ANTIOXIDANTS AND ANTI-INFLAMMATORY ACTIVITIES OF CHINESE HERBS: HUANG QIN, HUANG LIAN, AND HUANG BAI

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ABSTRACT

The three Chinese herbs such as *Scutellaria baicalensis* Georgi (Huang Qin), *Coptis chinensis* Franch (Huang Lian), *Phellodendron chinense* Scheid (Huang Bai) and their combination are associated with antioxidant and anti-inflammatory effects based on their contain of flavonoids and alkaloids. The antioxidants, cytotoxicity, anti-inflammatory properties, and synergistic interaction were conducted to investigate the effect of these plant extracts. The antioxidant activities of individual, binary, and triple combinations were determined using 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), and Ferric reducing antioxidant power (FRAP) assays. The results indicated that the paired and triple combinations exhibited superior antioxidant activities than individual extracts with the synergistic index (SI) confirmations, which both paired and triple indicated synergistic or strong synergistic interaction, particularly the triple combination, with IC_{50} values of 168.79 ± 21.31 µg/mL (DPPH) and 210.34 ± 9.14 µg/mL (ABTS) and FRAP value of 307.37±6.07 mg TE/g extract.

The cytotoxicity effect of individual extract was performed using HaCaT, MRC-5, L6, RAW 264.7, and Vero monkey cells. Huang Qin exhibits the highest toxicity compared to Huang Lian and Huang Bai. The cell survival of all extracts showed a dose-dependent relationship across all treatments. Additionally, all the combinations demonstrated stronger toxicity to the cell viability, particularly the L6 and Vero cell lines, than the single extract.

The anti-inflammatory effects of single, paired, and triple combinations of these plants were evaluated by measuring NO production in RAW 264.7 macrophages. The

combination of Huang Qin and Huang Bai has the most potent anti-inflammatory effect, with an IC_{50} of 44.88 ± 1.34 µg/mL, which was not significantly different from that of Huang Qin alone with an IC_{50} of 61.48 ± 9.94 µg/mL ($p < 0.05$).

The study's results indicated that the combinations of Huang Qin, Huang Lian, and Huang Bai, particularly the triple combination exhibited heightened antioxidant activities, while the pairing of Huang Qin and Huang Bai showed the most potent anti-inflammatory effects. This data may facilitate the selection of a suitable plant extract for the application. Specifically, it may function as a supporting component in pharmaceutical formulations and active compounds in cosmetics, addressing issues such as oxidative stress, skin inflammation, and aging-related conditions.

Keywords: Huang Qin, Huang Lian, Huang Bai, Antioxidant, Cytotoxicity, Anti-inflammatory, Synergistic Interaction

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ABBREVIATIONS AND SYMBOLS

Kg	Kilogram
^{o}C	Degree Celsius
min	Minute
hr	Hour
%	Percentage
mM	Micromolar
M	Molar
N	Mole
g	Gram
ABTS	2,2′-Azino-bis (3-ethybenzothiazoline-6-sulfonic acid)
DPPH	1,1 -diphenil-2-picryl-hydrazil
mg	Milligram
mL	Milliliter
GAE	Gallic acid equivalent
TE	Trolox equivalent
AE	Atropine equivalent
FRAP	Ferric reducing antioxidant power
v/v	Volume by volume
µg	Microgram
µL	Microliter
nm	Nanometer

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Free radicals are molecules with one or more unpaired electrons in the external shell, which is extremely reactive and unstable (Bhanpur, 2015). It plays a major role in various pathologies via oxidative stress and disrupted cell structure. Reactive oxygen species (ROS), superoxide anions ($O_2^{\bullet -}$), peroxyl radicals (ROO•), hydroxyls (OH•), hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS), including nitric oxide (NO•) and peroxynitrite (NO3•), are significant types of free radicals that play a crucial role in both physiological and pathological contexts (Apak et al., 2022; Tumilaar et al., 2024). The excessive ROS and RNS can be generated via environmental impact, such as UV light, heat stress, radiative exposure, etc. ROS and RNS rapidly interact with lipids, proteins, and nucleic acids, which results in tissue damage and leads to a condition known as oxidative stress (Ozcan & Ogun, 2015). During the inflammation reaction, macrophages produce the excessive level of NO, which has been well known as pro-inflammation (Sharma et al., 2007). The imbalance of NO leads to oxidative damage and is involved with various pathologies (Bogdan, 2001). NO can generate peroxynitrite, which is a potent oxidant, by interacting with free radicals such as superoxide, which results in tissue damage (Kamat, 2006).

To address oxidative damage and inflammation, natural products from plants offer a significant effect based on their richness in bioactive compounds such as phenolics, flavonoids, and alkaloids, making them catch the attention of many researchers (Ravipati et al., 2012; Rodríguez-Yoldi, 2021; Talhouk et al., 2007; Xie et al., 2023). This study has selected three Traditional Chinese Medicine (TCM) herbs to identify efficient natural antioxidant and anti-inflammatory activities.

The selection of *Scutellaria baicalensis* Georgi, known as Huang Qin, *Coptis chinensis* Franch (known as Huang Lian), and *Phellodendron chinense* Scheid, known as Huang Bai, comes from their significant role in herbal medicines of Traditional

Chinese Medicine (TCM) as well as modern drug discovery. In TCM, these three herbs are either used individually or in combination and are well known as “Three Yellows” (*San Huang*) despite their functions in clearing the heat, detoxifying the body, and addressing inflammation-based ailments (Qi et al., 2019).

Huang Qin, derived from the root of *Scutellaria baicalensis*, is frequently used for diarrhea, dysentery, hypertension, hemorrhaging, insomnia, inflammation, and respiratory infection (Zhou et al., 2016). The major bioactive compounds found in this plant are flavonoids, such as baicalein, baicalin, and wogonoside. These are capable of free radical scavenging and reducing inflammation reactions via various pathways (Wang et al., 2018).

Huang Lian is derived from the dried root of *Coptis chinensis* Franch. In TCM, it is frequently used for clearing heat, eliminating dampness, purging fire, and detoxification (Li et al., 2020). The major bioactive compounds found in this plant are alkaloid compounds such as magnolia, berberine, coptisine, jatrorrhizine, and palmatine. These compounds can be able to donate electrons or hydrogen atoms to neutralize free radicals and contribute to antioxidant activities. Additionally, in the activation of lipopolysaccharide (LPS) RAW 264.7 macrophages, coptisine has been demonstrated to decrease nitric oxide (NO) via blocking the protein and mRNA expression of inducible nitrite oxide synthase (iNOS) via NF-κB and MAPK pathways

Huang Bai is derived from *Phellodendron chinense* Scheid. In TCM, this plant is popularly used for diarrhea, jaundice, leucorrhea, stranguries, and knee swelling (Lee et al., 2018). The main bioactive compounds that are found in the cortex of Huang Bai are alkaloids, including berberine, palmatine, and phellodendron. The antioxidant and anti-inflammatory activities of this plant are well documented and come from their ability to neutralize free radicals and also reduce pro-inflammatory mediators via many pathways (Ekeuku et al., 2020; Xin et al., 2024).

The rationale for combining Huang Qin, Huang Lian, and Huang Bai is based on their TCM formulation, particularly following Huanglian Jiedu Tang and Gegen Qinlian Tang, which is known as Jun-Chen-Zuo-Shi (Emperor-Minister-Assistant-Envoy) in TCM formulation principles, where Huang Qin affects the upper part of the body, especially taking away the heat from the lung and upper energizer; Huang Lian

affects the middle part of the body, particularly purging fire from the middle energizer, and Huang Bai affects the lower part of the body by removing fire from the lower energizer. The combination ratio of these herbs was based on the condition and treatment purpose, which common ratio in many formulations is 3:2:2 or 1:1:1 (Qi et al., 2019; Wang et al., 2012).

Huang Qin, Huang Lian, and Huang Bai can be used singly or in mixtures in TCM. However, a comparison study between singles or mixtures has not yet been performed. Hence, this study aims to compare the antioxidant and anti-inflammatory activities of the single, paired, and triple combinations of Huang Qin, Huang Lian, and Huang Bai, providing evidence-based scientific credibility for their traditional role in modern cosmeceutical and nutraceutical innovations.

1.2 Objective of the Study

1.2.1 To determine the antioxidant activities of single, paired, and triple combination of Huang Qin, Huang Lian, and Huang Bai.

1.2.2 To determine the synergistic effect of antioxidant activities of the single, paired, and triple combinations of Huang Qin, Huang Lian, and Huang Bai.

1.2.3 To determine cytotoxicity effect of single, paired, and triple combinations of Huang Qin, Huang Lian, and Huang Bai.

1.2.4 To determine the anti-inflammatory activities of single, paired, and triple combinations of Huang Qin, Huang Lian, and Huang Bai.

1.2.5 To determine the synergistic effect of anti-inflammatory activities of single, paired, and triple combinations of Huang Qin, Huang Lian, and Huang Bai.

1.3 Scope of Study

The current study aimed to investigate the antioxidant, cytotoxicity, anti-inflammatory, and synergistic effects of Huang Qin, Huang Lian, Huang Bai, and their mixtures. The phytochemical content was determined on total phenolic, flavonoid, and alkaloid contents. The antioxidant activities were conducted using DPPH and ABTS

radical scavenging and FRAP antioxidant reducing power assays. The cytotoxicity of single extracts was performed on the HaCaT, MRC-5, L6, RAW 264.7, and Vero cell lines, and the combinations were conducted on the L6, RAW 264.7, and Vero cell lines. The anti-inflammatory effects of single, paired, and triple combinations were determined by measuring NO production in RAW 264.7 cells. The synergistic interactions between the paired and triple mixtures were investigated for both antioxidant and anti-inflammatory activities.

All experimental data underwent compositional analysis using GraphPad Prism software version 9.2. The synergistic effect (SE) of the combination of antioxidants and anti-inflammatory activities was utilized to determine the SE effect among the plant mixture, whether synergistic, additive, or antagonistic.

1.4 Outcome of the Study

1.4.1 To determine the antioxidant activities of single, paired, and triple combination of Huang Qin, Huang Lian, and Huang Bai.

1.4.2 To determine the synergistic effect of antioxidant activities of the single, paired, and triple combinations of Huang Qin, Huang Lian, and Huang Bai.

1.4.3 To determine cytotoxicity effect of single, paired, and triple combinations of Huang Qin, Huang Lian, and Huang Bai.

1.4.4 To determine the anti-inflammatory activities of single, paired, and triple combinations of Huang Qin, Huang Lian, and Huang Bai.

1.4.5 To determine the synergistic effect of anti-inflammatory activities of single, paired, and triple combinations of Huang Qin, Huang Lian, and Huang Bai.

CHAPTER 2

LITERATURE REVIEW

2.1 Free Radicals

Free radicals are chemicals with unpaired electron that are extremely reactive and unstable. As a consequence of unpaired electrons, it is preferable to capture other electrons to archive stability (Halliwell & Gutteridge, 2015; Lobo et al., 2010). As a result, the target molecule loses its electron and turns into free radicals, starting the series reaction that ultimately harms the living cell (Phaniendra et al., 2015). Free radicals are normally generated via endogenous, such as metabolic cellular, and exogenous, such as exposure to x-rays, ozone, smoke, pollution, and chemicals (Bagchi & Puri, 1998). The main categories of free radicals are reactive oxygen species (ROS) including superoxide anions ($O_2•^-$), peroxyl radicals (ROO•), hydroxyls (OH•), and hydrogen peroxide (H_2O_2), and reactive nitrogen species (RNS) including nitric oxide (NO•), and peroxynitrite (NO3•) radicals (Apak et al., 2022; Tumilaar et al., 2024).

2.1.1 Free Radical Generation

ROS and RNS can be generated via enzymatic and non-enzymatic reactions. By enzymatic pathway including respiratory reaction, phagocytosis, prostaglandin synthesis, and cytochrome P450 chain (Pham-Huy et al., 2008). For instance, the generation of superoxide, which is the most common by-product in biological mechanisms in mitochondria cells. It is generated via phagocytic cells (neutrophiles and macrophages), the cell acts as phagocytosis; it tends to increase the O_2 intake and start the oxidation of NADPH oxidase, converting O_2 into $O_2•-$ with the presence of catalyse (Bahorun et al., 2006). By the oxidation of xanthine oxidase, hypoxanthine converted to xanthine, then xanthine to uric acid. These reactions produced superoxide and hydrogen peroxide, respectively (Valko et al., 2004). Hydroxyl radical is one of the most reactive molecules and extremely harmful. When excessive superoxide radicals are produced, it leads to a cluster-forming enzyme that produces Fe^{2+}. The Fe^{2+} starts

the Fenton reaction, which forms hydroxyl radicals, especially under stress conditions (Valko et al., 2007).

Nitric oxide (NO•), the most commonly free radical found in the body mainly in macrophages. It plays an important role in the signalling of the oxidation system, such as neurotransmission, blood pressure regulation, protection from the infection, muscle dilatation, and immune homeostasis (Beneš et al., 1999; Valko et al., 2007). However, the activity of NO also concerns the target cell, oxidation-reduction system, NO concentration, and the length of exposure linked to a certain reaction (Ridnour et al., 2005). NO is produced by L-arginine and oxygen with the participation of nitric oxide synthases (NOS). The alternate process of NO production, defined as NOS-independent, generated the NO by the conversion of nitrate and nitrite (Besco et al., 2012; Kiani et al., 2022).

2.1.2 Targets of Free Radical

The unbalancing within the free radical generation (ROS/RNS) and defensive mechanism, antioxidants tend to produce an excessive concentration of free radicals, which results of oxidative stress and nitrosative stress. Because free radicals are extremely reactive, they harm the primary target of macromolecules such as nucleic acids, proteins, and lipids (Droge, 2002; Phaniendra et al., 2015). The phenomenon is caused by oxidative stress, which contributes to the pathogenesis of several chronic diseases, including cardiovascular diseases, diabetes, cancer, and neurological disorders (Bhanpur, 2015).

2.1.2.1 Lipids

Free radicals cause lipid damage via the oxidation of phospholipids, especially polyunsaturated fatty acids, which leads to lipid peroxidation. Free radicals, such as hydroxyl and hydroperoxyl species, oxidize peroxidation of long-chain polyunsaturated fatty acids, such as arachidonic acid and docosahexaenoic acid, which lead to production of metabolites, including endoperoxides, isoprostanes, and neuroprostanes, which these byproducts may be involved in various pathologies (Catalá & Díaz, 2016).

2.1.2.2 Nucleic acids

The increase of free radicals can lead to nucleic acid damage through nucleic acid base modification. Primarily, the increasing of hydroxyl radicals can produce 8-hydroxyguanine by guanine modification, resulting in adenine impair. This reaction led to transition mutation. Additionally, RNS such as peroxynitrous acid can interact with deoxyribose of DNA, which leads to single- and double-bond damage, which results in DNA base mutation (Reddy et al., 2007).

2.1.2.3 Proteins

Various amino acids in protein were compromised by free radical oxidation, resulting in impaired protein crosslinking, dysfunctional enzyme activation, and malfunctions in receptors and transporters. Methionine and cysteine, are the amino acid, both containing in sulfur, are prone to oxidation by free radicals, resulting in the formation of disulfides and methionine sulfoxide (Phaniendra et al., 2015).

2.1.3 Activities of Free Radical

ROS and RNS are important for the development of cellular at low or moderate concentrations. They can protect cells, which function as the host defense system. Additionally, free radicals produce phagocytes (neutrophile, macrophages, and monocytes) to kill the infectious cell, which act as bactericidal, anti-viral, and anti-tumour (Lander, 1997; Pham-Huy et al., 2008). The excessive production of ROS, known as oxidative stress, causes damage to all important biological cellular components, such as membranes, proteins, and nucleic acids, which contribute to many diseases, including cardiovascular disease, degenerative disease, diabetes, inflammation, cancer, anemia, and ischemia (Jomova et al., 2023; Labunskyy & Gladyshev, 2013; Simpson & Oliver, 2020). Especially, skin aging can be made worse by oxidative stress and the oxidative damage it causes, which leads to wrinkle, sagging, dryness, and roughness (Clatici et al., 2017). The overproduction of RNS, especially NO, known as nitrosative stress tends to be involved with diseases including the inflammatory system, neurological damage, and ischaemia (Klatt & Lamas, 2000).

2.1.4 Human Skin Aging

Aging processes are associated with all parts of the body, including skin aging. There are mainly two factors that contribute to skin aging: intrinsic and extrinsic. The intrinsic is generally concerned with physiological aging. While intrinsic is associated with environmental exposure such as smoking, radiation, alcohol, and other lifestyle

factors. The overproduction of free radicals in the skin is closely concerned with both intrinsic and extrinsic skin aging processes (Poljšak et al., 2012).

2.1.5 Oxidative Stress and Human Skin Aging

The concept of free radicals associated with aging is involved in almost every organ of the biological system, including the skin (Rinnerthaler et al., 2015). Oxidative stress is one of the main factors involved in aging progression (Harraan, 1955). The mitochondrial cell is attacked by oxidative stress which leads to mitochondrial malfunction associated with ROS overproduction. This reaction increases the aging process gradually (Lapointe & Hekimi, 2010).

2.1.6 Nitrosative Stress and Skin Inflammation

NO plays a significant function in multiple physiological processes of the skin, such as skin cell reproduction, collagen production, and defense systems. However, the excessive use of NO leads to a significant impact on skin health, especially skin inflammation (Mowbray et al., 2008; Tampa et al., 2024).

2.2 Antioxidant Defenses

The antioxidants are molecules that can reduce and inhibit oxidative stress (Halliwell, 1990). The excessive production of free radicals can be counteracted by antioxidants. They can collect and neutralize the oxidative damage agents by different reactions (Flieger et al., 2021). The reaction of antioxidants is according to its mechanisms, (i) substance reduction; (ii) the capacity of transition metals to chelate; (iii) the availability of antioxidants and their bioavailability; and (iv) the relationship within free radicals and antioxidant molecules (Andrés et al., 2024).

2.2.1 Antioxidants Classifications

Antioxidants are generally generated via endogenous and exogenous. They are mainly classified into enzymatic and non-enzymatic antioxidants. The enzymatic substances are peroxiredoxins (Prxs), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), and superoxide dismutase (SOD) (Irato & Santovito, 2021; Mirończuk-Chodakowska et al., 2018). The non-enzymatic antioxidants are categorized into metabolic antioxidants such as glutathione reduction, lipoid acid, L-

arginine, enzyme Q10, melatonin, uric acid, bilirubin, metal-chelating protein, transferrin, etc. and nutrient antioxidants, which are abundantly found in plants, including vitamin C, carotenoids, vitamin E, flavonoids, omega, and others (Andrés et al., 2024; Rudenko et al., 2023).

2.2.2 Antioxidants Mechanism

2.2.2.1 Enzymatic Antioxidants

Superoxide dismutase catalyzes the neutralization of superoxide radicals with the formation of hydrogen peroxide and oxygen (i). CAT converted hydrogen peroxides into water and oxygen (ii). Additionally, peroxidases also reduce hydrogen peroxide to water, however it works by donating the electron (iii). GPx, use glutathione to detoxicate the hydrogen peroxide (iv) (Goiris et al., 2015).

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \text{ (i)}$$

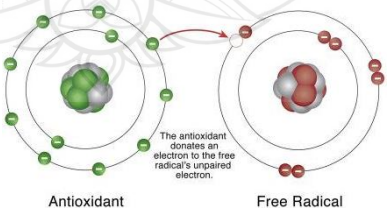
$$2H_2O_2 \rightarrow 2H_2O + O_2 \text{ (ii)}$$

$$H_2O_2 + 2e^- + 2H^+ \rightarrow 2H_2O \text{ (iii)}$$

$$2GSH + H_2O_2 \rightarrow GSSG + 2H_2O \text{ (iv)}$$

2.2.2.2 Non-enzymatic antioxidants

Non-enzymatic antioxidants act to oxidative stress closely to the enzymatic antioxidant, which react to different cell part, in water-soluble includes vitamin C, glutathione, and flavonoids, and membrane components such as flavonoids and isoprenoids (Rudenko et al., 2023). They act as radical scavengers by donating a hydrogen atom or electron to free radicals, so neutralizing them and preventing cellular damage, which was indicated in Figure 2.1 (Khammash et al., 2023; Satish & Dilipkumar, 2015).



Source Khammash et al. (2023)

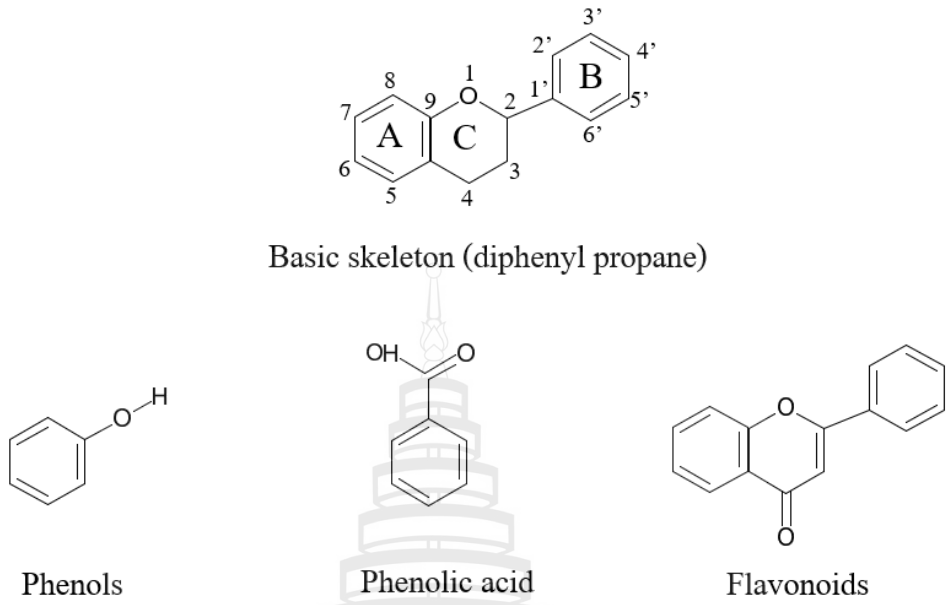
Figure 2.1 Basic mechanism of antioxidants in free radical defence mechanisms

2.3 Phenolic Compounds

Phenolic compounds, commonly found in the plant kingdom, are substances that contain one or more hydroxyl groups with a phenyl ring, which was indicated in Figure 2.2 (Ayad & Akkal, 2019; Rahman et al., 2021). Phenolics are essential to various biological advantages. They can serve as natural antioxidants, antimicrobials, and anti-inflammatory agents (Al Mamari, 2021). There exist two principal paths of phenolic mechanisms: non-specific and specific mechanistic activities. The non-specific activities include the capacity to neutralize free radicals and chelate metals due to the presence of a phenolic group, 2-3 double bonds, and a 4-keto group in ring C (direct impact). The specific activities are due to the structure of phenolic compounds interacting with biomolecules, including proteins, lipids, and other constituents. Antioxidant properties of phenolic compounds may derive from (i) the catechol moiety on ring B, (ii) the double bond between C2 and C3 in ring C, and (iii) the hydroxyl groups located at the C3 and C5 positions of ring A in polyphenols, which was showed in Figure 2.2 and 2.3 (Kruk et al., 2022). Furthermore, phenolic substances can reduce the inflammatory response by suppressing pro-inflammatory mediators such as cytokines, cyclooxygenase-2, and nitric oxide (Prasad et al., 2023). Phenolic compounds can inhibit inducible nitric oxide inducible synthetase (iNOS), which reduce NO production (Lee et al., 2015). The main class of phenolic compounds are flavonoids and non-flavonoids (Ayad & Akkal, 2019; Rahman et al., 2021).

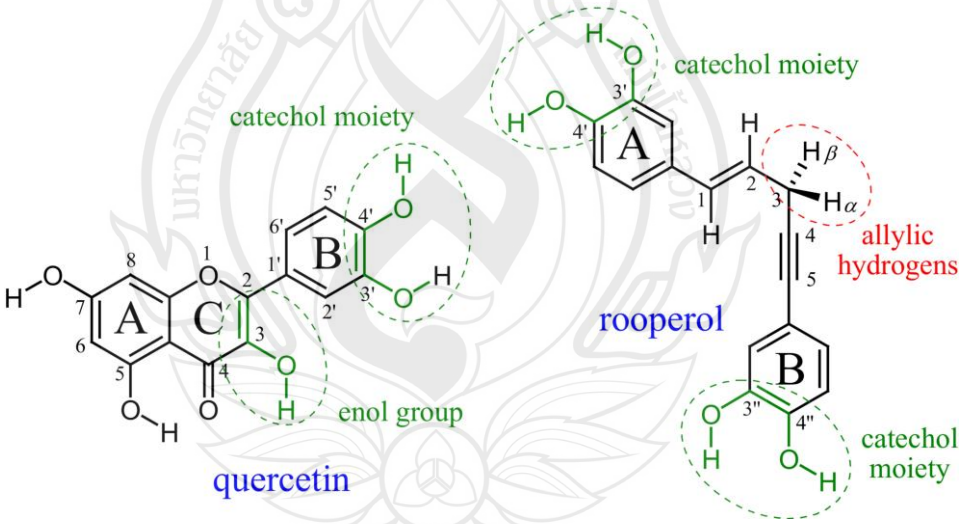
2.3.1 Flavonoid Compounds

Flavonoids compounds comprise of two phenyl rings with oxygen, which was indicated in Figure 2.3. It is classified into flavones, flavonoids, anthocyanins, and isoflavones, according to hydrolysis reaction and cyclic compound structure (Rahman et al., 2021). The antioxidant capacity of flavonoid compounds mostly depends on the number and position of hydroxyl groups and other substituents, including glycosylation. The flavonoid molecules contain hydroxyl groups that influence antioxidant action. Additionally, the antioxidant activity may be influenced by the B-ring, A-ring, 2,3-double bond, and the 4-oxo group in the C-ring (Cai et al., 2006).



Source Jideani et al. (2014)

Figure 2.2 Basic structure of phenolic and flavonoid compounds

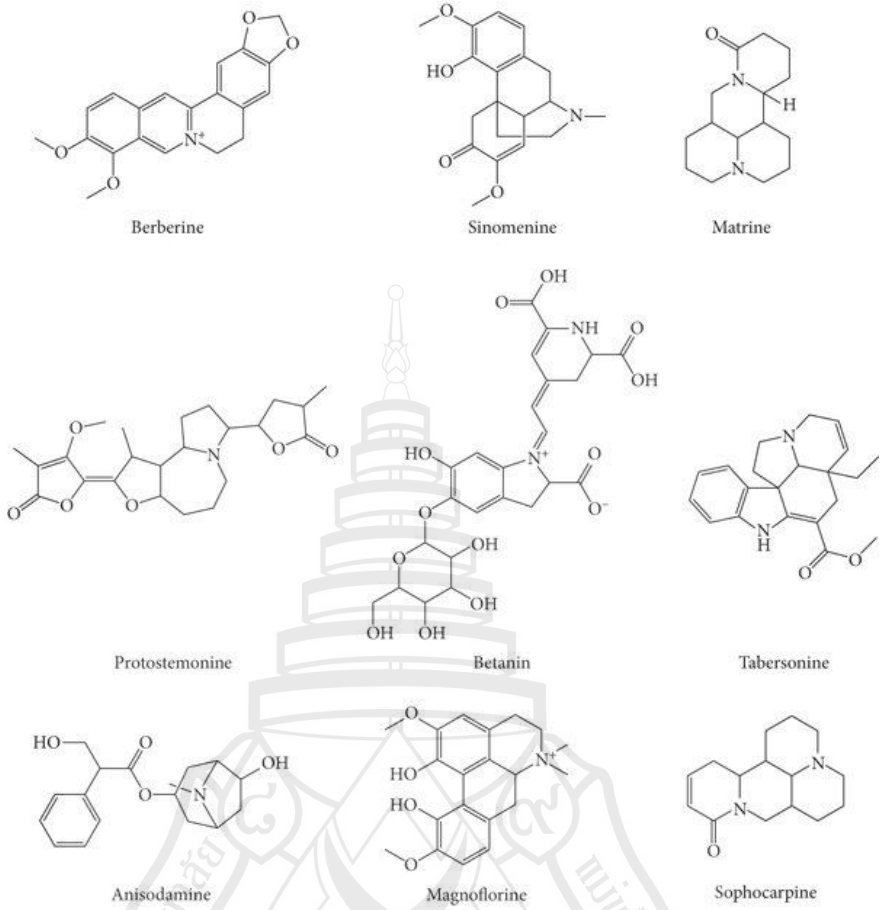


Source Amić and Mastiľák Cagardová (2022)

Figure 2.3 Structure, atom numbering, and antiradical moieties (shown in green) of quercetin and rooperol

2.4 Alkaloid Compounds

Alkaloid compounds are abundantly found in plants, which commonly contain atoms of nitrogen or hydrogen, carbon, and alkaloids, which was indicated in Figure 2.4 (Dey et al., 2020). In the heterocyclic ring, it comprises at least one nitrogen atom, which is generated from amino acid (Uzor, 2020). Alkaloids have been demonstrated to have antioxidant activities, which have a positive impact on human health because of their bioactive compounds. It has potential use in many diseases, such as neurodegenerative, cardiovascular inflammatory, and some chronic disorders related to oxidative stress, which are a result of the negative effect of free radicals (Atpadkar et al., 2023). The N atom and hydroxyl radicals play a crucial role in contributing to antioxidants activity. For example, the nitrogen atom situated in pyrrole contributes to the formation of an iminoquinone structure by donating a hydroxyl radical or an electron to a free radical (Khan & Chaudhary, 2024). Moreover, alkaloid compounds can suppress the inflammatory response by inhibiting the NF-κB pathway, hence decreasing inducible nitric oxide synthase (iNOS), which results in reduced nitric oxide synthesis and less inflammatory reactions (Zhang et al., 2019).



Source Amić and Mastiľák Cagardová (2022)

Figure 2.4 Chemicals structure of alkaloid compounds

2.5 Traditional Chinese Medicine (TCM)

TMC is the original medical science of the Chinese people. It can reveal the laws of human health and the development of diseases from a macroscopic, systematic, and holistic perspective. TCM also emphasizes the Chinese people's logical style. TCM has been strongly integrated into people's lives and productivity, developing a particular health culture and application (Meng et al., 2020). Conventional herbal remedies provide an intriguing, mainly unexplored method for the study of possible new medications. Recently, traditional herbal medicines have been revealed to have the benefits effect that could be used in innovative skin-care products (cosmetic applications of specific traditional Chinese herbal medicines). Additionally, traditional herbal medicines have a particular combination of chemical ingredients (Jia et al.,

2004). These components act as a solid foundation for the mechanism of action (MOA) and effect in the TCM recipe. The composition of the compounds could provide a better understanding of the pharmacological mechanism of the TCM formula (Wang et al., 2020). The *vivo* and *vitro* assays are the conventional methods to investigate the pharmacological effects of one or more TCM components.

2.5.1 Huang Qin, Huang Lian, Huang Bai

The water decoction of Chinese medicinal formulas that contain *Radix Scutellariae*, called Huang Qin *Rhizoma Coptidis*, called Huang Lian, or/and *Cortex Phellodendri*, called Huang Bai, are commonly known as "medicine pairs". Huang Qin and Huang Lian are frequently used in Chinese medicinal formulas such as "Shigao Tang", Huanglian Jiedu Tang", "Huanglian Ejiao Tang", and "Gegen Qinlian Tang" because their combined use improves therapeutic effects. Additionally, this "medicine couple" is frequently used in "herbal tea", which Southern Chinese people regularly consume as beverages (Wang et al., 2012). It acts as detoxifying and eliminating heat. The heat clearing is referred to as enhancing the internal pattern or disorders of extra heat, which are caused by infections from the environment and the interior organs. Heat occurs as an increase in body temperature above normal or as an irrational sensation of fever. Detoxifying refers to the process of eliminating a pathogen's toxicity and virulence. In Chinese medicine, infections can take the form of heat or poison. In accordance with the stern guidelines of "sovereign, minister, assistant, and courier", which was developed from "Huangdi's Internal Classic", to increase the potency of Chinese medicinal herbs and lessen toxins or adverse effects by combination of different types of herbs. Huang Qin acts as the ministerial medication by taking away the heat from the lungs and the fire from the upper energizer. As the middle energizer and sovereign medicine, Huang Lian reduces the fire in the heart. Lower energizer fire is eliminated by Huang Bai as the auxiliary medication (Qi et al., 2019). The herbal combination is thought to be better than a single herbal remedy because its different parts can work together to target more than one place at once.

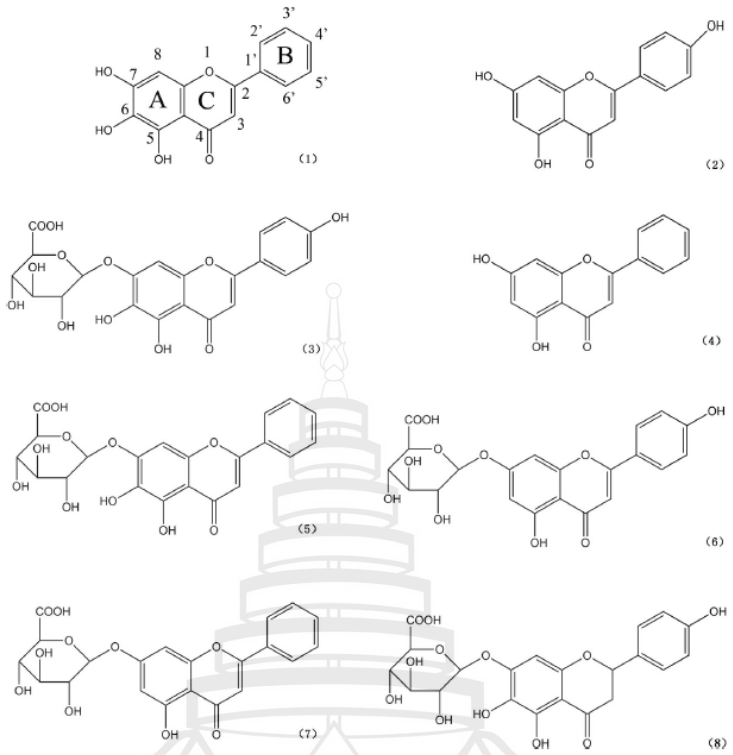
2.5.1.1 Huang Qin

Huang Qin (HQ), the dried root of *Scutellaria baicalensis* Georgi, which was showed in Figure 2.5. It is commonly prepared by decoction or tincture, which is used in treatment such as diarrhea, dysentery, hypertension, hemorrhaging, insomnia,

inflammation, and respiratory infection in TCM (Zhou, Xuelin et al., 2016). The major bioactive compounds found in HQ are flavones such as baicalin, wogonoside, and their aglycone baicalein, which was shown in Figure 2.6. They are responsible for anti-viral, anti-bacteria, antioxidant, anti-inflammatory, hepatoprotective, and neuroprotective activities (Wang et al., 2018). Antioxidant and anti-inflammation effects have been shown in many diseases' models, such as diabetes, cardiovascular disease, inflammatory bowel disease, gout, rheumatoid arthritis, asthma, neurodegenerative diseases, liver and kidney disease, cerebrospinal inflammation, and cancer (Liao et al., 2021). In addition, the study of the effectiveness of baicalein and wogonin isolated from *Scutellaria baicalensis* roots on skin damage found that wogonin and baicalein can inhibit irradiation-induced skin damage in hairless mice that are irradiated by UVB (Kimura & Sumiyoshi, 2011). The antioxidant capacity of Huang Qin is impacted by structure relationship activity (SRA). The flavonoids revealing double bond and phenolic hydroxyls on rings A and B, which serve as augmentors, while the sugar moiety acts as an attenuator that contributes to its antioxidant activity (Li et al., 2018). Furthermore, hydroxyl radical on benzene ring of flavonoids demonstrated radicals donating, which aids in neutralizing free radicals. The anti-inflammatory characteristics of Huang Qin have been evidenced by the capacity of flavonoids to decrease pro-inflammatory mediators, including NO generation, IL-6 β, and IL-1, in LPS-induced RAW 264.7 macrophages (Lee et al., 2014).

Source Amić and Mastiľák Cagardová (2022)

Figure 2.5 (A) *Scutelliaria baicalensis* Georgi plant; (B) dried root of *Scutelliaria baicalensis* Georgi used in TCM; (C) TCM prepared slide of *Scutelliaria baicalensis*.



Source Li et al. (2018)

Figure 2.6 Chemical structures of the main bioactive compounds of flavonoid. (1) baicalein; (2) apigenin; (3) scutellarin; (4) chrysin; (5) baicalin; (6) apigenin-7-O-glucuronide; (7) chrysin-7-O-glucuronide; (8) isocarthamidin-7-O-glucuronide

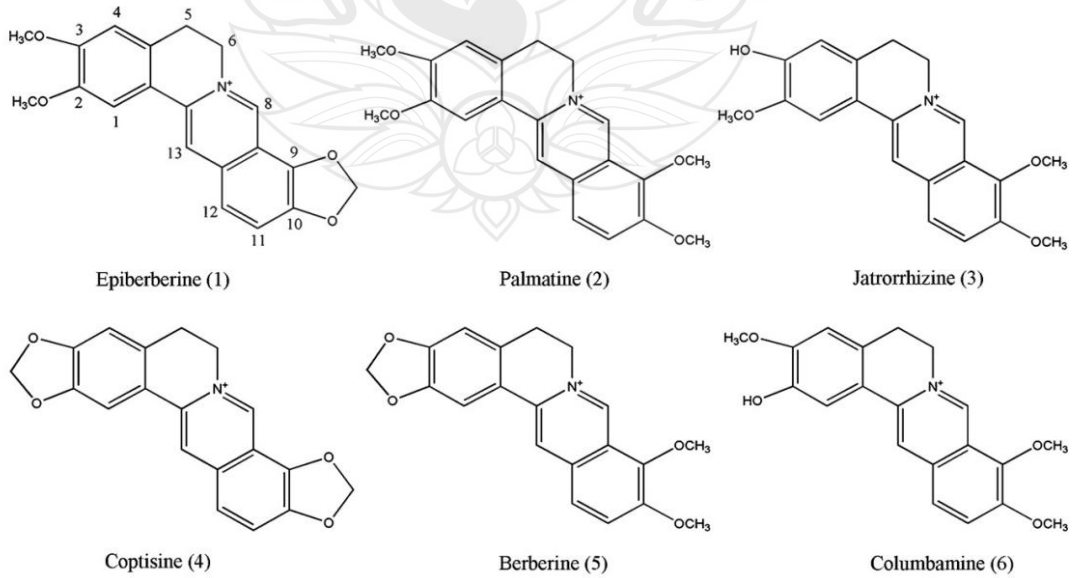
2.5.1.2 Huang Lian

Huang Lian (HL), the dried root of *Coptis chinensis* Franch, which was demonstrated in Figure 2.7 (Ma et al., 2012). It is widely used in food and pharmacological applications. The dried root was often prepared by decoction in TCM. In TCM, it is often used for clearing heat, eliminating dampness, purring fire, and detoxification (Li et al., 2000; Pan et al., 2023). The main components found in *Coptis chinensis* Franch were alkaloids, such as magnolia, berberine, coptisine, jatrorrhizine, epiberberine, columamine and palmatine, which was showed in Figure 2.8. They have potential anti-carcinogen, anti-microbial, anti-inflammatory, and antioxidant properties (Han et al., 2019; Meng et al., 2018; Wang et al., 2022). Significantly, berberine plays a significant role in the scavenging of various free radicals, potentially through one of the following mechanisms: (i) formal hydrogen transfer, (ii) single electron transfer-

proton transfer, and (iii) sequential proton-loss electron transfer and/or radical adduct formation. The variation is contingent upon the hydroxy group substituents at the C-9 and C-12 locations of the berberine backbone's structure (García-Muñoz et al., 2024; Liu et al., 2021; Nam et al., 2022). The phenolic -OH group of berberine is significantly augmenting free radical neutralization and facilitating antioxidant activity. Moreover, the methyl group on the benzene ring of alkaloids can scavenge free radicals by donating an electron, which may enhance antioxidant activity (Liu et al., 2021). Additionally, the alkaloids of Huang Lian extract can be based on their ability to increase antioxidant agents such as MDA and superoxide dismutase. The alkaloids of Huang Lian demonstrated the anti-inflammatory agent by their ability to reduce pro-inflammatory mediators such as IL-6 and NF-kB of LPS-stimulated cells (Meng et al., 2018).

Source Wang et al. (2019)

Figure 2.7 The whole plant and rhizome of *Coptis chinensis* Franch



Source Fan et al. (2012)

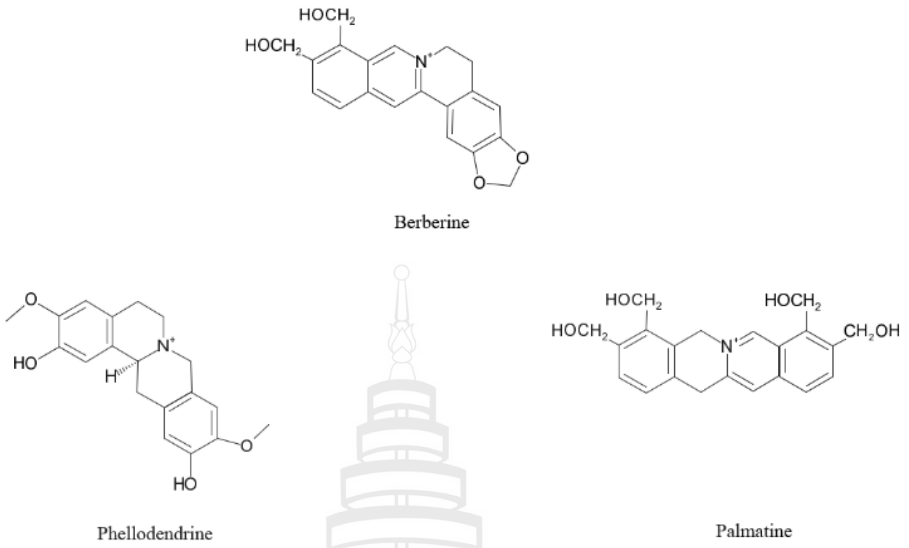
Figure 2.8 Chemical structure of main bioactive compounds of Huang Lian

2.5.1.3 Huang Bai

Huang Bai (HB), the dried bark of *Phellodendron chinense* Scheid, which was shown in Figure 2.9 (Ryuk et al., 2012). The cortex part of HB is frequently prepared in TCM use in treatment of diarrhea, jaundice, leucorrhea, stranguries, and knee swelling (Lee et al., 2018). In pharmacological activities, it is used in treatments such as inflammation, anti-diarrhea, anti-tumor, anti-viral, and pneumonia (Lin et al., 2017). The main bioactive compounds that are found in the cortex of HB are alkaloids such as berberine, palmatine, and phellodendrine, which was shown in Figure 2.10 (Kim et al., 2017; Pan et al., 2023), which are known as anti-inflammatory agents are used in pharmacological anticancer, antimicrobial, and neuroprotective activities (Cao et al., 2018; Jung et al., 2009). The polyphenol group of alkaloids has been shown the antioxidant and anti-inflammatory activities via various mechanisms, such as radicals scavenging, iron metal chelation, inhibiting specific receptor, and protein binding (Mridula et al., 2021). Berberine well known for antioxidant and anti-inflammatory agent by their ability to scavenge free radical and inhibiting pro-inflammatory, such as NO (Xin et al., 2024). Similar to berberine, palmatine acts as radical scavenging which neutralize DPPH, NO, H_2O_2, lipid peroxidation and FRAP, which contribute to antioxidant (Mridula et al., 2021). Palmatine provide the inflammatory activities by inhibit NO production via inhibiting inducible NO synthase (iNOS) and decrease cyclooxygenase-2 (COX-2). Additionally, they have ability to increase heme oxygenase-1 (HO-1) levels in human aortic endothelial cells, which contribute to antioxidant and anti-inflammatory activities (Ekeuku et al., 2020)

Source Zhang et al. (2021)

Figure 2.9 Dried bark of *Phellodendron chinense* Scheid



Source Kim et al. (2017)

Figure 2.10 Chemical structure of main bioactive compounds of Huang Bai

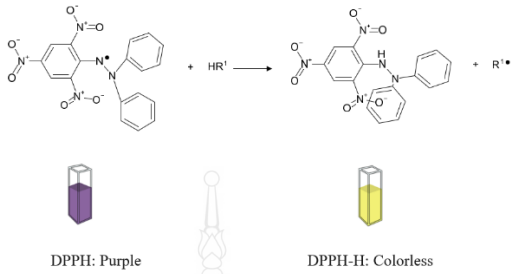
2.6 Determination of Antioxidant Activity

There are multiple methods to determine the capacity of antioxidants. Many chemical tests related to extremely accurate and automatic detection equipment have been applied to measure antioxidant capacity with particular approaches, such as free radical scavenging, reduction of ROS power, and chelation of metal (Munteanu & Apetrei, 2021).

2.6.1 1,1-Diphenyl-2-Picrylhydrazyl (DPPH) Assay

The antioxidant assay by free radicals 2,2-Diphenyl-1-picrylhydrazyl (DPPH), which have been frequently used for evaluation of antioxidant's compound of natural product based on free radical scavenging activity (Baliyan et al., 2022). DPPH contains the unpaired electron on an atomic structure of the nitrogen bridge; this change is accountable for the violet-colored DPPH radical. Phenolic, flavonoid, and alkaloid compounds of natural product can act as antioxidant agents by donating electrons to DPPH, which neutralize DPPH radical. The evaluation of the capacity of an antioxidant is performed by the reduction in violet coloration based on the reaction of DPPH radicals detectable at 517 nm, which was shown in Figure 2.11. The free radicals have

been captured by the antioxidant (hydrophilic or lipophilic molecules), which stabilize the unpaired electron (Bouabid et al., 2020; Gulcin & Alwasel, 2023).

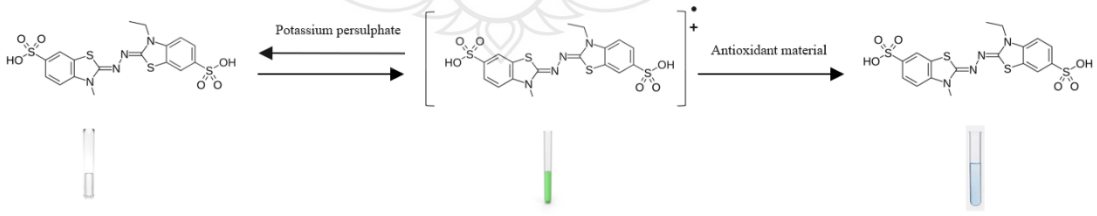


Source Arce-Amezquita et al. (2019)

Figure 2.11 The reaction of DPPH with antioxidant compounds

2.6.2 2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic Acid (ABTS) Radical Scavenging Assay

The ABTS assay is widely used to determine the antioxidants activity of natural product, which is based on the capacity of antioxidant substances to capture ABTS radicals in the water phase. ABTS is formed by the reaction of highly oxidant substances, such as potassium permanganate or potassium persulfate, with ABTS salt. It is measured based on the characteristic of decreasing the blue or green color of ABTS and broad spectrum, which was indicated in figure 2.12. Bioactive chemicals in natural products, including phenolics, flavonoids, and alkaloids, can give hydrogen atoms or electrons to ABTS radicals, therefore neutralizing these radicals into stable molecules. The assay is timesaving and can be applied to a large range of pH, especially organic and water solvents (Dong et al., 2015; Floegel et al., 2011; Ratnavathi & Komala, 2016).

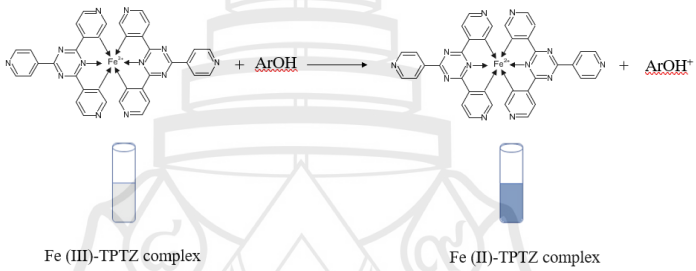


Source Üstündaş et al. (2018)

Figure 2.12 The reaction of ABTS radicals with antioxidant compounds

2.6.3 Ferric Reducing/Antioxidants Power Assay (FRAP)

The FRAP antioxidants assay is widely used to determine the antioxidant activity of natural product. This assay is unlike the others; it is not reacted with free radicals. However, it is measured the ability of a substance to decrease ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), which was shown in Figure 2.13 (Floegel et al., 2011). The hydroxyl and other groups of phenolic, flavonoid, and alkaloid chemicals function as electron donors, capable of reducing ferric to ferrous ions. Trolox, ascorbic, catechin, and gallic acid are popular standards to express the results of antioxidant substances. The link between antioxidant capacity and the calibration substances is applied to describe the standards equivalent of antioxidant substances (Hwang & Lee, 2023).



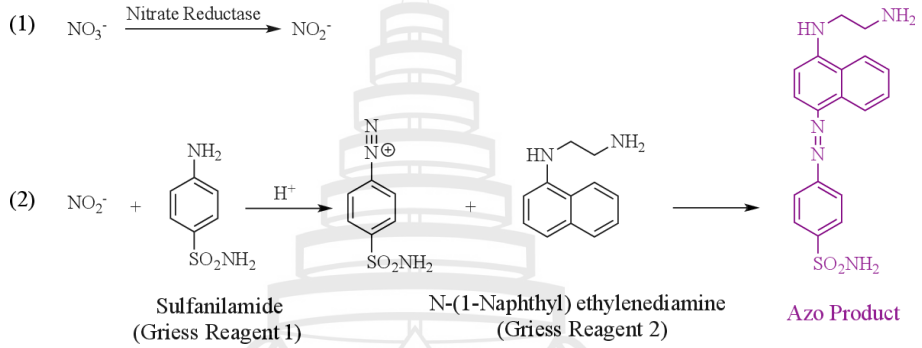
Source Wojtunik-Kulesza (2020)

Figure 2.13 The reaction of Fe (III)-TPTZ complex with antioxidant compounds

2.7 Determination of Anti-inflammatory Activities

In the process of inflammation, the free radicals are generated, especially NO, which is a main pro-inflammatory in the body. It is also known as an inflammation mediator, which is involved in skin inflammation in many circumstances, including dermatitis and allergies related to dermatitis. It is mostly produced by macrophages to react with the inflammatory system. So that NO production is mainly a primary factor related to inflammation and oxidation (Lueangsakulthai et al., 2018; Man et al., 2022). To determine the production of nitric oxide in living organisms is a very significant consideration. NO is easily reacted with oxygen and quickly forms nitrite and/or nitrate. The lifespan of NO in the tissue matrix is extremely brief, about lower than 1 second with the presence of red blood cells to 30 seconds. So that determination of the amount of nitrate/nitrite is widely used as an indicator of NO generation. Griess

reaction assay is the well-known method to measure the level of nitrite in biological systems, which is associated with the transformation of nitrite from nitrate. It is a colorimetric assay, which relies on the diazotization process of nitrite reacting with sulfanilamide (SAM) and N-1-naphthylethylenediamiine dihydrochloride (NED) in condition of acidity. To determine the nitrate concentration, the resultant pink azo dye is measured at 540 nm, which was indicated in Figure 2.14 (Reger, 2017; Sun et al., 2003; Wang et al., 2016).



Source Reger (2017)

Figure 2.14 The Grisse reaction assay is used to evaluate NO production. (1) Nitrate converts into nitrite; (2) a chromophoric chemical is formed, which can be defined

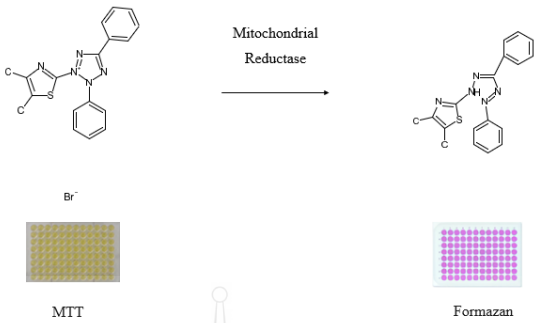
2.7.1 Cytotoxicity Evaluation

Cytotoxicity evaluation is important to assess the maximum safety dose or the highest dose that is compatible with cell existence of compound, such as drug, cosmetic, and chemicals (Tolosa et al., 2015). To assess the effect of single, paired, and triple mixtures of Huang Qin, Huang Lian, and Huang Bai on cell viability, an MTT assay was conducted. It is a colorimetric procedure, which the reaction was followed by color transformation of tetrazolium salt utilizing purple crystal and reducing the yellow color of the MTT. This reaction is caused by the diminishing of cell growth, which leads to the rising of the enzyme of mitochondrial dehydrogenase (Karatop et al., 2022). In other to assess the safety of Huang Qin, Huang Lian, and Huang Bai, HaCaT cell, MRC-5 cell, L6 cell, RAW 264.7 cell, and Vero cell line were selected. The HaCaT cells are immortalized human keratinocyte, which is commonly used in system in vitro activity and safety assessment (Hollywood et al., 2015; Maciel et al., 2019). MRC-5 cells are

human diploid fibroblast cell culture, which is generated from the healthy lung tissue of a 14 weeks old fetus (McSharry et al., 2001). They are widely used in development of vaccine, making reliable to study the safety of human cell (McSharry et al., 2001). L6 cells line is isolated from the rat skeletal muscle, which make them potential use in the substance that may impact the muscle cell (Das & Devi, 2015). Additionally, It was used in the study of irritation of injectable product, which make them reliable to study the cytotoxicity of the product (Laska et al., 1991). The murine macrophage cell line, RAW 264.7 cells, has been widely used and indicated that it is suitable for a macrophage model despite it can act as a pinocytosis and phagocytosis (Taciak et al., 2018). Vero cells are originated from the kidney epithelial cell of an Africa green monkey, which makes the cell model more reliable and frequently used in human experiment s (Ammerman et al., 2008). Hence, these cells can provide relevant information of various human tissue and mechanisms, especially the study of cytotoxicity.

2.7.2 MTT Assay

The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is used to determine cell viability. It is most frequently used to evaluate the cytotoxicity of drugs, products, and substances at various concentrations. According to the MTT assay's concept, mitochondrial activity is constant for most viable cells, meaning that changes in the number of viable cells are directly proportional to changes in mitochondrial activity. The mitochondrial activity of the cells is indicated based on the transformation of the tetrazolium salt into formazan crystals that can be dissolved for uniform measurement. Mitochondrial dehydrogenases convert MTT, a water-soluble yellow tetrazolium dye, into purple formazan crystals, which are then dissolved in dimethyl sulfoxide [DMSO (Sigma-Aldrich)] and subjected to spectrophotometric analysis, which was indicated in Figure 2.15. So that rise or reduction of cell viability value might be detected by observing the concentration of formazan expressed in optical density (OD) through a plater reader at 540 and 720 nm (Bahuguna et al., 2017; Van Meerloo et al., 2011).



Source Karumuri and Tech (2013)

Figure 2.15 The reaction of MTT to formazan crystals

CHAPTER 3

METHODOLOGY

3.1 Sample Collection and Extraction

The dried raw material of Huang Qin, (root), Huang Lian (root), and Huang Bai (bark) were obtained in their commercially air-dried form, as commonly prepared in clinical herbal medicine. The plant was prepared using a Hermetic decoction apparatus: one kilogram of each plant was soaked in 8 litters of deionized water for one hr at room temperature before extraction. The soaked sample of each plant is extracted at 120 degrees Celsius under pressure (1300 watts) for one hr (YJ 13 B-G, Donghuayuan Medical, China). The extract is filtered, evaporated, freeze-dried, and stored at 4 degrees Celsius before further experiments.

The extraction method was performed by decoction due to it the most frequently used and widely clinically accepted practice of herbal preparation in Chinese medical system. This method can be used for both hydrophilic and medium lipophilic compounds and commonly used in therapeutic applications based on their safety and capacity. The study of Zhang and Gao (2019) has reported that high pressure and temperature decoction can provide greater chemicals yield than conventional boiling method. The extraction was conducted at 120 ℃ under pressure for 1 hr per cycle, for two cycles. This condition was linked to clinical practice which has been demonstrated to be greater stability of complex phytochemicals including flavonoids and alkaloids compounds (Song et al., 2020). Furthermore, previous research has documented that Huang Qin (e.g., baicalein and baicalin) and Huang Lian (e.g., berberine) maintained their pharmacological stability up to 130 ℃ (Zhou et al., 2016). Additionally, the freeze-drying phase helps to protect the structural integrity of thermosensitive and prevent post extraction degradation

3.2 Ratio of Paired and Triple Combinations

The designed of paired and triple combinations were included as Huang Qin: Huang Lian (1:1, v/v), Huang Qin: Huang Bai (1:1, v/v), Huang Lian: Huang Bai (1:1, v/v), and Huang Qin: Huang Lian: Huang Bai (1:1:1, v/v/v) (Table 3.2).

Table 3.2 The ratio of the mixture of Huang Qin, Huang Lian, and Huang Bai

The paired and triple combination	The ratio of mixture
Huang Qin:Huang Lian (HQ:HL)	1:1, v/v
Huang Qin:Huang Bai (HQ:HB)	1:1, v/v
Huang Lian:Huang Bai (HL:HB)	1:1, v/v
Huang Qin: Huang Lian: Huang Bai (HQ:HL:HB)	1:1:1, v/v/v

3.3 Determination of Bioactive Compounds

3.3.1 Total Phenolic Content (TPC) Assay

The TPC of each plant extract and their combinations was assessed using the Folin-Ciocalteu technique, which was modified from (Silva et al., 2024). Twenty microliters of diluted sample were combined with twenty microliters of Folin-Ciocalteu reagent, thereafter, augmented by one hundred microliters of 7.5% (w/v) Na_2CO_3 solution into a 96-well microplate. The combination solution was maintained at ambient temperature for 30 min in the absence of light. The absorbance of the test solution was assessed at 760 nm utilizing a microplate reader. The outcome was recorded as milligrams of gallic acid equivalent (GAE) per gram of extract (mg GAE/g extract), employing gallic acid as the benchmark.

3.3.2 Total Flavonoid Content (TFC) Assay

The TFC was defined based on (Fattahi et al., 2014). Following this, 40 microliters of the diluted specimen for testing was combined with 15 microliters of 5% sodium nitrite, and then the mixture was subjected to incubation in darkness for 5 min. Subsequently, incorporate 15 microliters of 10% $AlCl_3$ and maintain the mixture in

darkness at ambient temperature for 6 min. The solution was combined with 100 microliters of 1M NaOH and maintained without light for 10 min. The outcome was quantified in milligrams of quercetin equivalent (QE) per gram of extract (mg QE/g extract), which quercetin was served as the calibration standard. The mixture was assessed at a wavelength of 510 nm utilizing a microplate reader.

3.3.3 Total Alkaloid Content (TAC) Assay

The TAC was assessed utilizing the methodology established by (Tabasum et al., 2016). A spectrophotometric method utilizing bromocresol green (BCG) has been developed to determine the alkaloid content in herbal remedies.

The plant extracts were prepared by solubilizing 100 mg of plant extract in 10 mL of 2 N HCl, then filtered. Each 1 mL of the solution was placed in the separatory funnel and rinsed three times with 10 mL of chloroform. Therefore, this solution was combined with 5 mL of phosphate buffer (pH 4.7) and 5 mL of BCG. 10 mL of chloroform was used to extract the solution. 10 mL of volumetric flask was used to gather the extracts and bring them up to final volume with chloroform. The chemicals in chloroform were evaluated at 470 nm. The atropine calibration plot was prepared with a specific concentration at (60, 80, 100, 120, and 150 µg/mL).

3.4 Antioxidant Activities

3.4.1 1,1-diphenyl-2-picryl-hydrazil (DPPH) Radical Scavenging Assay

The DPPH scavenging capabilities were assessed following the modified methodology of (Kantangkul et al., 2015). Absolute ethanol was used to solubilize the DPPH reagent. In brief, 50 µL of various concentrations of the test solution were mixed with 50 µL of 0.89 mM DPPH solution and maintained for 30 min without light at ambient temperature. The test solution was assessed at 517 nm by utilizing a microplate reader. Ascorbic acid was served as the reference standard. The potential of the test sample to scavenge radicals was measured by quantifying the reduction in DPPH absorbance and calculating it with the following formula: $\text{Inhibition}\,(\%) = \left[\left(\mathrm{A}_{\mathrm{control}} - \mathrm{A}_{\mathrm{sample}}/\mathrm{A}_{\mathrm{control}}\right)\right] \times 100$

3.4.2 2,2'-azino-bis-3-ethylbenthiazoline-6-sulphonic Acid (ABTS)

The $ABTS^{\bullet+}$ radical scavenging assay was assessed based on the methodology of (Polbuppha et al., 2017). $ABTS^{\bullet+}$ was generated by the interaction of a 7 mM ABTS concentrated solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) in a 1:1 ratio and maintained for 16 hr without light at an ambient temperature. The working solution was made by mixing deionized water in a ratio of 1:10. In summary, 10 µL of various concentrations of test samples were inserted into 100 µL of $ABTS^{\bullet+}$ solution and maintained in the absence of light for 30 min. The mixture was assessed at 734 nm utilizing a microplate reader. Ascorbic acid was applied as the calibration standard. The antioxidant activity was determined by the formula: $\text{Inhibition}\ (\%) = \left[\left(A_{\text{control}} - A_{\text{sample}}/A_{\text{control}}\right)\right] \times 100$

3.4.3 Ferric Reducing Antioxidants Power Assay (FRAP)

The FRAP assay was assessed based on the methodology of (Phosri et al., 2022), with certain modifications. The FRAP solution was generated by the reaction of 30 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tri(2-pyridyl)-s-triazine) in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ in a 10:1:1 proportion. The FRAP solution was prepared immediately before use. Ten microliters of various concentrations of the test sample were added into 200 microliters of fresh FRAP solution and maintained at ambient temperature for four min. The test solution was assessed at 593 nm utilizing a microplate reader. The outcome was computed and represented as the FRAP value, indicated in mg of Trolox equivalent (TE)/g of plant extract.

3.5 The Synergistic Scavenging Effect (SE) of the Compounds on DPPH and ABTS Radicals

The synergistic effect (SE) of the paired and triple combinations of Huang Qin, Huang Lian, and Huang Bai was investigated according to the methodology of (Chou, 2006; Wagner & Ulrich-Merzenich, 2009), which commonly accepted in phytopharmacology and synergistic research. It was calculated based on the Synergistic Index (SI), by comparing the observed IC_{50} of the combination with the expected additive IC_{50} from each single extract.

The paired combination in 1:1 ratio:

$$Expected\ IC_{50}(DPPH\ and\ ABTS)\ =\ \frac{IC_{50}plant\ A + IC_{50}plant\ B}{2}$$

The triple combination in 1:1:1 ratio:

$$Expected\ IC_{50}(DPPH\ and\ ABTS)\ =\ \frac{IC_{50}plant\ A + IC_{50}plant\ B + IC_{50}plant\ C}{3}$$

The Synergistic Index (SI) was calculated as:

$$SI(DPPH\ and\ ABTS)\ =\ \frac{Expected\ IC_{50}}{ObservedIC_{50combination}}$$

The synergistic effect expressed the result as:

- SI >1: indicated a synergistic interaction
- SI =1: indicated an additive interaction
- SI< 1: indicated antagonistic interaction

3.6 Cell Viability of HaCaT Cells, MRC-5 Cells, L6 Cells, Mouse Macrophage (RAW 264.7) Cells and Vero Monkey Cells

The cell viability of the single extract and their combinations was evaluated using an MTT assay with slight modifications (Phosri et al., 2014). The HaCaT cells, MRC-5 cells, L6 cells, RAW 264.7 cells and Vero monkey cells were seeded onto 96-well plates at densities of 1×10^4, 5×10^4, 6×10^4, 6×10^4 and 1×10^4 cells per well, respectively, in fresh DMEM medium and incubated under a humidified atmosphere with 5% CO_2 at 37°C for 24 h. The cells were then treated with either a single extract or combination of extracts. Subsequently, 150 µL of MTT solution (0.5 mg/mL) was added to each well, and the plates were incubated for 1 h. in CO_2 incubator Following this,100 µL of DMSO (100 µL) was added to each well to dissolve the formazan crystals. The absorbance of the resulting solution was measured at 570 nm. using microplate reader (EnSight, PerkinElmer, USA). The cell viability of each extract was evaluated by comparison to the untreated control.

3.7 Anti-inflammatory Activities

3.7.1 Measurement of Nitric Oxide

The determination of NO pro-inflammatory mediator production was carried out following the methodology described by (Phosri et al., 2014), using LPS-stimulated RAW 264.7 macrophage cell lines. Briefly, RAW 264.7 cells were seeded into a 96-well plate at a density of 6×10^4 cells/well and incubated for 24 hr at 37°C in a CO_2 incubator. Subsequently, the cells were treated with plant extracts at concentrations of 12.5, 25, 50, 100, and 200 µg/mL, along with co-incubation with 100 ng/mL of LPS for 24 h. Following the incubation, 100 µL of each test solution from the culture medium was gently mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. The absorbance was measured at 540 nm using microplate reader (EnSight, PerkinElmer, USA). The production of NO was expressed as a percentage of the control. Cell viability was assessed using an MTT assay, with LPS-treated cells alone considered to have 100% viability.

The IC_{50} was used to evaluate the level of anti-inflammatory activities of Huang Qin, Huang Lian, Huang Bai, and their combinations, which lower IC_{50} means stronger anti-inflammatory capacity.

To evaluate the interaction of extract combination, the Synergistic Index (SI) calculated, which was follow the method of (Chou, 2006; Wagner & Ulrich-Merzenich, 2009) by comparing observed IC_{50} of each combination with its expected IC_{50}, which derived from the mean IC_{50} values of the individual components:

$$SI = \frac{Expected\ IC_{50}}{Observed\ IC_{50}}$$

The interaction expresses the result as:

- SI > 1: Synergistic interaction
- SI = 1: Additive interaction
- SI < 1: Antagonistic interaction

3.8 Statistical Analysis

The experiment of the study was conducted in triplicate. Prism 9.5.0 software was executed to analyze the data of the experiment. The evaluation process was performed using a One-way ANOVA to compare more than two groups of test samples on bioactive compounds, antioxidant activity, cell viability, and anti-inflammatory activity. The confidence level was required as $p<0.05$. Mean ± standard deviation was assessed to indicate the entire data set.

CHAPTER 4

RESULTS

4.1 Characteristics of Plant Extracts

The characteristic of plant extracts of Huang Qin, Huang Lian, and Huang Bai was showed in table 4.1.

Table 4.1 Characteristic of plant extracts of Huang Qin, Huang Lian, Huang Bai

Plant extracts	Pictures	Color	Consistency	Odor	Solubility
Huang Qin		Light brown-yellowish	Crystalline solid	Sweet and aromatic floral odor	Poorly soluble in water
Huang Lian		Light dark	Crystalline solid	Slight, bitter odor	Poorly soluble in water
Huang Bai		Deep brown	Granular solid	Bitter and slight woody odor	Poorly soluble in water

4.2 Determination of Bioactive Compounds

4.2.1 Total Phenolic Contents (TPC) Determinations

The TPC was evaluated in comparison with the gallic acid standard curve expressed as mg of gallic acid equivalent per gram of extract (mg GAE/g extract). The gallic acid curve was plotted with various concentrations (15.625, 31.25, 62.5, 125, 250, and 500 µg/mL, $y = 0.0031x - 0.0549$, $R^2 = 0.9951$), as shown in Figure 4.2. The result indicated that the Huang Qin, Huang Lian, Huang Bai, Huang Qin:Huang Lian, Huang Qin:Huang Bai, Huang Lian:Huang Bai, and Huang Qin:Huang Lian:Huang Bai contained TPC of 193.00 ± 9.77 mg GAE/g extract, 74.81 ± 3.74 mg GAE/g extract,

138.85 ± 2.76 mg GAE/g extract, 268.57 ± 10.61 mg GAE/g extract, 286.56 ± 16.15 mg GAE/g extract, 257.81 ± 4.88 mg GAE/g extract, and 292.07 ± 10.92 mg GAE/g extract, respectively. The experiment showed that all the combinations contained a greater level of TPC than individual extracts. The triple combination of HQ:HL:HB had the highest TPC at 292.07 ± 10.92 mg GAE/g extract, which was not significantly different from HQ:HB at 286.56 ± 16.15 mg GAE/g extract, as indicated in Figure 4.1 and Table 4.2.

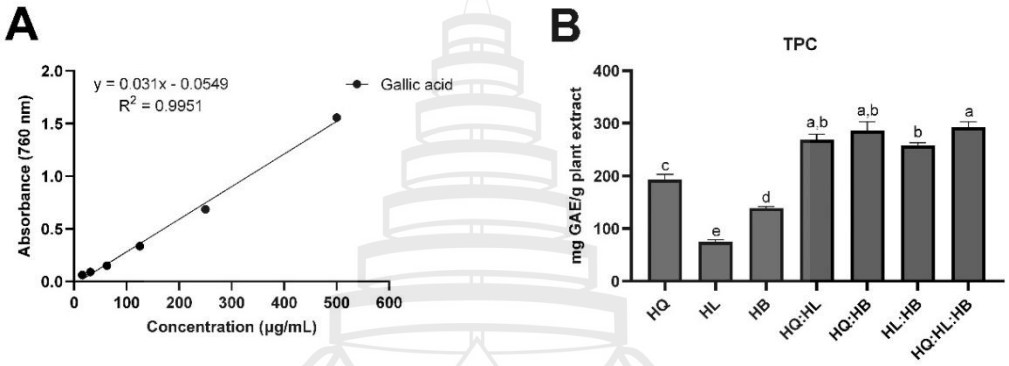


Figure 4.1 (A) Gallic Acid calibration curve, (B) TPC of single, pair, and triple combinations of Huang Qin, Huang Lian, and Huang Bai. The experiment was expressed as mean ± SD (n = 3) and $p < 0.05$

4.2.2 Total Flavonoid Contents (TFC) Determination

The TFC was determined using a quercetin standard curve and report as mg QE/g extract. The data was plotted with various concentrations (62.5, 125, 250, 500, and 1000 µg/mL, y = 0.0008x - 0.0038, R^2 = 0.9854), which was indicated in Figure 4.2. Huang Qin, Huang Lian, Huang Bai, Huang Qin:Huang Lian, Huang Qin:Huang Bai, Huang Lian:Huang Bai, and Huang Qin:Huang Lian:Huang Bai contained TFC of 122.53 ± 16.60 mg QE/g extract, 26.30 ± 11.10 mg QE/g extract, 43.16 ± 1.33 mg QE/g extract, 223.23 ± 9.06 mg QE/g extract, 141.94 ± 11.53 mg QE/g extract, 280.84 ± 9.47 mg QE/g extract, and 263.5 ± 28.28 mg QE/g extract, respectively. The experiment indicated that the paired and triple combinations had higher levels of TFC compared to individual plant extracts. The triple combination of HQ:HL:HB showed the highest level of TFC of 263.55 ± 28.25 mg QE/g extract, which was not significantly different

from the pair HL:HB of 280.84 ± 9.47 mg QE/g extract, as indicated in Figure 4.2 and Table 4.2.

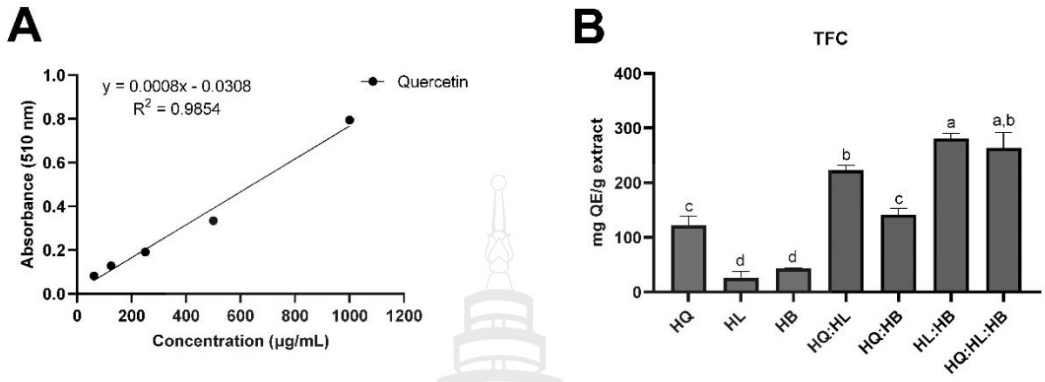


Figure 4.2 Quercetin calibration standard curve, (B) TFC of single, pair, and triple combinations of Huang Qin, Huang Lian, and Huang Bai. The experiment was expressed as mean ± SD (n = 3) and $p < 0.05$

4.2.3 Total Alkaloid Contents

The TAC was determined using the atropine calibration standard curve with a specific concentration at (60, 80, 100, 120, and 150 µg/mL, y = 0.003x-0.0086, R^2 = 0.9904), which was shown in Figure 4.1.3 A and expressed the result as mg atropine equivalent (AE) per g extract (mg AE/g extract). The TAC in Huang Qin, Huang Lian, Huang Bai, Huang Qin:Huang Lian, Huang Qin:Huang Bai, Huang Lian:Huang Bai, and Huang Qin:Huang Lian:Huang Bai was as follow: not detected (ND), 15.82 ± 0.46 mg AE/g extract, 9.86 ± 0.06 mg AE/g extract, 8.52 ± 0.06 mg AE/g extract, 5.31 ± 0.08 mg AE/g extract, 13.26 ± 0.10 mg AE/g extract, and 9.48 ± 0.11 mg AE/g extract, respectively. The experiment indicated that HL showed the highest TAC of 15.82 ± 0.46 mg AE/g extract. The paired HL:HB was the second highest TAC of 13.26 mg AE/g extract; however, it was undetectable in HQ, as shown in Figure 4.3 and Table 4.2.

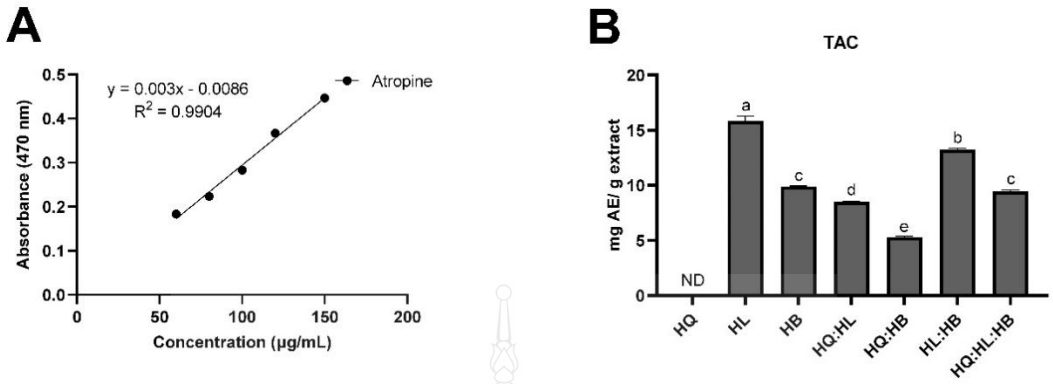


Figure 4.3 (A) Atropine calibration curve, (B) TAC of single, pair, and triple combinations of Huang Qin, Huang Lian, and Huang Bai. The experiment was expressed as mean ± SD (n = 3) and $p < 0.05$

Table 4.2 Total phenol, total flavonoid, and total alkaloid content of single, paired, and triple combinations of Huang Qin, Huang Lian, and Huang Bai

Sample or Standard	**TPC (mg GAE/g extract)**	**TFC (mg QE/g extract)**	**TAC (mg AE/g extract)**
HQ	193.00 ± 9.77^{c}	122.53 ± 16.60^{c}	ND
HL	74.81 ± 3.74^{e}	26.30 ± 11.10^{d}	15.82 ± 0.46^{a}
HB	138.85 ± 2.76^{d}	43.16 ± 1.33^{d}	9.89 ± 0.06^{c}
HQ:HL	268.57 ± 10.61^{b}	223.23 ± 9.06^{b}	8.52 ± 0.06^{e}
HQ:HB	286.56 ± 16.15^{a}	141.94 ± 11.53^{c}	5.31 ± 0.08^{f}
HL:HB	257.81 ± 4.88^{b}	280.84 ± 9.47^{a}	13.26 ± 0.10^{b}
HQ:HL:HB	292.07 ± 10.92^{a}	263.55 ± 28.28^{a}	9.48 ± 0.11^{d}

Note The data are represented as mean ± SD (N=3). Different letters (a-f) within each column indicate statistically significant differences ($p < 0.05$)

4.3 Antioxidant Activities

The antioxidant activities of single, paired, and triple mixtures of Huang Qin, Huang Lian, and Huang Bai are shown in Table 4.3.

Table 4.3 Antioxidant activities of single, paired, and triple mixtures of Huang Qin, Huang Lian, and Huang Bai

Sample or Standard	DPPH	ABTS	FRAP
	(IC_{50} ± SD, µg/mL)		(mg TE/g extract)
HQ	366.99 ± 17.83^{c}	598.60 ± 32.51^{c}	149.95 ± 2.09^{e}
HL	655.86 ± 17.64^{a}	808.73 ± 24.81^{a}	127.51 ± 5.22^{f}
HB	567.56 ± 23.74^{b}	654.65 ± 15.24^{b}	135.58 ± 4.02^{f}
HQ:HL	289.73 ± 5.49^{d}	228.16 ± 12.61^{d}	265.04 ± 4.56^{c}
HQ:HB	303.86 ± 12.24^{d}	222.54 ± 7.11^{d}	289.92 ± 6.91^{b}
HL:HB	299.55 ± 22.16^{d}	223.62 ± 9.02^{d}	249.28 ± 4.91^{d}
HQ:HL:HB	168.79 ± 21.31^{e}	210.34 ± 9.14^{d}	307.37 ± 6.07^{a}
Ascorbic acid	33.02 ± 2.85^{f}	54.02 ± 9.28^{e}	-

Note The data are indicated as mean ± SD (n = 3). Different letters (a-f) within each column indicate statistically significant differences *($p < 0.05$)*

4.3.1 The 1,1-diphenyl-2-picryl-hydrazil (DPPH) Radical Scavenging Assay

DPPH radical scavenging activity was determined by measuring the antioxidant capacity through the reduction of $DPPH^{\cdot+}$ radical. Ascorbic acid was served as a calibration standard. The antioxidant activity was investigated based on IC_{50} value. The lower IC_{50} indicates stronger antioxidant activity. The experiments demonstrated that the IC_{50} values of Huang Qin, Huang Lian, Huang Bai, Huang Qin:Huang Lian, Huang Qin:Huang bai, Huang Lian:Huang Bai, and Huang Qin:Huang Lian:Huang Bai were 366.99 ± 17.83 µg/mL, 655.86 ± 17.64 µg/mL, 567.56 ± 23.74 µg/mL, 289.73 ± 5.49 µg/mL, 303.86 ± 12.24 µg/mL, 299.55 ± 22.16 µg/mL, and 168.79 ± 21.31 µg/mL, respectively. The finding showed that paired and triple combinations inhibited greater

antioxidant capability than the individual extracts. Notably, the triple combination of HQ:HL:HB exhibited the lowest IC_{50} value of 168.79 ± 21.31 µg/mL, with statically significant difference $p < 0.05$, as shown in Figure 4.4 and table 4.3. The DPPH radical scavenging activity was assessed by measuring the antioxidant capacity through the decrease of the DPPH radical. Ascorbic acid was utilized as a calibration standard.

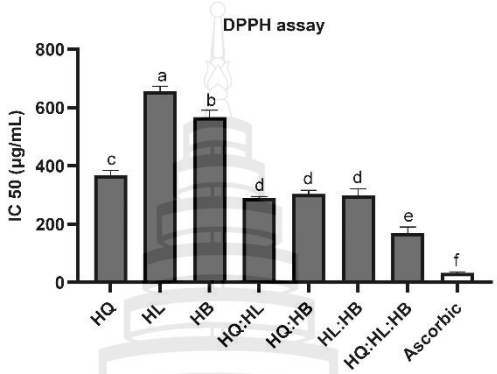


Figure 4.4 IC_{50} value of antioxidant capacity DPPH, the data indicated as mean ± SD (n = 3). Different letters (a-f) within each column indicate statistically significant differences ($p < 0.05$)

4.3.2 The 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic Acid) (ABTS) Radicals Scavenging Assay

The ABTS radical scavenging assay was determined by the antioxidant activity of single, paired, and triple combinations of Huang Qin, Huang Lian, and Huang Bai. Ascorbic acid was used as standard calibration, and the outcome was described based on the value of IC_{50}, which the lower IC_{50} value indicates stronger antioxidant activity. The results demonstrated that the IC_{50} value of Huang Qin, Huang Lian, Huang Bai, Huang Qin:Huang Lian, Huang Qin:Huang bai, Huang Lian:Huang Bai, and Huang Qin:Huang Lian:Huang Bai were 598.50 ± 32.15 µg/mL, 808.73 ± 24.81 µg/mL, 654.65 ± 15.24 µg/mL, 228.16 ± 12.61 µg/mL, 222.54 ± 7.11 µg/mL, 223.62 ± 9.02 µg/mL, and 210.34 ± 9.14 µg/mL, respectively. IC_{50} value of ascorbic acid was 54.02 ± 9.28 µg/mL. The experiment revealed that the paired and triple combinations inhibited stronger antioxidant capacity, and all the combinations were not significant $p < 0.05$, as indicated in Figure 4.5 and Table 4.3.

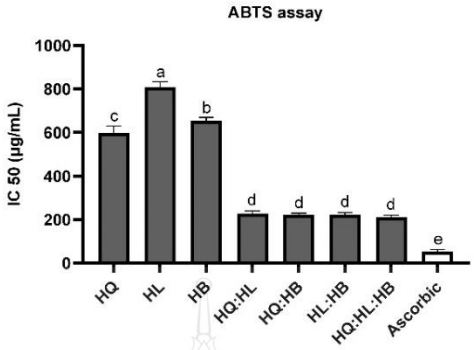


Figure 4.5 IC_{50} value of antioxidant capacity ABTS of single, paired, and triple combinations. Data indicated as mean ± SD (n = 3). Different letters (a-f) within each column indicate statistically significant differences ($p < 0.05$)

4.3.3 Ferric Reducing Antioxidant Power Assay (FRAP)

The FRAP assay was investigated for antioxidant activity based on the capacity to reduce ferric iron to ferrous iron through electron donation. Trolox was served as a standard calibration curve, plotted at different concentrations at (7.8125, 15.625, 31.25, 62.5, 125, and 250 µg/mL, y = 0.005x + 0.0707, R^2 = 0.9984). The outcome was described as mg Trolox equivalent per gram of plant extract (mg TE/g extract). The results demonstrated that Huang Qin, Huang Lian, Huang Bai, Huang Qin:Huang Lian, Huang Qin:Huang bai, Huang Lian:Huang Bai, and Huang Qin:Huang Lian:Huang Bai were 149.95 ± 2.09 mg TE/g extract, 127.51 ± 5.22 mg TE /g extract, 135.58 ± 4.03 mg TE/g extract, 265.04 ± 4.56 mg TE/g extract, 289.92 ± 6.91 mg TE/g extract, 249.28 ± 4.91 mg TE/g extract, and 307.37 ± 6.07 mg TE/g extract, respectively. The result of the experiment indicated that the paired and triple combinations had greater capacity to donate electrons compared to the single plant extracts. Particularly, the triple combination (HQ:HL:HB) demonstrated the greatest antioxidant activity of 307.37 ± 6.07 mg TE/ g extract, which indicated in Figure 4.6 and Table 4.3.

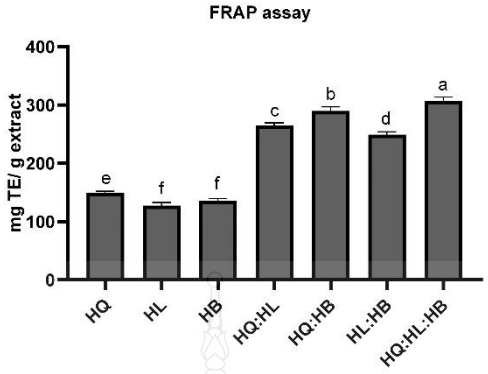


Figure 4.6 FRAP value expressed as mg TE/g extract. Data indicated as mean ± SD (n = 3). Different letters (a-f) within each column indicate statistically significant differences ($p < 0.05$).

4.4 The Synergistic Scavenging Effect (SE) of the Compounds on DPPH and ABTS Radicals

The synergistic effects (SE) of the paired and triple combinations of Huang Qin, Huang Lian, and Huang Bai were determined by comparing observed antioxidant activity (as IC_{50} value) with the expected additive antioxidant activity. DPPH and ABTS were selected to find the SE of plant extract by calculating SI, where SI > 1 mean synergistic effect, SI = auditive effect, and SI < 1 antagonistic effect. The results, which showed in Table 4.4 and 4.5, illustrated that the SE value of DPPH and ABTS of Huang Qin:Huang Lian, Huang Qin:Huang Bai, Huang Lian:Huang Bai, and Huang Qin:Huang Lian:Huang Bai were (1.77 and 3.08), (1.54 and 2.82), (2.04 and 3.27), and (3.14 and 3.27), respectively. All combinations inhibited synergistic interaction greater than 1, therefore synergistic effect was found. Significantly, triple combination indicated the highest SI both DPPH and ABTS, suggesting that the triple mixture could provide simultaneous presence than single and paired component. This result may provide the supplemented data to support the concept that combining traditional medicinal plants may have synergistic effect.

Table 4.4 The SE using IC_{50}value of DPPH of paired and triple mixtures of Huang Qin, Huang Lian, and Huang Bai

Combination	Expected	Observed	SI	Effect
		IC_{50} (µg/mL)		
HQ:HL	511.43	289.73	1.77	Synergistic
HQ:HB	467.28	303.86	1.54	Synergistic
HL:HB	611.71	299.55	2.04	Synergistic
HQ:HL:HB	530.14	168.79	3.14	Synergistic

Note SI > 1: Synergistic effect found, SI < 1: antagonistic effect found, and SI = 1: indicating an additive interaction

Table 4.5 The SE using IC_{50}value of ABTS of paired and triple mixtures of Huang Qin, Huang Lian, and Huang Bai

Combination	Expected	Observed	SI	Effect
		IC_{50} (µg/mL)		
HQ:HL	703.67	228.16	3.08	Synergistic
HQ:HB	626.63	222.54	2.82	Synergistic
HL:HB	731.69	223.62	3.27	Synergistic
HQ:HL:HB	687.33	210.34	3.27	Synergistic

Note SI > 1: Synergistic effect found, SI < 1: antagonistic effect found, and SI = 1: indicating an additive interaction

4.5 The Effect of Huang Qin, Huang Lian, and Huang Bai on Different Cells Viability

The cell viability was investigated following incubation with Huang Qin, Huang Lian and Huang Bai by the MTT assay. The HaCaT cells, MRC-5 cells, L6 cells, RAW 264.7 cells and Vero monkey cells were used to evaluate the effect of plant extracts at various concentrations. ISO 10993-5 say that the level of cell viability higher than 80%

are not cytotoxic, 80% to 60% are less toxic, 60% to 40% is medium, and less than 40% are high potential cytotoxicity , respectively (Silva et al., 2024).

4.5.1 The Effect of Huang Qin, Huang Lian, and Huang Bai on Cell Viability of HaCaT Cells

The impact of Huang Qin, Huang Lian, and Huang Bai on cell viability of HaCaT cells was conducted at concentrations ranging from 6.25 to 100 µg/mL. The result indicated that the percentages of cell survival that being treated with Huang Qin, Huang Lian, and Huang Bai were 95.39% - 52.57%, 93.81% - 46.74%, and 96.81% - 81.16%, respectively, as indicated in Figure 4.7. Additionally, these results demonstrated that at the dose of 100 µg/mL, Huang Qin and Huang Lian decreased cells survival by 52.57% and 46.78%, respectively, demonstrating medium toxicity to HaCaT cells. Although Huang Bai exhibited an inhibition rate of 81.16%, it did not showed cytotoxicity to these cells.

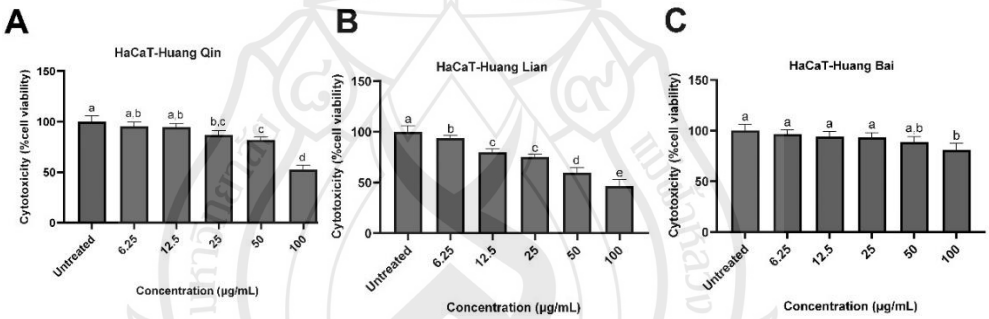


Figure 4.7 The effect of Huang Qin (A), Huang Lian (B), and Huang Bai (C) at the dose ranging from 6.25 to 100 µg/mL on cell viability of HaCaT cells. Each bar chart is indicated as mean ± SD (n = 3). Different letters represent significant at $p < 0.05$

4.5.2 The Effect of Huang Qin, Huang Lian, and Huang Bai on Cell Viability of MRC-5 Cells

The effect of Huang Qin, Huang Lian, and Huang Bai on cell viability of MRC-5 cells was conducted at concentrations of 2.5, 5, 10, 20, and 40 µg/mL, as well as 6.25, 12, 25, 50, and 100 µg/mL. The cell survival percentages following treatment treated with Huang Qin were 93.59%, 86.29%, 86.37%, 83.42%, and 83.95%, as well as 98.87%, 95.28%, 85.90%, 73.78%, and 35.87%, respectively. The percentages of cell

survival percentages following Huang Lian treatment were 80.64%, 80.16%, 81.77%, 74.75%, and 69.97%, as well as 85.47%, 80.97%, 62.90%, 54.96%, and 52.49%, respectively. The percentages of cell survival following treatment with Hiang Bai were 90.35%, 95.17%, 97.37%, 104.99%, and 97.44%, as well as 101.23%, 104.16%, 104.61%, 89.28%, and 72.98%, respectively, which indicated in Figure 4.8. These finding showed that at the concentration of 100 µg/mL, the percentages of cell survival of Huang Qin, Huang Lian, and Huang Bai are 35.87% (high toxicity), 52.49% (medium toxicity), and 72.98% (low toxicity), respectively.

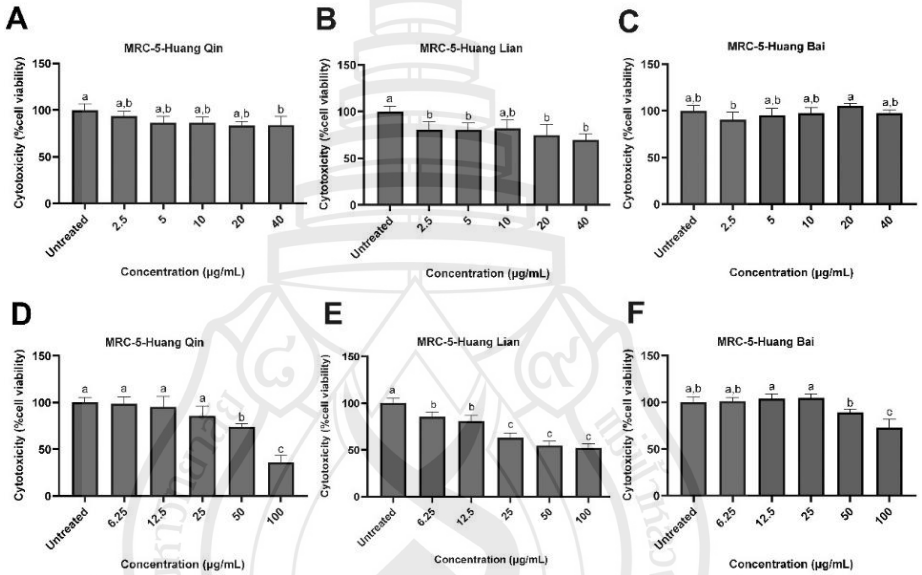


Figure 4.8 The effect of Huang Qin (A, D), Huang Lian (B, E), and Huang Bai (C, F) at the concentration of (2.5 - 40µg/mL) and (6.25 - 100 µg/mL), respectively, on cell viability of MRC-5 cells. Each bar chart indicated as mean ± SD (n = 3). Different letters represent significant at $p < 0.05$

4.5.3 The Effect of Huang Qin, Huang Lian, and Huang Bai on Cell Viability of L6 Cells

The impact of Huang Qin, Huang Lian, and Huang Bai as well as their combinations, on cell viability of L6 cells were conducted at concentrations ranging from 12.5 to 200 µg/mL. Following treatment with Huang Qin, Huang Lian, Huang Bai, Huang Qin:Huang Lian, Huang Qin:Huang Bai, Huang Lian:Huang Bai, and Huang Qin:Huang Lian:Huang Bai, the percentages of cell survival were 104.51% - 18.60%, 93.98% - 16.82%, 107.53% - 84.96%, 111.93% - 18.90%, 101.25% - 21.46%,

110.25% - 19.17%, and 96.65% - 19.8%, respectively, as shown in Figure 4.9. The results indicated that at the concentration of 200 µg/mL, all extracts were high toxicity to the cell, except for Huang Bai, which had not affected to the cell.

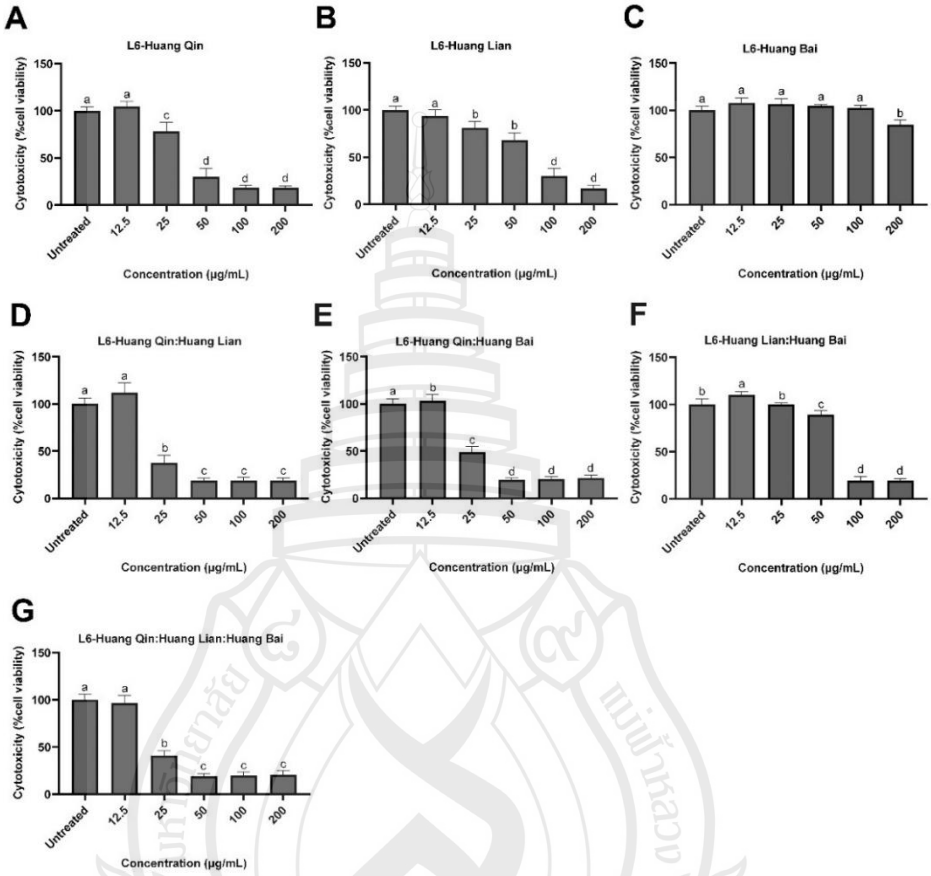


Figure 4.9 The effect of Huang Qin (A), Huang Lian (B), Huang Bai (C), Huang Qin:Huang Lian (1:1, v/v) (D), Huang Qin:Huang Bai (1:1, v/v) (E), Huang Lian:Huang Bai (1:1, v/v) (F), and Huang Qin:Huang Lian:Huang Bai (1:1:1, v/v/v) (G) at the concentration of 12.5 - 200 µg/mL on cell viability of L6 cell. Each bar chart is indicated as mean ± SD (n = 3). Different letters represent significant at $p < 0.05$

4.5.4 The Effect of Huang Qin, Huang Lian, and Huang Bai on Cell Viability of RAW 264.7 Cells

Mouse macrophage RAW 264.7 cells were co-incubated with LPS and extracts at concentration of 12.5, 25, 50, 100, and 200 µg/mL for 24h. The cells were

subsequently discarded, and cell viability was evaluated using the MTT assay. The percentages of cell survival following treatment with Huang Qin, Huang Lian, Huang Bai, Huang Qin:Huang Lian, Huang Qin:Huang Bai, Huang Lian:Huang Bai, and Huang Qin:Huang Lian:Huang Bai were 132.07% - 144.10%, 133.68% - 93.55%, 105.57 - 95.06%, 114.47% - 95.71%, 114.01% - 100.31%, 93.78% - 78.37%, 100.24% - 92.46%, respectively, as shown in Figure 4.10. At the concentrations ranging from 12.5 to 200 µg/mL, all extracts were less and no toxic to the cell viability of RAW 264.7 cells. Consequently, these concentrations were selected to evaluate the anti-inflammatory activities by production of NO.

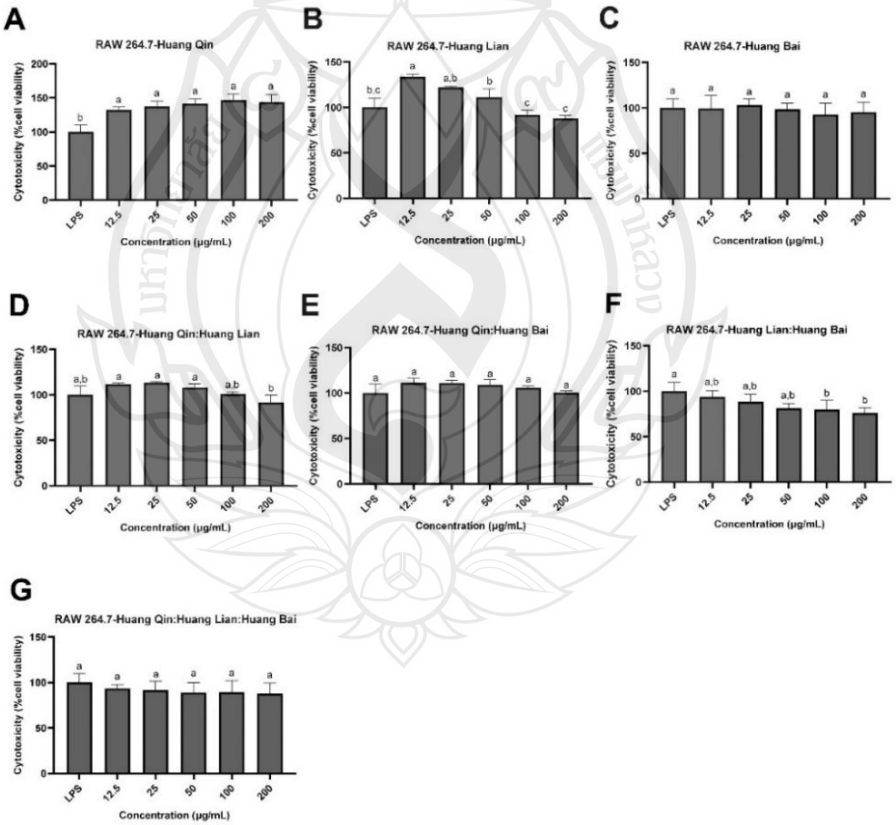


Figure 4.10 The effect of Huang Qin (A), Huang Lian (B), Huang Bai (C), Huang Qin:Huang Lian (1:1, v/v) (D), Huang Qin:Huang Bai (1:1, v/v) (E), Huang Lian:Huang Bai (1:1, v/v) (F), and Huang Qin:Huang Lian:Huang Bai (1:1:1, v/v/v) (G) at the concentration of 12.5 - 200 µg/mL on cell viability of RAW 264.7 cell. Each bar chart is indicated as mean ± SD (n = 3). Different letters represent significant at $p < 0.05$

4.5.5 The Effect of Huang Qin, Huang Lian, and Huang Bai on Cell Viability Vero Cells

The impact of Huang Qin, Huang Lian, and Huang Bai, as well as their mixtures on cell viability of Vero cells was conducted at concentrations of 12.5, 25, 50, 100, and 200 µg/mL. The cell survival percentages following treatment with Huang Qin, Huang Lian, Huang Bai, Huang Qin:Huang Lian, Huang Qin:Huang Bai, Huang Lian:Huang Bai, and Huang Qin:Huang Lian:Huang Bai were 109.65% - 57.08% medium, 103.56% - 95.38%, 105.87% - 105.05%, 103.25% - 23.65%, 102.64 - 34.26%, 105.19% - 83.05% and 102.73% - 29.35%, respectively, as indicated in Figure 4.11. The results indicated that at a concentration of 200 µg/mL, Huang Qin exhibited moderate toxicity, with cell survival at 57.8%. In contrast, Huang Lian and Huang Bai shown no toxicity, with survival rates of 95.38% and 105.87%, respectively. All combinations exhibited severe toxicity, with cell survival rates below 40%, except for the combination of Huang Lian and Huang Bai, which showed negligible toxicity, maintaining cell survival above 80%.

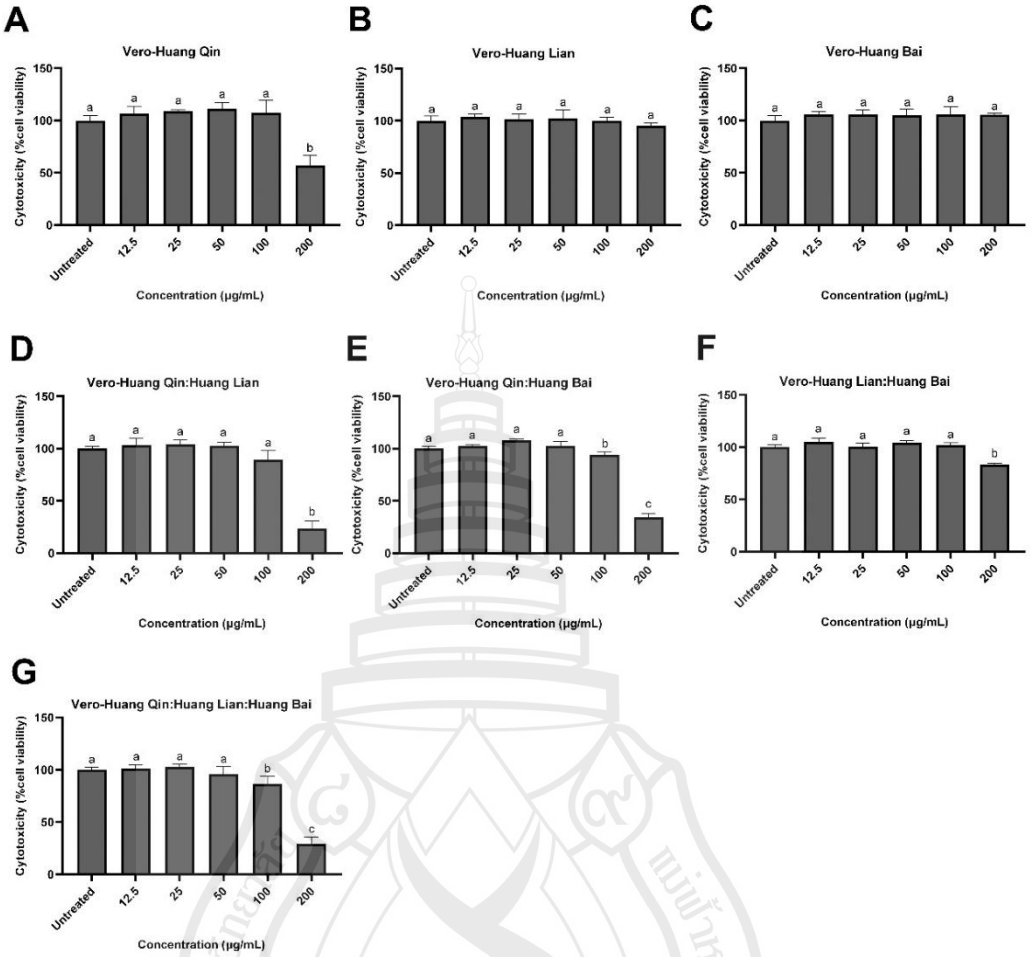


Figure 4.11 The effect of Huang Qin (A), Huang Lian (B), Huang Bai (C), Huang Qin:Huang Lian (1:1, v/v) (D), Huang Qin:Huang Bai (1:1, v/v) (E), Huang Lian:Huang Bai (1:1, v/v) (F), and Huang Qin:Huang Lian:Huang Bai (1:1:1, v/v/v) (G) at the concentration of 12.5 - 200 µg/mL on cell viability of Vero cell. Each bar chart is indicated as mean ± SD (n = 3). Different letters represent significant at $p < 0.05$

4.6 Anti-inflammatory Activities

4.6.1 Effect of Single, Paired, and Triple Combination on NO Production

Nitric Oxide (NO) was generated by a mouse macrophage RAW 264.7 cells after a 24-hr co-incubated with lipopolysaccharide (LPS) and extracts at the concentrations of 12.5, 25, 50, 100, and 200 µg/mL. Subsequently, 100 µL was gentle mixed with an equal amount of Griess reagent. The anti-inflammatory activities of

Huang Qin, Huang Lian, and Huang Bai, as well as their combinations was investigated via NO production. The results showed that the percentages of NO production of Huang Qin (HQ), Huang Lian (HL), Huang Bai (HB), Huang Qin:Huang Lian (HQ:HL), Huang Qin:Huang Bai (HQ:HB), Huang Lian:Huang Bai (HL:HB), and Huang Qin:Huang Lian:Huang Bai (HQ:HL:HB) were 75.58% - 20.49%, 74.20% - 29.05%, 91.44% - 74.53%, 86.73% - 23.36%, 73.53% - 21.84%, 113.65% - 32.98% and 78.18% - 23.57%, respectively. Among single extract, HQ and HL showed moderated NO inhibition, with IC_{50} of 61.48 ± 9.93 µg/mL and 105.44 ± 2.85 µg/mL, respective. HB inhibited the weakest activity, with IC_{50} of 414.36 ± 1.63 µg/mL ($P < 0.05$). In the combination, it showed that the combination of HQ:HB inhibits the highest NO production, with the lowest an IC_{50} of 44.88 ± 1.34 µg/mL, indicated a synergistic effect (SI = 5.30). The paired mixture of HL:HB also demonstrated synergistic effect with SI of 1.69 and IC_{50} of 92.31 ± 4.43 µg/mL. The triple combination also showed a strong synergistic effect, reducing NO production significantly with IC50 of 78.31 ± 4.77 µg/mL and SI of 2.47. This result was summarized in tables 4.6 and 4.7.

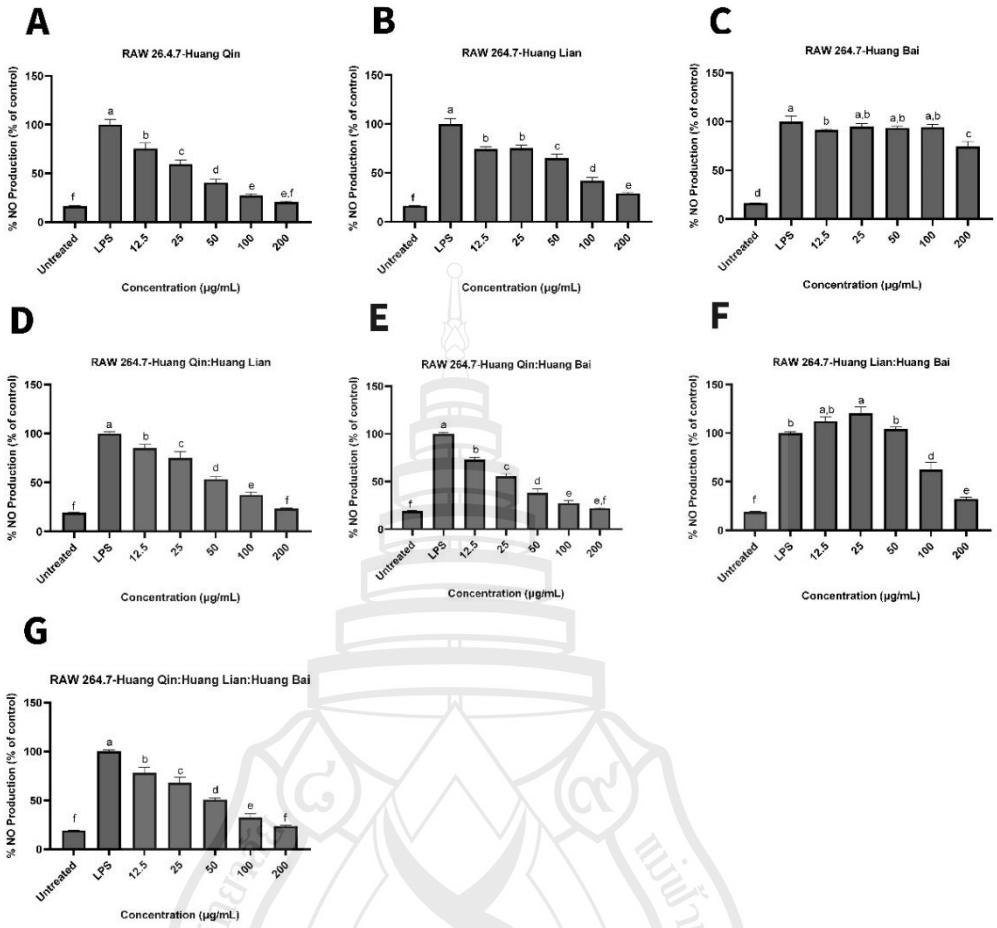


Figure 4.12 The effect of Huang Qin, Huang Lian, Huang Bai, and their combinations at the concentration of 12.5 - 200 µg/mL on NO production of RAW 264.7 cells. Each bar chart is indicated as mean ± SD (n = 3). Different letters represent significant at $p < 0.05$

Table 4.6 The effect of single, paired, and triple combination of Huang Qin, Huang Lian, Huang Bai on NO production of LPS-stimulated RAW 264.7 cell

Plant extract	**% NO Production**	**IC_{50} (µg/mL)**
HQ	75.58 – 20.49	61.48 ± 9.93^{e}
HL	74.20 – 29.05	105.44 ± 2.85^{c}
HB	91.44 – 74.53	414.36 ± 1.63^{a}
HQ:HL	86.73 – 23.36	$92.31 \pm 4.43^{c,d}$
HQ:HB	73.53 – 21.84	44.88 ± 1.34^{e}
HL:HB	113.65 – 32.98	153.47 ± 6.87^{b}
HQ:HL:HB	78.18 – 23.57	78.31 ± 4.77^{d}

Note The data are represented as mean ± SD (N=3). Different letters (a-e) within each column indicate statistically significant differences ($p < 0.05$)

Table 4.7 The SE using IC_{50}value of NO production of paired and triple mixtures of Huang Qin, Huang Lian, and Huang Bai

Combination	**Expected**	**Observed**	**SI**	**Effect**
	IC_{50} (µg/mL)			
HQ:HL	83.46	92.31	0.90	Antagonistic
HQ:HB	237.92	44.88	5.30	Synergistic
HL:HB	259.9	153.47	1.69	Synergistic
HQ:HL:HB	193.76	78.31	2.47	Synergistic

Note SI > 1: Synergistic effect found, SI < 1: antagonistic effect found, and SI = 1: indicating an additive interaction

CHAPTER 5

DISCUSSIONS

Oxidative stress and inflammation are associated with various pathologies such as inflammation disorder, cardiovascular disease, diabetes, respiratory disorder, and cancer, especially aging (Gambini & Stromsnes, 2022). By that reduction, the mechanism of oxidative stress and inflammation can have a great impact on several diseases. Various plant extracts have been demonstrated to have the curative and protective effects of oxidative stress and inflammation despite their bioactive compounds such as polyphenols and alkaloids (Rodríguez-Yoldi, 2021; Santiago et al., 2021). Significantly, traditional Chinese medicine herbs, such as Huang Qin, Huang Lian, and Huang Bai, have demonstrated a great potential for antioxidant and anti-inflammatory activity, which has been used in many diseases since ancient times. Furthermore, these plants are either used singly or in pairs to treat diseases depending on the condition of the patient. In this study the antioxidant and anti-inflammatory activities of single, paired, and triple combinations were investigated, emphasizing their bioactive constituents, such as total phenolic, flavonoid, and alkaloid levels.

5.1 Determination of Bioactive Compounds

The results demonstrated that amount the individual extract, Huang Qin had greater yields of TPC and TFC, whereas Huang Lian and Huang Bai contained greater yields of TAC significantly different $p < 0.05$. As previous mentioned, flavonoids are the main bioactive compounds remaining in Huang Qin, especially wogonoside, baicalein, and oroxylin A, whereas Huang Lian had the highest level of alkaloids, significantly berberine and coptisine. While Huang Bai possessed the second highest amount of alkaloids, such as berberine and phellodendrine (Cui et al., 2016; Lv et al., 2017; Meng et al., 2018; Sun et al., 2019). However, all the combinations had greater yields of phenolics and flavonoids, especially the triple combinations of Huang Qin, Huang Lian, and Huang Bai compared to all individual extracts, except for alkaloids,

which Huang Lian had the greatest yields of TAC. This result can be predicted based on the complimentary chemicals profile found in the combinations; this could be based on the bioactive compounds of individual extracts contributing to unique compounds, which may provide a great comprehensive range of bioactive compounds when they combine. However, this study showed that Huang Lian had the highest yields of TAC and the paired Huang Lian and Huang Bai was the second highest compared to both individual and combinations. This could be based on the compounds that presented in extract which Huang Lian and Huang Bai significantly contained phenolic and flavonoid contains, whereas alkaloids were not detected in Huang Qin. In a previous study, it mentioned that the alkaloid compounds were not in significant amounts in Huang Qin (Wagner et al., 2011), hence when combine with other plants it could be effect the total amount of alkaloids.

5.2 Antioxidant Activities

The results of this study showed that Huang Qin inhibits the strongest antioxidant activity than Huang Lian and Huang Bai. This result can be predicted based on it being rich in flavonoids such as baicalin, baicalein, wogonoside, and wogonin (Jang et al., 2023; Liu et al., 2024). The flavonoids, showing that double bonds and containing phenolic hydroxyls on rings A and B are augmentors, and sugar moieties as attenuators impacting antioxidant capacities scavenge DPPH, ABTS, and FRAP (Gao et al., 2000; Li et al., 2018). Similar to previous studies, flavonoids of Huang Qin, such as baicalein, oroxylin, wogonoside, and baicaleinyl 7-*O*-sulfate, have shown antioxidant activity by the ability to scavenge free radicals, such as superoxide radicals, HO radicals, Cu^{2+}, and DPPH radicals, and inhibit linoleic acid oxidation (Huang et al., 2006; Li et al., 2015). Whereas, Huang Lian and Huang Bai are rich in alkaloid compounds, (Ma et al., 2013; Rolle et al., 2021; Xin et al., 2024), which have the capability to defend against oxidative stress by the ability to scavenge the free radical and enhance the antioxidant enzyme, such as glutathione reductase, catalase, superoxide dismutase, and hydroxyproline (Ban et al., 2011; Kim et al., 2017; Ma et al., 2013). As previous mentioned, berberine significantly contributes to the scavenging of diverse free radicals, potentially via one of the following mechanisms: (i) formal

hydrogen transfer, (ii) single electron transfer-proton transfer, and (iii) sequential proton-loss electron transfer and/or radical adduct formation. The variance depends on the hydroxy group substituents at the C-9 and C-12 positions of the berberine backbone's structure (García-Muñoz et al., 2024; Liu et al., 2021; Nam et al., 2022). The phenolic -OH group of berberine plays a crucial role enhancing free radical neutralization and promoting antioxidant activity. Furthermore, the methyl group on the benzene ring of alkaloids can neutralize free radicals by donating an electron, thereby augmenting antioxidant activity (Liu et al., 2021). However, the paired and triple combinations of Huang Qin, Huang Lian, and Huang Bai showed superior effects compared to individual extracts in antioxidant activity, especially the triple combination. This result can be estimated from the notable compounds that presented in the plants, such as flavonoids (baicalin, wogonoside) of Huang Qin and alkaloids (berberine) of Huang Lian and Huang Bai. Especially, baicalin which is the most counted, then berberine and wogonoside, respectively (Qi et al., 2019). It is predicted that different compounds can hit multiple targets, which can have a synergistic effect.

5.3 The Synergistic Scavenging Effect (SE) of the Compounds on DPPH and ABTS Radicals

There are various methods has been assigned to study the interaction of the compounds in combination therapies, such as combination indices and principle of dose equivalence (Duarte & Vale, 2022). This experiment was performing herb extract's dose effect interaction and principle of dose equivalence. This result can be found based on the ratio of the combination and mechanistic model derived from the law of mass action (Tallarida, 2011). The result demonstrated that all the plant combinations inhibited synergistic interaction in both DPPH and ABTS antioxidant assays, with SI value greater than 1. Particularly, triple combination indicated the strongest synergism, supporting that co-presence of phytochemicals from the three plants contribute antioxidant capacity more than single and individual plant. This finding may provide the potential of combining traditional medicine plants.

5.4 The Effect of Huang Qin, Huang Lian, and Huang Bai on Different Cells Viability

HaCaT cells, MRC-5 cells, L6 cells, RAW 264.7 cells, and Vero cells line was used to determine the cytotoxicity effect of the single, paired, and triple combinations of Huang Qin, Huang Lian, and Huang Bai at various concentration. The result indicated that the cell survival of each cell line was dose-dependent, with lower concentrations resulting in less inhibition of cell survival and higher concentrations leading to greater inhibition. Moreover, past research indicates a dose-dependent relationship, wherein low quantities of the extract result in reduced cell viability, while high amounts yield increased cell viability (Bzinkowska & Sarnowska, 2024; Senthilraja & Kathiresan, 2015).

The results of the study showed that HaCaT cells, MRC-5 cells, L6 cells, and Vero cells were intended to have greater toxicity than Raw 264.7 cells, whose cell survival was significantly reduced with low concentrations. This could be predicted based on RAW 264.7 cells being macrophage-like cells, which can function as immune responses. They act as phagocytes, which capture the pathogen, antigen presentation cell, and secretion of pro-inflammatory mediators. (Kong et al., 2019; Rhule et al., 2006; Yang et al., 2016). Hence making them less sensitive compared to other cells.

The results showed that at the concentrations of 12.5 to 200 µg/mL, all mixtures of extracts were more toxic to the L6 cells and Vero cells than single extracts. However, all the extracts were not affecting the cell viability of RAW 264.7 cells, except for the mixture of Huang Lian and Huang Bai, which less toxic at 200 µg/mL. Similarly to previous study which mentioned that cytotoxic effect of the mixture of plant extract was based on the cell line (Silva et al., 2024). Various bioactive compound such as alkaloids and flavonoids could affect differently approaching apoptotic signaling mechanisms (Sun et al., 2019), which may making them highly impact the cell survival of cell model.

5.5 Anti-inflammatory Activities

The anti-inflammatory effect of single, paired, and triple combination of this study was evaluated by measuring nitric oxide (NO) production in LPS-stimulated macrophage RAW 264.7 cells. Our finding demonstrated that among the single extract, HQ inhibited the highest NO production with the IC_{50} of 61.48 ± 9.93 μg/mL, which also aligns with previous study which mentioned that high flavonoids contents, such as baicalin, baicalein, wogonoside, and chrysin are the most common presented in Huang Qin, may potentially high impact on anti-inflammatory activity by suppress iNOS expression via the NF-κB pathway (Huang et al., 2006; Xu et al., 2019; Yoon et al., 2009). On the other hand, HB demonstrated the lowest effect to inhibit NO production with IC_{50} of 414.36 ± 1.63 μg/mL. This effect could come from alkaloid compounds, which are the main bioactive chemicals in HB, such as coptisine, berberine, and palmatine. In previous study reported that these substances potentially reduce pro-inflammatory mediators such as NO and IL-6 by their ability to inhibit iNOS. This reaction involved the upstream mechanism of inflammation pathway, particularly NF-κB and MAPKs pathway (Choi et al., 2013; Lee et al., 2008; Shi et al., 2024; Wu et al., 2016).

In the combination showed that paired mixture of HQ:HB demonstrated the strongest NO production inhibition among all treatment, with IC_{50} of 44.88 ± 1.34 μg/mL, which also indicated synergistic interaction with the Synergistic Index (SI) of 5.30. However, it is not significantly different from HQ alone. This result suggested that while HB alone is weak, when combined with HQ, this could contribute to anti-inflammatory capacity and this reaction could come from complimentary mechanisms of action between flavonoids and alkaloids, as multiple bioactive compounds can approach various targets (Zhou, Xian et al., 2016). In a previous study, it was mentioned that the Huanglian Jiedu Decoction (HLJDD) (Huang Qin, Huang Lian, Huang Bai, and Zhi Zi) provides better antioxidant and anti-inflammation effects than berberine alone. Flavonoids are the major function in the inflammation defense of HLJDD, and the collaboration of flavonoids and alkaloids might decrease adverse effects of overuse of alkaloids and iridoids, which notably influence the anti-inflammation response of alkaloids (Lv et al., 2017). Furthermore, the alkaloids and flavonoid aglycones of

HLJDD, showed the anti-inflammatory response via the reduction of NO and IL-6 in LPS-stimulated RAW cells (Lu et al., 2011).

The triple combination (HQ:HL:HB) also demonstrated strong inhibitory effect, with an IC_{50} of 78.31 ± 4.77 μg/mL and SI of 2.47, indicating a synergistic interaction. This interaction may provide the evidence to support multi-herbal of Traditional Chinese Medicine (TCM) could hit multi-target of inflammatory pathway and enhance bioactivity superior therapeutic effect.

CHAPTER 6

CONCLUSION

The current study indicated that bioactive compounds of Huang Qin, Huang Lian, and Huang Bai are linked with bioactivities. The result showed that the combinations contained higher phenolic and flavonoid content than the singles extract. However, Huang Lian remained the greatest total alkaloid content (TAC) than all extracts, while it was detectable in Huang Qin. Hence, when combined with Huang Qin, it could reduce the alkaloid content of other extracts. The paired and triple mixtures indicated stronger antioxidant capacity than the individual extract, as indicated by the DPPH, ABTS, and FRAP assays, especially the triple combination. The cell survival of HaCaT, MRC-5, L6, RAW 264.7, and Vero cells were inhibited in a dose-dependent manner. The experiment demonstrated that Huang Qin inhibited the strongest toxicity than other single herbs. Additionally, the combinations shown increased toxicity to the viability of L6 and Vero cell lines in comparison to the individual extracts, with the exception of RAW 264.7 cells, where all extracts exhibited less toxicity and no harmful impacts on the cells across all the treatments.

Anti-inflammatory activity was evaluated by inhibition of nitric oxide (NO) production in LPS-stimulated RAW 264.7 macrophage cells, demonstrating that the combination of Huang Qin and Bai demonstrated the most potent anti-inflammatory effects, with the lowest IC_{50} value, and also confirming a synergistic effect by SI greater than 1, which was not significantly different from that of Huang Qin alone.

In summary, the triple mixture of Huang Qin, Huang Lian, and Huang Bai demonstrated the strongest antioxidant activities, while the pairing of Huang Qin and Huang Bai offers the most effective inhibition of NO production and contributes to anti-inflammatory activities.

We believe that this research would furnish the necessary information to support the development of multi-herb formulations for the advancement of supplemental products in the food, pharmaceutical, and cosmetic industries addressing oxidative stress and inflammation related.

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