Final volume
1	2.2 mL + 0.3 mL	0.5 mL	3.0 mL
2	4.5 mL + 0.5 mL	−	5.0 mL
3	4.5 mL + 0.5 mL	−	5.0 mL

The treatment mixtures of series 1 and 2 were removed after 6-h treatment and cell monolayers were washed once with 5 mL of Ca^{2+} and Mg^{2+} free Dulbecco's phosphate buffered saline. Cells were then refed with 5 mL of fresh medium and incubated until collecting the mitotic cells. Series 3 was incubated without washing. Each culture was observed for any abnormal color change due to pH or precipitation at the start and end of the treatment. Approximately 22 h after the start treatment, 50 μL of colchicine solution was added to each culture (final concentration of 1 μM) and incubated for 2 h for mitotic arrest. The mitotic cells were detached by gentle shaking. The medium containing mitotic cells were centrifuged, and the cell pellets were resuspended in 75 mM potassium chloride solution for hypotonic treatment. Then cells were fixed with fixative (methanol : glacial acetic acid = 3 : 1, by volume) three times and slides were prepared by the air-drying method. Slides were stained with 5% Giemsa stain solution. Two slides were prepared for each culture. After harvesting mitotic cells, the remaining cell monolayer of each flask was trypsinized and counted to calculate RCC.

4.5.3. Evaluation of chromosomal aberration

Morphological classification and counting of chromosomal aberration was done according to the principle of Atlas of chromosomal aberration by chemicals [202]. One well-stained slide was selected out of the two slides from each culture. Slides were coded, for blind reading, and

were examined under an optical microscopy at 1000× magnification. Well-spread 100 metaphases per slide were evaluated. Metaphases with 23~27 centromeres were evaluated for chromosomal aberration, and if any, the type, number of aberration and location in the slide were recorded. Chromosomal aberrations were classified into chromosome type deletion/exchange and chromatid type deletion/exchange. The frequency of metaphases with aberrations of each culture, both inclusive and exclusive of gaps, was presented. A metaphase with more than 10 aberrations (multiple aberrations including gaps) or with chromosome fragmentation was classified as 'other' and counted as one aberration. Regardless of the presence of aberration, 100 metaphases/culture were examined to determine the frequencies of diploid (23~26 centromeres), polyploid (37 ≤ centromeres) and endoreduplication. The numbers of aberrant metaphase and total aberration, both inclusive and exclusive gaps, and RCC were presented. The result was regarded as positive if there was a dose-related increase in the number of aberrant metaphases or a reproducible increase in at least one dose level. However, the statistical significance was not the only determining factor for a positive response. The biological relevance, frequency of aberrant metaphases, and cytotoxicity were also considered. The statistical analyses were performed using the SPSS software (ver. 10.1K).

4.6. *In vivo* micronucleus assay

4.6.1. Test system

SPF male Hsd:ICR (CD-1®) mice (29.3~32.4 g) were obtained from Koatech at 6 weeks of age. Mice were used in experiments after 1 week of quarantine and acclimation. The present study was approved by IACUC of Preclinical Research Center, Chemon (Serial no., 12-M116). Animals were individually allowed to acclimate in polycarbonate cages (W170 × L235 × H125 mm) on aspen chip bedding for a week to the controlled environment (temperature, 23 ± 2°C; humidity,

55 ± 15%; ventilation of 10~20 air change/h; 150~300 lux luminous intensity; and 12 h light cycle from 8:00 to 20:00). Animals were offered Harlan Laboratories irradiation-sterilized pellet diet as described earlier, *ad libitum*. Groundwater disinfected by ultraviolet sterilizer and ultrafiltration was given via water bottle.

To determine the range of doses to be used, a preliminary dose range-finding test was conducted. Three males and females per group were dosed by oral administration with test substance at dose of 1000, 2000, and 5000 mg/kg/day (dose volume: 10 mL/kg/day) for two consecutive days. Animals were observed for 4 days including the days of administration. As a result, there was no special abnormal findings were observed in all animals and no substantial differences between sexes in toxicity. Therefore, male mice were selected for the present study. The high dose was set at 5000 mg/kg/day and two lower doses were added with a common ratio of 2 (2500 and 1250 mg/kg/day). Cyclophosphamide monohydrate (CPA) (Sigma-Aldrich) was used as a positive control substance. CPA was selected one of the positive control substances listed in the OECD guideline TG 474. CPA was dissolved in isotonic sodium chloride injection (Daihan pharm.) just before use. CPA was given at a dose of 70 mg/kg intraperitoneal injection just before the dose on the second day of dosing.

4.6.2. Observations and examinations

All animals were daily observed for any clinical signs, and the observation was recorded individually. Individual BWs were recorded just before each dosing and at necropsy. Bone marrow samples were prepared as described by Schmid [197]. The sampling was done about 24 h after the final dosing. This sampling time has been generally adopted for the multiple dosing regimens. Mice were sacrificed by carbon dioxide inhalation. The right (or left) femoral bone marrow cells were flushed out using a 23G syringe with 2 mL of FBS and collected in a centrifuge

tube. After centrifugation at 1000 rpm for 5 min, the resuspended cells were smeared onto a clean glass slide. The slides were sufficiently dried and fixed in methanol for 5 min. Two smears (specimens) were made per animal. The specimens were kept in a cabinet before the staining and counting.

One well-smeared specimen per animal was selected and coded for blind counting. Acridine orange stock solution was prepared based on the method of Hayashi [203] using acridine orange base (Sigma-Aldrich). The stock solution was 1:4 (v/v) diluted with Sorensen's buffer (pH 6.8) to make a working solution for staining. Specimens were stained just before counting. Working solution were dropped onto the specimen and covered with a cover glass. After 2 min, the fluorescence was checked, and counting was started after confirming a vivid fluorescence. Cells were observed and counted using a Ni-U (with a B-2A fluorescence filter set) fluorescence microscope (Nikon, Tokyo, Japan) at 400× magnification. The identification of micronuclei was done according to the method of Hayashi [203]. Polychromatic erythrocyte (PCE) appeared red, and normochromatic erythrocyte (NCE) appeared dark grey. Micronucleus was observed as a green spot on the red background. The micronucleated polychromatic erythrocyte (MNPCE) was expressed as means ± stds of number of MNPCE per 2000 PCEs per animal. The PCE : RBC ratio, an indicator of cytotoxicity, was determined by counting at least 500 erythrocyte (PCE + NCE) per animal. The ratio was calculated by PCE / (PCE + NCE). The statistical analyses were performed using the SPSS software (ver. 10.1K). Parametric and nonparametric multiple comparison procedures were used, and the significance level was set at $P < 0.05$.

4.7. Effect of OF-80E on the central nervous system in ICR mice

4.7.1. Test system

This study was performed in accordance with International Council for Harmonization

(ICH) guideline S7A, 'Safety Pharmacology Studies for Human Pharmaceuticals' [204] and approved by IACUC of Preclinical Research Center, Chemon (serial no., 12-M110). SPF male Hsd:ICR (29.7~32.9 g, Koatech) at 6 weeks of age were used in the experiments after 1 week of quarantine and acclimation. In a single oral administration toxicity test of OF-80E, red urine, compound-mixed stool, and diarrhea were observed in males and females with 5000 mg/kg BW or more dose groups. Temporary salivations were observed in males and females with 10000 mg/kg BW. No abnormal changes in body temperature and general behavior were observed for 2 h after dosing, when the test substance was preliminarily administered to two ICR mice/group at 1250, 2500, and 5000 mg/kg BW. In the consideration of dose regimen in clinical study as oral administration (below 10 mg/kg) and base on the above results, the high dose of present study was set at 5000 mg/kg, which is corresponding to about 500 times of intended clinical dose. Two lower doses were added with the common ratio of 10, and vehicle control group treated with 1% MC solution for oral administration was also set up. 4 animals per group were dosed, single dose was administered 1st day and 2nd day, respectively. Dose volume was calculated as 10 mL/kg based on the BW measured on the day of administration. After fasting for 3~4 h before administration, test substance was administered directly into the stomach using a sonde. Feed was re-supplied about 2 h after administration.

4.7.2. Observations and examinations

The parameters in the Table 10 were observed before dosing and at the time points of 15, 30, 60, 120, 240, and 360 min (± 1 min) after dosing (ICH guideline S7A). The collected data were statistically analyzed with the commercial program SPSS software (ver. 10.1K), and the significance level was set at $P < 0.05$.

Table 10. Observation parameter, method, and score in effect of CNS

No	Parameter	Method	Score
1	Body temperature	Measuring the body temperature (rectal temperature)	°C
2	Catalepsy	Observing whether animal hanged down on the meshed cylinder (10 cm in diameter) moves with four feet within 8 sec	0: Moving with four legs within 8 sec / normal 1: No movement within 8 sec / abnormal
3	Traction	Observing whether animal hanged on the horizontal bar (30 cm high) fall within 5 sec	0: Being suspended more than 5 sec / normal 1: Not being suspended more than 5 sec / abnormal
4	Tremors	Observing the tremors	0: Tremor is absent / normal 1: Tremor is present / abnormal
5	Convulsion	Observing the convulsion	0: Convulsion is absent / normal 1: Convulsion is present / abnormal
6	Exophthalmos	Observing the abnormal protrusion of the eyeball in the orbit	0: Exophthalmos is absent / normal 1: Exophthalmos is present / abnormal
7	Piloerection	Observing the status of hair erection	0: Piloerection is absent / normal 1: Piloerection is present / abnormal
8	Salivation	Observing the secretion of saliva	0: Salivation is absent / normal 1: Salivation is present / abnormal
9	Lacrimation	Observing the shedding tears	0: Lacrimation is absent / normal 1: Lacrimation is present / abnormal
10	Diarrhea	Observing the diarrhea	0: Diarrhea is absent / normal 1: Diarrhea is present / abnormal
11	Skin color	Observing the ear and tail color changes	0: Discoloration of skin is absent / normal 1: Discoloration of skin is present / abnormal

No	Parameter	Method	Score
12	Pinna reflex	Confirming reflex to stimulation on auricle	0: Present / normal 1: Absent / abnormal
13	Righting reflex	Rolling animal	0: Instantly correct the posture back 2: Slowly correct the posture back 4: No reaction
14	Tail elevation	Observing the degree of the tail raising	0: Tail is on the bottom 2: Tail is stiffened and elevated at an angle of 0~90 degrees 4: Tail is stiffened and elevated over an angle of 0~90 degrees
15	Palpebral closure	Observing the drooping degree of the upper eyelid.	0: Normally eyes are open 2: Eyelids slightly lowered 4: Eyelids are closed
16	Abdominal tone	Massaging the abdominal region of animals	0: No resistance. Abdominal walls do not get back to normal 2: Decreased resistance. Abdominal walls slowly get back to normal 4: Normal resistance. Abdominal walls quickly get back to normal 6: Increased resistance. Abdominal walls are very hard to be pressed 8: Extremely increased resistance like wooden board
17	Locomotion	Observing the animal motion	0: No movement despite of stimulus 2: Slow or decreased movement 4: Normal movement 6: Increased movement 8: Extremely increased or continuous movement
18	Respiratory rate	Observing the respiration rate and frequency	0: Distinctly abnormal breathing and long breathing interval 1: Slow and difficult breathing, dyspnea 2: Moderately slow but regular

No	Parameter	Method	Score
			breathing, bradypnea 4: Normal and regular breathing 8: Extremely rapid breathing, polypnea
19	Death	Occurrence of death	0: Survival 1: Death

4.8. Effect of OF-80E on the respiratory rate and tidal volume in Sprague Dawley rats

4.8.1. Test system

This study was performed in accordance with ICH guideline S7A as described above, and approved by IACUC of Preclinical Research Center, Chemon (Serial no., 12-R187). SPF male Hsd:SD rats (99.8~120.7 g at 5 weeks of age) were used in experiments after 1 week of quarantine and acclimation. In a single oral administration toxicity test of OF-80E, no abnormal changes in respiratory rate, tidal volume, and minute volume were observed for 3 h after dosing, when the test substance was preliminarily administered to 4 rats/sex/group at 2500 and 5000 mg/kg. Two lower doses were added with the common ratio of 10, and vehicle control group treated with 1% MC solution for oral administration was also set up. Two animals/group/day in consecutive order were dosed for 4 days. Dose volume was calculated as 10 mL/kg based on the BW measured on the day of administration. After fasting for 16~20 h before administration, test substance was administered directly into the stomach using a sonde. Feed was re-supplied about 6 h after administration.

4.8.2. Observations and examinations

Clinical signs were checked at least once during the period of measuring respiration rate from the dosing day. From the animal receipt day to the day before dosing, all animals were

adapted once daily for approximately 30 min in the whole-body plethysmography chamber (Buxco Electronics, Wilmington, NC, USA). The measurement was performed with two animals/group, daily. The animals were individually weight on the dosing day for calculating the dose volume. Before dosing, once animals were completely stabilized in the chamber over 30 min, the respiratory rate, tidal volume, and minute volume (calculated) were measured for 10 min. A mean for each parameter was calculated. After dosing, the parameters were measured for 10 minutes beginning at time points of 30, 60, 120, 240, and 360 min (± 10 min), and a mean for each parameter was also calculated. The collected data were statistically analyzed with the commercial program SPSS software (ver. 10.1K), and the significance level was set at $P < 0.05$.

4.9. Effect of OF-80E on human ether-a-go-go-related gene potassium channel expressed in Chinese hamster ovary cells

4.9.1. Cells cultures

This study was performed in accordance with ICH guideline S7B, 'The non-clinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals' [205]. Chinese hamster ovary (CHO) cells stably expressing the human ether-a-go-go-related gene (hERG) was purchased from bSys GmbH Technology Center (Witterswill, Switzerland). CHO-hERG-Duo cells are stably transfected with hERG cDNA and widely used to measure potassium channel current in safety pharmacology studies and a large database exists for this cell line, thus these data can be utilized in the interpretation and evaluation of the results of the study. Cell culture media components included in the Dulbecco's Modified Eagle Medium (Sigma-Aldrich) with 10% heat inactivated FBS containing Ham's F12 (Welgene, Gyeongsan, Gyeongbuk, Korea), 100 IU/mL penicillin (Sigam-Aldrich), 100 μg/mL streptomycin (Sigam-Aldrich), and 50 μg/mL Hygromycin B (Invitrogen, Carlsbad, CA, USA). Cells were

cultured at humidified incubator with 5% CO_2 in air 37ºC.

4.9.2. Preparation of test substances and test solution

OF-80E was dissolved in DMSO to make the 1 mg/mL stock solution. The experimental doses were prepared by diluting in 1× normal Tyrode's solution (Sigma-Aldrich) at the day of experiment. Positive control substance (E-4031) (Sigma-Aldrich) was dissolved with distilled water to make the 100 μg/mL stock solution. The experimental doses were prepared by diluting in 1× normal Tyrode's solution at the day of experiment. The 1× normal Tyrode's solution was prepared by diluting 10× normal Tyrode's solution, which had been prepared prior to the present study and stored at refrigerator. The 1× normal Tyrode's solution was composed of 143 mM NaCl, 5.4 mM KCl, 5.0 mM HEPES, 0.33 mM NaH_2PO_4, 0.5 mM $MgCl_2$, 10.0 mM glucose, and 1.8 mM $CaCl_2$ (pH adjusted to 7.3~7.5 with NaOH) and checked for osmolarity (300 ± 10 mOsm) with Osmette osmometer (Precision System, Natick, MA, USA). Internal pipette solution for whole cell patch clamp recordings was composed of 130 mM KCl, 1 mM $MgCl_2$, 5 mM EDTA, 5 mM MgATP, and 10 mM HEPES (pH adjusted to 7.25~7.35 with KOH). Internal pipette solution was aliquoted and stored frozen, and a fresh aliquot was thawed on each day of use. All chemicals were obtained from Sigma-Aldrich.

4.9.3. Measurement and analysis

In a preliminary hERG assay study, 1, 10, and 100 μg/mL of test substance showed −0.1, −1.3, and 2.5% inhibition in the hERG channel current, respectively. There were no effects observed of hERG channel current related test substance. Based on these results, dose in present study is set at 0.5, 5, 50, and 500 μg/mL in 1× normal Tyrode's solution. Vehicle control (0.1% distilled water) group was added to compare the effects of test substance on potassium channel

current. The positive control (0.1 μM E-4031) was treated at least one cell per day for credible data. hERG currents were measured after confirmation of steady recording during 2~3 min on perfusion of 1× normal Tyrode's solution. Peak current was measured during the test ramp. A steady state was maintained for at least 2~3 min before applying test substance. Electrophysiological measurement method was used for evaluation of hERG channel. Cultured cells were prepared from flask using trypsin-EDTA solution and perfused with normal Tyrode's solution after stabilization on perfusion chamber for at least 20~30 min. Microelectrode was filled internal pipette solution and fixed into holder. Microelectrode was approached to cell using a micromanipulator and Molecular devices Axopatch 200B patch clamp amplifier was converted to voltage clamp mode. Microelectrode was made to giga seal through negative pressure and was made to whole cell condition through strong negative pressure more than giga seal. Cells were held at −80 mV. Onset and steady state of hERG potassium current by test substance was measured using a pulse pattern with fixed amplitudes (hyperpolarization pulse, −90 mV for 0.1 sec; depolarization pulse, +20 mV for 1 sec; and repolarization pulse, −40 mV for 3 sec) repeated at 20 sec intervals (1 sweep). hERG channel currents were observed and measured using pClamp 10 and Notocord-hem evolution® software platform version 4.2 (Notocord, Newark, NJ, USA) on normal Tyrode's solution at 37°C.

4.9.4. Data analysis

Data acquisition and analysis were performed using the Notocord-hem program. The steady states (tail current plus leak current) before and after test substance application were used to calculate the percentage of current inhibited (suppression rate, %) at each concentration as follows:

Relative current = tail current/leak current

Suppression rate (%) = (1 – relative current) × 100

Compensated suppression rate (%) = (A − B) / (100 – B) × 100

A: suppression rate of test substance, positive control (%)

B: mean suppression rate of vehicle control (%)

The collected data were statistically analyzed with the commercial program SPSS software (ver. 10.1K), and the significance level was set at $P < 0.05$.

IV. Results

1. Gastro-protective activity of *O. ficus-indica* fruits in an *in vitro* model

1.1. Activity comparisons of various *O. ficus-indica* fruit ethanol extracts on aspirin-induced cytotoxicity in AGS cells

The protective effects of *O. ficus-indica* fruit ethanol extracts (0~95%) against aspirin-induced apoptosis were assessed (Table 11). Based on the IC50 values, 80% ethanol extract was most effective (Table 11).

Table 11. Effects of *O. ficus-indica* fruit ethanol extracts on aspirin-induced cytotoxicity in AGS cells and fruit constituents

Ethanol (%)	IC_{50}[a] (μg/mL)	Narcissin contents (%)	Aromadendrin contents (%)	Betanin contents (%)
0 (Water)	> 100	0.05	0.01	4.21
10	> 100	0.05	0.02	4.02
20	> 100	0.02	0.01	3.85
30	> 100	0.04	0.02	3.67
40	> 100	0.10	0.03	3.78
50	> 100	0.10	0.03	3.89
60	> 100	0.10	0.03	3.64
70	7.1	0.11	0.02	3.45
80	< 0.4	0.14	0.04	3.56
90	> 100	0.13	0.08	0.51
95	> 100	0.13	0.08	0.03

[a] The 50% inhibition concentration (μg/mL) on aspirin induced cytotoxicity in AGS cells.

1.2. Chemical constituent of *O. ficus-indica* fruit ethanol extracts

Contents of narcissin, aromadendrin, and betanin were analyzed by HPLC (Table 11). Contents of narcissin and aromadendrin increased as the ethanol contents of extract solvent increased. At high ethanol contents of extract solvent (90% and 95%), content of betanin was rapidly decreased.

1.3. Bioassay-guided fractionation and identification of *O. ficus-indica* fruits

The protective effects of *O. ficus-indica* fruit ethanol extract fractions against aspirin-induced apoptosis were assessed. Active guided fractionations were performed. The fractions obtained from solvent partitioning of the ethanol extract of *O. ficus-indica* fruits were tested for protective activity against aspirin-induced cytotoxicity in AGS cells (Table 12). Significant differences in the activity were observed among the fractions and were used to identify the peak activity fractions for the next purification step (Table 13). As judged by the IC_{50} values, the butanol-soluble fraction was the most potent fraction, whereas no activity was obtained using the hexane- or chloroform- soluble fractions.

Table 12. Effects of each fraction obtained from the solvent partitioning of the ethanol extract of the *O. ficus-indica* fruits on aspirin-induced cytotoxicity in AGS cells

Fraction name	IC_{50}[a] (μg/mL)
Ethanol extract	> 10
Hexane soluble fraction	> 10
Chloroform soluble fraction	> 10
Butanol soluble fraction	2.1
Water soluble fraction	> 10

[a] The 50% inhibition concentration (μg/mL) on aspirin induced cytotoxicity in AGS cells.

Table 13. Effects of each fraction obtained from the butanol-soluble fraction on aspirin-induced cytotoxicity in AGS cells

Fraction name	IC_{50}[a] (μg/mL)
B-1	> 10
B-2	> 10
B-3	**0.8**
B-3-1	>10
B-3-2	8.2
B-3-3	3.3
B-3-4	**0.2**
B-3-5	**< 0.04**
B-3-6	2.6
B-3-7	> 10
B-3-4-1	> 10
B-3-4-2	> 10
B-3-4-3	0.9
B-3-4-4	**< 0.04**
B-3-4-5	3.3
B-3-5-1	0.9
B-3-5-2	**< 0.04**
B-3-5-3	0.7

[a] The 50% inhibition concentration (μg/mL) on aspirin induced cytotoxicity in AGS cells.

Bioassay-guided fractionation of the *O. ficus-indica* fruits led to the identification of two active compounds through spectroscopic analyses, including ESIMS and NMR. Compound **1** was obtained as pale yellowish amorphous powder and $[D]^{25}_{D}$ + 58.6 (c=0.30, MeOH). The molecular formula, $C_{15}H_{12}O_6$, was established by negative mode ESIMS (*m/z* 287 $[M-H]^-$), the ^{1}H-NMR and the ^{13}C-NMR spectrum (Figure 6). Based on above data, compound **1** was identified to aromadendrin (Figure 7).

Aromadendrin: pale yellowish amorphous powder; $C_{15}H_{12}O_6$; $[D]^{25}_{D}$ + 58.6 (c=0.30, MeOH); UV (λ_{max} nm) = 290; ESIMS *m/z* : 287 $[M-H]^-$, 269, 259, 243, 215, 201, 151, 125; ^{1}H-NMR (DMSO-*6d*, 400 MHz) and the ^{13}C-NMR (DMSO-*6d*, 100 MHz): see Table 14.

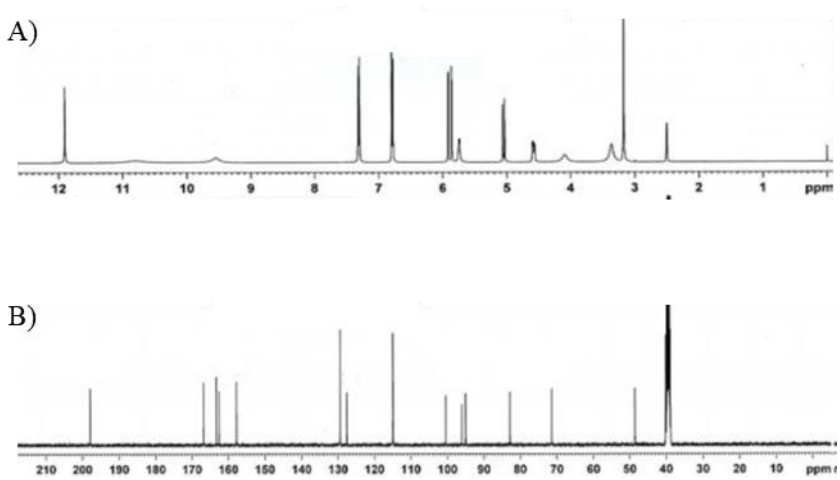


Figure 6. ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of compound 1.

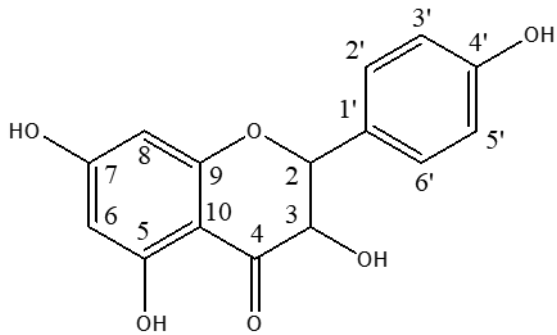


Figure 7. Structure of compound 1. This compound was identified in the fruits of *O. ficus-indica*. The chemical formular of the flavone, aromadendrin is $C_{15}H_{12}O_6$, with a molar mass of 288.26 g/mol.

Table 14. ^{1}H-NMR and ^{13}C-NMR data of compound **1**

No	δ_C (ppm)[a]	δ_H (ppm)[b]
2	84.7	5.01, d (*J* = 11.6 Hz)
3	73.6	4.56, dd (*J* = 11.8, 5.2 Hz)
4	198.6	
5	165.3	
6	97.0	5.87, d (*J* = 2.0 Hz)
7	167.8	
8	95.9	5.91, d (*J* = 2.0 Hz)
9	164.6	
10	101.0	
1'	128.6	
2'	130.9	7.33, d (*J* = 8.4 Hz)
3'	115.9	6.82, d (*J* = 8.4 Hz)
4'	158.7	
5'	115.9	6.82, d (*J* = 8.4 Hz)
6'	130.5	7.33, d (*J* = 8.4 Hz)

[a] ^{13}C data was measured at 100 MHz in DMSO-d_6.

[b] ^{1}H data was measured at 400 MHz in DMSO-d_6.

Compound **2** was obtained as pale yellowish amorphous powder. The molecular formula, $C_{28}H_{32}O_{16}$, was established by negative mode ESIMS (*m/z* 623 $[M-H]^-$) and the ^{1}H-NMR and the ^{13}C-NMR spectrum (Figure 8). The structure of compound **2** was defined as narcissin (Figure 9).

Narcissin: pale yellowish amorphous powder; $C_{28}H_{32}O_{16}$; UV (λ_{max} nm) = 254, 353; ESIMS *m/z* : 623 $[M-H]^-$, 593, 314 $[M-Rha-Glc]^-$; ^{1}H-NMR (methanol-d_4, 300 MHz) and the ^{13}C-NMR (methanol-d_4, 75 MHz): see Table 15.

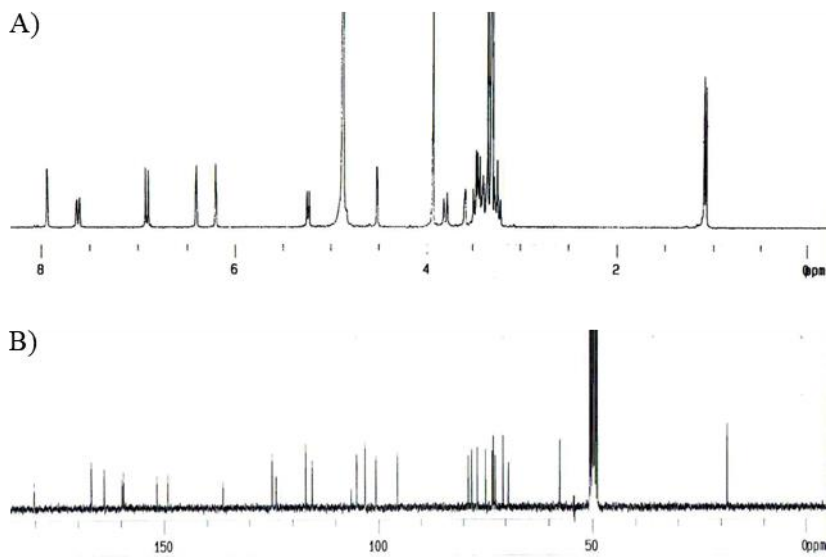


Figure 8. ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of compound 2.

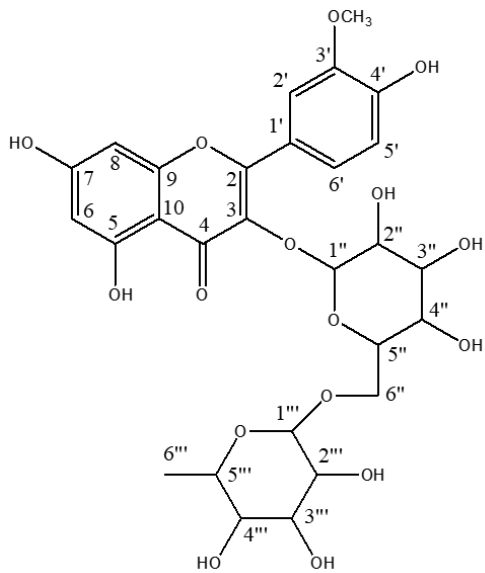


Figure 9. Structure of compound 2. This compound was identified in the fruits of *O. ficus-indica*. The chemical formular of the flavonol, narcissin is $C_{28}H_{32}O_{16}$, with a molar mass of 624.54 g/mol.

Table 15. ^{1}H-NMR and ^{13}C-NMR data of compound **2**

No	δ_C (ppm)[a]	δ_H (ppm)[b]
2	158.5	
3	135.4	
4	179.0	
5	162.8	
6	99.7	6.21, d (J = 2.0 Hz)
7	165.5	
8	94.7	6.41, d (J = 2.0 Hz)
9	158.1	
10	104.9	
1'	122.6	
2'	114.2	7.95, d (J = 2.0 Hz)
3'	147.9	

4'	150.3	
5'	115.7	6.92, d (*J* = 8.9 Hz)
6'	123.9	7.63, dd (*J* = 8.8, 2.0 Hz)
OCH_3	56.4	3.96
O-glucopyranosyl		
1"	104.1	5.24, d (*J* = 7.4 Hz)
2"	76.6	3.47, m
3"	78.0	3.36, m
4"	72.8	3.24, m
5"	77.2	3.45, m
6"	68.1	3.81, 3.41, m
O-rhamnopyranosyl		
1'"	102.1	4.52, d (*J* = 1.1 Hz)
2'"	72.6	3.60, m
3'"	71.8	3.48, m
4'"	74.1	3.23, m
5'"	69.6	3.41, m
6'"	17.7	1.10, d (*J* = 6.2 Hz)

[a] ^{13}C data was measured at 75 MHz in methanol-d_4.

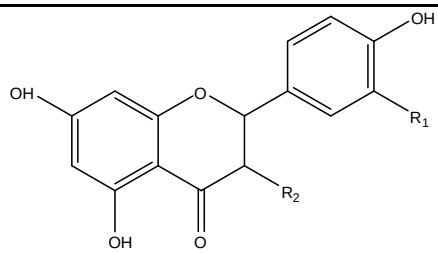
[b] ^{1}H data was measured at 300 MHz in methanol-d_4.

1.4. Effect of the isolated flavonoids on aspirin-induced cytotoxicity in AGS cells

Because of the potent protective activity of the flavone, aromadendrin, and the flavonol, narcissin, against aspirin-induced cytotoxicity in AGS cells, the protective activity of the three flavones (eriodictyol, naringenin, and taxifolin) and four flavonols (isokaempferol, kaempferol, 3-*O*-methyl quercetin, and quercetin) was evaluated (Table 16). Based on the IC_{50} values, aromadendrin, 3-*O*-methyl quercetin, and narcissin were more effective than other tested compounds. Moderate activity was observed with naringenin and taxifolin. No activity was obtained from the other four compounds.

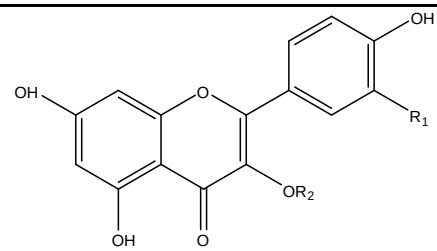
Table 16. Effects of flavonoids in *O. ficus-indica* fruits on aspirin-induced cytotoxicity in AGS cells

Flavone



Compound	R_1	R_2	IC_{50}[a] (μM)
Aromadendrin	H	OH	< 0.5
Eriodictyol	OH	H	> 10
Naringenin	H	H	5.9
Taxifolin	OH	OH	1.1

Flavonol



Compound	R_1	R_2	IC_{50} (μM)
Isokaempferol	H	CH_3	> 10
Kaempferol	H	H	> 10
3-*O*-methyl quercetin	OH	CH_3	< 0.5
Narcissin	OH	Glc-Rut	< 0.5
Quercetin	OH	H	> 10

[a] The 50% inhibition concentration (μM) on aspirin induced cytotoxicity in AGS cells.

1.5. Effect of OF-80E on ethanol-induced cytotoxicity in AGS cells

The protective effects of OF-80E on ethanol-induced apoptosis were assessed (Figure 10A). Ethanol (control group) significantly reduced the cell viability as compared to the vehicle group (Figure 10A). Pretreatment with OF-80E at concentrations of 100, 200, and 500 μg/mL significantly inhibited ethanol-induced cell death with mean viability of 41.1, 51.8, and 58.2%, respectively.

The protective effects of betanin on ethanol-induced apoptosis were also assessed (Figure 10B). Ethanol (control group) significantly reduced the cell viability as compared to the vehicle group (Figure 10B). Pretreatment with betanin at concentrations of 1, 2, 5, 10, and 50 μM significantly inhibited ethanol-induced cell death with mean viability of 34.1, 62.9, 48.4, 52.8, and 70.6%, respectively.

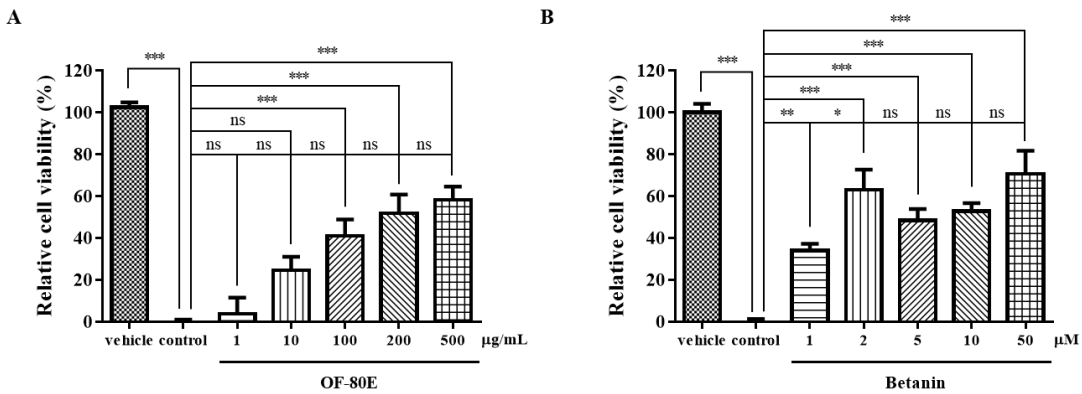


Figure 10. Effects of OF-80E on ethanol-induced cytotoxicity in AGS cells. The cells were treated with (**A**) OF-80E (1, 10, 100, 200, and 500 µg/mL) or (**B**) betanin (1, 2, 5, 10, and 50 µM). After 1 h, the medium was added with 9% ethanol for 30 min (except vehicle group). The cell viability was determined using MTT assay, as described in the Materials and Methods section. Each bar represents the mean ± SEM of triplicate samples ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, no significant difference, using Tukey's test).

1.6. Effect of OF-80E on aspirin-induced cytotoxicity in AGS cells

The protective effects of OF-80E on aspirin-induced apoptosis were determined (Figure 11A). Aspirin (control group) significantly reduced the cell viability as compared to the vehicle group (Figure 11A). Pretreatment with OF-80E at concentrations of 10, 100, 200, and 500 µg/mL significantly inhibited aspirin-induced cell death with mean viability of 63.9, 70.6, 97.8, and 68.9%, respectively.

The protective effects of betanin on aspirin-induced apoptosis were also determined (Figure 11B). Aspirin (control group) significantly reduced the cell viability as compared to the vehicle group (Figure 11B). Pretreatment with betanin at concentrations of 1, 2, 5, 10, and 50 µM significantly inhibited aspirin-induced cell death with mean viability of 74.1, 88.8, 113.2, 103.9,

and 101.3%, respectively.

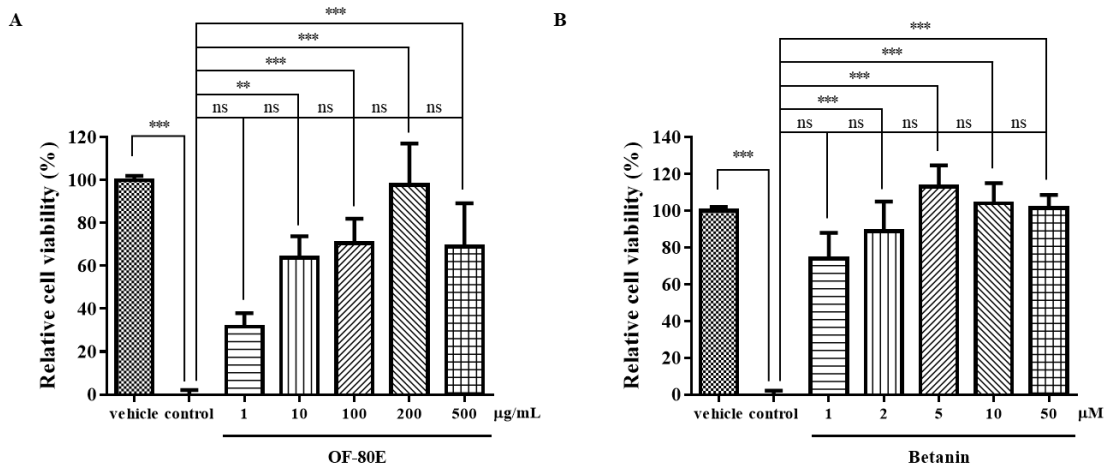


Figure 11. Effects of OF-80E on aspirin-induced cytotoxicity in AGS cells. The cells were treated with (**A**) OF-80E (1, 10, 100, 200, and 500 µg/mL) or (**B**) betanin (1, 2, 5, 10, and 50 µM). After 1 h, the medium was added with aspirin (4 mM) for 24 h (except vehicle group). The cell viability was determined using MTT assay, as described in the Materials and Methods section. Each bar represents the mean ± SEM of triplicate samples ($^{***}P < 0.001$; $^{**}P < 0.01$; ns, no significant difference, using Tukey's test).

1.7. Reduced glutathione and prostaglandin E_2 levels in AGS cells treated with OF-80E

Free radical generation promotes the appearance of gastric ulcers, explaining the ulcerogenic effect of aspirin. Intercellular GSH is an important factor contributing to gastric mucosal protection against aspirin-induced damage [206]. A significant reduction of 48.5% in the total cellular GSH content was observed in AGS cells treated only with aspirin (control group) compared to without aspirin (vehicle group) (Figure 12). Pretreatment with OF-80E (Figure 12A) and betanin (Figure 12B) significantly increased GSH levels reduced by aspirin, mean level of 61.2 and 67.6 %, respectively.

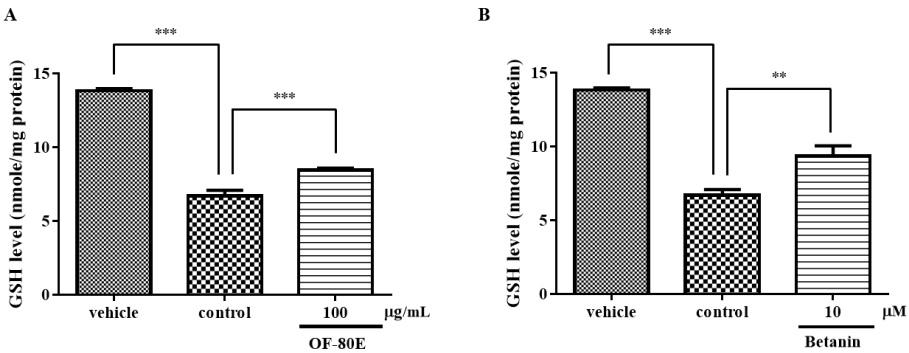


Figure 12. Reduced glutathione level of OF-80E on aspirin-induced cytotoxicity in AGS cells. The cells were treated with (**A**) OF-80E at a concentration of 100 µg/mL or (**B**) betanin at a concentration of 10 µM. After 1h, the medium was added with aspirin (4 mM) for 24 h (except vehicle group). GSH content was expressed a nmole/mg protein, determined by the Bradford assay, as described in the Materials and Methods section. Each bar represents the mean ± SEM of triplicate samples ($^{***}P < 0.001$; $^{**}P < 0.01$, using Tukey's test).

Crucial gastro-protective mechanism involves PG that stimulate multiple defense factors of the gastric mucosa. Prostaglandins accelerate ulcer healing, possibly via angiogenesis, epithelial cell proliferation, production of growth factors, reconstruction of extracellular matrices, and suppression of inflammatory cell infiltration. Prostaglandin E_2 is involved in the synthesis of mucus and bicarbonate, and in the regulation of acid secretion and gastric mucosal blood flow [207]. A significant reduction of 57.5% in the cellular PGE_2 level was observed in AGS cells treated only with aspirin (control group) compared to the without aspirin (vehicle group) (Figure 13). Pretreatment with OF-80E significantly stimulated PGE_2 synthesis inhibited by aspirin, mean level of 92.7% (Figure 13A). Pretreatment with betanin stimulated PGE_2 synthesis inhibited by aspirin, mean level of 79.1%, but it was not statistically significant (Figure 13B).

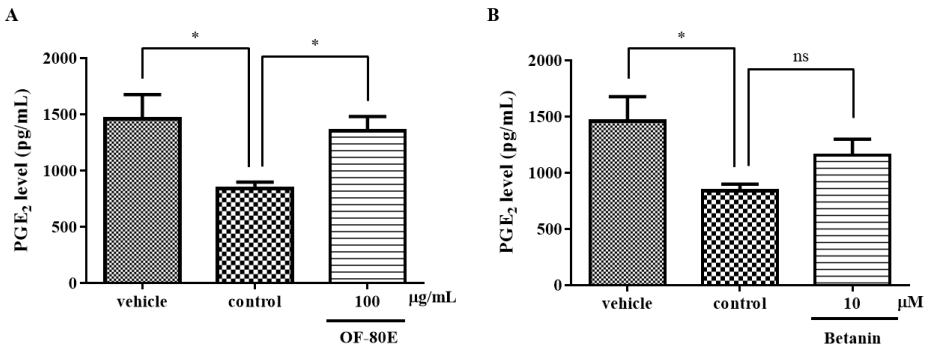


Figure 13. Prostaglandin E_2 level of OF-80E on aspirin-induced cytotoxicity in AGS cells. The cells were treated with (**A**) OF-80E at a concentration of 100 μg/mL or (**B**) betanin at a concentration of 10 μM. After 1 h, the medium was added with aspirin (4 mM) for 24 h (except vehicle group). PGE_2 levels were determined in the medium using an ELISA kit, as described in the Materials and Methods section. Each bar represents the mean ± SEM of triplicate samples ($^*P < 0.05$; ns, no significant difference, using Tukey's test).

2. Gastro-protective activity of *O. ficus-indica* fruits in an *in vivo* model

2.1. Gastro-protective activity of OF-80E in an ethanol-induced gastritis rat

An oral administration of ethanol in rat accelerates gastric mucosal necrosis and apoptosis by damaging the gastric mucosal and producing acute hemorrhagic gastric erosion [208]. Ethanol directly stimulates the gastric mucosa and causes swelling in the submucosal muscle layer, temporary ischemia, cell death due to oxidative damage, and HCl directly stimulates the gastric mucosa and promotes gastric movement, resulting in acute gastritis [208].

A single dose of 60% ethanol in 150 mM HCl (5 mL/kg; p.o.) produced severe elevated mucosal hemorrhagic lesions (lesion area was 168.9 mm^2) (Figure 14). Pretreatment with OF-80E (50, 100, 150, and 200 mg/kg; p.o.) showed a dose-dependent and significant reduction in mucosal hemorrhagic lesions with an inhibition of 48.9, 55.5, 57.1, and 62.7%, respectively, as compared with ethanol/HCl alone-treated group (control group) (Figure 14). OF-80E was well

tolerated and there was no mortality in the rats. These results indicate that OF-80E displays an antiulcer effect related to cytoprotective activity, since it significantly reduced the ethanol/HCl-induced intraluminal bleeding. The rats pre-treated with 100 mg/kg of Mucosta® tablet and 60 mg/kg of Stillen® tablet exhibited percentage of inhibition of mucosal hemorrhagic lesions, 21.9 and 45.5%, respectively.

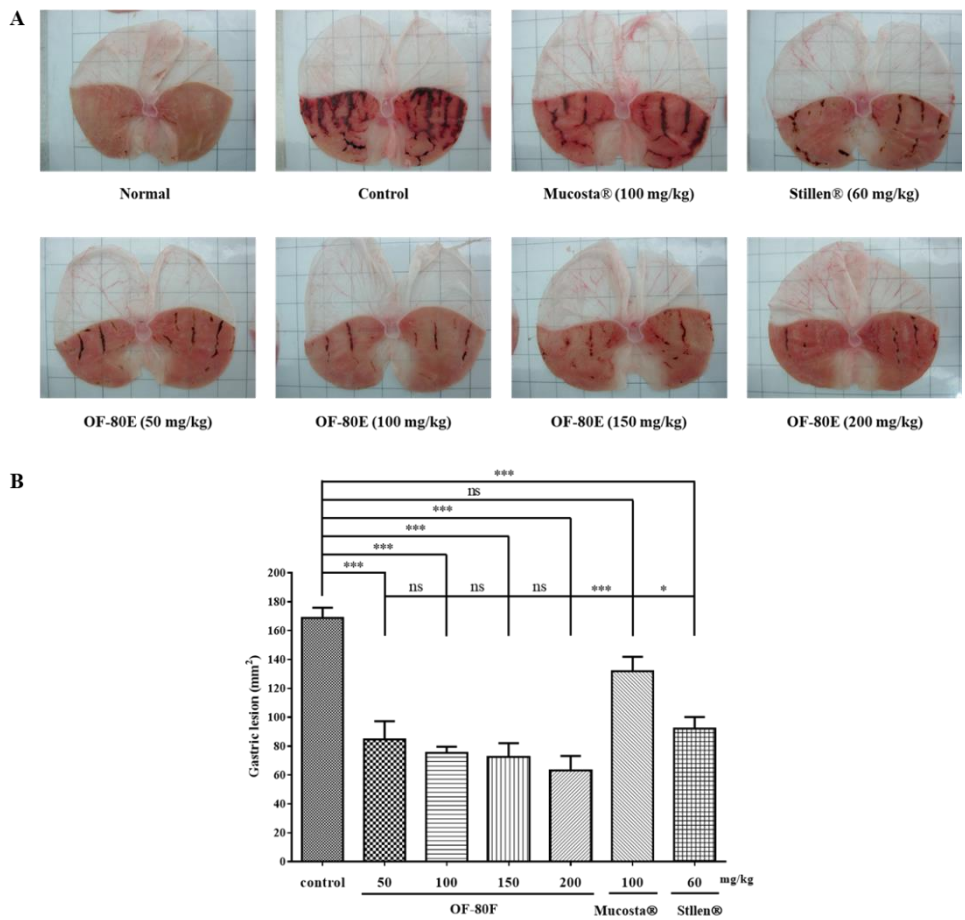


Figure 14. Gastro-protective effects of OF-80E on ethanol-induced gastric lesions in rats. The rats (8 weeks old, n = 10/group) were pre-treated orally with OF-80E (50, 100, 150, and 200 mg/kg BW), Mucosta® tablet (100 mg/kg BW), Stillen® tablet (60 mg/kg BW), and vehicle (1% HPMC). 1 h after tested sample treatments, the rats were received 5 mL/kg acidified

ethanol (150 mM HCl in 60% ethanol, p.o.). 1 h later, the rats had been euthanized, the stomachs were removed and then the area of the lesions was measured, as described in the Materials and Methods section. (**A**) Representative gastric mucosal lesion photo of each group in the experiment. (**B**) Graph of gastric lesion in the experiment. Each bar represents the mean ± SEM of samples ($^{***}P < 0.001$; $^{*}P < 0.05$; ns, no significant difference, using Tukey's test).

A single dose of 60% ethanol in 150 mM HCl (5 mL/kg; p.o.) produced severe elevated mucosal tissue damage (histology index was 34.4) (Figure 15). Pretreatment with OF-80E (50, 100, 150, and 200 mg/kg; p.o.) showed a significant reduction in histology index with an inhibition of 25.0, 62.2, 46.7, and 54.9%, respectively, as compared with ethanol/HCl alone-treated group (Figure 15). The rats pre-treated with 100 mg/kg of Mucosta® tablet and 60 mg/kg of Stillen® tablet exhibited 22.9 and 15.9% of inhibition of histology index, respectively. These results indicated that OF-80E in ethanol/HCl-induced ulcer rat model displays gastro-protective effects and significantly reduced the ethanol/HCl-inducted gastric tissue damage. In addition, OF-80E displayed a higher level of potency than both Mucosta® tablet and Stillen® tablet in this model.

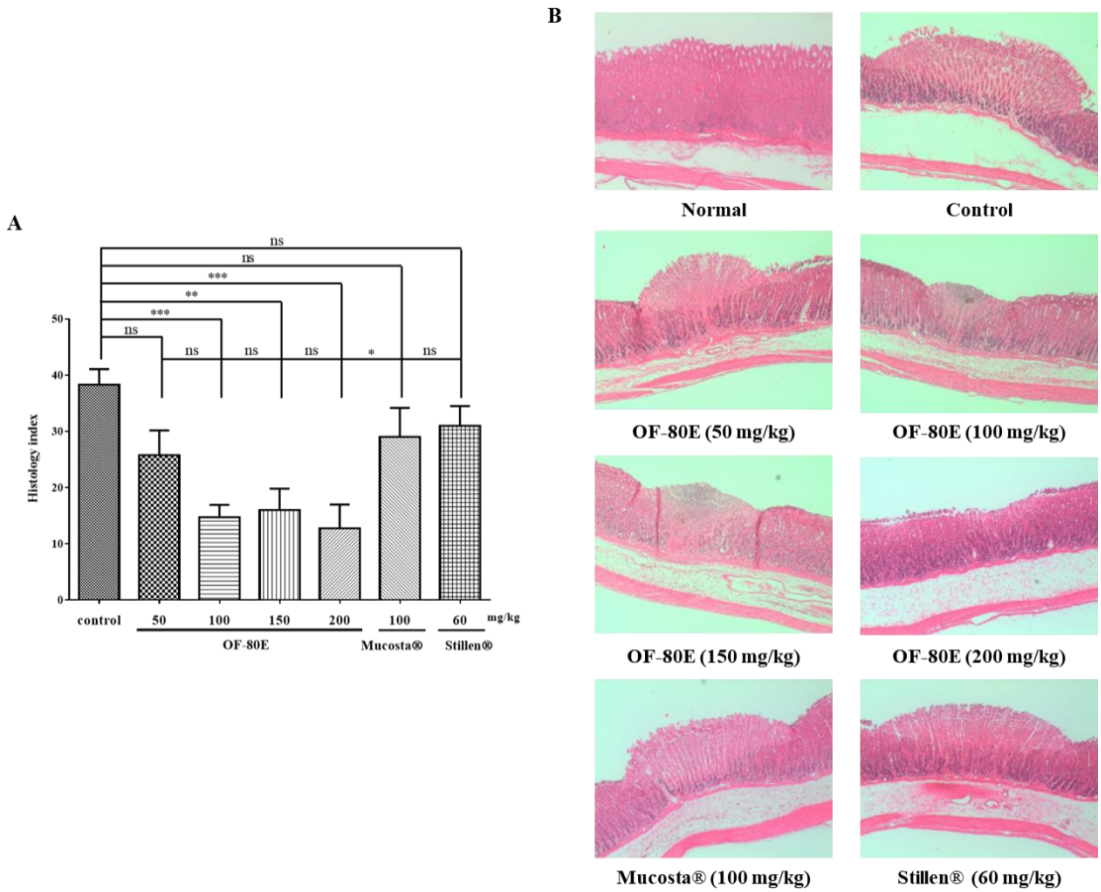


Figure 15. Gastro-protective effects of OF-80E on ethanol-induced gastric histology index in rats. The rats (8 weeks old, n = 10/group) were pre-treated orally with OF-80E (50, 100, 150, and 200 mg/kg BW), Mucosta® tablet (100 mg/kg BW), Stillen® tablet (60 mg/kg BW), and vehicle (1% HPMC). 1 h after tested sample treatments, the rats were received 5 mL/kg acidified ethanol (150 mM HCl in 60% ethanol; p.o.). 1 h later, the stomachs were removed and then the histology index was measured, as described in the Materials and Methods section. (**A**) Graph of histology index in the experiment. (**B**) Representative histological photo of each group in the experiment. The tissues were stained by hematoxylin and eosin. Each bar represents the mean ± SEM of samples ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, no significant difference, using Tukey's test).

2.2. Gastro-protective activity of OF-80E in an indomethacin-induced gastritis rat

NSAIDs cause gastric ulcer by oxidative damage and PG deficiency and administration of indomethacin significantly induced ulcerative lesions [190]. After administration of indomethacin (100 mg/kg; p.o.) in rats, ulcerative lesions were observed as an average of 37.1 mm^2 (Figure 16A). OF-80E at 50, 100, 150, and 200 mg/kg significantly inhibited mucosal hemorrhagic lesions by 62.0, 65.1, 65.9, and 63.1%, respectively, compared to the indomethacin-treated control group (Figure 16A). OF-80E was well tolerated and there was no mortality in the rats. In comparison, pretreatment with comparator agents, Mucosta® tablet (100 mg/kg; p.o.) and Stillen® tablet (60 mg/kg; p.o.), reduced mucosal lesions by 18.3 and 35.5%, respectively.

After administration of indomethacin in rats, mucosal tissue damages were observed as a histology index of 17.9 (Figure 16B). OF-80E at 50, 100, 150, and 200 mg/kg significantly reduced histology index by 57.3, 76.2, 56.6, and 65.7%, respectively, compared to the indomethacin-treated control group (Figure 16B). In comparison, pretreatment with comparator agents, Mucosta® tablet and Stillen® tablet, reduced histology index by 19.6 and 37.8%, respectively.

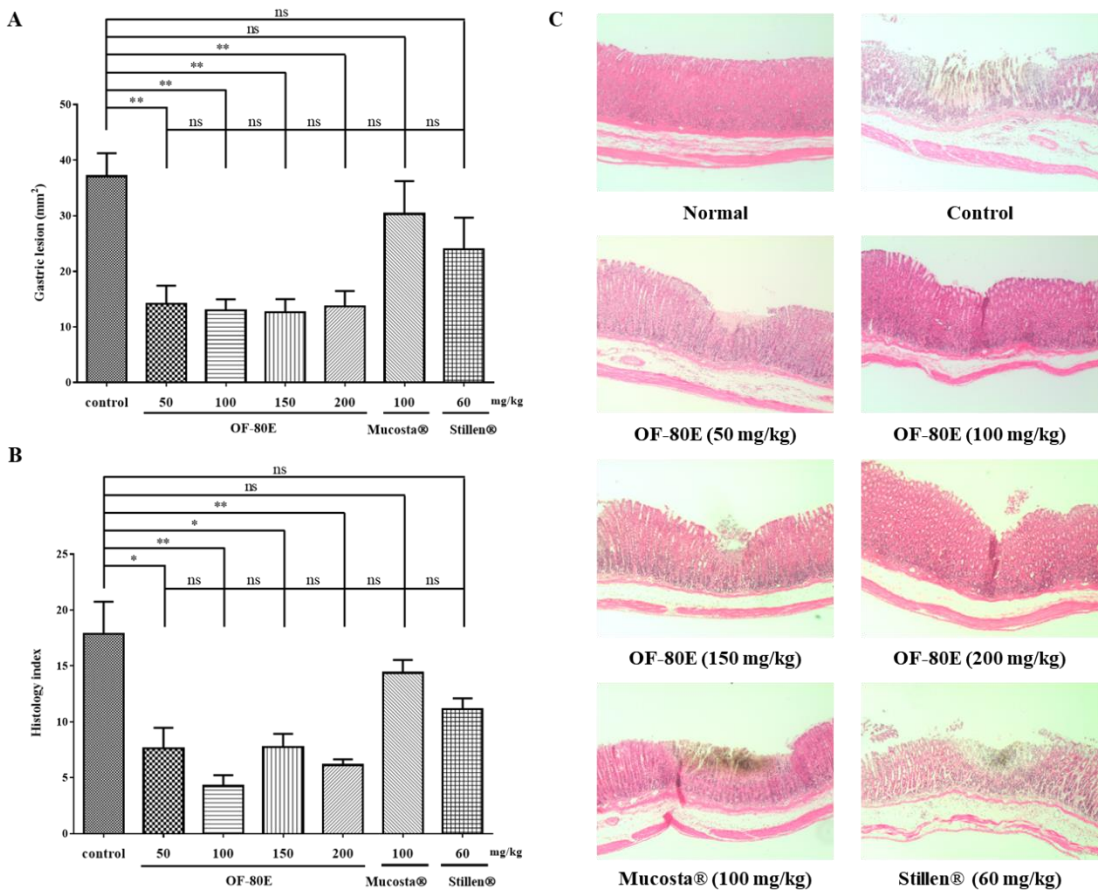


Figure 16. Gastro-protective effects of OF-80E on indomethacin-induced gastric ulcers in rats. The rats (8 weeks old, n = 10/group) were pre-treated orally with OF-80E (50, 100, 150, and 200 mg/kg BW), Mucosta® tablet (100 mg/kg BW), Stillen® tablet (60 mg/kg BW), and vehicle (1% HPMC). 1 h after tested sample treatments, the rats were received indomethacin in 0.5% CMC-Na (100 mg/kg; p.o.) to induce acute gastric ulcer. 6 h later, the stomachs of the rats were removed and then the area of the lesions and the histology index were measured, as described in the Materials and Methods section. (**A**) Graph of gastric lesion in the experiment. (**B**) Graph of histology index in the experiment. (**C**) Representative histological photo of each group in the experiment. The tissues were stained by hematoxylin and eosin. Each bar represents the mean ± SEM of samples ($^{**}P < 0.01$; $^{*}P < 0.05$; ns, no significant difference, using Tukey's test).

2.3. Gastro-protective activity of OF-80E in an aspirin-induced gastritis rat

Oxygen radical-mediated lipid peroxidation and neutrophil activation are involved in the development of NSAID-induced gastric mucosal injury [191]. After administration of aspirin (200 mg/kg; p.o.) in rats, ulcerative lesions were observed as an average of 87.2 mm^2 (Figure 17A). OF-80E at 100, 150, and 200 mg/kg significantly inhibited mucosal hemorrhagic lesions by 69.2, 80.6, and 93.4%, respectively, compared to the aspirin-treated control group (Figure 17A). OF-80E was well tolerated and there was no mortality in the rats. In comparison, pretreatment with comparator agents, Mucosta® tablet (100 mg/kg; p.o.) and Stillen® tablet (60 mg/kg; p.o.), reduced mucosal lesions by 44.2 and 58.7%, respectively.

After administration of aspirin in rats, mucosal tissue damages were observed as a histology index of 23.5 (Figure 17B). OF-80E at 100, 150, and 200 mg/kg significantly reduced histology index by 73.0, 81.6, and 83.0%, respectively, compared to the aspirin-treated control group (Figure 17B). In comparison, pretreatment with comparator agents, Mucosta® tablet and Stillen® tablet, reduced histology index by 57.4 and 69.5%, respectively.

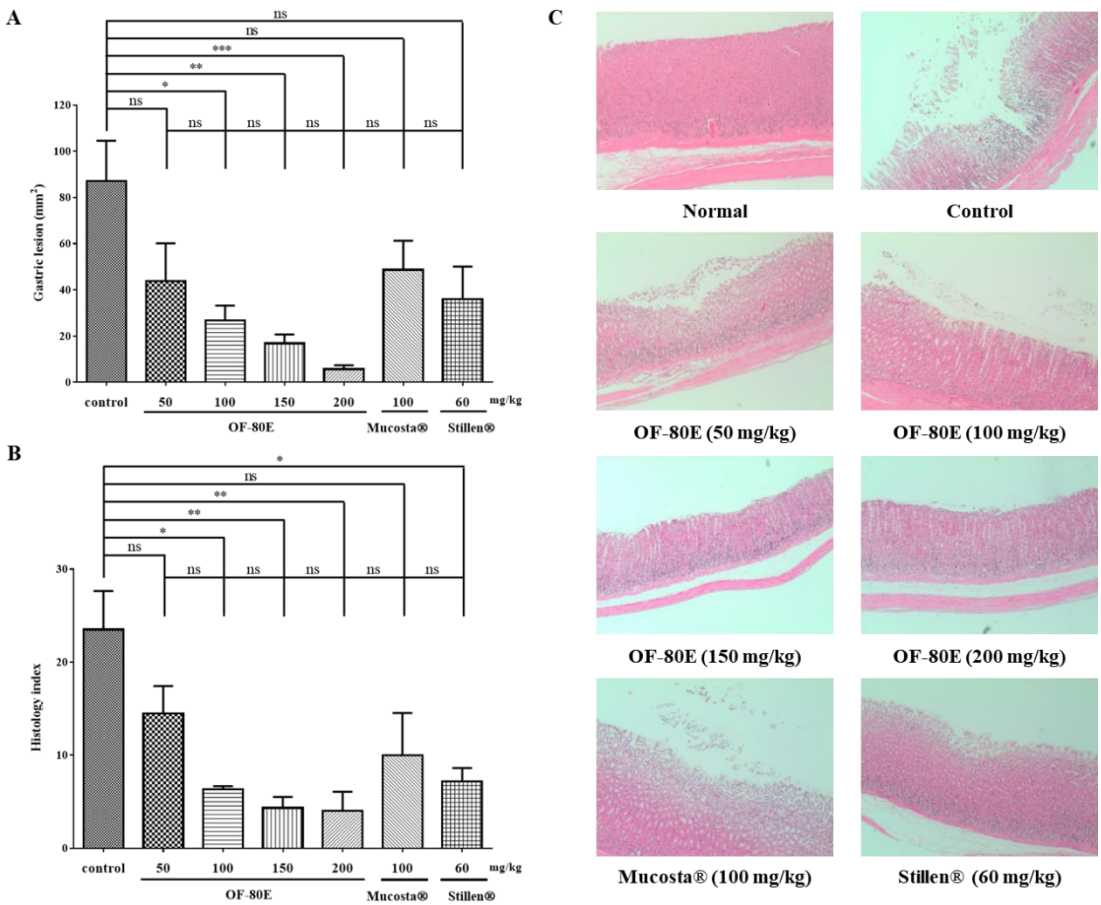


Figure 17. Gastro-protective effects of OF-80E on aspirin-induced gastric ulcers in rats. The rats (8 weeks old, n = 7/group) were pre-treated orally with OF-80E (50, 100, 150, and 200 mg/kg BW), Mucosta® tablet (100 mg/kg BW), Stillen® tablet (60 mg/kg BW), and vehicle (1% HPMC). Thirty minutes after, rats were anesthetized, and the pyloric end of the stomach was ligated. The rats received aspirin in 0.5% CMC-Na (200 mg/kg; p.o.) to induce acute gastric ulcer. 6 h later, the stomachs of the rats were removed and then the area of the lesions and the histology index were measured, as described in the Materials and Methods section. (**A**) Graph of gastric lesion in the experiment. (**B**) Graph of histology index in the experiment. (**C**) Representative histological photo of each group in the experiment. The tissues were stained by hematoxylin and eosin. Each bar represents the mean ± SEM of samples ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, no significant difference, using Tukey's test).

2.4. Gastro-protective activity of OF-80E in a stress-induced gastritis rat

Both physical and psychological stresses can cause gastric ulceration in humans and experimental animals. WIRS is widely accepted for studying stress ulcers and can also mimic the clinical acute gastritis ulcerations caused by trauma, surgery, or sepsis [209]. It has been reported that oxidative stress generated by infiltrated neutrophils and the xanthine-oxidase system and its subsequent lipid peroxidation is involved in the development of gastric mucosal lesions induced by WIRS [210]. Six hours of WIRS produced severe elevated mucosal hemorrhagic injury in glandular stomach (lesion area was 124.4 mm^2) (Figure 18A). Pretreatment with OF-80E (50, 100, 150, and 200 mg/kg; p.o.) showed a dose-dependent and significant reduction in mucosal hemorrhagic lesions with an inhibition of 54.3, 50.1, 70.1, and 75.0%, respectively, as compared with WIRS-treated group (Figure 18A). OF-80E was well tolerated and there was no mortality in the rats. These results indicate that OF-80E displays an antiulcer effect related to cytoprotective activity, since it significantly reduced the stress-induced intraluminal bleeding. The rats pre-treated with 100 mg/kg of Mucosta® tablet and 60 mg/kg of Stillen® tablet exhibited 14.3 and 28.2% of inhibition of mucosal hemorrhagic lesions, respectively.

WIRS produced severe elevated mucosal tissue damage (histology index was 11.7) (Figure 18B). Pretreatment with OF-80E (50, 100, 150, and 200 mg/kg; p.o.) showed reduction in histology index with an inhibition of 38.6, 31.4, 68.6, and 80.0%, respectively, as compared with WIRS-treated group (Figure 18B). The rats pre-treated with 100 mg/kg of Mucosta® tablet and 60 mg/kg of Stillen® tablet exhibited 15.7 and 4.3% of inhibition of histology index, respectively. These results indicated that OF-80E in WIRS-induced rat ulcer model displays gastro-protective effects and significantly reduced the stress-induced gastric tissue damage. In addition, OF-80E displayed a higher level of potency than both Mucosta® tablet and Stillen® tablet in this model.

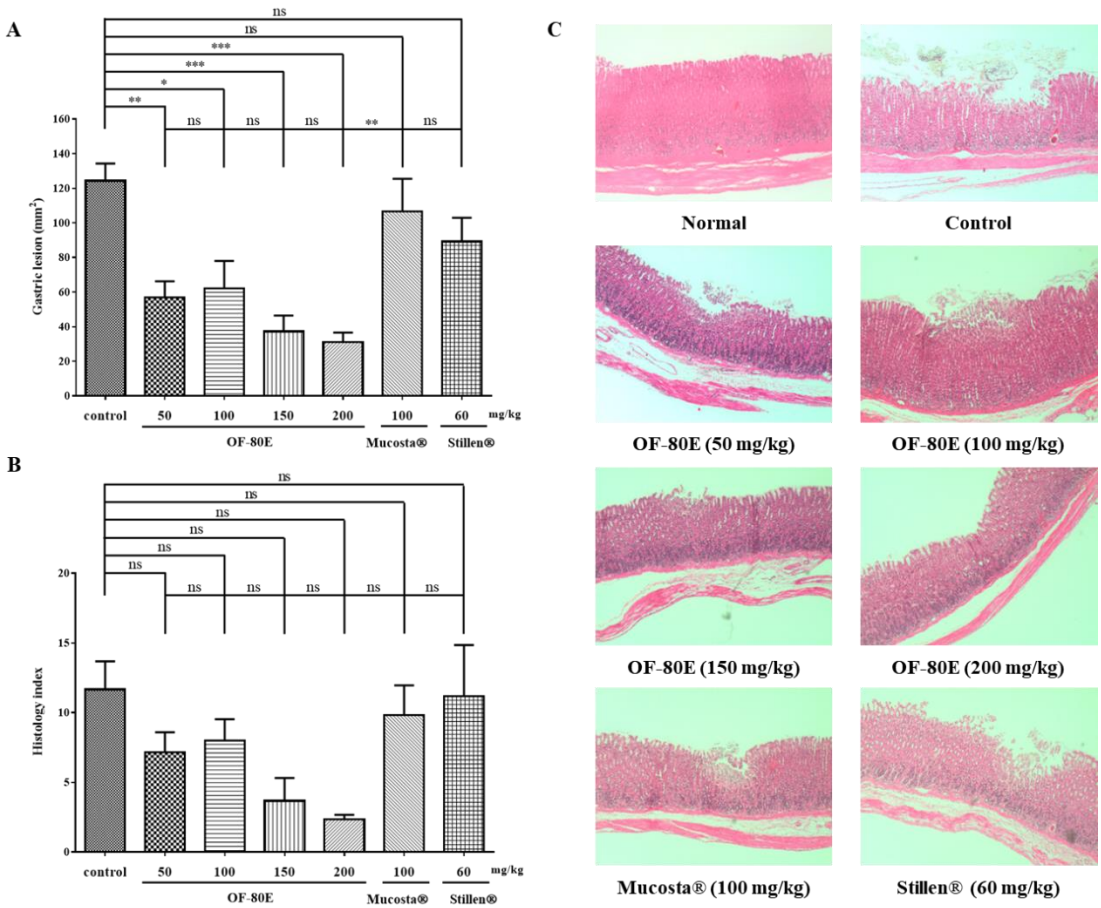


Figure 18. Gastro-protective effects of OF-80E on stress-induced gastric ulcers in rats. The rats (8 weeks old, n = 7/group) were pre-treated orally with OF-80E (50, 100, 150, and 200 mg/kg BW), Mucosta® tablet (100 mg/kg BW), Stillen® tablet (60 mg/kg BW), and vehicle (1% HPMC). Thirty minutes after tested sample treatments, the rats were placed in individual restraint cages and vertically immersed in a water bath (20ºC) to the level of the xyphoid process for 6 h. The stomachs of the rats were removed and then the area of the lesions and the histology index were measured, as described in the Materials and Methods section. (**A**) Graph of gastric lesion in the experiment. (**B**) Graph of histology index in the experiment. (**C**) Representative histological photo of each group in the experiment. The tissues were stained by hematoxylin and eosin. Each bar represents the mean ± SEM of samples ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, no significant difference, using Tukey's test).

2.5. Gastro-protective activity of OF-80E in a diclofenac-induced gastritis rat

NSAIDs-induced stomach damage occurs due to increased gastric movement, increased gastric juice secretion, and decreased gastric blood flow [57]. As people age, the gastro cells-damage caused by NSAIDs intensified, so old rats (20~25 weeks old) were used in this experiment. Rats were administered test substances while chronically inducing gastritis with diclofenac. After administration of diclofenac (50 mg/kg; p.o.) for 14 days in rats, ulcerative lesions were observed as an average of 4.8 mm^2 (Figure 19A). OF-80E at 50, 100, 150, and 200 mg/kg significantly inhibited mucosal hemorrhagic lesions by 58.5, 86.9, 73.0, and 91.3%, respectively, compared to the diclofenac-treated control group (Figure 19A). OF-80E was well tolerated and there was no mortality in the rats. In comparison, pretreatment with comparator agents, Mucosta® tablet (100 mg/kg; p.o.) and Stillen® tablet (60 mg/kg; p.o.), reduced mucosal lesions by 30.4 and 39.5%, respectively.

After administration of diclofenac (50 mg/kg; p.o.) in rats, mucosal tissue damages were observed at a histology index of 2.5 (Figure 19B). OF-80E at 50, 100, 150, and 200 mg/kg reduced histology index by 60.0, 60.0, 66.7, and 53.3%, respectively, compared to the diclofenac-treated control group (Figure 19B). In comparison, pretreatment with comparator agents, Mucosta® tablet (100 mg/kg; p.o.) and Stillen® tablet (60 mg/kg; p.o.), reduced histology index by 40.0 and 60.0%, respectively.

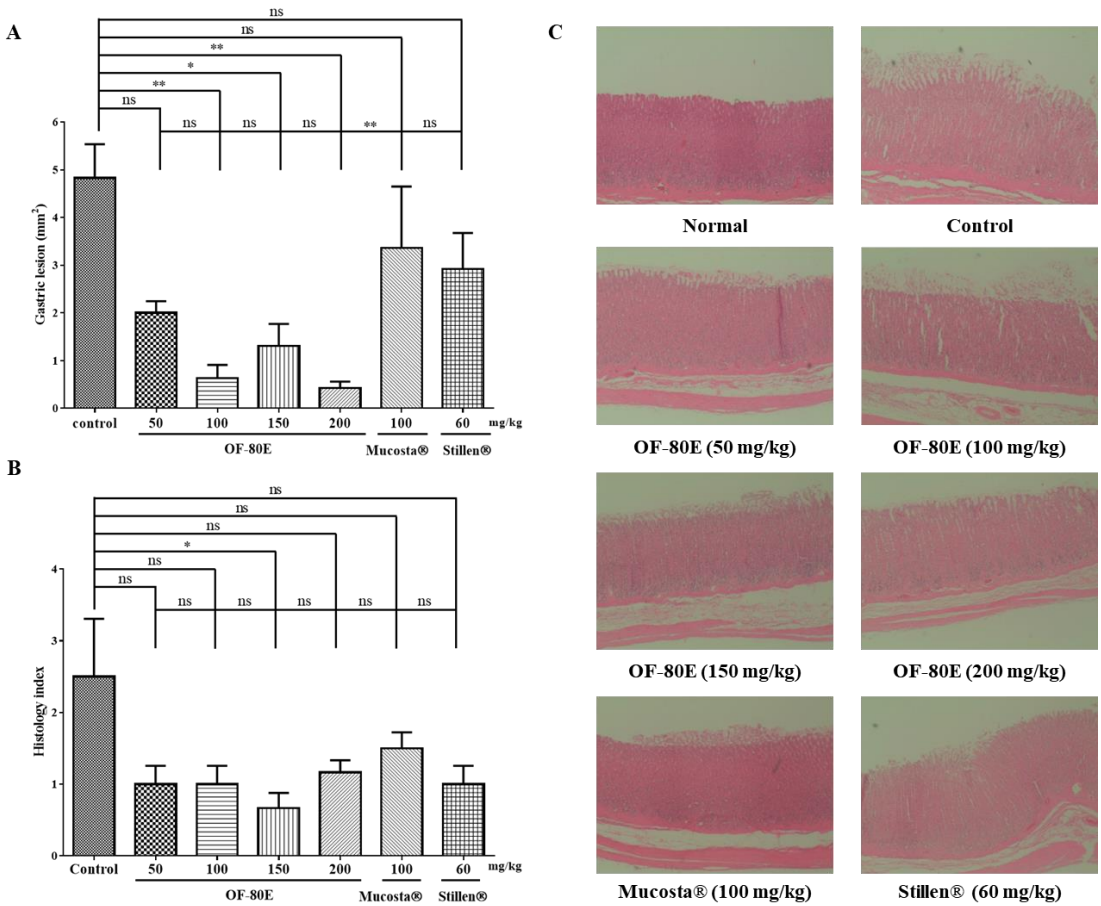


Figure 19. Gastro-protective effects of OF-80E on diclofenac induced gastric ulcers in rats. Daily for 14 days, the rats (20~25 weeks old, n = 7/group) were pre-treated orally with OF-80E (50, 100, 150, and 200 mg/kg BW), Mucosta® tablet (100 mg/kg BW), Stillen® tablet (60 mg/kg BW), and vehicle (1% HPMC). 1 h after tested sample treatments, the rats received diclofenac sodium 0.5% CMC-Na (50 mg/kg; p.o.) to induce gastric ulcer. After treated 14 day, the stomachs of rats were removed and then the area of the lesions and the histology index were measured, as described in the Materials and Methods section. (**A**) Graph of gastric lesion in the experiment. (**B**) Graph of histology index in the experiment. (**C**) Representative histological photo of each group in the experiment. The tissues were stained by hematoxylin and eosin. Each bar represents the mean ± SEM of samples ($^{**}P < 0.01$; $^{*}P < 0.05$; ns, no significant difference, using Tukey's test).

2.6. Anti-inflammatory and antioxidative activity of OF-80E

2.6.1. Effect of OF-80E on membrane-bound myeloperoxidase activity

An assay of gastric mucosal MPO was used to quantify the degree of neutrophil infiltration. MPO activity was measured in indomethacin-induced gastritis rat model (Figure 20A). The NSAIDs induced increase in MPO activity in the gastric mucosa was suppressed by OF-80E, Mucosta® tablet, and Stillen® tablet (Figure 20A). Stress induced increase in MPO activity in the gastric mucosa was significantly suppressed by betanin (Figure 20B).

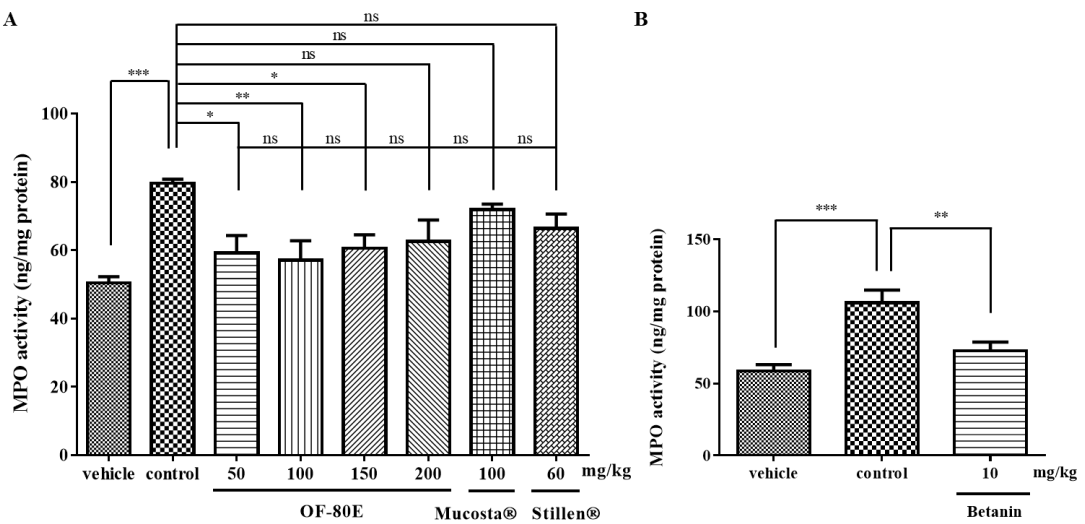


Figure 20. Effects of OF-80E on gastric mucosal concentration of membrane-bound myeloperoxidase. (**A**) MPO activity was measured in indomethacin-induced gastritis rats. (**B**) MPO activity was measured in stress-induced gastritis rats. Gastric mucosal MPO concentration was measured using ELISA kit, as described in the Materials and Methods section. Each bar represents the mean ± SEM of samples ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, no significant difference, using Tukey's test).

2.6.2. Effect of OF-80E on tumor necrosis factor-α level

The gastric mucosal concentration of TNF-α was increased significantly after 6h of stress. Betanin at 10 mg/kg showed suppressive effect on stress induced increase in TNF-α (Figure 21).

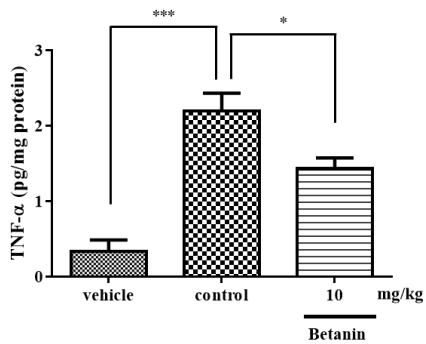


Figure 21. Effects of betanin on gastric mucosal concentration of tumor necrosis factor-α. TNF-α level was measured in stress-induced gastritis rats. The concentration of TNF-α was measured using an ELISA kit, as described in the Materials and Methods section. Each bar represents the mean ± SEM of samples ($^{***}P < 0.001$; $^{*}P < 0.05$, using Tukey's test).

2.6.3. Effect of OF-80E on reduced glutathione level

Intracellular GSH is an important factor that contributes to the protection of the gastric mucosa against NSAIDs induced damage. The NSAIDs induced decrease in GSH concentration in the gastric mucosa was significantly increased by OF-80E (50 mg/kg and 100 mg/kg) (Figure 22).

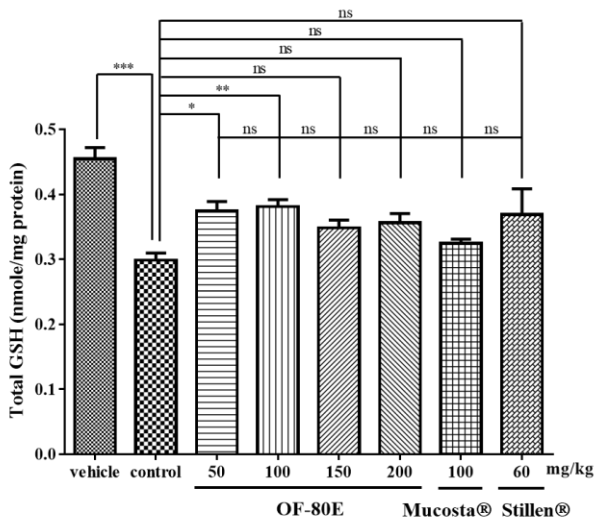


Figure 22. Effects of OF-80E on gastric mucosal concentration of reduced glutathione. GSH level was measured in indomethacin-induced gastritis rats. GSH content was expressed a nmole/mg protein, determined by the Bradford assay, as described in the Materials and Methods section. Each bar represents the mean ± SEM of samples ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, no significant difference, using Tukey's test).

2.7. Effect of OF-80E on adherent mucus level

Gastric mucosa is continuously exposed to many noxious factors and substances. A continuous layer of mucus covers gastric mucosa and serves as a physiological barrier and primary pre-epithelial defense [194]. Rats treated with indomethacin had a significant decrease in gastric adherent mucus concentration when compared with indomethacin-untreated rats (vehicle group) (Figure 23A). Pre-administration of OF-80E significantly attenuated the decreased gastric adherent mucus concentration (Figure 23A). Stress-induced decrease in gastric adherent mucus concentration in the gastric mucosa was significantly increased by betanin (Figure 23B).

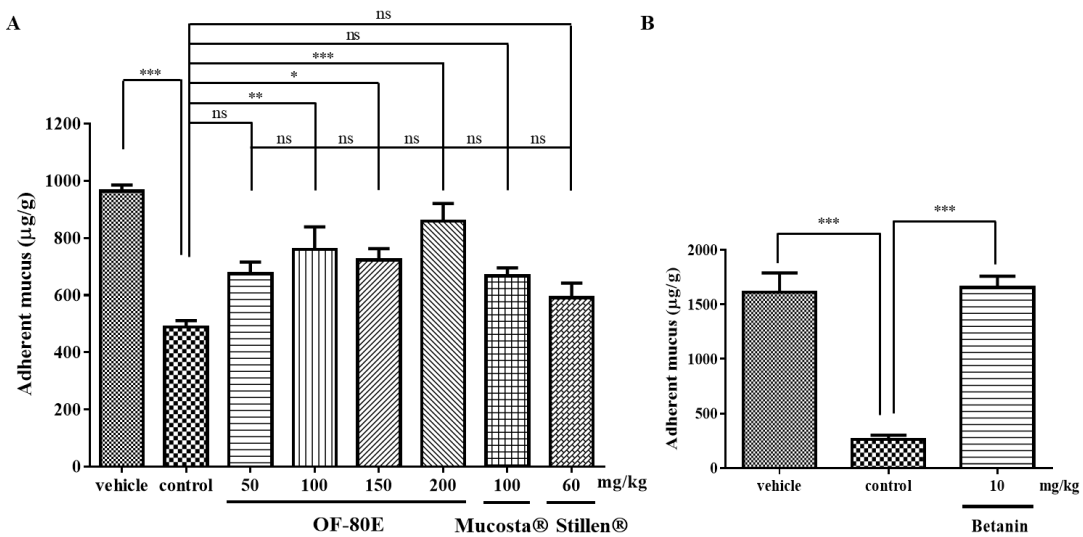


Figure 23. Effects of OF-80E on adherent mucus concentration in gastric mucosa. (**A**) Adherent mucus concentration was measured in indomethacin-induced gastritis rats. (**B**) Adherent mucus concentration was measured in stress-induced gastritis rats. The glandular portion of the stomach was excised and stained by alcian blue solution, as described in the Materials and Methods section. The results were expressed as the concentration (μg/g tissue) of alcian blue adhering to the gastric mucosal surface. Each bar represents the mean ± SEM of samples ($^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; ns, no significant difference, using Tukey's test).

3. Toxicity studies of OF-80E

3.1. Single dose oral toxicity study in Sprague Dawley rats

This study was carried out to evaluate the toxicity of OF-80E in SD rats after a single oral administration. In male rats, in the 5000 mg/kg administration group (n = 5), 2 cases of reddish urine temporarily occurred at 2 h after administration, 4 cases of compound-colored stool at 3 h, 3 cases of soft stool, 2 cases of soiled perineal region, and 1 case of diarrhea were observed. On day 2, 4 cases of soiled perineal region were observed. In the 10000 mg/kg administration group (n = 5), red urine was observed in all animals within 1 h after administration, and all of them

recovered within 4 h after administration. Compound-colored stool, diarrhea, and soiled perineal region were observed in 1 case within 1 h after administration and were observed until 6 h after administration. Salivation was temporarily observed in 1 case immediately after administration. Perineal contamination was observed in all animals (Table 17). In female rats, in the vehicle control group, 2500 and 5000 mg/kg administration groups (n = 5/group), 1, 2, and 1 cases of soft stool were observed within 2 h after administration, respectively. In the 10000 mg/kg administration group (n = 5), red urine was observed in all animals within 1 h after administration on the day of administration, and all of them recovered within 3 h after administration. Soft stool was temporarily observed in 2 cases immediately after administration, and soft stool was observed in 2 cases within 3 h after administration. Diarrhea, compound-colored stool, and soiled perineal region were observed in 2 cases at 2~5 h after administration, and all of them were recovered until 1 hour later. On day 2, perineal contamination was observed in 2 cases (Table 17).

There were no dead animals observed during the experimental period. There were no treatment-related effects on body weights were noted (Table 18). In necropsy, there were no treatment-related abnormal findings were observed. Based on the above result, the approximate lethal dose of OF-80E in SD male and female rats was higher than 10000 mg/kg after a single oral administration.

Table 17. Clinical signs after oral administration of OF-80E in a single dose oral toxicity study

Day[a]	Sign	OF-80E (mg/kg)			
		0	2500	5000	10000
Male					
1	Normal	5 / 5[b]	5 / 5	0 / 5	0 / 5
	Soft stool	0 / 5	0 / 5	3 / 5	0 / 5
	Reddish urine	0 / 5	0 / 5	2 / 5	5 / 5
	Diarrhea	0 / 5	0 / 5	1 / 5	1 / 5
	Compound colored stool	0 / 5	0 / 5	4 / 5	1 / 5
	Soiled perineal region	0 / 5	0 / 5	2 / 5	1 / 5
	Salivation	0 / 5	0 / 5	0 / 5	1 / 5
2	Normal	5 / 5	5 / 5	1 / 5	0 / 5
	Soiled perineal region	0 / 5	0 / 5	4 / 5	5 / 5
3-14	Normal	5 / 5	5 / 5	5 / 5	5 / 5
15	Normal	5 / 5	5 / 5	5 / 5	5 / 5
	Terminal sacrifice	5 / 5	5 / 5	5 / 5	5 / 5
Female					
1	Normal	4 / 5	3 / 5	4 / 5	0 / 5
	Soft stool	1 / 5	2 / 5	1 / 5	2 / 5
	Reddish urine	0 / 5	0 / 5	0 / 5	5 / 5
	Diarrhea	0 / 5	0 / 5	0 / 5	2 / 5
	Compound colored stool	0 / 5	0 / 5	0 / 5	2 / 5
	Soiled perineal region	0 / 5	0 / 5	0 / 5	2 / 5
	Salivation	0 / 5	0 / 5	0 / 5	2 / 5
2	Normal	5 / 5	5 / 5	5 / 5	3 / 5
	Soiled perineal region	0 / 5	0 / 5	0 / 5	2 / 5
3-14	Normal	5 / 5	5 / 5	5 / 5	5 / 5
15	Normal	5 / 5	5 / 5	5 / 5	5 / 5
	Terminal sacrifice	5 / 5	5 / 5	5 / 5	5 / 5

[a] The day of administration was designated as day 1.

[b] Number of animals with the sign / number of animals examined.

Table 18. Body weights after oral administration of OF-80E

Day[a]	Body weight, g (mean ± std)			
	OF-80E			
	0	2500	5000	10000
Male				
1	223.2 ± 03.91	222.9 ± 04.91	222.9 ± 04.05	221.2 ± 04.90
2	251.0 ± 05.11	249.3 ± 06.00	250.7 ± 08.34	247.0 ± 04.50
4	266.9 ± 09.58	264.9 ± 08.46	263.5 ± 04.29	261.1 ± 02.60
8	291.8 ± 12.95	284.3 ± 11.65	294.1 ± 05.84	285.6 ± 00.66
15	327.6 ± 15.06	324.1 ± 11.21	334.7 ± 08.65	322.9 ± 07.14
Weight gain[b]	104.4 ± 11.97	100.2 ± 09.89	111.8 ± 06.11	101.7 ± 08.34
Female				
1	154.5 ± 03.20	156.8 ± 03.17	155.7 ± 03.99	154.7 ± 05.45
2	173.1 ± 03.03	173.7 ± 02.27	174.1 ± 03.88	173.9 ± 07.63
4	179.4 ± 05.60	181.0 ± 05.96	182.2 ± 03.08	180.3 ± 06.37
8	187.8 ± 07.62	190.4 ± 06.53	191.2 ± 02.87	192.7 ± 10.43
15	206.7 ± 08.30	205.7 ± 06.97	206.1 ± 03.81	209.7 ± 15.84
Weight gain[b]	52.2 ± 06.67	49.0 ± 04.34	50.4 ± 04.97	55.0 ± 12.12

[a] The day of administration was designated as day 1.

[b] Body weight on day 15 – body weight on day 1.

3.2. Thirteen-week repeated-dose oral toxicity study in Sprague Dawley rats

The present study was carried out to investigate the toxicity of OF-80E after repeating oral administration over a period of 13 weeks against rats to determine no observed adverse effect level (NOAEL) and target organ (orangs with test-substance related toxicity), and to investigate the recovery potential during a 4-week recovery period.

3.2.1. General clinical signs

During the administration period, black compound-colored stool was observed in the male and female groups administered with OF-80E. There was no significant difference in the color of

the stool, but the ratio of the color of the test substance was a dose dependent. During the 4-week recovery period, color stools in the male and female recovery group in the 2000 mg/kg/day group were observed on day 92~94 and 93~94, respectively, but were then recovered (Table 19).

Table 19. Clinical signs after oral administration of OF-80E in a 13-week repeated dose oral toxicity study

Day[a]	Sign	OF-80E (mg/kg/day)			
		0	500	1000	2000
Male					
1	Normal	15 / 15[b]	10 / 10	10 / 10	15 / 15
2~90	Normal	15 / 15	0 / 10	0 / 10	0 / 15
	Compound colored stool	0 / 15	10 / 10	10 / 10	15 / 15
91	Normal	15 / 15	0 / 10	0 / 10	0 / 15
	Compound colored stool	0 / 15	10 / 10	10 / 10	15 / 15
	Terminal sacrifice	10 / 15	10 / 10	10 / 10	10 / 15
92~94	Normal	5 / 5	–[c]	–	0 / 5
	Compound colored stool	0 / 5	–	–	5 / 5
95~119	Normal	5 / 5	–	–	5 / 5
120	Normal	5 / 5	–	–	5 / 5
	Terminal sacrifice	5 / 5	–	–	5 / 5
Female					
1	Normal	15 / 15	10 / 10	10 / 10	15 / 15
2~91	Normal	15 / 15	0 / 10	0 / 10	0 / 15
	Compound colored stool	0 / 15	10 / 10	10 / 10	15 / 15
92	Normal	15 / 15	0 / 10	0 / 10	0 / 15
	Compound colored stool	0 / 15	10 / 10	10 / 10	15 / 15
	Terminal sacrifice	10 / 15	10 / 10	10 / 10	10 / 15
93~94	Normal	5 / 5	–	–	0 / 5
	Compound colored stool	0 / 5	–	–	5 / 5
95~119	Normal	5 / 5	–	–	5 / 5
120	Normal	5 / 5	–	–	5 / 5
	Terminal sacrifice	5 / 5	–	–	5 / 5

[a] The day of administration was designated as day 1.

[b] Number of animals with the sign / number of animals examined.

[c] Animals, except for recovery group, subjected to necropsy on the scheduled day.

3.2.2. Body weights

There were no test substance-related changes in BW of rats. However, on day 117, the BW in females at recovery group in the 2000 mg/kg/day group was significantly higher than the vehicle control recovery group (Table 20). However, it was within the normal range of physiological parameters [211], although BW change was not related to the test substance.

3.2.3. Food consumption

There were no test substance-related changes in food consumption of rats (Table 21). On day 22, the average food consumption of males in the 2000 mg/kg/day group was significantly lower than that in the vehicle control group. On day 64, the average food consumption of males in the 500 mg/kg/day group was significantly higher than that of the vehicle control group. However, the above statistically significant results appeared to be a one-time change observed during the administration period and is not due to the effect of the test substance. On day 117, the average food consumption of male in the recovery group in the 2000 mg/kg/day group was significantly higher than that of the vehicle control group. However, it was within the normal range of physiological parameters [211], although the change in food consumption was not related to the test substance.

Table 20. Body weights after oral administration of OF-80E

Day[a]	Male rat body weight, g (mean ± std)			
	OF-80E (mg/kg/day)			
	0	500	1000	2000
1	180.7 ± 06.80	179.7 ± 06.60	178.7 ± 06.12	179.0 ± 07.03
8	227.5 ± 08.74	226.1 ± 04.96	225.0 ± 06.83	228.1 ± 09.50
15	276.4 ± 12.80	276.1 ± 07.69	275.0 ± 07.05	277.9 ± 09.97
22	310.0 ± 15.10	314.6 ± 08.66	310.9 ± 09.98	317.2 ± 11.60
29	339.5 ± 19.42	343.9 ± 08.93	337.1 ± 12.74	346.1 ± 13.67
36	360.0 ± 21.84	366.3 ± 12.49	337.4 ± 14.44	371.4 ± 15.63
43	381.1 ± 23.20	387.7 ± 15.25	376.5 ± 16.36	391.3 ± 19.44
50	395.1 ± 27.26	402.4 ± 16.12	389.7 ± 19.83	404.8 ± 21.60
57	405.8 ± 28.72	414.5 ± 15.76	400.6 ± 21.53	417.4 ± 21.16
64	420.2 ± 30.34	429.1 ± 16.57	415.2 ± 23.36	431.9 ± 25.72
71	425.6 ± 32.57	433.8 ± 15.83	421.3 ± 24.38	437.5 ± 26.49
78	431.4 ± 31.06	436.3 ± 14.96	426.4 ± 25.03	440.8 ± 26.46
84	440.8 ± 31.94	445.7 ± 15.39	432.2 ± 27.11	449.5 ± 25.55
90	450.9 ± 33.59	453.9 ± 17.38	439.2 ± 25.41	459.1 ± 25.42
Weight gain[b]	270.2 ± 30.05	274.2 ± 18.37	260.6 ± 22.97	280.1 ± 22.76
n[c]	15	10	10	15
90	454.8 ± 26.95	–[d]	–	461.2 ± 17.27
99	455.8 ± 27.18	–	–	461.6 ± 18.00
105	464.9 ± 24.76	–	–	467.6 ± 20.30
111	469.8 ± 26.79	–	–	474.7 ± 20.03
117	475.7 ± 27.43	–	–	485.9 ± 19.77
Weight gain[b]	20.9 ± 04.37	–	–	24.7 ± 07.70
n[c]	5			5

[a] The day of first treatment was designated day 1.

[b] Body weight on day 90 – body weight on day 1 or body weight on day 117 – body weight on day 90.

[c] Number of animals examined.

[d] Animals, except for recovery group, subjected to necropsy on the scheduled day.

Table 20. (continued)

Day[a]	Female rat body weight, g (mean ± std)			
	OF-80E (mg/kg/day)			
	0	500	1000	2000
1	135.7 ± 05.43	136.8 ± 04.28	135.1 ± 05.47	135.7 ± 05.59
8	152.0 ± 06.19	153.3 ± 09.53	155.2 ± 05.39	152.7 ± 04.60
15	175.9 ± 09.42	172.2 ± 10.46	178.1 ± 06.64	176.2 ± 08.71
22	191.6 ± 08.58	191.5 ± 15.57	195.8 ± 08.04	191.7 ± 10.11
29	203.9 ± 07.83	203.8 ± 15.14	206.3 ± 06.19	203.7 ± 14.50
36	217.7 ± 09.52	216.4 ± 16.00	220.3 ± 08.56	215.8 ± 11.82
43	227.2 ± 08.70	228.9 ± 20.51	229.6 ± 07.94	224.4 ± 14.86
50	232.9 ± 12.18	235.2 ± 18.79	235.9 ± 06.91	230.4 ± 11.58
57	235.0 ± 10.14	239.7 ± 18.78	241.3 ± 09.16	236.9 ± 10.75
64	243.0 ± 09.17	246.5 ± 16.94	248.1 ± 11.36	241.4 ± 11.88
71	242.7 ± 11.73	243.6 ± 16.00	246.9 ± 06.68	239.1 ± 13.51
78	243.4 ± 09.09	244.6 ± 15.83	250.4 ± 08.97	240.0 ± 16.91
84	247.4 ± 09.28	245.5 ± 15.11	250.8 ± 10.53	242.1 ± 14.51
90	254.9 ± 10.54	254.5 ± 15.07	261.8 ± 10.33	249.4 ± 13.49
Weight gain[b]	119.2 ± 09.14	117.6 ± 11.58	126.8 ± 12.56	113.7 ± 12.48
n[c]	15	10	10	15
90	248.3 ± 08.72	–[d]	–	259.8 ± 10.33
99	245.8 ± 05.84	–	–	256.8 ± 09.38
105	249.6 ± 06.43	–	–	263.0 ± 08.90
111	252.1 ± 04.37	–	–	263.5 ± 11.15
117	255.7 ± 06.13	–	–	265.5 ± 11.90*
Weight gain[b]	7.5 ± 05.36	–	–	5.72 ± 04.81
n[c]	5			5

[a] The day of first treatment was designated day 1.

[b] Body weight on day 90 – body weight on day 1 or body weight on day 117 – body weight on day 90.

[c] Number of animals examined.

[d] Animals, except for recovery group, subjected to necropsy on the scheduled day.

* Significant difference at $P < 0.05$ level compared with the vehicle control group.

Table 21. Food consumption after oral administration of OF-80E

Day[a]	Male rat, daily food consumption, g (mean ± std)			
	OF-80E (mg/kg/day)			
	0	500	1000	2000
1	17.7 ± 2.01	17.7 ± 1.70	17.9 ± 0.63	18.1 ± 1.22
8	19.5 ± 1.26	19.5 ± 0.98	19.2 ± 0.82	17.9 ± 6.64
15	20.3 ± 1.13	20.0 ± 1.16	19.6 ± 0.55	20.4 ± 1.90
22	21.4 ± 1.18	20.7 ± 1.42	19.9 ± 0.78	19.3 ± 1.77*
29	19.6 ± 3.18	20.6 ± 1.42	20.7 ± 1.51	20.6 ± 1.28
36	21.0 ± 1.42	20.6 ± 1.90	18.8 ± 0.63	20.1 ± 1.62
43	20.6 ± 1.67	20.9 ± 1.70	19.8 ± 1.05	19.1 ± 1.65
50	20.4 ± 3.06	20.1 ± 1.22	17.9 ± 1.66	18.3 ± 1.43
57	18.8 ± 2.05	18.4 ± 0.62	18.4 ± 1.46	18.2 ± 1.52
64	20.1 ± 1.10	22.0 ± 1.05*	20.2 ± 1.03	20.0 ± 0.89
71	17.7 ± 1.12	18.1 ± 1.09	18.5 ± 0.49	16.7 ± 2.03
78	17.7 ± 1.65	17.5 ± 0.96	16.5 ± 0.82	16.6 ± 0.87
84	19.8 ± 2.21	18.8 ± 1.68	19.3 ± 0.44	19.1 ± 1.58
90	18.9 ± 0.83	18.9 ± 1.61	18.1 ± 0.62	18.0 ± 1.37
n[b]	15	10	10	15
99	21.4 ± 1.24	–[c]	–	21.4 ± 0.99
105	21.6 ± 1.96	–	–	20.8 ± 1.11
111	21.8 ± 1.22	–	–	21.2 ± 1.49
117	19.8 ± 0.87	–	–	22.7 ± 0.95*
n[b]	5	–	–	5

[a] The day of first treatment was designated day 1.

[b] Number of animals examined.

[c] Animals, except for recovery group, subjected to necropsy on the scheduled day.

* Significant difference at $P < 0.05$ level compared with the vehicle control group.

Table 21. (continued)

Day[a]	Female rat, daily food consumption, g (mean ± std)			
	OF-80E (mg/kg/day)			
	0	500	1000	2000
1	12.6 ± 1.19	12.2 ± 0.60	12.2 ± 0.87	12.4 ± 0.87
8	14.0 ± 0.91	12.7 ± 1.32	13.3 ± 0.92	12.6 ± 1.60
15	13.6 ± 1.31	13.0 ± 1.83	13.0 ± 0.59	12.7 ± 1.40
22	12.5 ± 0.94	12.6 ± 0.44	13.2 ± 1.49	12.7 ± 1.82
29	12.7 ± 1.98	13.8 ± 2.41	14.2 ± 0.60	12.8 ± 1.29
36	13.9 ± 0.56	13.1 ± 1.99	13.2 ± 0.87	13.0 ± 1.05
43	13.7 ± 0.91	12.1 ± 3.05	11.8 ± 0.55	11.9 ± 1.76
50	13.0 ± 1.54	12.5 ± 1.47	13.1 ± 1.36	12.3 ± 0.79
57	11.6 ± 1.13	11.8 ± 1.81	12.3 ± 1.15	12.2 ± 1.20
64	13.6 ± 1.73	12.9 ± 0.81	13.0 ± 0.98	12.1 ± 2.25
71	12.5 ± 2.20	13.0 ± 1.84	13.2 ± 1.91	10.9 ± 1.27
78	13.0 ± 1.41	13.2 ± 1.21	13.2 ± 1.29	11.9 ± 1.05
84	13.5 ± 1.43	13.7 ± 0.95	13.1 ± 1.06	12.06 ± 1.71
90	12.5 ± 1.42	12.8 ± 0.87	12.7 ± 1.45	12.6 ± 1.20
n[b]	15	10	10	15
99	13.3 ± 2.61	–[c]	–	13.4 ± 0.50
105	13.7 ± 2.58	–	–	14.4 ± 1.31
111	15.1 ± 1.26	–	–	13.7 ± 1.62
117	14.3 ± 1.93	–	–	13.8 ± 0.81
n[b]	5	–	–	5

[a] The day of first treatment was designated day 1.

[b] Number of animals examined.

[c] Animals, except for recovery group, subjected to necropsy on the scheduled day.

3.2.4. Water consumption

There were no test substance-related changes in water consumption (Table 22). However, on day 99, 111, and 117, the average water consumption of females in the recovery group in the 2000 mg/kg/day group was significantly higher than that in the vehicle control group. Change

during administration period was not observed. It was within the normal range of physiological parameters [211], although the change in water consumption was not related to the test substance.

Table 22. Water consumption after oral administration of OF-80E

Day[a]	Male rat, daily water consumption, g (mean ± std)			
	OF-80E (mg/kg/day)			
	0	500	1000	2000
1	24.5 ± 3.05	23.6 ± 2.00	24.7 ± 1.52	24.4 ± 2.51
8	22.8 ± 2.09	25.1 ± 3.18	24.9 ± 2.05	23.1 ± 0.95
15	24.3 ± 3.99	25.9 ± 3.72	24.5 ± 2.55	24.2 ± 2.61
22	27.2 ± 4.92	26.3 ± 3.63	24.9 ± 3.17	24.6 ± 1.31
29	24.7 ± 3.61	25.5 ± 4.00	22.0 ± 4.73	22.7 ± 1.73
36	26.1 ± 3.18	27.3 ± 7.12	23.3 ± 2.34	22.5 ± 1.79
43	25.8 ± 6.10	27.2 ± 5.35	22.3 ± 1.91	22.7 ± 2.70
50	24.6 ± 3.53	27.4 ± 6.50	22.2 ± 2.85	20.6 ± 2.12
57	24.4 ± 3.87	24.9 ± 5.53	24.8 ± 6.59	21.5 ± 2.55
64	26.9 ± 4.93	30.9 ± 9.69	25.7 ± 5.50	23.5 ± 2.93
71	23.2 ± 8.94	19.4 ± 3.99	17.4 ± 2.89	16.5 ± 2.32
78	19.7 ± 4.43	21.4 ± 4.77	17.5 ± 3.11	18.7 ± 2.07
84	23.8 ± 5.38	25.4 ± 4.85	22.8 ± 1.94	20.6 ± 3.64
90	23.7 ± 2.84	24.6 ± 7.77	19.6 ± 2.45	21.1 ± 3.60
n[b]	15	10	10	15
99	28.6 ± 6.91	–[c]	–	27.7 ± 0.82
105	28.3 ± 7.11	–	–	24.5 ± 4.37
111	27.4 ± 3.86	–	–	27.0 ± 3.31
117	29.1 ± 7.42	–	–	23.3 ± 2.09
n[b]	5	–	–	5

[a] The day of first treatment was designated day 1.

[b] Number of animals examined.

[c] Animals, except for recovery group, subjected to necropsy on the scheduled day.

Table 22. (continued)

Day[a]	Female rat, daily water consumption, g (mean ± std)			
	OF-80E (mg/kg/day)			
	0	500	1000	2000
1	19.8 ± 1.54	18.0 ± 2.00	20.3 ± 2.19	20.4 ± 3.62
8	18.4 ± 2.03	16.0 ± 2.99	20.5 ± 2.24	17.6 ± 3.48
15	19.1 ± 2.66	17.2 ± 2.83	21.0 ± 2.91	18.6 ± 2.63
22	20.5 ± 3.24	18.6 ± 1.77	24.4 ± 2.80	21.1 ± 3.25
29	19.7 ± 2.07	19.1 ± 2.67	24.3 ± 5.86	18.8 ± 3.65
36	21.2 ± 4.34	17.9 ± 2.38	23.0 ± 3.63	20.5 ± 2.91
43	21.5 ± 3.69	18.6 ± 2.70	22.5 ± 3.93	20.1 ± 3.92
50	19.9 ± 3.34	17.8 ± 3.77	22.3 ± 3.37	20.0 ± 1.72
57	22.3 ± 6.53	19.5 ± 2.33	23.5 ± 3.64	21.0 ± 2.71
64	23.3 ± 4.05	19.9 ± 2.99	24.0 ± 2.94	22.7 ± 2.92
71	16.4 ± 3.08	15.2 ± 2.65	16.9 ± 2.73	15.6 ± 2.90
78	19.2 ± 5.06	18.2 ± 4.02	22.0 ± 4.68	17.8 ± 2.56
84	23.0 ± 4.59	20.9 ± 3.26	25.0 ± 7.80	19.5 ± 3.79
90	24.2 ± 6.12	24.1 ± 8.83	23.2 ± 2.47	20.2 ± 2.46
n[b]	15	10	10	15
99	22.1 ± 1.02	–[c]	–	26.9 ± 2.87*
105	22.8 ± 0.60	–	–	28.2 ± 3.75
111	24.4 ± 0.04	–	–	27.4 ± 0.94**
117	23.0 ± 0.68	–	–	29.1 ± 1.75**
n[b]	5	–	–	5

[a] The day of first treatment was designated day 1.

[b] Number of animals examined.

[c] Animals, except for recovery group, subjected to necropsy on the scheduled day.

*/** Significant difference at $P < 0.05$ / $P < 0.01$ level compared with the vehicle control group.

3.2.5. Urinalysis

In the present study, the judgement criterions of urinalysis parameters and urine sediments parameters are shown in the following Table 23 and Table 24, respectively.

Table 23. Judgement criterions of urinalysis parameters

Result	Grade	Glucose (mg/dL)	Bilirubin	Ketone (mg/dL)	Protein (mg/dL)	Urobilinogen (EU/dL)	Nitrite	Occult blood (EA/μL)	Clarity
−	0	Negative	Negative	Negative	Negative	0.2	Negative	Negative	Clear
+/−	1	Trace	Trace	Trace	Trace	1.0	NA	Trace	Trace
+	1	NA[a]	NA	NA	NA	NA	Positive	NA	NA
1+	2	250	Small	15	30	2.0	NA	Small	Cloudy
2+	3	500	Moderate	40	100	4.0	NA	Moderate	Turbid
3+	4	≥ 1000	Large	≥ 80	≥ 300	≥ 8.0	NA	Large	Other

[a] NA: Not applicable.

Table 24. Judgement criterions of urine sediments

Result	Grade	RBC (mean/field)	WBC (mean/field)	Epithelial cell (number/field)	Casts (mean/field)
−	0	0	0	0	0
+/−	1	≤ 4	≤ 5	Few / 20 field	1
+	1	5~8	6~20	Around 1 / few field	2~5
1+	2	9~30	21~50	Few	6~10
2+	3	≥ 30 (local)	≥ 51 (local)	10~20	11~30
3+	4	All over the field	All over the field	≥ 30	≥ 31

In main group (13-week administration group), ketone body, protein, and WBC of males were significantly higher in 2000 mg/kg/day group than that in vehicle control group (Tables 25 and 26). It was considered test substance related change because the above change had a dose-response correlation observed. In addition, the urinary specific gravity of males in the 1000 mg/kg/day group was significantly higher than vehicle control group, and the occult blood was significantly lower, but dose-response correlation was not observed. In addition, because of histopathological examination, no related changes were observed in the urinary system. It was within the normal range of physiological parameters [211], although the change in urinalysis was not related to the test substance.

In recovery group, urinary specific gravity of female in the 2000 mg/kg/day group was significantly higher than that in vehicle control group (Tables 27 and 28). However, change in specific gravity was not observed during administration period (Table 25), and change in the electrolyte in blood biochemical test was not observed. It was within the normal range of physiological parameters [211], although the change in urinalysis was not related to the test substance.

Table 25. Summary of urinalysis in main group

Tests	Result	Grade	Male rat, OF-80E (mg/kg/day)			
			0	500	1000	2000
Glucose	−	0	5	5	5	5
Bilirubin	−	0	5	5	5	5
	+/−	1	0	0	0	0
	1+	2	0	0	0	2
Ketone	−	0	4	3	2	0
	+/−	1	1	2	2	2
	1+	2	0	0	1	3*

Specific gravity	≤ 1.005	0	0	0	0	0
	1.010	1	2	3	0	1
	1.015	2	3	1	1	0
	1.020	3	0	1	4*	1
	1.025	4	0	0	0	1
	≥ 1.030	5	0	0	0	2
pH	7.0	0	0	0	0	0
	7.5	1	0	0	1	0
	8.0	2	3	1	1	3
	8.5	3	2	3	3	1
	≥ 9.0	4	0	1	0	1
Protein	−	0	1	0	2	0
	+/−	1	1	1	0	0
	1+	2	2	3	2	0
	2+	3	1	1	1	4
	3+	4	0	0	0	1*
Urobilinogen	0.2	0	5	5	5	4
	1.0	1	0	0	0	1
Nitrite	−	0	4	2	1	1
	+	1	1	3	4	4
Occult blood	−	0	1	1	5*	0
	+/−	1	4	4	0	3
	1+	2	0	0	0	1
	2+	3	0	0	0	1
Clarity	−	0	5	5	5	5
Volume (mL)			15.0 ± 3.2	13.4 ± 3.3	12.4 ± 2.4	14.4 ± 2.8
Color: yellow			5	5	5	5
Number of animals			5	5	5	5

* Significant difference at $P < 0.05$ level compared with the vehicle control group.

Table 25. (continued)

Tests	Result	Grade	Female rat, OF-80E (mg/kg/day)			
			0	500	1000	2000
Glucose	−	0	5	5	5	5
Bilirubin	−	0	5	5	5	5
Ketone	−	0	5	5	5	5
Specific gravity	≤ 1.005	0	2	3	0	1
	1.010	1	2	1	3	0
	1.015	2	1	0	1	4
	1.020	3	0	1	1	0
pH	7.0	0	0	2	0	0
	7.5	1	0	2	0	0
	8.0	2	3	0	3	3
	8.5	3	2	1	2	2
	≥ 9.0	4	0	0	0	0
Protein	−	0	5	3	3	5
	+/−	1	0	2	1	0
	1+	2	0	0	1	0
Urobilinogen	0.2	0	5	5	5	5
Nitrate	−	0	1	1	0	2
	+	1	4	4	5	3
Occult blood	−	0	4	4	4	4
	+/−	1	1	0	1	1
	1+	2	0	0	0	0
	2+	3	0	0	0	0
	3+	4	0	1	0	0
Clarity	−	0	5	5	5	5
Volume (mL)			19.2 ± 4.1	15.8 ± 4.3	19.0 ± 3.9	13.2 ± 3.0
Color: yellow			5	5	5	5
Number of animals			5	5	5	5

Table 26. Summary of urine sediments in main group

Tests[a]	Result	Grade	OF-80E (mg/kg/day)			
			0	500	1000	2000
Male						
RBC	−	0	2	3	5	3
	+/−	1	3	2	0	1
	1+	2	0	0	0	1
WBC	−	0	2	2	0	0
	+/−	1	1	2	2	1
	1+	2	2	1	1	4*
	2+	3	0	0	2	0
Epithelial cell	−	0	3	2	2	2
	+/−	1	2	2	1	2
	1+	2	0	1	1	1
	2+	3	0	0	1	0
Casts	−	0	5	5	5	5
Number of animals			5	5	5	5
Female						
RBC	−	0	5	3	5	5
	+/−	1	0	2	0	0
WBC	−	0	5	5	4	5
	+/−	1	0	0	1	0
Epithelial cell	−	0	5	5	5	4
	+/−	1	0	0	0	1
Casts	−	0	5	5	5	5
Number of animals			5	5	5	5

[a] RBC: red blood cell; and WBC: white blood cell.

* Significant difference at $P < 0.05$ level compared with the vehicle control group.

Table 27. Summary of urinalysis in recovery group

Tests	Result	Grade	OF-80E (mg/kg/day)			
			Male		Female	
			0	2000	0	2000
Glucose	−	0	5	5	5	5
Bilirubin	−	0	5	5	5	5
Ketone	−	0	0	0	5	5
	+/−	1	2	3	0	0
	1+	2	3	2	0	0
Specific gravity	≤ 1.005	0	0	0	0	0
	1.010	1	1	0	4	1
	1.015	2	2	3	1	1
	1.020	3	2	2	0	2
	1.025	4	0	0	0	1*
pH	7.0	0	0	0	1	3
	7.5	1	0	0	2	1
	8.0	2	0	0	0	1
	8.5	3	5	5	2	0
Protein	−	0	0	0	4	3
	+/−	1	0	0	1	0
	1+	2	1	1	0	1
	2+	3	4	4	0	1
Urobilinogen	0.2	0	5	5	5	5
Nitrate	−	0	3	4	2	1
	+	1	2	1	3	4
Occult blood	−	0	2	3	5	4
	+/−	1	3	2	0	1
Clarity	−	0	5	5	5	5
Volume (mL)			11.0 ± 1.6	13.0 ± 3.2	16.8 ± 3.3	15.8 ± 4.1
Color: yellow			5	5	5	5
Number of animals			5	5	5	5

* Significant difference at $P < 0.05$ level compared with the vehicle control group.

Table 28. Summary of urine sediments in recovery group

Tests[a]	Result	Grade	OF-80E (mg/kg/day)	
			0	2000
Male				
RBC	−	0	4	4
	+/−	1	1	1
WBC	−	0	1	0
	+/−	1	1	3
	1+	2	3	2
Epithelial cell	−	0	3	3
	+/−	1	1	1
	1+	2	1	1
Casts	−	0	5	5
	N		5	5
Female				
RBC	−	0	5	5
WBC	−	0	5	4
	+/−	1	0	1
Epithelial cell	−	0	5	5
Casts	−	0	5	5
	N		5	5

[a] RBC: red blood cell; and WBC: white blood cell.

3.2.6. Hematological test

The mean platelet volume in main group was statistically significantly higher in females in the 2000 mg/kg/day group (4.8 fluid ounce) than that in the vehicle control group (4.7 fluid ounce), but dose-response correlation was not observed. It was within the normal range of physiological parameters [211], although the change in hematological test was not related to the test substance. In recovery group, basophil of male in the 2000 mg/kg/day group (0.5%) was significantly higher

than that in vehicle control group (0.3%). Change in the WBC count related basophil was not observed. It was within the normal range of physiological parameters [211], although the change in hematological test was not related to the test substance.

3.2.7. Clinical blood biochemistry test

In main group, blood chloride ion in females was statistically significantly lower in the 1000 mg/kg/day group (101.79 nM/L) than that in the vehicle control group (102.76 nM/L), but dose-response correlation was not observed. Change in the WBC count related basophil was not observed. Morphological change with the kidney in histopathological examination related to chloride ion was not observed. It was within the normal range of physiological parameters [211], although the change in blood biochemistry was not related to the test substance.

In recovery group, glucose of male in the 2000 mg/kg/day group (131.1 mg/dL) was significantly higher than that in vehicle control group (115.7 mg/dL). Blood urea nitrogen (17.0 mg/dL) and creatinine (0.56 mg/dL) of female in the 2000 mg/kg/day group in recovery group was significantly lower than that in vehicle control group (blood urea nitrogen: 21.1 mg/dL, and creatinine: 0.64 mg/dL). These changes were not observed during administration period. Morphological change with the kidney in histopathological examination related to blood urea nitrogen and creatinine was not observed. It was within the normal range of physiological parameters [211], although the change in blood biochemistry was not related to the test substance.

3.2.8. Organ weights

In main group, relative weight of right kidney of males was statistically significantly higher in the 2000 mg/kg/day group (1.32 g) than that in the vehicle control group (1.30 g). Relative weight of left adrenal was statistically higher in females in the 2000 mg/kg/day group (0.0300 g)

than that in the vehicle control group (0.0282 g). The absolute weight of lungs was statistically significantly lower in all females in the test substance administered group (500 mg/kg group: 1.27 g, 1000 mg/kg group: 1.26 g, and 2000 mg/kg group: 1.23 g) than that in the vehicle control group (1.35 g). Morphological change with histopathological examination related above organs was not observed. It was within the normal range of physiological parameters [211], although the change in organ weight was not related to the test substance. The BW before necropsy was statistically significantly lower in females in the 2000 mg/kg/day group (229.01 g) than that in the vehicle control group (240.95 g). But, it was within the normal range of physiological parameters [211], although the change in BW was not related to the test substance.

In recovery group, absolute and relative weight of right adrenal (0.027 g) of male and absolute weight of lungs (1.345 g) of female in the 2000 mg/kg/day group were significantly higher than that in vehicle control group (male right adrenal: 0.022 g, and female lungs: 1.288 g). Morphological change with histopathological examination related above organs was not observed. Also, it was within the normal range of physiological parameters [211], although the change in organ weight was not related to the test substance.

3.2.9. Necropsy finding

In main group, protuberance onto part of the middle lobe and adhesion with diaphragm were observed in the liver of one male in the vehicle control group and one male in the 1000 mg/kg/day group. The retention of clear fluid in uterus was observed in each 2 case of females in vehicle control, 1000 mg/kg/day, and 2000 mg/kg/day groups. The above-mentioned changes were not observed with dose-response correlation and considered as spontaneous changes independent of the test substance. In recovery group, retention of clear fluid in uterus was observed in 3 cases of

females in vehicle control group. This change was not observed with dose-response correlation and considered as spontaneous changes independent of the test substance.

3.2.10. Histopathological examination

In the vehicle control group and 1000 mg/kg/day group in male, the protuberance of a part of the mesenchyme of the liver and adhesion with the diaphragm were confirmed as a hepatic diaphragm nodule. It was accidentally distributed, considering spontaneous changes. The retention of clear fluid in uterus in females of the vehicle control, 1000 mg/kg/day, and 2000 mg/kg/day groups and vehicle control in recovery group were observed. The above-mentioned changes were confirmed as dilation of the uterine lumen. These changes were related to the sexual cycle, considering independent of the test substance.

Test substance-related changes in BW, food and water consumption, urinalysis, ophthalmological examination, hematological test, clinical biochemistry, organ weights, and histopathological examination were not observed. In clinical sign, the black color stool like test substance color were observed in male and female in all administration groups indicating a change in the excretion of some components or its metabolites through the urinary or digestive tract. However, considering not to be a toxicological effect as it does not accompany any abnormalities in related blood and blood biochemical tests and organ weight. In urinalysis, ketone body, urine protein, and leukocytes of males were observed high in 2000 mg/kg/day group. It was considered as test substance related change because this change had a dose-response correlation. No changes in related blood, hematological test, and organ weights were observed. No related morphological changes in liver and kidney were also observed. Therefore, it was considered not to be a toxicological effect, considering the recovery during the 4-week recovery period.

The effects of the test substance on general symptoms and urinalysis under this test condition were observed. These effects were not observed after the recovery period and were not accompanied by changes in related indicators. Thus, it was considered a non-toxicological change. Therefore, the NOAEL of OF-80E was 2000 mg/kg/day for SD rats of both sexes and the target organ was not observed.

3.3. Four-week repeated-dose oral toxicity study in beagle dogs

This study was performed to evaluate the toxicity of the OF-80E after 4-week repeated oral administration in beagle dogs (Table 29). There were no mortalities in both sexes. Compound-colored stool in the form of diarrhea was observed intermittently 10 times from day 4 to day 14 in female in the 300 mg/kg/day group. Diarrhea was temporarily observed in female administration with 300 and 1500 mg/kg/day group at 2 cases (days 16 and 18) and 1 case (day 20), respectively. In addition, in the vehicle control group, 600, and 1500 mg/kg/day group, the remaining of food in male and female was observed sporadically without a dose-response relationship. This was a change that could be naturally observed in experiments with beagle dog.

In BWs, food consumption, ophthalmological examination, hematological test, clinical biochemistry test, and necropsy findings, test substance-related changes in both sexes were not observed. In urinalysis, when each group with reference to the pre-administration was compared, light brownish urine was observed in males and females administered at 1000 mg/kg/day or more. In organ weights, the absolute and relative weights of the thymus glands of males were tended to be higher in the 1000 mg/kg/day group (10.51 g) compare to the vehicle control group (6.59 g). The absolute and relative weights of the spleen of females were tended to be lower in the 300 mg/kg/day group (18.85 g) compare to the vehicle control group (52.42 g). These changes were identified as general changes due to individual difference.

The soft stool was observed intermittently 10 times from day 2 to day 14 in male administration with 1000 mg/kg/day group and 7 times from day 17 to day 28 in female administration with 300 mg/kg/day group. The vomiting was observed in female administration with 300, 1000, and 1500 mg/kg/day group at 1 case (day 7), 4 cases (days 4, 6, 7, and 14), 3 cases (days 2, 3, and 21), respectively. All were observed within 1 h after administration.

In general symptoms, the major changes caused by the test substance administration included the compound-colored stool, diarrhea, soft stool, and vomiting. Generally, vomiting in dogs is also caused by mild GI irritation without toxic effects due to the development of the vomiting center as described previously [212]. Since vomiting was observed at a low frequency without systemic abnormalities in BW and related tests, the toxicological significance was considered insignificant. The change seemed to be due to physical properties such as taste and smell of the substance. The temporary diarrhea observed in females in the administration group was presumed to be a change due to stimulation of the GI tract of the test substance.

The compound-colored stool was considered as a result the coloration of the test substance or its metabolite. However, the abnormal changes were at a slight level in the observed degree, and no abnormalities were observed in the necropsy findings of the GI tract. There was no systemic abnormality in weight and related tests. The toxicological significance was considered to be insignificant. In urinalysis, brownish urine was observed. It was considered to be a change in the excretion of the test substance or its metabolite in the urinary tract in relation to the above-mentioned compound-colored stool, indicating a toxicologically meaningless change.

As a result of the above, under this test condition, when OF-80E was repeatedly orally administered to a beagle dog for 4-week, no toxicological significant change was observed due to the administration of the test substance. Therefore, the maximum tolerance dose (MTD) of OF-

80E was 1500 mg/kg/day for both male and female beagle dogs based on the dose at which no toxicological change of OF-80E was observed.

Table 29. Individual clinical signs after oral administration of OF-80E

Sex	Dose (mg/kg/day)	Sign	Observed day
Male	0	Normal	Day 1~4, 6~29
		Remaining of food	Day 5
	300	Normal	Day 1~29
	600	Normal	Day 1~5, 9~14, 16~29
		Remaining of food	Day 6~8, 15
	1000	Normal	Day 1, 3~4, 7, 15~29
		Soft stool	Day 2, 5~6, 8~14
	1500	Normal	Day 1, 3~29
		Remaining of food	Day 2
Female	0	Normal	Day 1, 4~14, 16~22, 25~27, 29
		Remaining of food	Day 2~3, 15, 23~24, 28
	300	Normal	Day 1~3, 6, 15, 21, 24~26, 29
		Diarrhea	Day 4~5*, 7~14*, 16, 18
		Compound colored stool	Day 4~5*, 7~14*
		Vomiting	Day 7
		Soft stool	Day 17, 19~20, 22~23, 27~28
	600	Normal	Day 1~6, 9~11, 13~15, 18~20, 23~26, 29
		Remaining of food	Day 7~8, 12, 16~17, 21~22, 27~28
	1000	Normal	Day 1~3, 4, 8~13, 15~29
		Vomiting	Day 4, 6~7, 14
	1500	Normal	Day 1, 4, 6~13, 18~19, 26~27, 29
		Vomiting	Day 2~3, 21
		Remaining of food	Day 5, 14~17, 22~25, 28
		Diarrhea	Day 20

* Mixed from of diarrhea and compound-colored stool.

3.4. Bacterial reverse mutation study

In the treatment with OF-80E, there was no increase in the number of revertant colony *S. typhimurium* test strains, TA100, TA1535, TA98, and TA1537 and *E. coli* WP2 *uvr*A both in the presence (+S9) and absence (−S9) of metabolic activation system, independently (Table 30). The growth inhibition was not observed in all *S. typhimurium* test strains and *E. coli* WP2 *uvr*A both in the presence (+S9) and absence (−S9) of metabolic activation system. In the positive controls, increases of revertant colonies in all positive control plates were observed (Table 30). There were no precipitation or other abnormalities observed in all plates. There were no colonies due to microbial contamination were observed in the plate to confirm the highest concentration of the test substance and the sterility of the S9 mixture. Therefore, OF-80E was considered to be non-mutagenic in the bacterial reverse mutation assay.

Table 30. Effect of OF-80E and six mutagens on reverse mutagenicity

Bacterial strain	Test materials	Treatment[a] (μg/plate)	Colonies/plate (factor)[b] (mean ± std)	
			With S9 mixture	Without S9 mixture
S. typhimurium TA100	OF-80E	0	120 ± 14 (1.0)	113 ± 05 (1.0)
		15	119 ± 10 (1.0)	108 ± 07 (1.0)
		50	116 ± 11 (1.0)	112 ± 09 (1.0)
		150	114 ± 07 (0.9)	100 ± 04 (0.9)
		500	107 ± 06 (0.9)	107 ± 04 (0.9)
		1500	109 ± 14 (0.9)	098 ± 05 (0.9)
		5000	139 ± 08 (1.2)	106 ± 04 (0.9)
	2-aminoanthracene	1.0	860 ± 12 (7.1)	−
	sodium azide	0.5	−	387 ± 18 (3.4)
S. typhimurium TA1535	OF-80E	0	015 ± 03 (1.0)	014 ± 01 (1.0)
		15	016 ± 04 (1.0)	016 ± 02 (1.1)
		50	011 ± 02 (0.7)	014 ± 02 (1.0)
		150	010 ± 01 (0.7)	015 ± 04 (1.0)

		500	012 ± 01 (0.8)	014 ± 02 (1.0)
		1500	015 ± 03 (1.0)	014 ± 01 (1.0)
		5000	012 ± 03 (0.8)	019 ± 03 (1.3)
	2-aminoanthracene	2.0	167 ± 19 (11.1)	−
	sodium azide	0.5	−	278 ± 21 (19.4)
S. typhimurium TA98	OF-80E	0	032 ± 01 (1.0)	021 ± 01 (1.0)
		15	028 ± 05 (0.9)	017 ± 02 (0.8)
		50	022 ± 01 (0.7)	016 ± 03 (0.8)
		150	022 ± 03 (0.7)	018 ± 04 (0.9)
		500	023 ± 03 (0.7)	017 ± 02 (0.8)
		1500	034 ± 01 (1.1)	018 ± 01 (0.9)
		5000	046 ± 01 (1.4)	021 ± 03 (1.0)
	benzo(a)pyrene	1.0	254 ± 46 (7.9)	−
	2-nitrofluorene	2.0	−	351 ± 18 (16.7)
S. typhimurium TA1537	OF-80E	0	015 ± 02 (1.0)	010 ± 02 (1.0)
		15	009 ± 01 (0.6)	009 ± 01 (0.8)
		50	010 ± 01 (0.7)	011 ± 03 (1.0)
		150	011 ± 02 (0.8)	008 ± 02 (0.8)
		500	012 ± 02 (0.8)	007 ± 01 (0.7)
		1500	017 ± 05 (1.2)	012 ± 02 (1.1)
		5000	014 ± 08 (0.9)	012 ± 03 (1.2)
	2-aminoanthracene	1.0	274 ± 25 (18.7)	−
	acridine mutagen	0.5	−	145 ± 35 (13.7)
E. coli WP2 *uvr*A	OF-80E	0	027 ± 04 (1.0)	021 ± 03 (1.0)
		15	024 ± 02 (0.9)	020 ± 03 (1.0)
		50	025 ± 03 (0.9)	019 ± 03 (0.9)
		150	023 ± 02 (0.8)	024 ± 03 (1.2)
		500	019 ± 02 (0.7)	021 ± 04 (1.0)
		1500	022 ± 03 (0.8)	018 ± 02 (0.9)
		5000	023 ± 03 (0.9)	022 ± 05 (1.0)
	2-aminoanthracene	6.0	146 ± 19 (5.4)	−
	4-nitroquinoline *N*-oxide	0.5	−	151 ± 20 (7.3)

[a] Three plates per dose were used.

[b] Number of colonies of treated plate/number of colonies of negative control plate.

3.5. Chromosome aberration test in CHL cells

3.5.1. Results in the presence of S9 mixture

In the 6h treatment with OF-80E, there was no precipitation and cytotoxicity in the treatments of any dose level in CHL cells. The frequency of metaphases with structural aberrations was 0.0 in all treated doses of the negative control and test substance (Table 31). There was no statistically significant increase in all doses treated with the test substance compared to the negative control. The mean frequency of metaphases with numerical aberrations in the negative control and all dose levels of the test substance were not more than 0.5, and there was no statistically significant increase at any dose level of test substance compared to the negative control. In the positive control [B(a)P], there was a statistically significant increase in the mean frequency of aberrant metaphase (39.0).

3.5.2. Results in the absence of S9 mixture

In the 6 h and 24 h treatments with OF-80E, there was no precipitation and cytotoxicity in the treatment of any dose level in CHL cells. The frequency of metaphases with structural aberrations was 0.0 in all treated doses of the negative control and test substance (Table 32). There was no statistically significant increase in all doses treated with the test substance compared to the negative control. The mean frequency of metaphases with numerical aberrations in the negative control and all dose levels of the test substances were 0.0. In the positive control (EMS), 6 h and 24 h treatment, there was a statistically significant increase in the mean frequency of aberrant metaphase (23.0 and 30.0, respectively). Therefore, it is concluded that OF-80E did not cause chromosomal aberration in CHL cells.

Table 31. Effect of OF-80E on chromosome aberration in the presence of S9 mixture in CHL cells

Treatment[a] (μg/mL)	Aberration						PP[b] + ER[c]		No. aberration metaphase[d]			RCC[e]
	Chromosome type		Chromatid type		Others[j]	Gaps[k]	no.	Decision	+Gaps	−Gaps		
	csd[f]	cse[g]	ctd[h]	cte[i]					no.	no.	Decision	
0	0	0	0	0	0	0	0	Negative	0	0	Negative	100
1250	0	0	0	0	0	0.5	0.5	Negative	0	0	Negative	99
2500	0	0	0	0	0	0.5	0.5	Negative	0.5	0	Negative	89
5000	0	0	0	0	0	1.5	0	Negative	1.5	0	Negative	90
B(a)P[l] (20)	0	1	1.5	84.5	0	2.5	0.5	Negative	41	39**	Positive	75

[a] 6 h treatment and 18 h recovery. Data are expressed as mean of duplicate cultures.

[b] PP: polyploid.

[c] ER: endoreduplication.

[d] Inclusive / exclusive gaps, 100 metaphases were examined per culture.

[e] RCC: relative cell counts = (cell count of treatment flask / cell count of control flask) × 100 %

[f] csd: chromosome type deletions.

[g] cse: chromosome type exchanges.

[h] ctd: chromatid type deletions.

[i] cte: chromatid type exchanges.

[j] Other: metaphases with more than 10 aberrations (including gaps) or with chromosome fragmentation.

[k] Gap: chromosome + chromatid types. Gap refers to a non-chromosomal lesion that is smaller than the width of a chromatid, and the chromatid is not aligned.

[l] B(a)P: Benzo (a) pyrene (positive control).

** Statistical comparison was performed using Fisher's exact test. $^{**}P < 0.01$ compare with negative control.

Table 32. Effect of OF-80E on chromosome aberration in the absence of S9 mixture in CHL cells

Treatment[a] (μg/mL)	Aberration						PP[b] + ER[c]		No. aberration metaphase[d]			RCC[e]
	Chromosome type		Chromatid type		Others[j]	Gaps[k]	no.	Decision	+Gaps	−Gaps		
	csd[f]	cse[g]	ctd[h]	cte[i]					no.	no.	Decision	
6 h treatment and 18 h recovery												
0	0	0	0	0	0	1.5	0	Negative	1.5	0	Negative	100
1250	0	0	0	0	0	1	0	Negative	1	0	Negative	97
2500	0	0	0	0	0	0.5	0	Negative	0.5	0	Negative	98
5000	0	0	0	0	0	0.5	0	Negative	0	0	Negative	95
EMS[l] *(800)*	0	1.5	1.0	24.5	0	0.5	0.5	Negative	23.5	23**	Positive	73
24h treatment and 0 h recovery												
0	0	0	0	0	0	0	0	Negative	0	0	Negative	100
1250	0	0	0	0	0	0	0	Negative	0	0	Negative	98
2500	0	0	0	0	0	1	0	Negative	1	0	Negative	94
5000	0	0	0	0	0	1.5	0	Negative	1.5	0	Negative	92
EMS[l] *(600)*	0.5	1.0	1.5	43	0	2	0	Negative	32**	30**	Positive	74

[a] 6 h treatment and 18 h recovery; or 24 h treatment and 0 h recovery. Data are expressed as mean of duplicate cultures.

[b] PP: polyploid.

[c] ER: endoreduplication.

[d] Inclusive / exclusive gaps, 100 metaphases were examined per culture.

[e] RCC: relative cell counts = (cell count of treatment flask / cell count of control flask) × 100 %

[f] csd: chromosome type deletions.

[g] cse: chromosome type exchanges.

[h] ctd: chromatid type deletions.

[i] cte: chromatid type exchanges.

[j] Other: metaphases with more than 10 aberrations (including gaps) or with chromosome fragmentation.

[k] Gap: chromosome + chromatid types. Gap refers to a non-chromosomal lesion that is smaller than the width of a chromatid, and the chromatid is not aligned.

[l] EMS: Ethyl methane sulfonate (positive control).

** Statistical comparison was performed using Fisher's exact test. $^{**}P < 0.01$ compare with negative control.

3.6. Frequency of micronucleated polychromatic erythrocyte and cytotoxicity in an *in vivo* micronucleus assay

The mean numbers of micronucleated polychromatic erythrocyte (MNPCE) per 2000 polychromatic erythrocytes (PCEs) were 1.0, 0.8, 1.7, and 0.8 at doses of 0 (negative control), 1250, 2500, and 5000 mg/kg/day mice group, respectively (Table 33). There was no statistically significant increase in frequencies of MNPCE at any dose level of the test substance compared to the negative control. The positive control, cyclophosphamide monohydrate, induced a statistically significant increase of MNPCE (62.8) when compared to the negative control group.

The PCE : RBC ratios were all 0.4 at doses 0 (negative control), 1250, 2500, and 5000 mg/kg/day mice group (Table 33). There was no statistically significant difference at any dose level of the test substance compared to the negative control. The positive control induced a statistically significant decrease in the PCE : RBC ratio (0.2) compared to the negative control group. There was no statistically significant difference in the BWs of mice in all groups. There was no abnormal clinical sign were observed in all treated animals (Table 34). It was considered that OF-80E did not induce micronuclei in the mammalian bone marrow cells used in this study under the present experimental conditions.

Table 33. Observations of micronucleus and polychromatic erythrocyte : red blood cell ratios of OF-80E

Dose[a] (mg/kg/day)	MNPCE[b] / 2000 PCE[c] (mean ± std)	PCE : RBC[d] ratio (mean ± std)	% ratio
0	1.0 ± 0.89	0.4 ± 0.03	100
1250	0.8 ± 0.98	0.4 ± 0.03	99
2500	1.7 ± 1.37	0.4 ± 0.02	103
5000	0.8 ± 0.98	0.4 ± 0.02	97
CPA[e] (70 mg/kg)	62.8 ± 8.01**	0.2 ± 0.02**	64

[a] Six rats per dose were used.

[b] MNPCE: Micronucleated polychromatic erythrocyte.

[c] PCE: polychromatic erythrocyte.

[d] RBC: red blood cells (polychromatic erythrocyte + normochromatic erythrocyte).

[e] CPA: cyclophosphamide monohydrate (positive control).

** Statistical comparison was performed using ANOVA followed by Levene test. $^{**}P < 0.01$ compare with negative control group.

Table 34. Effect of OF-80E on body weights of mice in an *in vivo* micronucleus assay

Dose[a] (mg/kg/day)	Body weight at the time, g (mean ± std)		
	1st administration	2nd administration	Sacrifice
0	35.4 ± 1.17	35.2 ± 1.48	34.6 ± 1.83
1250	35.0 ± 1.66	35.6 ± 1.20	34.6 ± 1.37
2500	35.2 ± 1.19	35.4 ± 1.23	34.8 ± 1.39
5000	34.9 ± 0.79	34.9 ± 0.78	34.5 ± 1.04
CPA[b] (70 mg/kg)	34.4 ± 0.90	35.0 ± 0.98	34.7 ± 0.84

[a] Six rats per dose were used.

[b] CPA: cyclophosphamide monohydrate (positive control).

3.7. Effect of OF-80E on the central nervous system in ICR mice

The effects of OF-80E on the CNS by measuring the body temperature and general behavior following a single oral administration in ICR mice. In body temperature, there were not abnormal changes related with the test substance in all mice groups (Table 35). In general behavior, there were not abnormal changes related with the test substance in all mice groups (Table 36). Based on the results, it is considered that single oral administration of OF-80E to ICR mice at below 5000 mg/kg does not affect the CNS under the present experimental condition.

Table 35. Effect of OF-80E on body temperature of mice

Dose[a]	Mice body temperature at the time (min), °C (mean ± std)					
(mg/kg)	0	30	60	120	240	360
0	38.3 ± 0.3	38.7 ± 0.6	38.7 ± 0.5	38.4 ± 0.6	38.6 ± 0.4	38.5 ± 0.4
50	37.9 ± 0.4	38.6 ± 0.5	38.6 ± 0.3	38.3 ± 0.3	38.3 ± 0.3	38.4 ± 0.4
500	38.1 ± 0.2	38.4 ± 0.4	38.5 ± 0.5	38.5 ± 0.3	38.4 ± 0.3	38.4 ± 0.6
5000	38.1 ± 0.4	38.5 ± 0.4	38.5 ± 0.4	38.4 ± 0.4	38.3 ± 0.4	38.3 ± 0.3

[a] Eight rats per dose were used.

Table 36. General behavior of OF-80E-treated mice

Response[a]	Dose (mg/kg) at the time (min)											
	0						50					
	0	30	60	120	240	360	0	30	60	120	240	360
1. Catalepsy	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
2. Traction	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
3. Tremors	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
4. Convulsion	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
5. Exophthalmos	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
6. Piloerection	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
7. Salivation	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
8. Lacrimation	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
9. Diarrhea	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
10. Skin color	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
11. Pinna reflex	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
12. Righting reflex	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
13. Tail elevation	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14. Palpebral closure	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15. Abdominal tone	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
16. Locomotion	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
17. Respiration rate	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
18. Death	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8

[a] For parameters 1~11 and 18, data reported as: number of animals with the sign / number of animals examined. For parameters 12~17, data reported as the mean score.

Table 36. (continued)

Response[a]	Dose (mg/kg) at the time (min)											
	500						5000					
	0	30	60	120	240	360	0	30	60	120	240	360
1. Catalepsy	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
2. Traction	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
3. Tremors	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
4. Convulsion	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
5. Exophthalmos	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
6. Piloerection	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
7. Salivation	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
8. Lacrimation	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
9. Diarrhea	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
10. Skin color	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
11. Pinna reflex	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
12. Righting reflex	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
13. Tail elevation	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
14. Palpebral closure	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
15. Abdominal tone	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
16. Locomotion	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
17. Respiration rate	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0	4.0
18. Death	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8

[a] For parameters 1~11 and 18, data reported as: number of animals with the sign / number of animals examined. For parameters 12~17, data reported as the mean score.

3.8. Effect of OF-80E on the respiratory rate and tidal volume in Sprague Dawley rats

The effects of OF-80E on the respiratory system following a single oral administration in SD rats were elucidated using whole body plethysmography. As a result of clinical signs, there were chromaturia observed at all rats in 5000 mg/kg group, from 3 to 6 h after administration. The colored urine was examined to be a change in the excretion of the test substance or its metabolite in the urinary tract in relation to the above-described single- and repeated-dose toxicity studies. Thus, it was considered as a toxicologically meaningless change. At the 5000 mg/kg/day group in above-described repeated toxicity study, compound-colored stool and red color urine were observed. However, abnormal changes in respiratory rate and volume were not observed in all animals in the 5000 mg/kg group in which pigmented urine was observed, therefore it was considered that this change did not effect on the respiratory system.

In respiration rate, there were no statistically significant changes in all test substance treatment groups compared to the vehicle control group (Table 37). In tidal volume, the mean respiratory rate was statistically significant higher in the 5000 mg/kg group at 30 min after administration compared to the vehicle control group, and significantly higher in the 50 mg/kg group at 360 min (Table 38). In the group administered 500 and 5000 mg/kg at 240 min after administration, it was significantly lower than that of the vehicle control group. Above-mentioned changes did not show a dose-response correlation and consistency over time. Therefore, the above changes were considered to be due to individual differences as changes not related to the test substance. In minute volume, there were no statistically significant changes in all test substance treatment groups compared to the vehicle control group (Table 39). Under this experimental condition, OF-80E treatment at single oral administration of at below 5000 mg/kg did not induce adverse effects on the respiratory system of SD rats.

Table 37. Effect of OF-80E on the respiratory rate of rats

Dose[a]	Respiratory rate at the time (min), breaths/min (mean ± std)					
(mg/kg)	0	30	60	120	240	360
0	89 ± 10.9	90 ± 18.5	89 ± 3.8	94 ± 11.6	106 ± 26.7	108 ± 32.8
50	91 ± 9.0	91 ± 17.4	83 ± 8.2	86 ± 15.0	91 ± 13.4	98 ± 17.9
500	96 ± 10.7	102 ± 21.7	103 ± 29.7	89 ± 7.5	113 ± 36.7	99 ± 12.0
5000	99 ± 10.5	97 ± 25.0	89 ± 9.2	97 ± 15.3	96 ± 6.2	90 ± 14.7

[a] Eight rats per dose were used.

Table 38. Effect of OF-80E on the tidal volume of rats

Dose[a]	Tidal volume at the time (min), mL (mean ± std)					
(mg/kg)	0	30	60	120	240	360
0	1.3 ± 0.10	1.4 ± 0.16	1.3 ± 0.10	1.3 ± 0.12	1.4 ± 0.11	1.4 ± 0.09
50	1.4 ± 0.08	1.4 ± 0.15	1.3 ± 0.10	1.3 ± 0.15	1.4 ± 0.07	1.5 ± 0.10*
500	1.4 ± 0.11	1.3 ± 0.11	1.3 ± 0.17	1.3 ± 0.08	1.3 ± 0.12**	1.4 ± 0.12
5000	1.3 ± 0.07	1.5 ± 0.11*	1.4 ± 0.13	1.3 ± 0.10	1.3 ± 0.12**	1.4 ± 0.10

[a] Eight rats per dose were used.

*/** : Represent significant difference at $P < 0.05$ / $P < 0.01$ level compared with vehicle control group.

Table 39. Effect of OF-80E on the minute volume of rats

Dose[a]	Minute volume at the time (min), mL/min (mean ± std)					
(mg/kg)	0	30	60	120	240	360
0	115 ± 10.6	115 ± 24.0	111 ± 9.0	118 ± 17.0	143 ± 32.9	138 ± 29.1
50	123 ± 13.9	117 ± 22.4	107 ± 8.8	107 ± 16.4	120 ± 15.3	141 ± 22.7
500	128 ± 16.1	123 ± 27.9	127 ± 23.1	114 ± 10.8	133 ± 32.7	134 ± 19.8
5000	123 ± 11.9	142 ± 24.2	118 ± 12.4	126 ± 17.1	117 ± 13.8	123 ± 18.9

[a] Eight rats per dose were used.

3.9. Effect of OF-80E on human ether-a-go-go-related gene potassium channel expressed in Chinese hamster ovary cells

The effects of OF-80E on the cardiovascular system by measuring a hERG potassium channel expressed in CHO hERG cells were evaluated using whole-cell patch-clamp technique (Figure 24). After stabilizing the hERG channel current, the run-down rate was recorded while flowing Tyrode's solution (vehicle control) containing 0.3% DMSO and OF-80E for about 5 min or more. Treatment with OF-80E (0.5, 5, 50, and 500 μg/mL) showed 0.4, −2.1, 0.9, and 0.9% inhibition in the hERG channel current, respectively (n = 3). At 5 μg/mL treated group, the inhibition was statistically significant compare to vehicle control group. Compared with the vehicle control data (−2.7~5.5%), −2.1% inhibition (at 5 μg/mL treated group) was not considered a drug related response because the −2.1% inhibition was within the range of influence of vehicle control group. When perfused with the positive control, E-4031 (0.1 μM), under the same condition, hERG channel current was inhibited by 75.3% compare to the vehicle control group. It was confirmed that the cells used in this experiment were suitable for measurement. Therefore, OF-80E did not effect on the hERG channel up to the concentration of 500 μg/mL, and the IC_{50} value appeared to be more than 500 μg/mL.

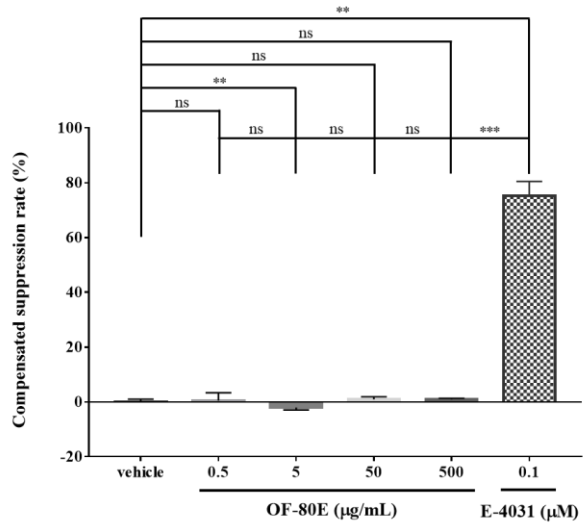


Figure 24. Effects of OF-80E on cloned human ether-a-go-go-related gene channel expressed in Chinese hamster ovary cells. CHO-hERG-Duo cells were used for evaluation of hERG channel. The cells were treated with medium containing the OF-80E (0.5, 5, 50, and 500 μg/mL), the positive control (0.1 μM E-4031), and vehicle control (0.1% distilled water). hERG channel currents were observed and measured using electrophysiological measurement method, as described in the Materials and Methods section. Each bar represents the mean ± SEM of triplicate samples ($^{**}P < 0.01$; ns, no significant difference, using Dunnett's test).

V. Discussion

Worldwide, the genus *Opuntia* is widely distributed throughout Mexico, Latin America, South Africa, Mediterranean countries, and Asia (also called nopal cactus). The cactus fruit has been used both as edible fruit (prickly pear) and folk medicine in Mexico, Latin America, and Korea. The plant species is regarded to be one of the many fruits rich in antioxidant components, and many of its functional effects were related to the antioxidants that it contains. The cactus plant fruit has been explored extensively for its phytochemical attributes. The cactus plant fruits have antidiabetic, antidiuretic, antihypercholesterolemic, antihyperlipidemic, antitumor, antistress, anti-inflammatory, hepatoprotective, and neuroprotective activity [152]. Cactus plant fruits contains various phytochemicals, such as flavonoids (e.g., myricetin and quercetin), phenolic acid (e.g., ferulic acid and syringic acid), betalain (e.g., orientin and vitexin), betaxanthin (e.g., muscaaurin and betanin) and volatile oil (e.g., linalool and terpinene) [213-215]. In this study, *O. ficus-indica* fruit 80% ethanol extracts and their butanol-soluble fractions exhibited good gastro-protective activity (IC_{50}, below 0.4 and 2.1 μg/mL). *Opuntia* species are also well known for its gastro-protective activities as demonstrated in various experimental model [158].

Generally, the biological activities of major phytochemical groups in *Opuntia* species plant tissues are closely linked with structure of compounds within the groups, working singly or synergistically within extracts. These phytochemicals are secondary metabolites whose structure consist of one or more hydroxyl groups linked to aromatic rings [216]. Most phenolic/bioactive molecules belong to tannins, stilbenes, phenolic acids, coumarins, quinones, flavonoids/flavonoid glycosides, and lignans. In this study, aspirin-induced cytotoxicity assay to identify the antiulcer constituents from the *O. ficus-indica* fruit extract was used. The active constituents were

determined to be the aromadendrin and narcissin from the fruits. The interpretations of the proton and carbon signals of compounds **1** and **2** were largely consistent with the findings of Jeon *et al.* [217] and Lee *et al.,* [10] respectively. The IC_{50} values of these constituents were below 0.5 μM. This original finding indicates that materials derived from *O. ficus-indica* fruit can hold promise for the development of novel, effective, naturally occurring antiulcer agent. Aromadendrin has been reported to possess antidiabetic, antitumor, anti-inflammatory, radical scavenging, and osteogenic activities [218]. Narcissin has been reported to possess antidiabetic, antihypertensive, antioxidant, anti-inflammatory, and hepatoprotective activities [219].

Quantitative structure-activity relationship (QSAR) analyses of phytochemicals of antioxidant activity are common. Not only can QSAR information contribute to the search for additional compounds with higher activities but it can also promote greater understanding of the mechanism of antioxidant of phytochemicals, as described by Amic *et al.* [220]. The *o*-dihydroxy (3', 4'-diOH, i.e., catechol) structure, which confers high stability to the flavonoid phenoxyl radicals *via* hydrogen bonding or by expanded electron delocalization. The C2-C3 double bond (in conjugation with the 4-oxo group), which determines the coplanarity of the heteroring and participates in radical stabilization *via* electron delocalization over all three-ring system. The presence of both 3-OH and 5-OH groups for the maximal radical scavenging capacity and the strongest radical absorption. In the absence of *o*-dihydroxy structure, the presence of both 5-OH and 7-OH are able to compensate and become a larger determinant of flavonoid antiradical activity. In this study, aromadendrin, 3-*O*-methyl quercetin, and narcissin were most effective compounds in the inhibition of aspirin-induced apoptosis. Moderate activity was observed with taxifolin. All tested compound has both 5-OH and 7-OH groups. Aromadendrin has both 3-OH and 5-OH groups. 3-*O*-methyl quercetin and narcissin have *o*-dihydroxy structure and C2-C3 double bond. Taxifolin has *o*-dihydroxy structure and 3-OH, and 5-OH groups.

At dose of 50, 100, 150, and 200 mg/kg, OF-80E significantly inhibited gastric hemorrhagic lesions compare to ethanol/HCl-treated group. Mucosta® tablet (100 mg/kg) and Stillen® tablet (60 mg/kg) also inhibited gastric hemorrhagic lesions compare to ethanol/HCl-treated group. Due to rapid ulcer induction and reproducibility, acidified ethanol (150 mM HCl in 60% ethanol, p.o.) induced gastric ulcer model has been used a common and reliable model for the pathogenesis of gastritis and gastric ulcer [221]. Ethanol is a noxious factor that can negatively influence gastric mucosa. Ethanol can solubilize the gastric wall mucus and penetrate the gastric mucosa rapidly. Therefore, the gastric mucosa can be easily damaged by aggressive factors like HCl and pepsin. Furthermore, Acidified ethanol induces necrotic lesions directly by reducing defensive factors, inducing bicarbonate secretions, and mucus productions [222]. These results suggest that OF-80E has gastro-protective effects against ethanol induced gastric mucosal injury.

NSAIDs, that are widely used to treat pain and inflammation, are involved in gastric epithelial cells damage and promote gastric mucosal damage by decreasing endogenous prostaglandins by systemic COX activity inhibition [223]. Since prostaglandins are important gastro-protective factors that are involved in the decrease of acid secretion, regulation of mucosal blood flow, mucus production and secretion, the use of NSAIDs cause gastric lesions in animals and humans [224]. Treatment with OF-80E significantly reduced indomethacin-, aspirin- or diclofenac-induced mucosal hemorrhagic lesions in rats. Particularly, it is well known that PGE_2 protects the gastric mucosa against various aggressive factors, such as ethanol, stress, and NSAIDs. These results suggest that OF-80E incurs gastro-protective effects in NSAIDs induced gastric mucosal injury.

Physical and psychological stresses can play a major risk factor for the occurrence of gastric ulceration. The WIRS model has been reported to mimic the clinical acute gastric ulcers due to trauma, surgery, or sepsis. It has been widely used in stress-induced gastric ulcer studies [209].

WIRS reduces the synthesis of PGE_2 in gastric mucosa and induces the production of reactive oxygen species [225]. These changes lead to a reduction of mucosal blood flow and mucus secretion in the gastric mucosa resulting in the gastric lesions [221]. In this study, treatment with OF-80E significantly decreased gastric hemorrhagic lesions that were induced WIRS. These results suggest that OF-80E has gastro-protective effects against stress induced gastric ulcer.

Histologically, an ulcer consists of two major structures; a distinct ulcer margin formed by the adjacent non-necrotic mucosa, the epithelial component and the granulation tissue at the ulcer base, the connective tissue component. The latter consists of fibroblasts, macrophages, and proliferating endothelial cells forming microvessels. Ulcer healing is a complex process, which involves cell migration, proliferation, re-epithelialization, angiogenesis, and matrix deposition, all ultimately leading to scar formation [226]. In this study, treatment with OF-80E significantly reduced ethanol-, NSAIDs-, or stress-induced gastric tissue damage in rats.

An investigation of the mechanisms of action and gastro-protective mechanisms of botanical drugs can provide useful information for the development of biorational gastric ulcer control alternatives with lower toxicities, as well as for determining the most appropriate formulations and delivery means to be adopted for their future commercialization. Intercellular GSH is an important factor contributing to gastric mucosal protection against aspirin-induced damage [206]. In this study, OF-80E and betanin significantly increased GSH level reduced by aspirin. Prostaglandin E_2 is involved in the synthesis of mucus and bicarbonate, and in the regulation of acid secretion and gastric mucosal blood flow [207]. OF-80E and betanin significantly increased PGE_2 level reduced by aspirin.

In the experiments of NSAID- and stress-induced gastritis rats, disturbance of gastric mucosal microcirculation is implicated in gastric lesions, which is followed by impaired gastric mucus synthesis and secretion. In this study, significant decrease of adherent mucus was observed

after 6 h of NSAIDs treatment or 6 h of WIRS. Decreased adherent mucus was restored by pretreatment of OF-80E or betanin. Since gastric mucus play a critical role in the primary defense of the gastric mucosa and provides a protective barrier in the gastric epithelium [227], these results suggested gastro-protective activity of OF-80E and betanin through preservation of gastric mucus synthesis and secretion.

MPO is an enzyme found primarily in the azurophilic granules of neutrophils and is used as a marker for quantification of neutrophil sequestration in tissue and the severity of inflammation. This enzyme also has enormous potential to inflict tissue damage through its ability to catalyze the production of a complex array of reactive oxidants [228]. Previously, betanin exhibited significant inhibitory effects on human MPO activity [228]. In this study, OF-80E effectively inhibited NSAIDs-induced MPO activity in the experimental condition. Also, betanin effectively inhibited stress-stimulated MPO activity, which implies the possible contribution of betanin to the action of OF-80E through its antioxidative and anti-inflammatory properties.

Increased in plasma TNF-α level after several hours of stress in rats has been observed in the study. TNF-α, a major proinflammatory cytokine, plays an important role in the development of acute inflammation, mediated by neutrophil infiltration of gastric mucosa [229]. In the Kwiencien's study, pretreatment with pentoxifilline, an inhibitor of TNF-α activity, resulted in a significant reduction in gastric lesions. The decreased gastric lesion by nitric oxide-releasing aspirin was accompanied by decreases in plasma TNF-α [230]. Although, it is not certain whether TNF-α is a cause or a result of gastro-protection, these studies suggest the involvement of TNF-α mediated inflammatory cascade in NSAID- or stress-induced gastric lesion. In this study, betanin reduced TNF-α in gastric mucosal.

In an attempt to find and effective treatment for various disease, the modern medicine turns to traditional medicine. There are decades of using specific plants or certain plant parts in the

treatment of several health conditions throughout the world. Many side effects because of using conventional antiulcer drugs has shifted the search for new drugs to folk and traditional medicines. Indeed, there is an effort in pharmacology to confirm the real benefits of traditionally used plants in antiulcer therapy and to identify active compounds responsible for their positive effects. There are few animal models developed in ulcers research, usually including rats. The growing interest in herbal medicines in partly derived from the results of many animal studies, indicating that plant extracts have lower toxicity than synthetic drugs [231]. However, for using specific plants extracts as antiulcer agents, the data of acute and chronic toxicity should be obtained. Acute toxicity studies, also known as single dose studies and chronic (or sub-acute) studies, also named repeat dose studies, are of crucial importance in case of testing extracts from plants which include high number of difference substances. There are few recommendations for toxicity studies and how to be performed, being one of them described by OECD [232]. During the examination of plants biological activities, the first step that should be provided is the detection of acute toxicity. In an acute toxicity study in SD rats, single dose limit is 2000 mg/kg or 5000 mg/kg BW and if the substance is not lethal on acute administration of 5000 mg/kg BW, according to toxicologists through the world, that substance is essentially considered non-toxic. Indeed, the benefits of plant products as antiulcer agents due to their low toxicity are clearly evident, particularly in cases where a single dose of 10000 mg/kg body weight do not evoke a change, as was observed in rat treated with OF-80E.

The subchronic toxicity test is used to assess the long-term safety of exogenous substance [232]. In this study, 13-week repeated oral dose toxicity study in SD rats, the effects of OF-80E were observed in general symptoms and urinalysis under the test condition. Red urine and compound-colored stool observed on the day of administration in male and female with 500 mg/kg or more, were like the color of the test substance (reddish brown). It was considered that

some of the components of the test substance were excreted into urine or feces through the urinary or digestive system. Above changes were not observed after the recovery period and accompanied by changes in related indicators of hematological and histopathological tests, thus they were considered a non-toxicological change. Results of this study clearly supported that administration of OF-80E did not exert adverse effect in male and female rats for most toxicological factors. Subchronic NOAEL of OF-80E was considered 2000 mg/kg for both male and female rats. In this study, when the OF-80E was repeatedly orally administered (300, 500, 1000, and 1500 mg/kg/day) to a beagle dog for 4 weeks, toxicological significant change was not observed due to the test substance. MTD was considered as 1500 mg/kg/day for both male and female beagle dogs.

Genotoxicity tests have been used mainly for the prediction of genotoxicity and carcinogenicity of chemicals because compounds that are positive in these tests have carcinogenic and/or mutagenic potential in humans [233]. However, most used herbal formulas have no indications of quality, safety, and efficacy. It was performed that tests to detect chromosome aberrations in CHL cells, a bacterial reverse mutation test using the *S. typhimurium* / *E. coli* incorporation assay (Ames test), and an *in vivo* micronucleus test. The Ames test uses amino acid-requiring strains of *S. typhimurium* and *E. coli* to detect point mutations involving substitution, addition, or deletions of one or more DNA base pairs [201]. In this study, there was not found positive mutagenic responses to OF-80E in any of the test strains compared with the concurrent vehicle control groups both with- and without-application of the metabolic activation system. This is a widely accepted short-term assay to identify substances that can produce genetic damage leading to gene mutations [234]. Chromosome aberrations are the classical genotoxic response to tumor initiation and development process. The purpose of the *in vitro* chromosome aberration test is to identify substances that cause structural chromosome aberrations in cultured mammalian cells [202]. CHL cells were selected as the test system because they are sensitive to mutagens,

their low chromosome number facilitates scoring, and they can be used for repeated measurements. In this study, the chromosome aberration assay demonstrated clearly that there were no significant increases in the number of metaphases with structural aberrations at any dose of OF-80E in the presence or absence of the metabolic activation system in CHL cells. The micronucleus test detects mutagenic substances, thus altering the equitable distribution of chromosomes during cell division [235]. In this study, the micronucleus assay in male mice showed no significant increases in the frequency of micronuclei at any dose of OF-80E (1250, 2500, and 5000 mg/kg/day) compared with the vehicle control group.

Safety pharmacology is a pharmacological method used to study drugs given at the recommended treatment doses, or higher doses, which may exert harmful pharmacological effects or adverse drug reactions [236]. Safety pharmacology research can provide information for clinical research and safety drug use, and can be used long-term toxicity testing as a reference for the design and development of new indications [237]. In this study, conducted according to safety pharmacology research technical guidelines of OECD, tested effects of OF-80E on critical body functions, such as central nervous, respiratory, and cardiovascular system activities. Based on the results of this study, OF-80E appeared to have exerted no adverse effects on the central nervous, respiratory, and cardiovascular systems.

In conclusion, OF-80E (*O. ficus indica* fruit 80% ethanol extracts) has an excellent efficacy and safety profile. Therefore, if it will be confirmed efficacy and safety by clinical study, OF-80E is a highly attractive option for the treatment of erosive gastritis, in which a balance between aggressive and defensive factors plays a significant role. Additional detailed tests are needed to understand how best to improve the antiulcer potency and stability of OF-80E for eventual commercial development.

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Abstract in Korean

백년초(*Opuntia ficus-indica*) 열매 유래물질의 항궤양 효과, 독성 및 작용 기전

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위염은 주로 짠 음식과 매운 음식을 주로 먹는 한국 성인들에게 흔한 질병이다. 일반적으로 증상에는 상복부 통증, 메스꺼움, 구토, 복통, 소화 불량 및 복부 팽만감이 있다. 위염은 병태생리학적으로 위 공격 인자 (산, 펩신, 헬리코박터 파일로리)와 위 점막 방어 인자 (위 점액, 중탄산염 분비, 프로스타글란딘 및 점막 세포의 선천적인 저항성) 사이의 불균형으로 발병한다. 위궤양 치료제로 여러 약물이 사용되고 있다. 그러나 이러한 치료제에는 부작용이 있다. 부작용이 적은 새로운 위염 치료제 개발이 절실히 요구되고 있다. 식물은 잠재적인 치료제를 가지고 있을 것으로 생각되어 약용 식물에서 새로운 화합물을 찾기 위한 노력이 계속되고 있다.

Opuntia ficus-indica (선인장과) (백년초)는 많은 국가에서 전통 의약품으로 사용되고 있다. 대한민국의 제주도에서는 건강 식품 제조 용도로 널리 재배되고 있다. 본 연구는 백년초 열매 추출물을 위염 치료 천연물의약품으로 개발하기 위해 수행

되었다. OF-80E (백년초 열매 80% 에탄올 추출물)의 항궤양 활성은 에탄올, 비스테로이드성 항염증제 (인도메타신, 아스피린 및 디클로페낙) 및 스트레스 유발 위염 랫트 모델을 사용하여 평가하였다. 그 결과는, 시판된 약물인 스티렌정 (Stillen® tablet) 및 뮤코스타정 (Mucosta® tablet)과 비교하였다. 또한, OF-80E의 급성독성, 아만성독성, 유전독성 및 안전성 약리 연구는 인간의 안전한 섭취를 위해 경제협력 개발기구 지침 및 우수실험실관리 규정에 따라 평가하였다. 마지막으로, 위염 모델에서 OF-80E의 항궤양작용의 가능한 메커니즘은 생화학 및 분자 분석을 사용하여 설명하였다.

AGS 세포를 이용한 아스피린 유도 세포독성 억제 시험을 기반으로 성분 분리를 통하여 백년초 열매로부터 분리하고, 전자이온화 질량분석법과 핵자기 공명 분광법을 포함한 분광 분석을 통해 두 종의 활성 화합물을 동정하였다. 두 종의 항궤양 성분은 플라보노이드인 aromadendrin과 narcissin으로 확인되었다. IC_{50} 값으로 보면, flavone인 aromadendrin (<0.5 μM)과 flavonol인 narcissin (<0.5 μM)은 다른 flavones인 naringenin (5.9 μM), eriodictyol (>10 μM), taxifolin (1.1 μM) 및 다른 flavonols인 kaempferol, quercetin 및 isokaempfride (>10 μM) 보다 AGS 세포를 이용한 아스피린 유도 세포독성 억제 효과가 우수하였다.

OF-80E는 시판된 약물보다 공격인자에 대해서 위 점막 손상을 보호하는 효과가 우수하였다. 에탄올, 비스테로이드성 항염증제 및 스트레스 유발 위염 랫트 모델에서, OF-80E는 시판된 약물에 비해 효과적으로 위 출혈성 병변과 조직학적 조직 손상을 억제하였다.

단회 경구투여 독성연구에서 OF-80E의 개략적인 치사량은 SD 랫트의 암, 수 모두에서 10000 mg/kg 이상으로 확인되었다. 13주 반복 경구투여 독성연구에서 OF-

80E의 무독성량은 SD 랫트의 암, 수 모두에서 2000 mg/kg/day로 확인되었다. 4주 반복 경구투여 독성 연구에서 OF-80E의 최대내성용량은 비글견 암, 수 모두에서 1500 mg/kg/day로 확인되었다. *Salmonella typhimurium*과 *Escherichia coli*를 이용한 복귀돌연변이 시험에서 OF-80E는 돌연변이를 유발하지 않았다. Chinese hamster lung 세포를 이용한 염색체 이상시험에서 OF-80E는 염색체 이상을 유발하지 않았다. 소핵시험에서 OF-80E는 동물 골수세포에서 소핵을 유발하지 않았다. OF-80E를 5000 mg/kg 이하로 설치류에 단회 경구 투여했을 때, ICR 마우스의 중추신경계에 영향을 미치지 않았고, SD 랫트의 호흡기계에 영향을 미치지 않았다. OF-80E는 500 μg/mL 농도까지 human ether-a-go-go related gene 채널에 영향을 미치지 않는 것으로 보아, OF-80E는 심혈관계에 미치는 영향이 낮을 것으로 확인되었다.

OF-80E는 AGS 세포에서 아스피린에 의해 감소되고, 랫트에서 인도메타신에 의해 감소한 glutathione을 증가시켰다. OF-80E는 AGS 세포에서 아스피린에 의해 감소한 prostaglandin E_2의 농도를 증가시켰다. 인도메타신 유도 위염 랫트에서 감소된 부착성 위점액은 OF-80E의 처리에 의해 합성되고 분비되었다. OF-80E는 인도메타신 유도 랫트의 위 점막에서 myeloperoxidase의 활성을 억제하고, 스트레스 유도 위염 랫트의 위 점막에서 tumor necrosis factor-α를 감소시켰다.

검색어: 위염, 백년초(*Opuntia ficus-indica*) 열매, 위보호 효과, 경구투여 독성연구, 유전독성, 안전성 약리

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