GASTROPROTECTIVE AND ANTIULCER ACTIVITIES OF POMELO (*CITRUS MAXIMA*) LEAF EXTRACTS

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GASTROPROTECTIVE AND ANTIULCER ACTIVITIES OF POMELO (*CITRUS MAXIMA*) LEAF EXTRACTS

by

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
ADI	Acceptable daily intake
AG	Acacia gum
AMDI	Advanced Medical and Dental Institute
ANOVA	Analysis of Variance
AP	*Aframomum pruinosum*
BD	*Baccharis dracunculifolia*
BP	*Bryophyllum pinnatum*
BT	*Baccharis trimera*
CA	Calcium Ascorbate
CB	*Cibotium barometz*
CCE	Counter-current extraction
CR	*Cyperus rotundus*
DL	*Desolvation line*
DPPH	2,2-diphenyl-1-picrylhydrazyl
DW	Distilled water
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionization
FDA	Food and Drug Administration
GRAS	Generally Recognized as Safe
HCl	Hydrochloric Acid
HG	*Hieracium gymnocephalum*
HPLC	High Performance Liquid Chromatography
HPMC	Hydroxypropyl methylcellulose
HQC	High quality control
HSD	Honest significant difference
HSV	Herpes simplex virus
IACUC	Institutional Animal Care and Use Committee
JECFA	Joint Expert Committee on Food Additives
LCMS	Liquid Chromatography Mass Spectrometry
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry

LC-PDA-QTOF	Liquid Chromatography with Photodiode Array and Quadrupole Time-of Flight detection
LOD	Limit of detection
LOQ	Limit of quantification
LQC	Low quality control
MD	Maltodextrin
MM	*Myristica malabarica*
MQC	Medium quality control
NIST	National Institute of Standards and Technology
NSAID	Non-steroidal anti-inflammatory drugs
OECD	Organisation for Economic Co-operation and Development
PDA	Photodiode array
PT	Pectin
PUD	Peptic ulcer disease
PY	Process yield
QC	Quality control
RE	Relative percentage of error
RF	Rhoifolin
RH	Relative Humidity
ROS	Reactive oxygen species
RSD	Relative standard deviation
SC	*Solidago chilensis*
SPH	Seksyen Penyelidikan Haiwan
SSR	Solid-to-solvent Ratio
TP	*Talinum portulacifolium*
UAE	Ultrasound Assisted Extraction
USM	Universiti Sains Malaysia
UV-VIS	Ultraviolet visible spectroscopy
WHO	World Health Organization
ZP	Zeta Potential

LIST OF APPENDICES

AKTIVITI GASTROPERLINDUNGAN DAN ANTI-ULSER EKSTRAK DAUN LIMAU BALI (*CITRUS MAXIMA*)

ABSTRAK

Kajian ini meneroka potensi kesan gastroprotektif dan aktiviti antiulser ekstrak daun limau bali (*Citrus maxima*) terhadap gangguan gastrousus. Walaupun *Citrus maxima*digunakan secara tradisional dalam pelbagai aplikasi perubatan, bukti saintifik tentang kesan gastroprotektif ekstrak daun limau bali adalah terhad. Penyelidikan ini bertujuan untuk mengatasi kekurangan ini dengan meneliti keberkesanan daun *Citrus maxima*dalam mencegah dan merawat ulser gastrik. Objektif kajian termasuk membangunkan kaedah HPLC yang menunjukkan kestabilan untuk mengenal pasti sebatian penanda, mengoptimumkan proses pengekstrakan dan pengeringan, menilai konstituen fitokimia, dan menilai kesan gastroprotektif menggunakan model tikus *in vivo*. Khususnya, hasil kajian ini boleh menyumbang kepada pembangunan rawatan alternatif untuk gangguan gastrousus dan meningkatkan pemahaman kita tentang mekanisme di sebalik kesan gastroprotektif ekstrak daun limau bali. Kajian ini berjaya mengembangkan dan memvalidasi kaedah HPLC-UV yang menunjukkan kestabilan untuk penentuan kandungan rhoifolin secara tepat dalam ekstrak daun *Citrus maxima*. Apabila dibandingkan antara ekstrak daun muda dan tua, segar dan kering, didapati ekstrak daun kering tua adalah yang paling optimum untuk keberkesanan terapeutik gastroprotektif dari segi pengeluaran dan pengendalian bahan mentah. Dengan menggunakan daun ini untuk pembangunan seterusnya, rhoifolin digunakan sebagai biomarker untuk penstandardan dan pembangunan formulasi. Formula serbuk dan gel yang dibangunkan didapati mempunyai isu kestabilan yang telah diatasi dengan teknik formulasi. Kajian haiwan tentang keberkesanan ekstrak mentah dan formulasi yang

sepadan telah dilakuka dan didapati formulasi gel menunjukkan kesan gastroprotektif yang lebih berkesan. Secara keseluruhan, parameter pengekstrakan yang dioptimumkan menghasilkan ekstrak daun *Citrus maxima* yang diingini, memperlihatkan aktiviti antasid dan sifat antioksidan dan anti-radang yang signifikan. Analisis menunjukkan kehadiran flavonoid, triterpen, tanin, dan glikosida jantung dalam ekstrak, memberikan pandangan tentang kestabilan dan aplikasi potensinya. Penemuan ini juga menyokong keberkesanan ekstrak daun *Citrus maxima* sebagai agen antiulser dan penyembuh yang berkesan serta menunjukkan kesan perlindungan dan terapeutik yang ketara pada tisu gastrik.

GASTROPROTECTIVE AND ANTIULCER ACTIVITIES OF POMELO (*CITRUS MAXIMA*) LEAF EXTRACTS

ABSTRACT

This study explores the potential gastroprotective effects and antiulcer activity of pomelo (*Citrus maxima*) leaf extract against gastric disorders. Despite the traditional use of *Citrus maxima* in various medicinal applications, scientific evidence on the gastroprotective effects of pomelo leaf extract is limited. The research aims to fill this gap by investigating the effectiveness of *Citrus maxima* leaves in preventing and treating gastric ulcers. The study objectives include the development of a stability-indicating HPLC method for identifying marker compounds, optimizing the extraction, and drying process, assessing phytochemical constituents, and evaluating gastroprotective effects using an *in vivo* rat model. This study successfully developed and validated a stability-indicating HPLC- UV method for precise determination of Rhoifolin in *Citrus maxima* leaves extract. Among the tested leaves, which include young, old, fresh, and dried leaves, it was found that the most optimum leaves extract for gastroprotective therapeutics efficacy is dried old leaves as far as manufacturing and raw material handling are concerned. Using these leaves for subsequent development, the biomarker of Rhoifolin was used for standardization and formulation development. Both developed powder and gel formulas were found to possess stability issues which have been overcome with formulation techniques. An animal study on the effectiveness of the raw extract and its corresponding formulation was carried out. It was noted that gel formulation indicated a more effective gastroprotective effect. In conclusion, the optimized extraction parameters resulted in a desirable *Citrus maxima* leaf extract, showcasing antacid activity and significant antioxidant and anti-inflammatory

properties. Phytochemical screening detected flavonoids, triterpenes, tannins, and cardiac glycosides in the extract, providing insights into its stability and potential uses for treating gastric conditions. The findings also support the efficacy of *Citrus maxima* leaf extract as an effective antiulcer and healing agent, demonstrating notable protective and therapeutic effects on gastric tissues.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

The relationship between humans and nature has existed for thousands of years and has led to the discovery and utilization of various plants with medicinal properties for the treatment and cure of numerous ailments. As noted by (Builders, 2019), the use of herbal medicine is the oldest form of healthcare known to humankind and has been used in all cultures throughout history. The World Health Organization (WHO) has reported that herbal medicine is still the mainstay therapeutic option for about 70-80% of the world's population due to the easy availability of these remedies (Organization, 2013). Gastrointestinal disorders, including gastric ulcers, are a growing concern and a major contributor to the global burden of diseases (Vijaylakshmi & Radha, 2015). Despite the availability of conventional medical treatments, gastrointestinal disorders continue to increase (Manousi, Sarakatsianos, & Samanidou, 2019). Thus, exploring alternative therapies is necessary because current options may not always be effective or may cause significant side effects. Alternative treatments could offer additional solutions and improve patient care.

1.1.1 Herbal medicine for gastrointestinal disorders

Plants and products derived from plants have long been utilized in folklore medicine all over the world. For many centuries, peptic ulcers, digestive diseases, and other conditions associated with these diseases have been treated with a variety of herbs and herbal remedies that have demonstrated anti-ulcer and gastroprotective benefits. Numerous herbal treatments recommended for ulcers and gastroprotection are shown in Table 1.1.

Table 1.1 Plant extracts with anti-ulcer or gastroprotective effects

Binomial name	Local name	Antiulcer and/or gastroprotective effects	References
Centella asiatica (CA)	Gotu Kola	CA strengthens the mucosal barrier and reduces free radicals that can cause stomach lesions.	(Cheng & Koo, 2000)
		CA extract prevents stomach ulcers induced by cold and restraint stress in rats.	(Chatterjee, Chakraborty, Pathak, & Sengupta, 1992)
		CA leaf extract (50 or 250 mg/kg) protected the stomach against ulcers caused by indomethacin in rats, possibly by reducing lipid peroxidation and increasing gastric mucus.	(Zheng et al., 2016)
		CA and its constituents, asiaticosides, act as anti-inflammatory agents that inhibit nitric oxide and promote ulcer healing.	(Guo, Cheng, & Koo, 2004)
Baccharis dracunculifolia (BD)	Alecrimdo-campo	BD is used as a treatment for ulcers, inflammation, and hepatic disorders.	(Cestari, Bastos, & Di Stasi, 2011)
		Essential oil from BD inhibited ethanol-induced ulcer formation.	(Klopell et al., 2007)
		In a study on rats with ulcer at colon area, 5 and 50 mg/kg of essential oil from BD reduced colon damage.	(Massignani et al., 2009)

Table 1.1 Continued

Binomial name	Local name	Antiulcer gastroprotective effects	Reference
Baccharis trimera (BT)	Carqueja	Treatment with BT in rats with ethanol-induced ulcers enhances antioxidant defenses, reduces oxidative damage, decreases inflammation and bleeding, and demonstrates remarkable gastroprotection.	(Rabelo et al., 2020)
		Ulcer-preventing or reversing effects of BT extract may be due to its impact on lipid peroxidation, flavonoids, and caffeoylquinic acid.	(Sabir et al., 2017)
		Essential oil of BT proved successful in halting and accelerating ulcer repair.	(Bueno et al., 2021)
Hieracium gymnocephalum (HG)	Hawkweeds	Chloroform extract of HG effectively treated induced acute ulcers in Wistar rats. Triterpene is believed to be the primary cause for the observed gastroprotective and anti-inflammatory actions.	(Petrović et al., 2008)
Solidago chilensis (SC)	Goldenrods	The methanolic extract of SC reduced both ethanol/HCl-induced gastric ulcers and indomethacin-induced gastric ulcers, while also displaying anti-secretory effects by decreasing gastric juice volume and acidity and increasing gastric pH.	(de Barros et al., 2016)
		SC leaf extract provides gastroprotection, including wound healing, antioxidant properties, anti-secretory effects, and increases mucus production, with no reported toxicity.	(Bucciarelli, Minetti, Milczakowskyg, & Skliar, 2010).

Table 1.1 Continued

Binomial name	Local name	Antiulcer gastroprotective effects	Reference
Aframomum pruinosum (AP)	Alligator pepper	Methanolic extract of AP has a mild anti-helicobacter effect, reduces ulceration in rats, and boosts stomach mucus and nitric oxide levels.	(Kouitcheu Mabeku, Nanfack Nana, Eyoum Bille, Tchuenteu Tchuenguem, & Nguepi, 2017)
Bryophyllum pinnatum (BP)	Panfuti	Aqueous extract of BP reduces ethanol-induced ulcers, decreases gastric volume and acidity, and prevents sub-mucosal layer swelling and white blood cell infiltration.	(A. L. Sharma, Bhot, & Chandra, 2014)
		Treatment of BP methanolic extract against indomethacin-induced gastric ulcers, significant ulcer inhibition was observed along with 100% mortality.	(Oladokun et al., 2007)
Cibotium barometz (CB)	Paku ayam mas	CB hair extract reduces ulcer size, balances gastric juice, and safeguards stomach wall mucus. The leaf extract of BP shows similar results as the hair extract.	(Al-Wajeeh, Hajerezaie, et al., 2017)
Cyperus rotundus (CR)	Nut grass	In Wistar rats, the methanol extract from CR decreased ulcers caused by aspirin. Additionally, it helped protect the stomach lining from oxidative harm by boosting antioxidant enzymes.	(Thomas et al., 2015)
		CR had significant anti-ulcer effects on pylorus ligation-induced ulcers, possibly due to inhibiting harmful free radical cascades and the release of oxidant radicals.	(Rajakrishnan et al., 2020)

Table 1.1 Continued

Binomial name	Local name	Antiulcer gastroprotective effects	Reference
Myristica malabarica (MM)	Malabar nutmeg	Malabaricone B isolated from MM balanced anti/pro-inflammatory cytokines, aiding in the healing of indomethacin-induced gastric ulcers by adjusting arginase and nitric oxide synthesis.	(Maity, Banerjee, Bandyopadhyay, & Chattopadhyay, 2009)
Talinum portulacifoliu m (TP)	Flame flower	Ethanolic extract of TP demonstrated anti-ulcer effects in three ulcer models (ethanol, pylorus ligated aspirin, and histamine), leading to increased stomach volume, improved gastric juice pH, reduced acidity, and improved ulcer severity.	(Gundamaraju, Maheedhar, & Hwi, 2014)
		Aqueous extract of TP reduced ulcer area and index in mice with various ulcer models (acetylsalicylic acid, HCl-ethanol, and cold restraint stress).	(Onwurah, Eke, & Anaga, 2013)

1.1.2 *Citrus maxima* Leaves

Citrus maxima (Pomelo) is a plant that is widely distributed in Southeast Asia and has been extensively used by people for the treatment of various ailments (L. B. Lim, Priyantha, Lu, & Zaidi, 2019). Pomelo trees often grow between 16 to 50 feet tall with big leaves size which are commonly between 2-12 cm wide and 15-23 cm long. The leaves are dark green with a glossy and slightly waxy texture surface. Other characteristics include acute apex, asymmetric base, and ambrosial smell (H. Xie, Zhang, & Pan, 2005). (Rajakrishnan et al., 2020) (Thomas et al., 2015)

In recent years, there has been growing scientific interest in the medicinal properties of pomelo leaves, and studies have been conducted to evaluate their efficacy and safety for various health applications. *Citrus maxima* leaf is believed to have several health advantages as traditional medicine in the Asian culture. It was found to exhibit gastroprotective effects which may be attributable to its anti-inflammatory activity (B Sapkota & Jain, 2021). According to (Tran et al., 2021) *Citrus maxima* leaves contain polyphenols and flavonoids that have potent antioxidant and anti-inflammatory properties. These secondary metabolites contribute to the gastroprotection activity by reducing gastric acid secretion and inhibiting the growth of *Helicobacter pylori* (B Sapkota & Jain, 2021). The methanolic extract of pomelo leaves has been shown to inhibit the formation of stomach ulcers and provide protection against ulcerogenic agents (Unanma, Anaduaka, Uchendu, Ononiwu, & Ogugua, 2021). *Citrus maxima* leaves have been employed to treat stomach pain caused by indigestion, epilepsy, and chorea in the traditional medicinal system of India. (Dinesh & Hegde, 2016). In a study involving rats with paracetamol-induced liver injury, it was determined that the leaf extract of *Citrus maxima* demonstrated a hepatoprotective effect (Abirami, Nagarani, & Siddhuraju, 2015). The antioxidant potential of methanolic *Citrus maxima* leaves

extracts can reduce reactive oxygen species (Baan *et al.*) that may lessen the oxidative damage to the hepatocytes and improve liver antioxidant enzyme activity (Feksa et al., 2018). A study investigated the anticancer properties of *Citrus maxima* leaf extract on mice that were treated with Ehrlich's ascites carcinoma cells. It was found that oral administration of the methanol *Citrus maxima* leaves extract at doses of 200 and 400mg/kg resulted in a significant decrease in tumor volume and viable tumor cell count, as well as an increase in hematological parameters and life span compared to the Ehrlich's ascites carcinoma control group. Flavonoids and limonin were identified as the compounds responsible for the anti-tumor and anti-inflammatory effects of the extract (KunduSen et al., 2011).

1.1.3 Chemical Constituents of *Citrus maxima* Leaves

Numerous phytochemicals including alkaloids and flavonoids have been reported from *Citrus maxima* leaves. Studies have reported that the presence of essential oil in *Citrus maxima* leaves is the main reason behind the flavor and aroma of leaves and they highly contribute to the medicinal properties (Prasad, Prasad, Prasad, Shetty, & Kumar, 2016; Saeb, Amin, Gooybari, & Aghel, 2016; Tsai et al., 2017). The essential oil extracted from *Citrus maxima* leaves contains a high percentage of limonene, α-phellandrene, β-ocimene, β-caryophyllene, nerol, citronellol, geraniol, geranial, and neral (Chi, Van Hung, Le Thanh, & Phi, 2020) with strong antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis,* and *Salmonella typhi* (Saeb et al., 2016). As one of the main constituents in essential oil from *Citrus maxima* leaves, limonene has been reported to contribute to its anti-ulcer activity and gastroprotection role by reducing the gastric ulcer area and increasing the mucus production and preserving the integrity of gastric mucosa (de Souza et al., 2019).

Another chemical constituent present in *Citrus maxima* leaves is flavonoids. According to (Panche, Diwan, & Chandra, 2016), flavonoids is a group of natural antioxidants present in plants and are famous for their beneficial effects on health. Flavonoids are chemical compounds that have a 15-carbon skeleton (C6-C3-C6) consisting of two aromatic carbon rings linked by a heterocyclic ring that contains embedded oxygen (Russo, 2018). The heterocyclic ring contains hydroxyl groups, which confer antioxidant activity to flavonoids (Heim, Tagliaferro & Bobilya, 2002). Flavonoids can be classified into subgroups based on their substitution patterns, such as flavanols, flavones, isoflavones, and anthocyanins (Karak, 2019). *Citrus maxima* leaves contain a substantial number of flavanones and anthocyanins, with the presence of common flavonoids such as naringenin and hesperidin suggesting a broad range of pharmacological activities, including anti-inflammatory, anti-cancer, anti-diarrheal, and anti-ulcer properties *(*Yunus, Putri, & Hafifah, 2018; Cordenonsi et al., 2017). Several outstanding reviews indicate that naringin, another flavonoid found in *Citrus maxima* leaves has anti-ulcer and gastroprotective effects on animals (Emin & Volkan, 2019; Galati et al., 1998; Martin, Marhuenda, Perez-Guerrero, & Franco, 1994).

Among the active phytochemical detected from *Citrus maxima* leaves, alkaloids have caught researcher's attention with their significance as therapeutic agent for medical and industrial uses due to the wide range of biological activities. A study shows that alkaloids are a class of natural products containing nitrogen, which possess significant anti-ulcer properties (Zhu, Liu, Chen, & Wang, 2012). In a study carried out by (Sharifi-Rad et al., 2018) on the anti-ulcer properties of 61 alkaloids in the gastric and duodenal regions, it was found that 55 of the tested alkaloids exhibited anti-ulcer effects, which is due to the superior solubility of alkaloids in acidic medium (stomach juice). Studies have proven that some alkaloids present in the *Citrus maxima* leaves such

as p-synephrine (M. D. Arbo et al., 2008) can reduce oxidative stress and inflammation in the gastrointestinal tract (Marcelo Dutra Arbo et al., 2009; Q. Wu et al., 2014).

Another chemical compound present in *Citrus maxima* leaves is phenolic compounds such as gallic acids and catechins which are powerful antioxidants and have potential anti-inflammatory, anti-cancer, and antiviral properties (Feksa et al., 2018; H.-Y. Huang et al., 2021).

1.1.4 Therapeutic Effects and Health Benefits of *Citrus maxima* Leaves

In recent years, there has been growing interest in investigating the potential health benefits and therapeutic effects of *Citrus maxima* leaves, as evidenced by numerous studies (Anmol et al., 2021; Das, Borah, & Ahmed, 2013; Jabamalairaj, Dorairaj, Yadav, & Bathrachalam, 2015). The above-mentioned findings about rich flavonoids and phenolic acids present in *Citrus maxima* leaves contribute to the potent antioxidant and anti-inflammatory properties (KunduSen et al., 2010; P. Singh et al., 2010). These antioxidants help protect the body from oxidative stress that occurs when there is an imbalance between ROS (Baan *et al*.) production and the body's antioxidant defense mechanisms (Adwas, Elsayed, Azab, & Quwaydir, 2019; Pamplona & Costantini, 2011). High vitamin content in *Citrus maxima* leaves, such as Vitamin C, as well as polyphenols and terpenoids enhances the immune system and helps fight against common respiratory illnesses like coughs, colds, and sore throats (Ding et al., 2022).

The anti-inflammatory properties found in *Citrus maxima* leaves are crucial in reducing inflammation within the body and alleviating symptoms associated with inflammatory conditions like eczema and arthritis (M.-H. Liu, Li, & Chen, 2021). Inflammation is a natural bodily response to injury or infection, but when it becomes chronic, it can lead to a host of diseases, such as cardiovascular disease, cancer, and neurodegenerative disorders (Furman et al., 2019). According to (Zhao et al., 2019) the

anti-inflammatory effects of *Citrus maxima* leaves have been studied by various scholars. Some studies have established that the compounds in pomelo leaves including flavonoids, terpenoids, and phenolic acids have anti-inflammatory properties that are essential in the reduction of inflammation in the body (Ishida, Takekuni, Nishi, & Sugahara, 2019; E.-J. Yang et al., 2009). One study conducted on acute and chronic inflammatory activities in rats where a dose of 300mg/kg extract from *Citrus maxima* leaves revealed significant anti-inflammatory activities (Rahman, Ali, Sharif, & Tajmim, 2017). *Citrus maxima* leaves have been found to have antimicrobial properties which help inhibit the growth of bacteria, viruses and other pathogens that may cause infection (Akther et al., 2021). Several studies have shown that pomelo leaf extract has antibacterial effects against a variety of pathogenic bacteria including *Pseudomonas aeruginosa*, *Staphylococcus aureus*, and *Escherichia coli* (Das et al., 2013; Biswash Sapkota, Devkota, & Poudel, 2022). Furthermore, *Citrus maxima* leaves are naturally rich in potassium, which helps to maintain normal blood pressure levels (Thamrin, Susanto, Susila, & Sutandi, 2014).

Citrus maxima leaves serve as an abundant source of vitamins from nature, which possess anti-inflammatory properties that help to reduce swelling in the bladder and improve women's health (Cox-Georgian, Ramadoss, Dona, & Basu, 2019). Additionally, *Citrus maxima* leaves with affluent antioxidant properties help to eliminate harmful free radicals and bacteria from the body, protecting healthy cells in the excretory system organs from oxidation. This ensures the proper elimination of waste and food residues from the body, preventing the buildup and accumulation in the system (Evans, Mendonca, & Soliman, 2022). Other studies discovered that pomelo leaf extract has antiviral effects against some viruses including the herpes simplex virus (HSV) and the human immunodeficiency virus (T. K. Lim, 2012). Reduction of immobility time in

forced swimming test and tail suspension test in mice demonstrate the noteworthy antidepression-like of *Citrus maxima* leaves extract (Potdar & Kibile, 2011).

1.1.5 Gastric Ulcers

Gastric diseases are a prevalent global issue affecting individuals of all ages. One common gastric disease is gastric ulcers, which are painful sores that develop in the stomach lining (Avinash, Abha, & Ganesh, 2011). Gastric ulcers refer to a type of acid-induced lesions in the gastrointestinal mucosa that may penetrate the muscular layer, usually occurs in the stomach, and is characterized by denuded mucosa with the defect extending into the muscular propria (Gadekar, Singour, Chaurasiya, Pawar, & Patil, 2010; Kuna et al., 2019).

Duodenal and gastric ulcer diseases are jointly referred to as peptic ulcer disease (Figure 1.1). Stomach ulcers tend to occur more frequently along the small curvature of the stomach, between the body and the antrum, while duodenal ulcers are more commonly found in the duodenal bulb or bulb (Kaufman & Levene, 1957; Malik, Gnanapandithan, & Singh, 2018; Oi et al., 1969). Peptic ulcer disease (PUD) affects around 10% of the global population and has a significant impact on both individuals and public health (X. Xie, Ren, Zhou, Dang, & Zhang, 2022). Men are more commonly affected by both gastric and duodenal ulcers, with younger individuals having a higher prevalence of duodenal ulcers and older adults being more likely to develop gastric ulcers (Groenen, Kuipers, Hansen, & Ouwendijk, 2009). Gastric ulcers develop as a result of a complex multifactorial process that is triggered by an imbalance between the aggressive and defensive components of the stomach mucosa. When the mucus layer that ordinarily protects the stomach from digestive fluids is thinner than usual, the tissues lining the stomach are more vulnerable to erosion by the digestive acids, which can result in an ulcer (Malik et al., 2018).

The human body has various natural mechanisms that help to shield stomach lining tissue from the harsh acidic environment in the stomach. Any disturbance to these protective measures may lead to the development of ulcers (Holton, 2013). Some of the known factors that harm the protective barriers include hydrochloric acid produced by the parietal cells in the stomach lining, lack of blood flow, low oxygen levels, and infection with the bacteria *Helicobacter pylori* (Søreide et al., 2015).

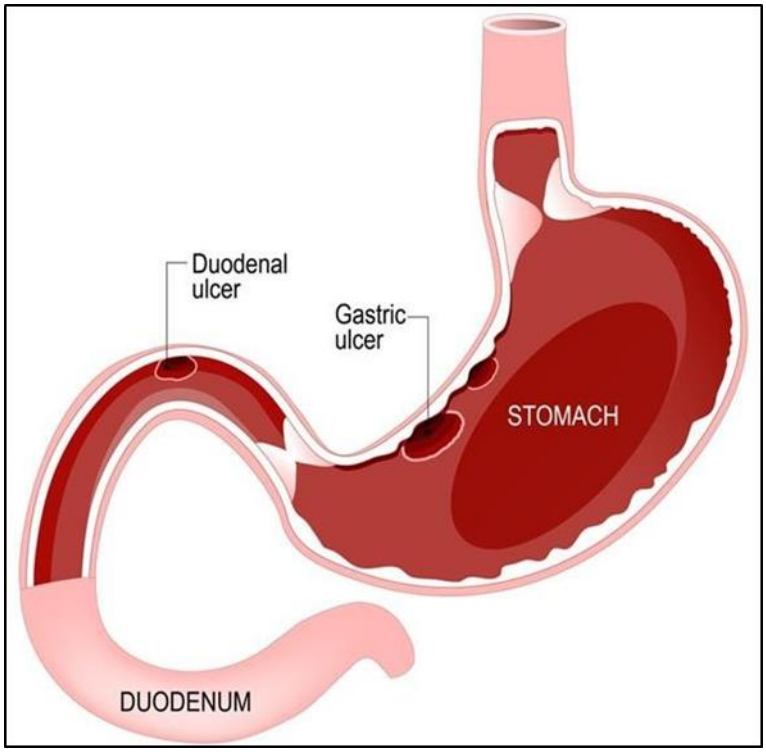

Figure 1.1 Duodenal and gastric ulcer (China Medical University Hospital, 2023)

The development of gastric ulcers may be attributed to several reasons. *Helicobacter* bacterial infection and gastric prostaglandin loss brought on by non-steroidal anti-inflammatory drugs (NSAIDs) are two of the most frequent causes of gastric ulcers (Milligan, Jain, Garrett, & Knutsen, 2012). Contrarily, NSAIDs and aspirin users have a four-fold and two-fold greater chance of developing peptic ulcers, respectively (Lanas et al., 2015). Other less frequent etiologies include viral infection and chemotherapy, gastric outlet obstruction, and hypergastrinemia which is important

to note that all the mentioned factors promote breakdown in the mucosal barriers and expose gastric mucosa to the damaging effects of acids (Venerito, Schneider, & Malfertheiner, 2016). Gastric ulcer carries a lifetime prevalence of 5 to 10% of patients, making it easy for many people to underestimate the disease given the fact that some patients may remain asymptomatic (Dionne, Vrins, Doucet, & Pare, 2003).

Numerous individuals afflicted by peptic ulcer disease experience discomfort, pain, or gastrointestinal distress, primarily characterized by epigastric pain. This pain is frequently described as a burning or gnawing sensation that typically occurs post-meal (Duggan & Duggan, 2006). Approximately 50-80% of individuals with a duodenal ulcer may experience pain at night, compared to 30-40% of those with gastric ulcer (Malfertheiner, Chan, & McColl, 2009). Pain that radiates to the back may indicate that the ulcer has penetrated deeply or may be related to pancreatic issues (Tana et al., 2017). Other symptoms may include dyspepsia (such as belching, bloating, and distension), heartburn, chest pain, hematemesis, and even anemia (Simadibrata, 2009).

In most cases, ulcers are treated through a prescription from a doctor with surgery requirements in very rare cases (Van Zanten, Dixon, & Lee, 1999). A variety of drugs including proton pump blockers (omeprazole and lansoprazole), prostaglandin analogs, histamine receptor antagonists, antacids, H_2 receptor inhibitors (ranitidine, famotidine), and cytoprotective agents are available for the treatment of peptic ulcer (Ariyphisi, 1986; Hoogerwerf & Pasricha, 2006; Wallace & Sharkey, 2011). Most of the drugs currently available in the market show limited efficacy against gastric diseases and are often associated with adverse effects (Oluwafisayo, Mlenzana, Shamila, Nesto, & Grace, 2014). Therefore, there is a rationale to develop a more effective and safer antiulcer agent. A study conducted on the activity of alkaloids on peptic ulcers established that natural products from plants and animals could be effective in regulating

the biological systems and may be used to treat gastric ulcers (do Nascimento et al., 2015).

1.2 Herbal Extraction

Extraction is a process of obtaining the active compounds and beneficial substances from a plant material. It has been used for centuries in many cultures around the world that serve as the first step to separate the desired natural products from the raw materials (Vidhu, 2020). Several methods, including maceration, ultrasonic-assisted extraction, expression, and distillation with selected solvents, are the most commonly used techniques for isolating and concentrating specific compounds from herbal materials (Savić, 2014). The choice of extraction method depends on the nature of the compounds to be extracted and the desired outcome; for example, maceration is ideal for heat-sensitive compounds, while ultrasonic-assisted extraction is effective for rapid processing (Abdullahi R Abubakar & Mainul Haque, 2020). Herbal extracts are commonly used in a variety of products, such as herbal supplements, medicinal products, cosmetics, and perfumes. Because they are frequently more concentrated than raw plant material, herbal extracts are prized for their medicinal and health benefits, making them a more effective source of the desired components (Espareh, Nejati, & Jafari, 2017). Herbal extraction should be carried out carefully and with consideration for the safety of the extract and the consumer. Using harmful solvents, such as benzene or chloroform, which are known for their high toxicity and potential health risks, can result in contaminated or ineffective extracts and pose serious health hazards (Vale & Sampayo, 2002).

1.2.1 Standardization of Herbal Medicine Using Marker Compounds

Standardization of herbal medication is the process of establishing a set of standards, including qualitative and quantitative values, for herbal medicine to ensure

their quality, efficacy, safety, and reproducibility (MJ, 2018). The fundamental condition of being safe and effective should be met by all medications, whether they are made synthetically or from plants (WHO, 1997). Standardization is important to guarantee the quality and therapeutic benefits of substances in each dose (Jain, 2019).

Due to lack of standardization and quality control in herbal products, herbal medicine has long been seen as an unreliable type of treatment, despite its widespread use (Ekor, 2014). A study has identified several factors, such as the technique employed for collecting, drying, storing, transporting, and processing herbal medicines that frequently impact their quality and safety (Raj, 2016).

The batch-to-batch consistency of herbal products can be impacted by a variety of factors, including geographical and environmental differences in cultivation conditions, in which the presence of marker compounds is useful to ensure the quality, safety, and efficacy of herbal medicine (Shi et al., 2014). A study conducted by (N. Yang et al., 2016) defined marker compounds as one or more constituents that chemically define herbal material and gain attention from researchers to serve as the reference/guide. Furthermore, it ensures that each batch of herbal product contains a consistent amount of active components in each unit dose (Kushwaha, Kushwaha, Maurya, & Awani, 2010). Marker compounds can help to ensure herbal medicines are produced consistently and reduce the risk of adverse reactions or health problems associated with inconsistent processing (Calixto, 2000).

However, there are some potential drawbacks to using marker compounds in the standardization of herbal medicine. Choosing the right marker compounds is difficult because researchers must identify a single compound from a complex mix of active chemicals. As a result, many herbal products are sold with limited information about their chemical ingredients (Rivera-Mondragón et al., 2017). Factors such as the

availability of analytical methods and standards, the importance of identifying the components in the extract, and the perceived therapeutic or health relevance must be considered when determining the most suitable marker compounds for a given herbal product (Ruiz et al., 2016).

i. *Qualitative identification:* Detection and characterization of unusual chemical entities in plants may be employed as a marker compound to establish. the identity of herbal raw materials or products, thus reduces the likelihood of mistakes during the collection, manufacturing, and packaging of herbal medicines.

ii. *Detection of adulteration:* To guarantee that known adulterants are not present in a batch of material, specific markers can be employed to check for their absence. On occasions, certain botanicals become adulterated with other plants; some of these adulterants are toxic and can lead to serious harm. Manufacturers can test for known components of the adulterating species (negative markers) to ensure the adulterant is absent to avoid this issue.

iii. *Indication of proper handling, packaging, and storage:* From pre-harvesting to product selling, markers are a helpful control for the transport and processing of botanical raw materials and finished goods. Each stage in this process can result in material degradation due to improper handling. Markers act as a check on these processes by giving a fair reflection of the material's past. If the anticipated markers are not present at the anticipated levels, it is possible that the manufacturing process could have gone wrong.

iv. *Control of pharmacological activity:* The presence of marker compounds in plants that are known to have pharmacological activity can help to assure that each batch of the product will have the same physiological impact.

v. *Evaluation of clinical trial data:* A marker compound or a set of markers is frequently used in the assessment of clinical trials. To extrapolates the trial's findings to other batches of the same product and other botanical items with a similar composition, it is critical that the material used in clinical trials are characterized thoroughly.

1.2.2 Selection of Appropriate Extraction Method

Extraction is the practice of utilizing solvents with specified processes to separate the biologically active components from plant or animal tissues. The result of an extraction process is usually a mix of crude and impure liquid, semi-solid, or powder that is meant for oral or topical use only (Mohammad Azmin et al., 2016). There are numerous ways to extract therapeutic herbs, and while some are evolving, others are being enhanced. It is crucial to select the optimum extraction method, and in many cases, this choice is determined by how the extract will be used (Fongang Fotsing Yannick, Bankeu Kezetas Jean, Gaber El-Saber, Iftikhar, & Lenta Ndjakou, 2021).

The preparation of the sample prior to the extraction of medicinal plants is also crucial, aspects like proper authentication and careful selection of the plant parts, the removal of any foreign matter, appropriate drying conditions, grinding of plant parts, utilizing suitable grinding equipment, and achieving the required particles size will directly influence the outcome of extract (Azwanida, 2015). Follow by is the selection of appropriate extraction method which is dependent on a variety of factors which is a crucial process for achieving the desired outcomes. When selecting the extraction technique, various criteria should be considered, including the fact that the chemical

composition of herbal medicines can differ significantly. Additionally, the polarity, volatility, and thermal stability of the target ingredient greatly influence the extraction process. Methods like decoction or infusion, which entail soaking the plant material in boiling water, may be more effective if the target compound is hydrophilic. Methods like solvent extraction or maceration, which involve the use of organic solvents, may be more efficient if the compound is more soluble in organic solvent (Sasidharan, Chen, Saravanan, Sundram, & Yoga Latha, 2011).

Also, care is given to the plant material's thermal stability, cold extraction e.g., maceration, percolation, and counter-current extraction (CCE) techniques could be used with thermolabile compounds. As opposed to this, two methods are preferred for organic solvents when working with heat-resistant compounds: the Soxhlet extraction method and the decoction method (Nandre, 2012). Optimized extraction time is another crucial factor that must be satisfied; otherwise, fewer active ingredients will be extracted if adequate time is not given for comprehensive extraction. In contrast, a prolonged extraction process may cause leaching out of unnecessary compounds. As an example, boiling tea leaves for an extended period may cause tannins to be extracted, which gives tea its bitter flavor (Sayyad, Randive, Jagtap, Chaudhari, & Panda, 2012).

Different extraction methods require different equipment, and the availability and cost of this equipment can influence which method is chosen. For instance, some extraction methods require expensive specialized equipment, such as rotary evaporators, soxhlet extractors, or supercritical fluid extractors. These methods can produce high-quality extracts, but they may not be practical for small-scale operations or those with limited resources. In contrast, simpler extraction methods, such as maceration or percolation, may require less specialized equipment and be more

accessible to a wider range of herbalists and manufacturers (Popović, Matić, Bojović, Stefanović, & Vidaković, 2016).

1.2.3 Maceration (Cold Aqueous Extraction)

Maceration (Cold Aqueous Extraction) is an old method used for herbal extraction that was also adopted in wine-making process. It requires combining a solvent with the raw herbal component and letting it rest for at least three days at room temperature. This method is regarded as a cost-effective means of obtaining natural thermolabile compounds from plant sources (J. Singh, 2008). Although being a convenient extraction technique, maceration has the drawbacks of a lengthy extraction process and poor extraction effectiveness (Zhang, Lin, & Ye, 2018). Maceration is composed of three basic steps. In the first step, plant material is griddled into powder form to produce small units with a wide surface area, increasing the solvent and solute's interaction to achieve the desired extraction effectiveness. In addition to allowing greater contact between the solvent and the solute, the small size and broken plant materials also enable the solvent to penetrate the deepest layers of cells for improved extraction results. A chosen solvent is put in a sealed vessel after the plant components have been grounded and allowed to soak in the solvent for at least 2-3 days at ambient temperature with occasional shaking for exhaustive extraction to be achieved. After a predetermined amount of time, the extract is filtered to remove the marc, and further filtration or decantation processes to obtain a clear liquid extract (Coşkun & Gülseren, 2020).

A vital influence is played by the solvent employed in the maceration process of soaking. Maceration is the most straightforward procedure employing straightforward tools. Skilled operators are not required and the process of extraction is energy saving.

Nonetheless, where a significant amount of solvent is employed, effective waste management is necessary (Hidayat & Wulandari, 2021).

According to an experimental study, maceration is a quick and efficient way to extract phenolic compounds. High yields of total phenols and total anthocyanins were obtained from chokeberry fruit under optimal conditions with 50% ethanol, a solid-solvent ratio of 1:20, and a particle size of 0.75 mm (Ćujić et al., 2016). In an experiment conducted to determine the effectiveness of maceration with different solvents, aqueous extraction of *Psidium guajava L.* leaves resulted in the most outstanding antioxidant ability and the highest concentration of phenolic compound as compared to pure ethanol and methanol extracts. However, the amount of phenolic compounds extracted using hydroethanolic solvent was higher than water, and 50% hydroethanolic was shown to be the most efficient solvent with the highest antioxidant capacity (Seo et al., 2014).

1.2.4 Decoction (Hot Aqueous Extraction)

Chinese herbal medicine is most frequently administered through decoction (Marshall, 2020). Decoctions are commonly consumed orally. Of all the conventional preparation methods, decoction has the quickest absorption rates and the most potent effects (Y. Yang & Ross, 2010). Decoction is a popular hot aqua extraction technique used to draw out soluble substances like flavorings and medicines from plant components (which may include stems, roots, backs, and rhizomes) that can withstand high heat (Azwanida, 2015; Grzegorczyk-Karolak & Kiss, 2018). The difference between the decoction technique and the maceration method lies in the length of time and temperature of the extraction process (H.-B. Li, Jiang, Wong, Cheng, & Chen, 2007). For tougher herbs like roots, barks, and seeds, decoctions are typically recommended. Before making the decoction, it is beneficial to ground or crush the whole root, bark, and seeds. When preparing a decoction, the required quantity of herbs and water is

heated to 100°C for about 30 minutes, or until roughly 50% of the water has evaporated (Bensky *et al.*, 2004). To avoid the evaporation of any vital components during heating, the vessel must be sealed. The extract is next taken off the heat and filtered through a filter; the decoction is then utilized either in its whole or after the proper dilution (Nagalingam, 2017). The ingredients should be pre-prepared before decoction to maximize solvent penetration into the solid matrix by providing wide surface area. This can be done by smashing, slicing, or cutting the materials into small pieces (Zhang et al., 2018). Decoction is more suitable for extracting plant components that are both thermostable and water-soluble (Manousi, Sarakatsianos, & Samanidou, 2019).

In traditional contexts, decoction was frequently used to treat a wide range of illnesses, including skin diseases, respiratory infections, and digestive issues. (Qasim et al., 2014). South Asians have traditionally extracted medicinal compounds from *Citrus maxima* leaves using decoction. The treatment of edema and ulcers is notably enhanced by using hot leaf's decoction (A. Singh & Navneet, 2017). Research indicates that a decoction of *Scutellaria baicalensis* roots, rich in compounds such as baicalin and wogonin, exhibits anti-inflammatory properties and may reduce the risk of cardiovascular disease (W.-H. Huang, Lee, & Yang, 2006; Yoon et al., 2009).

Although decoction has been used by many cultures throughout the world and has been passed down through the ages, there are still certain drawbacks, such as the fact that the extract from decoction contains a significant number of water-soluble contaminants. The extraction of volatile or thermolabile substances cannot be accomplished using decoction (Zhang et al., 2018). But despite that, decoction can provide liquids with more rich chemical contents than maceration because temperature or preparation differences may lead to more oil-soluble compounds in decoction (Gharby, 2022). A study manifested those 11 constituents—benzoylaconine, benzoylhypaconine,

benzoylmesaconine, berberine, coptisine, palmatine, jatrorrhizine, aloe-emodin and emodin, baicalin, and wogonoside—had significantly larger concentrations in the decoction than in the maceration (Zhang et al., 2018).

1.2.5 Advanced Extraction Techniques

1.2.5(a) Ultrasound-assisted Extraction (UAE) Method

Another technique for extracting bioactive substances from plant materials to manufacture herbal medicines is ultrasound-assisted extraction (UAE) method which involves ultrasonic wave energy in the extraction process, also known as ultrasonic extraction or sonication (Zhang et al., 2018). UAE has been used more frequently in recent years to extract polyphenols from various plant sources (Dahmoune et al., 2014; Qun et al., 2017; Shirzad, Niknam, Taheri, & Ebrahimzadeh, 2017). Ultrasound wave with a frequency range of 20 to 2000kHz is employed in the extraction process, these waves, which are made up of a series of compression and rarefaction cycles, can travel through solid, liquid, or gaseous media and cause the molecules to be displaced and knocked out of their initial locations. During rarefaction at high sound wave intensities, the attractive force that holds molecules together is outweighed by the negative pressure, which pulls the molecules apart and produces cavitation bubbles. The hot spot and intense local conditions are produced by these bubbles as they increase through coalescence and then collapse during the compression phase which creates high temperatures of up to 5000K and increases the pressure up to 1000 atm (Chemat et al., 2017). The propagation of ultrasonic pressure waves and subsequent cavitation processes have been linked to the augmentation of extraction by ultrasound. Increased mass transfer of extractants results from high shear stresses (Roselló-Soto et al., 2015; Šic Žlabur et al., 2015; Vilkhu, Mawson, Simons, & Bates, 2008; Zinoviadou et al., 2015). By causing cavitation in the cell walls, this increases the permeability of cell walls and

improves mass transit from crude drug to menstruum by increasing the sample's surface area for extraction solvent. (Oreopoulou, Tsimogiannis, & Oreopoulou, 2019).

Ultrasonic baths and probes, which rely on piezoelectric transducers as their power source for ultrasound, have been used in UAE procedures generally (Lavilla & endicho, 2017). An ultrasonic bath is built up by treating the solid matrix in the solvent in a stainless-steel tank attached to a transducer. Although more practical friendly and affordable, ultrasonic baths are limited in their application in the extraction process by their poor extraction rate. A transducer and a probe or horn make up an ultrasonic probe. With the least amount of energy loss, the probe provides ultrasound to the media while submerged in the extraction vessel. Due to their higher ultrasonic intensity, probe-based systems are frequently chosen over bath-based systems for the extraction of bioactive chemicals (Chemat et al., 2017).

According to research, compared to other extraction methods, the UAE offers a faster and better extraction of polyphenols with minimal chemical degradation (Vinatoru, 2001). For instance, traditional extraction methods have been proven to be less effective than UAE for obtaining rosmarinic and carnosic acids. Liquid-based extraction methods typically achieve an extraction efficiency of less than 40%, whereas ultrasonic-assisted extraction (UAE) can exceed 40% efficiency (Zu et al., 2012). In fact, previous research indicates that UAE can achieve extraction efficiencies as high as 115 ± 0.42 mg dried extract/g plant powder, significantly outperforming traditional methods such as maceration, which has a maximum efficiency of 41 ± 0.37 mg dried extract/g plant powder (Salarbashi et al., 2014). According to a recent study, the greatest yield of polyphenols that could be extracted from spruce wood bark was 13.20 mg/g dry weight using the UAE method (Ghitescu et al., 2015). Also, it was claimed that using the UAE increased the output of anthocyanin from purple sweet potatoes (Z. Cai et al., 2016).

The use of ultrasound-assisted extraction, which improves the rate of compound solubility into the extraction solvent and lowers the necessary solvent volume to achieve 100% phytochemical recovery. Smaller solvent volume requirements, large sample volume, and short extraction time give rise to UAE as an economy-friendly extraction method. The extraction of polyphenols and preservation of the thermolabile chemicals might benefit from shorter sonication times and lower temperatures (Alara, Abdurahman, & Ukaegbu, 2021). Nevertheless, it has been stated in certain research that prolonged sonication lasting longer than 40 minutes at an energy level above 20 kHz may negatively impact the extracted phytochemicals due to a decreased rate of diffusion area/rate and an increased diffusion distance (Annegowda, Anwar, Mordi, Ramanathan, & Mansor, 2010; Wang et al., 2008).

1.2.5(b) Supercritical Fluid Extraction (SFE)

Supercritical fluid extraction (SFE), microwave-assisted extraction (MAE), and pressurized liquid extraction (PLE) are modern techniques used to extract valuable compounds from natural sources in an efficient and environmentally friendly way.

SFE employs a supercritical fluid as the solvent, often carbon dioxide (CO2). When CO2 is heated and pressurized to a supercritical state, it has properties of both a gas and a liquid. This unique combination allows CO2 to move through materials like a gas but dissolve compounds like a liquid (Uwineza and Waśkiewicz, 2020). This approach is quick, precise, and effective at removing delicate substances like bioactive chemicals or essential oils without the use of high heat, which could damage them (Herrero et al., 2010).