BIOLOGICAL ACTIVITIES AND CHEMICAL ANALYSIS OF A THAI TRADITIONAL REMEDY CALLED TRI-PHON-THAT AND ITS PLANT INGREDIENTS EXTRACTS

BY

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ABSTRACT

Herbal medicines are currently widely utilized to treat, alleviate, or rehabilitate various ailments. However, many herbs and remedies still lack sufficient research or validation. The Tri-Phon-That remedy, comprising the roots of three herbal plants: *Zingiber zerumbet* (ZZ), *Zingiber montanum* (ZM), and *Cymbopogon nardus* (CN), is one such formulation that has not been extensively studied. This remedy has documented properties for resolving fever, reducing swelling, and alleviating muscle pain. Previous research has indicated its efficacy in reducing pain in rats' hind paws and alleviating lower back pain in patients through oil application. Nevertheless, studies concerning the mechanisms of reducing pro-inflammatory mediators and antipyretic effects remain limited. Therefore, this study aimed to investigate the anti-inflammatory activity by examining the reduction of various mediators involved in inflammation, including nitric oxide, IL-6, PGE_2, and TNF-α. Additionally, the study explored the antibacterial activity against potential fever-inducing bacteria, analyzed the chemical constituents of the remedy extract and its individual herbal components, and assessed

the stability under accelerated conditions. Furthermore, due to the concern of cytotoxicity and skin irritation associated with CN, a comparative study was conducted between CN and *Cymbopogon citratus* (CC) as a substitute in remedy. The findings revealed that the extracts of the remedy and its individual herbal components obtained through maceration with 95% ethanol exhibited superior antibacterial and anti-inflammatory activities compared to those extracted via decoction.

Regarding antibacterial activity, the ethanolic extracts demonstrated inhibitory effects against bacteria, while the aqueous extracts showed no such activity. The Tri-Phon-That remedy extract and its individual herbal components exhibited greater inhibitory effects against gram-positive bacteria (MIC = 39–2500 µg/mL) compared to gram-negative bacteria (MIC > 5000 µg/mL). Furthermore, the extracts effectively inhibited *Streptococcus pyogenes*, a significant causative agent of fever, with MIC values ranging from 39 to 312.5 µg/mL, and demonstrated good inhibitory activity against *Streptococcus pneumoniae* with MIC values ranging from 39 to 625 µg/mL.

Regarding anti-inflammatory activity, the ethanolic extracts of the formulation containing *C. nardus* (TPECN) and the formulation containing *C. citratus* (TPECC) exhibited significant inhibitory effects on nitric oxide, IL-6, and PGE_2 dose-dependently. The IC_{50} values of TPECN are 5.35 ± 0.77, 7.48 ± 0.26, and 24.75 ± 0.33 µg/mL, respectively. The IC_{50} values of TPECC are 7.02 ± 0.61, 10.46 ± 0.08, and 17.50 ± 0.03 µg/mL, respectively. Conversely, TNF-α inhibition was observed for both formulations, but exhibited IC_{50} values exceeding 100 µg/mL. Considering the cytotoxicity, it revealed that TPECN and TPECC showed cytotoxicity above 25 and 50 µg/mL, with safe concentrations at 30.81 ± 12.55 and 60.41 ± 11.40 µg/mL, respectively.

Chemical analysis of the remedy extract and its herbal components revealed compound D, DMPBD, and zerumbone as key constituents, abundant in ethanolic extracts of the remedy. Zerumbone had the highest content, followed by DMPBD and compound D. They have 13.31 ± 0.87, 4.6 ± 0.30, and 3.16 ± 0.17 % w/w,

respectively, for TPECN extract. The TPECC extract has 10.06 ± 0.31, 3.05 ± 0.08, and 2.38 ± 0.02 % w/w, respectively. Furthermore, the ZM ethanolic extract contained substantial amounts of compound D and DMPBD, quantified at 6.97 ± 0.28 % w/w and 8.58 ± 0.24 % w/w, respectively. Zerumbone was most concentrated in the ZZ ethanolic extract, measuring 36.05 ± 1.67 % w/w. Conversely, the chemical analysis of the CN and CC extracts in this study did not detect those chemical constituents investigated. Stability testing TPECN and TPECC under accelerated conditions for six months indicated a decline in anti-inflammatory activity, suppressing NO and IL-6 activity. This corresponded with a significant reduction in DMPBD and zerumbone levels, as well as a decrease in compound D levels.

In conclusion, this study suggests that *C. citratus* may replace *C. nardus* in the Tri-Phon-That remedy. Further research is warranted to comprehensively evaluate the remedy's cytotoxicity and long-term storage stability to preserve bioactive components and sustained anti-inflammatory efficacy. Additionally, future studies should assess the long-term safety, cytotoxicity, and stability of these formulations under various conditions and their potential therapeutic benefits in clinical applications.

Keywords: Tri-Phon-That, Anti-inflammation, Anti-bacterial activity, Chemical analysis, Stability testing, Cytotoxicity activity

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Natthakan Chitkrachang

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LIST OF ABBREVIATIONS

Symbols/Abbreviations	Terms
IL-1β	Interleukin-1β
IL-6	Interleukin-6
TNF-α	Tumor necrosis factor-α
COX-2	Cyclooxygenase-2
PGE2	Prostaglandin E2
TP	Tri-Phon-That remedy
NOX	NADPH oxidase
iNOS	Inducible nitric oxide synthase
COX	Cyclooxygenase
MTT	3-(4,5-dimethyl-2-thiazolyl)-2-5, diphenyl-2H-tetrazolium bromide
BHI broth	Brain Heart Infusion broth
BHI agar	Brain Heart Infusion agar
DMEM	Dulbecco' modified eagle medium
DMSO	Dimethylsulfoxide
LPS	Lipopolysaccharide from *Escherichia coli*
MHB	Mueller Hinton Broth
NA	Nutrient Agar
PBS	Phosphate-buffered saline

CHAPTER 1
INTRODUCTION

1.1 Introduction

Thai traditional medicine involves the knowledge, skills, and practices based on traditional scriptures, theories of folk doctors, beliefs, and experiences from different cultures (Disayavanish & Disayavanish, 1998). Its purpose is to promote well-being and protect against, identify, enhance, or treat both mental and physical conditions. Thai traditional medicine frequently uses herbal remedies to treat and prevent illnesses, enhance health, and enhance both longevity and quality of life (Kanjanahattakij et al., 2019; Barkat et al., 2021). Herbal therapy is now widely recognized as a viable healthcare option for treating a range of ailments, including diarrhea, skin problems, and infections (Shenefelt, 2011; Prempeh, Akwetey, Ankamah, Amofah-Serwaa, & Bekoe, 2024). Moreover, scientific approaches are applied to examine and create innovative pharmaceuticals derived from traditional herbal medicine formulations as substitutes for conventional therapies (Kwon, Jin, & Cho, 2019). The Thai herbal composition, known as Tri-Phon-That remedy (TP), is composed of three rhizomes of medicinal plants: *Zingiber zerumbet*, *Zingiber montanum*, and *Cymbopogon nardus* (Foundation for the Promotion of Thai Traditional Medicine, 2005). Thai traditional scripture explicitly states that it possesses antipyretic, anti-inflammatory, and swelling and bruising alleviation properties. The traditional method of preparing TP entails boiling its constituents in equal proportions and using it as an internal drug for treating illness. Thai traditional theory explains that inflammation in the body can arise from both internal and external factors, as well as behavioral factors. Internal and behavioral factors, such as excessive physical activity, chronic illness, and dietary habits, contribute to an increase in the fire element (Chiranakkhee), ultimately resulting in the body's decline. The external factors related to the presence of the condition include infection (Ki-mi-chat) and the impact of the environment on the fire elements (Santappakkhee). The increased levels of these fire elements

stimulate symptoms such as elevated body temperature, inflammation, and increased redness in the affected area with pathology. Inflammation is the result of the retention of the wind element (Angkhamangkhanusareevata) in that location. This results in discomfort and suffering throughout the entire body. In addition, the wind element continues to impact the water element (Lohitthang), resulting in swelling. Finally, it could have an impact on earth elements such as Mangsang and Naharoo (as shown in Figure 1.1). Therefore, it reactivates the illness mechanism. Inflammation, pain, and fever are immunological responses triggered by bacteria, antigens, or foreign substances. These reactions aim to kill pathogens while promoting tissue repair and recovery. Microorganism infections, particularly bacterial infections caused by *Shigella dysenteriae*, *Salmonella* Typhi, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and *Streptococcus pneumoniae*, are the most common causes of inflammation (El-Radhi, 2018).

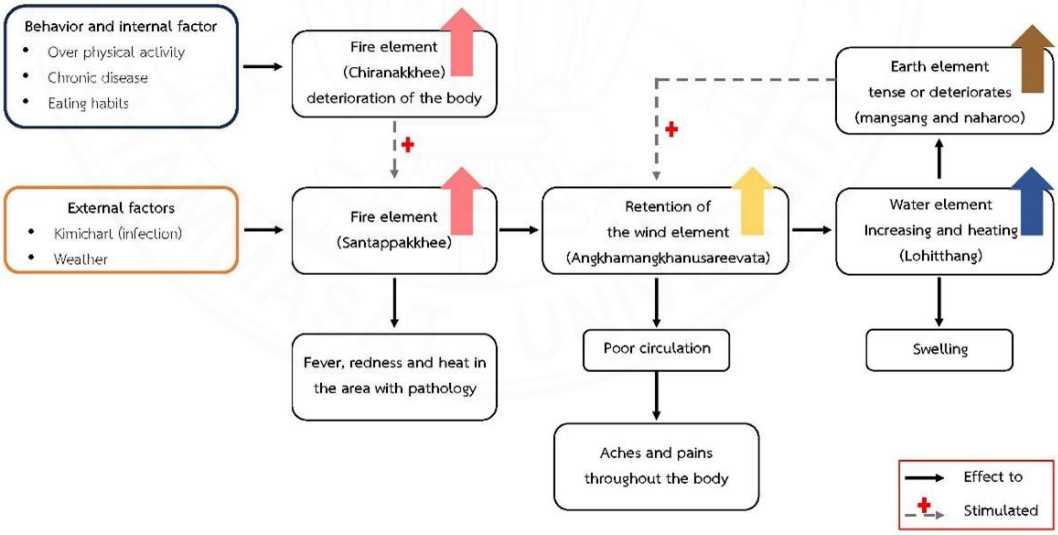

Figure 1.1 Thai traditional theories causing inflammation, fever, and pain

Following immunological sensitization, the immune system generates chemokines and inflammatory cytokines, such as nitric oxide, interleukin-1ß (IL-1ß), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), cyclooxygenase (COX)-2, and

prostaglandin E2 (PGE_2) (Chen et al., 2018; Furman et al., 2019). These cytokines initiate an inflammatory response, resulting in pain, swelling, redness, and an elevated body temperature or fever. Inflammation can progress from acute to chronic, resulting in tissue damage, persistent pain, and difficulty with treatment (Voscopoulos & Lema, 2010).

Although TP has been traditionally used in Thai medicine to treat pain, inflammation, and fever, scientific investigations into its biological activities and chemical composition remain limited. The previous report revealed that TP's aqueous and ethanolic extracts are antinociceptive in rats using the hot-plate, formalin, and acetic acid-induced writhing tests (Deeoun, Phuneerub, Palanuvej, Ruangrungsi, & Towiwat, 2014). There have been no scientific reports on the anti-inflammatory, antipyretic effects or chemical analysis of TP. On the other hand, many previous reports of TP plant ingredients show anti-inflammatory activity. *Z. zerumbet* contains several chemical compounds, including zerumbone, the primary compound, and another chemical constituent (Rawat et al., 2023). The 80% ethanolic extract of *Z. zerumbet* significantly reduced TNF-α, IL-1β, PGE_2, and COX-2 secretion in macrophages (Haque, Jantan, Harikrishnan, & Ghazalee, 2019). Additionally, the aqueous and ethanolic extracts of *Z. zerumbet* reduced fever in experimental animals at doses of 25, 50, and 100 mg/kg (Yob et al., 2011). Furthermore, it is antimicrobial against many pathogens such as *B. cereus, E. coli, S.* Typhi*, and P. aeruginosa* (Kader, Nikkon, Rashid, & Yeasmin, 2011). The ethanolic extract of *Z. montanum* exhibited strong inhibitory efficacy against the production of nitric oxide and PGE_2 (Jaiaree, Itharat, & Ruangnoo, 2016). According to Panthong A et al. (1997), component D, the active component found in *Z. montanum*, can lower body temperature when administered at a dose of 75 mg/kg. The composition of *Cymbopogon nardus* includes citronellal, geraniol, citral, nerol, and elemol (Phuneerub, 2014; Kaur, Bhardwaj, & Kaur, 2021). Citral exhibited a substantial antipyretic effect in rats, reducing both body temperature and the levels of TNF-α and IL-1β in the hypothalamus. This effect was observed at 300 mg/kg (Emilio-Silva et al., 2020). Thus, TP may potentially decrease the production

of inflammatory cytokines and prevent the growth of disease-causing bacteria that lead to fever.

Z. zerumbet and *Z. montanum* demonstrate toxicity at dosages of 3,000 mg/kg and 1,125 mg/kg, respectively, when subjected to toxicity tests. (Chang, Tzeng, Liou, Chang, & Liu, 2012; Koontongkaew et al., 2014). The essential oil derived from *C. nardus* has demonstrated no risk to animals regarding their longevity and reproductive capabilities. However, it has been found to be irritating to the skin, eyes, and can cause sensitization when applied topically (European Food Safety Authority et al., 2024). Therefore, the application of a food plant to replace *C. nardus* is interesting in this investigation. *Cymbopogon citratus*, sometimes known as lemongrass, belongs to the same genus as *C. nardus*. It is frequently consumed as food and applied as a seasoning in Southeast Asia. (Do, 2004). It did not cause skin irritation (Lulekal et al., 2019). In addition, it was found that *C. citratus* presented no significant risks for a period of 90 days in individuals with chronic kidney disease (Wongmanit et al., 2023). This study compares TP's biological activity and chemical analysis between *C. nardus* and *C. citratus*. Moreover, the biological activity and chemical analysis of TP and plant ingredients that are anti-inflammation and antipyretic are investigated.

1.2 Research problems

1.2.1 Do Tri-Phon-That remedy and its plant ingredient extracts show the antibacterial-related fever caused?

1.2.2 Do Tri-Phon-That remedy and its plant ingredient extracts show anti-inflammatory activity?

1.2.3 What chemical compounds are in Tri-Phon-That remedy and its plant ingredient extracts?

1.2.4 Can *C. citratus* be used instead of *C. nardus* in the Tri-Phon-That remedy?

1.3 Aims of this study

1.3.1 To investigate antibacterial-related fever caused by Tri-Phon-That remedy and its plant ingredient extracts.

1.3.2 To investigate the anti-inflammatory activity of Tri-Phon-That remedy and its plant ingredient extracts.

1.3.3 To quantify the chemical components included in the Tri-Phon-That remedy and its plant ingredient extracts.

1.3.4 To compare the antibacterial and anti-inflammatory effects of *C. citratus* and *C. nardus* and determine whether they can be used as substitutes.

1.4 Conceptual framework

This study started with the authentication of the herbal materials, followed by quality control, following the standards and criteria established in the Thai Herbal Pharmacopoeia (THP) 2024. The herbs are then extracted using maceration and decoction techniques. The resulting extracts are evaluated for their biological activities, including antibacterial effects against fever-related pathogens and anti-inflammatory properties, assessed through the inhibition of nitric oxide (NO), tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and prostaglandin E2 (PGE_2). In addition, cytotoxicity is assessed using human keratinocyte cell lines. Chemical profiling of the extracts is conducted via High-Performance Liquid Chromatography (HPLC). Based on the bioactivity results, the most potent extracts are selected for stability testing under accelerated conditions. During the stability study, both the concentrations of active compounds and the inhibitory activity against nitric oxide production are monitored. The overall workflow of the study is illustrated in the conceptual framework presented in Figure 1.2.

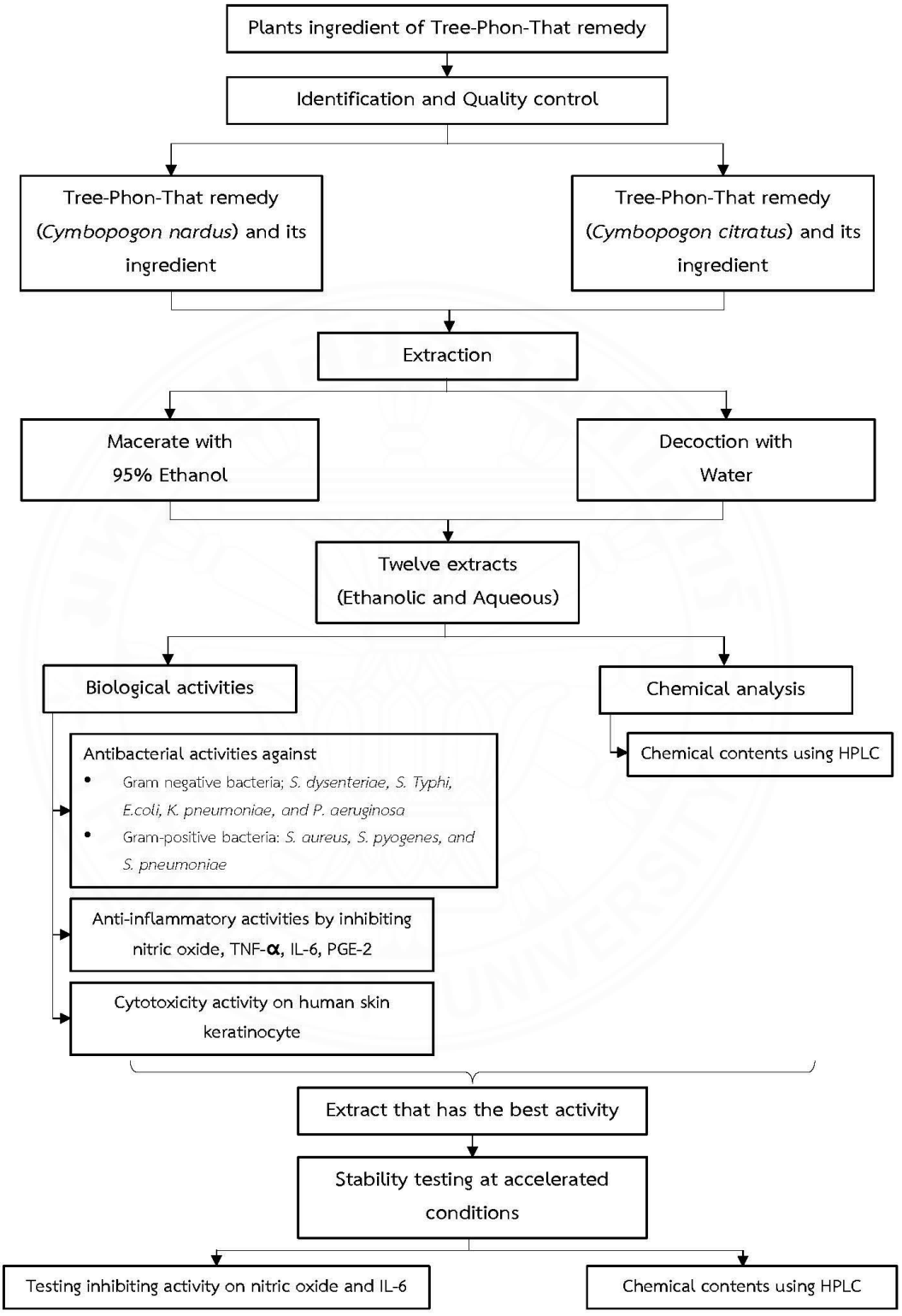

Figure 1.2 Conceptual framework of this study

CHAPTER 2
REVIEW OF LITERATURE

2.1 Inflammation

Inflammation is the immune system's response to harmful objects in the body, such as pathogens, damaged cells, and toxic compounds. The inflammatory response activates signaling pathways, releasing inflammatory cytokines, enzymes, and proteins, and also triggering immune cell responses, as illustrated in Figure 2.1.

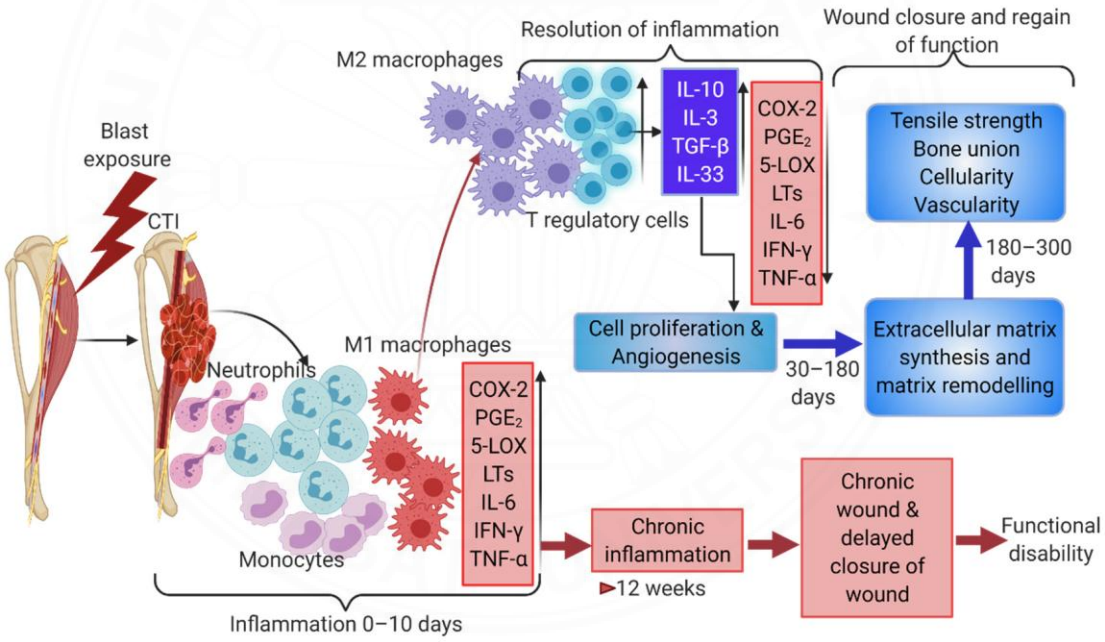

Figure 2.1 Inflammation process (Janakiram et al., 2021)

Inflammatory cytokines, including interleukin-1ß (IL-1ß), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α). Activation of inflammatory proteins and enzymes, including NADPH oxidase (NOX), inducible nitric oxide synthase (iNOS), and cyclooxygenase-2 (COX-2). The immune cell responses, including neutrophils, are followed by monocytes, lymphocytes (natural killer cells), T cells, B cells, and mast

cells. Inflammation is characterized by redness, swelling, heat or fever, and pain (Janakiram et al., 2021). These responses occur to restore tissue homeostasis and acute inflammation. However, uncontrolled acute inflammation may become chronic. Then, they cause many diseases, such as cardiovascular diseases, bowel diseases, diabetes, arthritis, and cancer. Following the above, cytokines, proteins, enzymes, and the release of immune cells can potentially serve as markers to determine the inflammatory process. (Chen et al., 2018; Menzel, 2021).

2.2 Fever

2.2.1 Definition of fever

In homeotherms (warm-blooded species), body temperature is maintained over a narrow range by both behavioral and physiologic mechanisms (Bernheim, Block, & Atkins, 1979). Normal body temperature is about 37 °C. Fever refers to a condition in which the body temperature is higher than 37.5 °C, when measured orally, or higher than 38 °C when measured rectally (Bene, 1990). The body should adapt to the balancing of behavioral and physiologic mechanisms to return the temperature to normal. Early behavioral adaptations include seeking a warmer environment or adding clothing, while physiologic mechanisms include vasoconstriction, shivering, and increasing some of the hormones to increase the body's metabolism (Aronoff & Neilson, 2001).

2.2.2 Causes of fever

There are many causes of fever, including infections and non-infectious causes. Firstly, infections are caused by viruses, bacteria, or parasites (Aronoff & Neilson, 2001). The most common infectious viruses leading to fever symptoms are influenza A and B, coronaviruses, rubella virus, and hepatitis A and B viruses. In parasites, the infection is usually transmitted by the genus *Plasmodium*, which causes malaria fever. Bacterial infections also play an important role in fever symptoms, such as *Shigella dysenteriae*, *Salmonella* Typhi, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogenes*, and

Streptococcus pneumoniae. These lead to fever and other systemic symptoms in the respiratory, gastrointestinal, and CNS systems (El-Radhi, 2018). Secondly, non-infectious causes of fever are associated with thermoregulation disorders leading to hyperthermia. CNS damage, such as subarachnoid hemorrhage, brain trauma, ischemic stroke, and hemorrhagic stroke, is an important cause of high fever. (Zawadzka, Szmuda, & Mazurkiewicz-Bełdziński, 2017). On the other hand, tissue destruction and inflammatory disorders, such as trauma and tumors, are also usually causes of fever (Aronoff & Neilson, 2001).

2.2.3 Mechanism of fever

When the body is exposed to pyrogens, it responds by sending leukocytes to the infected area, resulting in the release of various inflammatory cytokines, including Interleukin-1β (IL-1β), Tumor Necrosis Factor (TNF-α), and Interleukin-6 (IL-6). These cytokines signal to the hypothalamus in the brain, prompting the release of the enzyme cyclooxygenase (COX) and stimulating the production of prostaglandin E2 (PGE_2). PGE_2 affects the body's thermoregulatory set point, raising the normal temperature set point in response to inflammation and infection, which leads to fever, as shown in Figure 2.2 (Aronoff & Neilson, 2001).

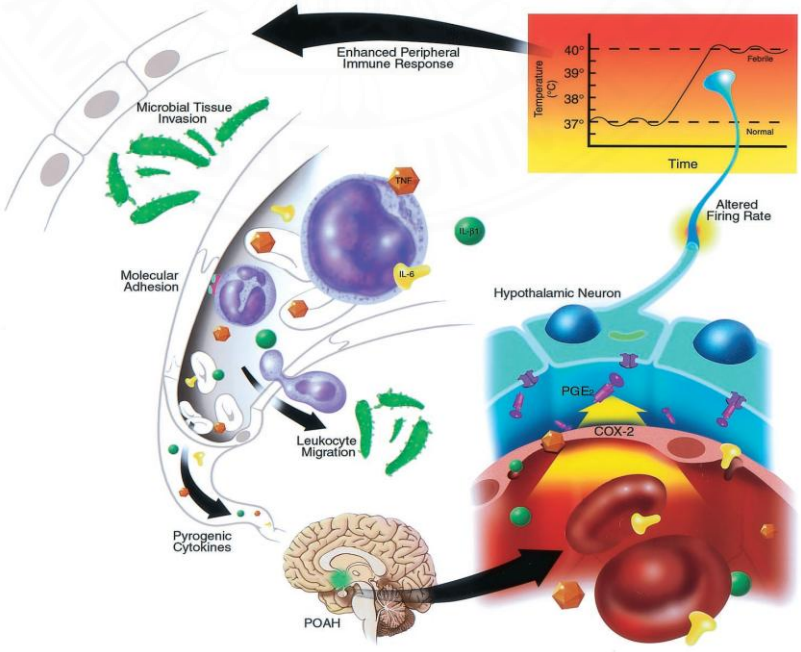

Figure 2.2 Mechanism of fever (Aronoff & Neilson, 2001).

2.2.4 Antipyretic drug

Antipyretic drugs such as acetaminophen, aspirin, and nonsteroidal anti-inflammatory drugs (NSAIDs) help reduce fever by decreasing inflammatory mediators in the peripheral nervous system of inflamed tissues and the central nervous system (the area regulating temperature). They inhibit the production of cytokines such as IL-1ß, TNF-**α**, and IL-6, as well as the adhesion molecule interactions between endothelial cells and leukocytes, or by blocking the production of COX and PGE_2 (Aronoff & Neilson, 2001).

2.2.5 Fever in Thai Traditional Medicine

According to Thai traditional medicine theory, the four elements (earth, water, wind, and fire) are balanced under normal conditions. The fever consists of symptoms such as a hot body and a sensation of internal or external heat. Additionally, it affects the mental state and all five senses: vision, hearing, smell, taste, and sensation of mind and body. A fever occurs when the fire element becomes imbalanced. These imbalances can be caused by factors such as excessive physical activity, chronic illness, dietary habits, including infection (Ki-mi-chat), and the impact of the environment on the fire elements, which includes Santappakkhee and Chiranakkhee (Lumlerdkij et al., 2020; Prommee et al., 2021). This imbalance occurs because of the previously mentioned causes. As a result, it affects the wind element (Angkhamangkhanusareevata), leading to heat retention in the body and poor blood (Lohitthang) circulation. Thus, it impacts the earth's elements (Mangsang and Naharoo), causing stiffness and abnormalities.

2.2.6 Principles for Selecting Herbs to Treat Fever

Various herbs are used to treat fevers, as mentioned in several medical texts, such as That-wi-won, Tak-ka-si-la, Chan-ta-sat, and Sap-pha-khun-ya Text. These texts describe using single herbs and herbal formulas for treatment. When analyzing traditional Thai medicine formulas for treating fever, it is found that they generally consist of ingredients derived from plants, animals, and minerals. The main ingredients are herbs with fever-reducing properties, while secondary ones are intended to aid digestion, expel wind, nourish the heart, strengthen the body, and

relieve fatigue. Consider using herbal formulas that combine multiple herbs to enhance effectiveness and address various aspects of fever. Ensure that the combination is appropriate for the individual's symptoms and condition. (Lumlerdkij et al., 2020).

2.3 Tri-Phon-That remedy

The Tri-Phon-That remedy is recorded in the book Pad-sart-song-khor. It has three rhizome components, including *Zingiber zerumbet, Zingiber montanum, and Cymbopogon nardus*, which are present in equal proportions (Foundation for the Promotion of Thai Traditional Medicine, 2005). It is used for antipyretics, anti-inflammation, swelling and bruising relief, and pain relief.

2.3.1 Biological activities of Tri-Phon-That remedy

Antibacterial activity: It has not been reported.

Anti-inflammation activity: The carrageenan-induced mouse paw edema model was used. The extract was prepared using ethanol and water fermentation. Mice treated with Indomethacin and Tri-Phon-That extract at 300 mg/kg showed a significant reduction in carrageenan-induced paw edema at 3-6 hours after the induction of swelling (Deeoun et al., 2014).

Anti-nociceptive: The extract was prepared using ethanol and water fermentation. It was found that oral administration of Tri-Phon-That extract at doses of 75, 150, and 300 mg/kg significantly alleviated pain in mice that were heated on their paws using the hot plate method and significantly reduced the number of writhing using acetic acid-induced writhing tests. In the Formalin test, the extract at 150 and 300 mg/kg doses significantly inhibited pain perception in the late phase, comparable to the standard drug Indomethacin (Deeoun *et al.*, 2014). Moreover, the study in patients with low back pain at Traditional Thai Medicine Hospital, Prince of Songkla University, revealed the significant Effect of reduced pain ($p < 0.05$) and influenced the muscular flexibility of Tri-Phon-That oil poultice treatment (Jarukitsakul et al., 2024).

Cytotoxicity: The brine shrimp were used to determine the toxicity of the Tri-Phon-That remedy. The remedy was found to be toxic to brine shrimp, with an LC_{50} of 576.66 µg/mL (Deeoun et al., 2014).

2.3.2 Plant ingredients of Tri-Phon-That remedy

2.3.2.1 *Zingiber zerumbet* (L.) Roscoe ex Sm.

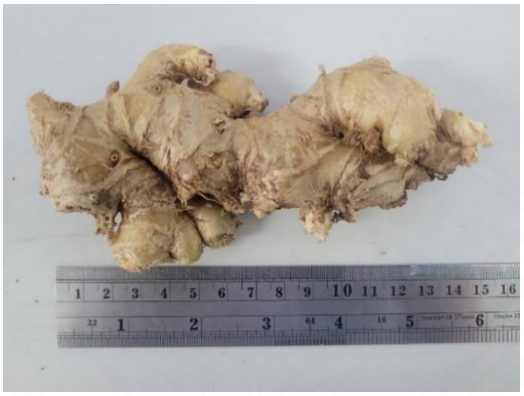

Figure 2.3 *Zingiber zerumbet* (L.) Roscoe ex Sm.

Scientific name: *Zingiber zerumbet* (L.) Roscoe ex Sm.

Family: Zingiberaceae

Used part: Rhizome

Ethnomedical use: treatment of inflammation, diarrhea, bacterial infections, fever, flatulence, allergies, joint pain, and stomach disease (Rawat et al., 2023).

Characteristic of plant: This elegant ginger is found in moist places in the forests of China, Taiwan, Cambodia, India, Laos, Malaysia, Myanmar, Sri Lanka, Thailand, and Vietnam. It is also cultivated to ornament gardens and parks. The rhizome is yellowish inside, edible, and tuberous. The stems are 1 m tall. The leaves are simple, sessile, and alternate. The ligule is entire and 1.5-2 cm long. The blade is oblong or elliptic, up to 40 cm - 310 cm, hairy below, narrow at base, and acuminate or acute at apex. The inflorescence is a conical spike which is up to 15 cm35 cm and obtuse on a 30 cm tall peduncle. The bracts are closely imbricate, glossy, obtuse, and reddish. The calyx is 2 cm long, membranaceous, and trifid. The corolla tube is 3 cm long and slender. The corolla lobes are pale yellow and lanceolate. The labellum is

yellow and 2.5 cm long. The lateral lobes are ovate and 1 cm long. The stamen is 1 cm long. The ovary is 0.5 cm long. The capsules are elliptic, 1 cm long, and enclose numerous black seeds (Wiart, 2020).

Chemical constituents: Zerumbone was found to be the major compound, and another chemical constituent was found: α-Humulene, humulene oxide-I, humulene oxide-II, β-Caryophyllene, and curzerenone (Rawat et al., 2023).

Biological activities:

Antibacterial activity:

Rhizomes: Fresh water and ethanol extracts of rhizomes can be used against *Enterococcus faecalis*, *Streptococcus mutans, Lactobacillus spp*., and *Staphylococcus spp*. using the Kirby-Bauer disk diffusion technique. Both extracts exhibited the best antibacterial activity against *S. mutans*, with the ethanol extract being more effective than the water extract, having inhibition zones of 18.2±0.28 mm and 15.5±0.5 mm, respectively. (Assiry et al., 2023). Furthermore, crude ethanol extract, petroleum ether, and chloroform fractions of the rhizome of *Z. zerumbet* had antibacterial activity within the MIC values of 128-256 µg/mL against *B. cereus, S. lBarkatutea, V. parahemolyticus, E. coli, S.* Typhi*, and P. aeruginosa* (Kader et al., 2011).

Anti-inflammation activity:

Rhizomes: The extract of 80% ethanol significantly reduced TNF-α and IL-1β secretion. Furthermore, the release of PGE_2 and COX-2 in macrophages decreased with the extract at concentrations of 4.68, 18.75, and 75 µg/mL, dose-dependent (Haque et al., 2019). Additionally, the methanol extract of *Z. zerumbet* demonstrates the ability to reverse both acute and chronic inflammation as assessed by the carrageenan-induced paw edema and cotton-pellet-induced granuloma tests. The extracts at 25, 50, and 100 mg/kg significantly reduced paw edema in rats and the weight of exudates and granuloma tissues in a concentration-dependent manner (Zakaria et al., 2010).

Antipyretic activity:

Rhizomes: The extracts from water and ethanol at doses of 25, 50, and 100 mg/kg can reduce fever in experimental animals when assessed for 8 hours using the Brewer's yeast-induced pyrexia test (Yob et al., 2011).

Anti-nociceptive activity:

Rhizomes: The antinociceptive profile of the methanol extract of *Z. zerumbet* showed significant activity at doses of ≥ 50 mg/kg in the hot plate and abdominal constriction tests. The 100 mg/kg doses produced an approximately 50% antinociceptive effect in the abdominal constriction test, comparable to a 100 mg/kg dose of acetylsalicylic acid (Zakaria et al., 2010).

Cytotoxicity:

Rhizomes: Tested for acute and sub-chronic toxicity in experimental animals at an oral dose of 3000 mg/kg/day of ethanol extracts of *Z. zerumbet* showed no significant changes in animal behavior, body weight, plasma urea, or creatinine levels, and no damage to the liver (Chang et al., 2012).

2.3.2.2 *Zingiber montanum* (J.Koenig) A.Dietr.

Figure 2.4 *Zingiber montanum* (J.Koenig) A.Dietr.

Scientific name: *Zingiber montanum* (J.Koenig) A.Dietr.

Synonym: *Zingiber cassumunar* Roxb.

Family: Zingiberaceae

Used part: Rhizome

Ethnomedical use: muscle and joint inflammation, menstrual cramping, infections, various skin ailments, and wound healing (Verma et al., 2018).

Characteristic of plant: It has a semi-erect plant growth direction, plant height 171.5-202.5 cm, diameter of pseudo-stem 15.8-18.1 mm, and the base of the pseudo-stem is reddish. The leaf consists of a blade, sheath, and petiole; the leaf blade shape is narrow-lanceolate, and green; the leaf apex is acute, the leaf base is acute; the leaf margin is entire; leaf venation is parallel (recliners), midrib is prominent on the abaxial surface. Number of leaves 46-57 blades, length of sheath 26.0-29.4 cm, length of ligule 0.3-1.0 cm, length of leaf blade 35.7-38.3 cm, leaf blade width 5.0-5.6 cm, leaf tip is acute, leaf base is acute; petiole is short, length 0.8-1.0 cm, grooves on the adaxial surface. True stem has a height of 147.0-175.8 cm, segmented into nodes and internodes with internode length of 5.7-9.8 cm. Rhizome is spherical, diameter 3.27-4.50 cm, rhizome surface is rough and brownish-yellow, rhizome flesh color is dark-yellow (Windarsih, Utami, & Yuriyah, 2021).

Chemical constituents: major components of the oils were sabinene, (E)-1-(3',4'-dimethoxyphenyl)buta-1,3-diene (DMPBD), terpinen-4-ol, γ-terpinene, β-phellandrene, (E)-1-(2',4',5'-trimethoxyphenyl)buta-1,3-diene (TMPBD), β-sesquiphellandrene, (E)-1-(3',4'-dimethoxyphenyl)but-1-ene (DMPBE), α-terpinene, β-pinene, α-pinene, *p*-cymene, α-zingiberene, (E)-4-(3,4-dimethoxyphenyl)but-3-en-1-ol (Compound D) and myrcene (Park et al., 2015; Verma et al., 2018).

Biological activities:

Antibacterial activity:

Rhizomes: The n-hexane and $CHCl_3$ extracts by Soxhlet extraction demonstrated antibacterial activity against MRSA, with MIC values from 64 to 256 μg/mL(Siddique, Pendry, & Rahman, 2019). The ethanol and 75% ethanol extracts had quite low efficiency against *E. coli,* with clear zones of 10.5 ± 1.53 and 12.0 ± 0.72 mm, respectively, and activity against *P. aeruginosa,* with clear zones of 12.0 ± 1.76 and 11.0 ± 0.58 mm, respectively (Thepthong et al., 2023). The oil of *Z. montanum* rhizome showed activities against *S.* Typhimurium*, S. epidermidis, E. coli,*

S. aureus, P. aeruginosa, S. mutans, and *K. pneumoniae*, with a zone of inhibition about 4.0 mm—13.0 mm. The MIC values ranged from 125 to 500 µg/mL, with the best activity on *S.* Typhimurium (125 µg/mL), followed by *S. epidermidis* and *E. coli* (250 µg/mL) (Verma et al., 2018).

Anti-inflammation activity:

Rhizomes: The extract reduced swelling in the ears of rats induced with ethyl phenylpropiolate in both the inner and outer ear. The Plai oil, extracted by frying with coconut oil at high temperatures (240-260 °C), significantly reduced swelling from the first hour, almost returning to normal by the fourth hour. This effect was more potent than that of Indomethacin. A 25% extract concentration was sufficient to significantly reduce swelling (Singharach, Thongpraditchote, Anantachoke, & Temsiririrkkul, 2020). The ethanolic extracts showed potent inhibitory nitric oxide production activity with IC_{50} values of 4.93+0.42 µg/mL and IC_{50} values of 7.45+0.01 µg/mL for PGE_2 inhibition (Jaiaree et al., 2016).

Antipyretic activity: It has not been reported.

Anti-nociceptive:

Rhizomes: Methanolic extracts at 3 g/kg significantly reduce the number of writhing induced by acetic acid in rats (Ozaki, Kawahara, & Harada, 1991).

Cytotoxicity:

Rhizomes: *Z. montanum* oil is safe and non-toxic to experimental rats when tested for chronic toxicity. Rats were administered *Z. montanum* oil orally at a dose of 1,125 mg/kg for 270 consecutive days, and the oil did not cause toxicity or abnormalities in the tissues of the experimental animals (Koontongkaew et al., 2014). The oral administration of *Z. montanum* capsules 200 mg/day over 12 weeks did not cause serious adverse effects or laboratory abnormalities in healthy volunteers, including complete blood count, kidney function, liver function, and fasting blood sugar (Pirompanich, Ayudhya, Koontongkaew, Poachanukoon, & Asia, 2022).

2.3.2.4 *Cymbopogon nardus* (L.) Rendle

Figure 2.5 *Cymbopogon nardus* (L.) Rendle

Scientific name: *Cymbopogon nardus (L.)* Rendle

Family: Poaceae

Used parts: essential oil, leaf, and rhizome.

Ethnomedical use: In Ayurveda, it's used in the treatment of redness, irritation, toothaches, fever, and inflammation. In Thailand and China, it is usually used to relieve the symptoms of gastrointestinal problems such as irritable bowel, stomach ache, gastritis, and flatulence (Kaur et al., 2021).

Characteristic of the plant: perennial from a stout rootstock. Culms tufted, robust, up to 2.5 m tall, 1-2 cm in diameter. Leave sheaths reddish purple at base, smooth, glabrous; leaf blades dark green or dark brown when dry, dropping for 1/3 of their length, 50-150 cm long 1-3 cm wide, glabrous, abaxial surface panicle large, narrow, congested, interrupted, 60-90 cm; spatheoles reddish brown, 1.2-2.5 cm; racemes 1-1.5 cm; rachis internodes and pedicels ciliate on margins; pedicel of homogamous pair not swollen. Sessile spikelet oblong-lanceolate, 3-4.5 x 1-1.2 mm; lower glume flat or slightly concave, reddish brown or purplish upward, sharply 2-keeled, keels narrowly winged, obscurely 0-3-veined between keels; upper lemma linear, entire or slightly 2-lobed, mucronate or very shortly awned. Pedicelled spikelet 3.5-7 mm (Nitangsam, 2012).

Chemical constituents: The chemical composition of *C. nardus* essential oil from the aerial part was studied, and its major constituents included camphene, β-caryophyllene, limonene, myrcene, terpinolene, borneol, citronellol, geraniol, linalool, piperitol, citral (*cis and trans*), citronellal, methyl heptenone, citronellic acid, piperitone, citronellyl acetate, caryophyllene oxide, geranyl acetate, geranyl butyrate, methyl eugenol, chavicol, eugenol, methyl isoeugenol, nerol, ocimene, elemol, η-propyl alcohol, 4-terpineol, menthane, α-Terpinene, α-Thujiene, α-Terpineol, α-Pinene and β-Pinene (Kaur et al., 2021). From the dried root part, the chemical constituents were revealed as elemol, α-Eudesmol, β- Eudesmol, cadinol (epi-gamma), elemene, germacrene D, cuparene, and other compounds (Phuneerub, 2014).

Biological activities:

Antibacterial activity:

Rhizome: It has not been reported.

Aerial part: The essential oil from *C. nardus* obtained from steam distillation has MIC values ranging from 0.244 µg/mL to 0.977 µg/mL against *E. coli and P. aeruginosa* (Wei & Wee, 2013).

Leaf: The application of *C. nardus* essential oil 0.12% v/v to 2% v/v inhibited human pathogens such as *Enterococcus faecalis, E. coli, K. pneumoniae, P. aeruginosa, S.* Typhimurium*, Serratia marcescens, and S. aureus* testing by broth microdilution assay. (Hammer, Carson, & Riley, 1999).

Anti-inflammation activity:

Rhizome: It has not been reported.

Aerial part: The study of the effect of IL-1ß-Induced IL-6, IL-8, and PGE_2 in Human Gingival Fibroblasts of *C. nardus* oil found a significant decrease in IL-1ß-induced IL-6 secretion at 15 µg/mL and a decrease in IL-1ß-induced IL-8 secretion in a dose-dependent manner. Whereas it had the synergistic effects of IL-1ß on the secretion of PGE_2 at 30 µg/mL ($p < 0.05$) (Ocheng et al., 2016).

Leaf: The essential oil of *C. nardus* extracted by hydro distillation exhibited a concentration-dependent inhibition of lipoxygenase type I-B.

There is inhibition at a higher concentration (2.2 mg/mL) with 25 ± 3% inhibition (Bayala et al., 2020).

Antipyretic activity: It has not been reported.

Anti-nociceptive:

Rhizome: It has not been reported.

Leaf: Essential oil from the leaf part has a significantly increased reaction time in rats, which was reported in the hot plate test and tail flick test; it was determined that it has an analgesic effect. Also, it can reduce the number of writhing in acid-induced writhing tests in rats. The most significant effect was observed with the dose of 4 ml/kg (Albaayit, Maharjan, Abdullah, & Noor, 2022).

Cytotoxicity:

Rhizome: It has not been reported.

Leaf: The essential oil from the leaf of *C. nardus* has a low cytotoxicity (IC_{50} >50 µg/mL) against the Chinese Hamster Ovary (CHO) cells and the human non-cancer fibroblast cell line (WI38) (Kpoviessi et al., 2014).

Essential oil: The study of *Cymbopogon genus* essential oil that contained citronellal showed slightly developed cardiac toxicity and shortened tails in the zebrafish model. Whereas the essential oil containing the highest amount of geraniol showed the most toxicity to the development of zebrafish (Piasecki, Biernasiuk, Skiba, Skalicka-Wozniak, & Ludwiczuk, 2021).

2.4 *Cymbopogon citratus* (DC.) Stapf

Figure 2.6 *Cymbopogon citratus* (DC.) Stapf

Scientific name: *Cymbopogon citratus* (DC.) Stapf

Family: Poaceae

Used parts: essential oil, leaf, and rhizome.

Ethnomedical use: a folk remedy for coughs, influenza, headache, arthritis, leprosy, malaria, and inflammatory disorders (Lawal, Ogundajo, Avoseh, & Ogunwande, 2017).

Characteristic of plant: It is a tufted herb that grows in tropical Asia to a height of 2 m from a rhizome. The leaves at the base form a sort of elongated bud, which is light yellow to pinkish and aromatic. The blade is lanceolate, dull light green, linear, and 15-60 cm X 1-2 cm. The inflorescences are racemes of spikelets, which are up to 2 cm long. (Wiart, 2020).

Chemical constituents: From aerial parts, *C. citratus* has been reported to contain a high percentage (about 80%) of citral, neral, and geranial. On the other hand, the rhizome oils displayed a different chemical profile. It was a high amount of selina-6-en-4-ol, α-cadinol, neointermediol, eudesma-7(11)-en-4-ol, myrcene, 4,5-epoxycarene, linalool, (S)-cisverbenol and undecan-2-one (Lawal et al., 2017; Lulekal et al., 2019)

Biological activities:

Antibacterial activity:

Rhizome: It has not been reported.

Leaf: *C. citratus* ethanol extract showed antimicrobial activity to *S. aureus* with a 7.00 ± 0.00 mm zone of inhibition, and MIC against *S. aureus* was 0.78 mg/mL (Nimrat, Soodsaweang, & Vuthiphandchai, 2018). The N-hexane, ethyl acetate, and ethanol extracts also showed antibacterial activity in *S. aureus* and *E. coli*. The highest activity was observed with hexane extract against *E. coli* with a zone of inhibition of 21 mm and *S. aureus* with a zone of inhibition of 14 mm (Hassan, Maigoro, & Shema, 2023).

Anti-inflammation activity:

Rhizome: It has not been reported.

Leaf: The leaf extract decreased the LPS-induced TNF-α mRNA levels by 64.89±5.04% by inhibiting the NF-κB activation pathway (Francisco et al., 2013).

Antipyretic activity:

Rhizome: It has not been reported.

Leaf: The leaf aqueous extract at doses of 200 mg/kg exhibited pyrexia inhibition of 95% on D-Amphetamine-induced pyrexia and 97% inhibition on brewer's yeast-induced hyperpyrexia, comparable to the standard drug, paracetamol (100%) (Tarkang et al., 2015).

Anti-nociceptive:

Rhizome: It has not been reported.

Leaf: In animals administered leaf aqueous extract, pain inhibition was observed at doses up to 600 mg/kg ($p < 0.05$) in the tail flick response. All doses of the extracts (300, 600, and 1200 mg/kg) can increase the reaction time of mice on the hot-plate response, though they have weak activity compared to piroxicam (Tarkang et al., 2015).

Cytotoxicity:

Rhizome: It has not been reported.

Leaf and stalk: In acute and sub-acute toxicity tests, rats treated with 2000 mg/kg of *C. citratus* essential oil had no mortality, and no signs of toxicity were observed (Lulekal et al., 2019).

2.5 Chemical compounds from plants ingredients of Tri-Phon-That remedy

2.5.1 Zerumbone

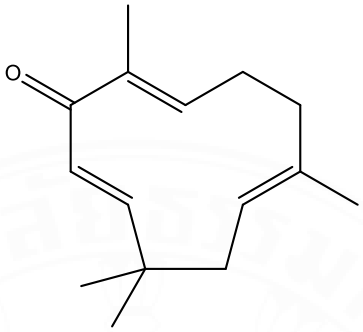

Figure 2.7 Chemical structure of zerumbone

Biological activities:

Antibacterial activity: Zerumbone can inhibit MRSA growth at 200 µg/mL; there was 80 ± 5% bacterial inhibition by the broth dilution method (Albaayit et al., 2022).

Anti-inflammation and anti-nociceptive activity: In the test of LPS-induced RAW 264.7 cells, zerumbone was obtained from the isolated rhizome of *Z. zerumbet,* inhibited inducible nitric oxide (NO) and PGE_2 production at doses 2.5-20 µM in a dose-dependent manner. At doses of 0.5-4 µM is significantly downregulated COX-2. Furthermore, it has analgesic and anti-inflammatory effects in rat experiments. on acetic acid-induced writhing response, a higher dose of 50 mg/kg of zerumbone inhibited the number of writhing, similar to morphine inhibition. In MIa-induced OA, zerumbone at doses of 5 mg/kg was orally administered to markedly reduce paw edema. Also, it significantly reduced the imbalance rate between hind-limb weights, indicating reduced joint discomfort in this model (Chien, Huang, Lee, Tsai, & Wang, 2016).

Antipyretic activity: It has not been reported.

Cytotoxicity: In the acute toxicity study, there was no death in the animals that received 100,200, 500, and 1000 mg/kg of single doses of zerumbone.

Then, the LC_{50} of zerumbone is determined to be 1.84 g/kg. The high dose of zerumbone at 500 mg/kg caused abnormal renal and hepatic function through a remarkable increase in serum creatinine and (BUN) and ALT, ALP, and GGT levels. Thus, doses of 100 and 200 mg/kg of zerumbone are safe and do not affect renal and liver function (Ibrahim et al., 2010).

2.5.2 E-4-(3′,4′-Dimethoxyphenyl) but-3-en-1-ol (Compound D)

Several compounds isolated from the rhizomes of *Zingiber montanum* also possess anti-inflammatory activity. Compound D is the major bioactive component (Khemawoot et al., 2016).

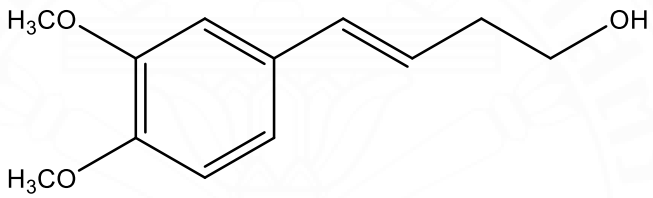

Figure 2.8 Chemical structure of compound D.

Biological activities:

Antibacterial activity: The antibacterial activity against *S. aureus, Bacillus cereus, E. coli, and P. aeruginosa* is observed in a dose of 50 µg/disc with the zone inhibition about 30.37 ± 2.85 mm, 14.67 ± 3.38 mm, 31.22 ± 3.60 mm, and 10.81 ± 47 mm, respectively. The MIC value determined the best activity against *S. aureus* and *E. coli* (32 µg/mL), followed by *B. cereus* (64 µg/mL) and (128 µg/mL) (Taechowisan, Suttichokthanakorn, & Phutdhawong, 2018).

Anti-inflammation activity: Compound D at a concentration of 100 µM tested in a human synovial sarcoma cell line, SW982, can downgrade the expression of interleukin-1ß-converting enzyme (ICE), thus degrading the newly synthesized pro-IL-1ß. Also, it can downregulate MMP-1, -2, -3, and -13, resulting in joint damage that is important in rheumatoid arthritis patients (Chaiwongsa et al., 2012).

Antipyretic activity: Compound D at a dose of 75 mg/kg can reduce fever when tested in yeast-induced hyperthermia in rats (Panthong, Kanjanapothi, Niwatananant, Tuntiwachwuttikul, & Reutrakul, 1997).

Anti-nociceptive activity: Compound D at a dose of 300 mg/kg, remarkable inhibitory activity on the writhing response of about 52.0% after acetic acid injection 1 h, as well as the standard drug aspirin (57.0%). It also produced a slight inhibition (11.7%) on the tail-flick response. (Panthong et al., 1997).

Cytotoxicity: There was no significant change after 24 h of IV or oral administration of Compound D at doses of 25 mg/kg IV and 25-250 mg/kg orally (Khemawoot et al., 2016).

2.5.3 (E)-1-(3',4'-Dimethoxyphenyl) buta-1,3-diene (DMPBD)

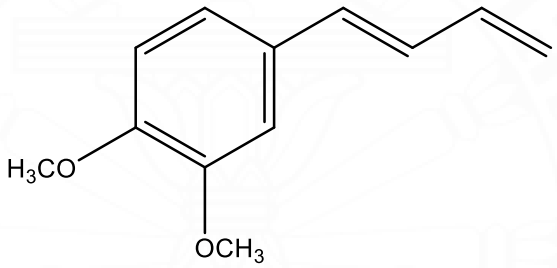

Figure 2.9 Chemical structure of DMPBD

Biological activities:

Antibacterial activity: It has not been reported.

Anti-inflammation activity: The inhibitory effect on NO production of DMPBD was reported with IC_{50} values of 56.3 mM using RAW264.7 cells (Kaewchoothong, Tewtrakul, & Panichayupakaranant, 2012). Additionally, DMPBD has reported inhibitory ear edema in rats that induced Ethyl phenylpropiolate (EPP), arachidonic acid (AA), and 12-*O*-tetradecanoylphorbol 13-acetate (TPA) with IC_{50} of 21 nmol, 60 nmol, and 660 pmol per ear, respectively. The topical application of DMPBD also showed an inhibitory paw edema induced by the carrageenan effect with an IC_{50} of 22 μmol per paw (Jeenapongsa, Yoovathaworn, Sriwatanakul, Pongprayoon, & Sriwatanakul, 2003).

Antipyretic activity: It has not been reported.

Anti-nociceptive activity: It has not been reported.

Cytotoxicity: It has not been reported.

2.5.4 Citronellal

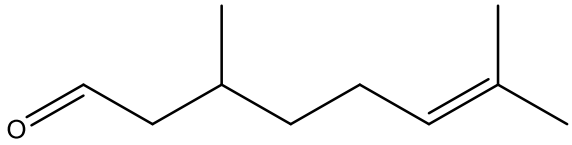

Figure 2.10 Chemical structure of citronellal

Biological activities:

Antibacterial activity: The micro broth dilution technique showed MIC values of 312.5 µg/mL on *B. cereus, S. epidermidis, Propionibacterium acnes, S. aureus, S. pyogenes, Serratia marcescens,* and *P. aeruginosa* (Dangol et al., 2023).

Anti-inflammation activity: Administration of citronellal at 50, 100, and 200 mg/kg inhibited leukocyte migration, and administration of 100 and 200 mg/kg inhibited rat hind paw edema in carrageenan-induced inflammation (Melo et al., 2011).

Antipyretic activity: It has not been reported.

Anti-nociceptive activity: citronellal 50, 100, and 200 mg/kg was recorded to reduce the number of writhing induced by Acetic acid dose-dependent with 61.4, 66.4, and 81.9% of inhibition, respectively. Comparable to the formalin test, citronellal significantly reduced the licking time in an early acute phase and a late phase (0–5 and 15–30 min after formalin injection, respectively) in a dose-dependent manner. While 100 and 200 mg/kg increased the latency time in the licking response. The higher doses have similar effects to the inhibition by morphine (5 mg/kg) (Melo et al., 2010).

Cytotoxicity: Citronellal at low concentrations (25, 50, and 75 µL/mL) did not significantly affect cell viability. However, higher concentrations above 100 µL/mL increased cell cytotoxicity towards HaCaT cells (Koba et al., 2009).

2.5.5 Citral

Citral (3,7-Dimethyl-2,6-octadienal) is a mixture of *cis*-isomer neral and *trans*-isomer geranial. It is the major constituent of the genus *Cymbopogon* (Nishijima et al., 2014).

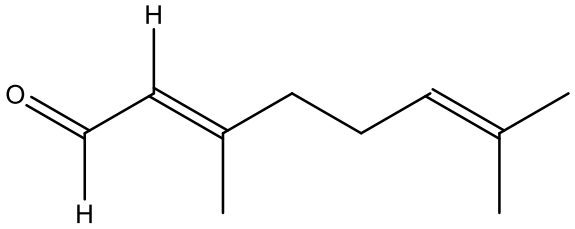

Figure 2.11 Chemical structure of citral

Biological activities:

Antibacterial activity: By disc diffusion assay, citral has the activity against *E. coli, E. coli* HB 101, *S.* Typhimurium, *Proteus vulgaris*, *P. aeruginosa*, *S. aureus*, S. *faecalis*, and *B. subtilis*. A high dose of citral (8 µl/disc) showed better activity. It has the best effects on *S.* Typhimurium and *S. aureus*, with an inhibition zone of 16-17 mm (Mangalagiri, Panditi, & Jeevigunta, 2021). The micro broth dilution technique showed the MIC values = 78.1 µg/mL on *S. aureus* and 156.3 µg/mL on *B. subtilis* and *S. pyogenes* (Dangol et al., 2023).

Anti-inflammation activity: The blood inflammatory cytokines were measured 90 minutes after inflammation was induced with LPS. The group given citral at 300 mg/kg had a reduced level of TNF-α. When measuring inflammatory substances in the hypothalamus of rats, it was found that administering citral at concentrations of 25 and 100 mg/kg can also reduce the level of IL-6 in the hypothalamus (Emilio-Silva et al., 2020).

Antipyretic activity: Administering citral at 300 mg/kg significantly reduced the rats' temperature, with the greatest reduction occurring between 60-120 minutes after LPS-induced inflammation and rise in temperature (Emilio-Silva et al., 2020).

Anti-nociceptive activity: The oral administration of citral can reduce the time spent licking the hind paw on formalin-induced nociception. It has the best inhibition activity at a dose of 300 mg/kg with 54% and 65% inhibitions in the neurogenic (0–5 min) and inflammatory (15–30 min) phases, respectively. Moreover,

citral at 100 mg/kg also reduced chronic pain in models of post-operative pain, chronic post-ischemic pain, and partial sciatic nerve ligation (Nishijima et al., 2014).

Cytotoxicity: It does not have cytotoxic to human Peripheral Blood Mononuclear Cells (PBMC) at all concentrations used to determine (2.5, 5, 10, 25, 50, 100, 250, 500, and 1000 mg/mL). it showed cell viability ≥88%. In contrast, the cytotoxic human Hepatoma cell line (HepG2) revealed that concentrations ≥5 mg/mL showed cell viability of less than 80% (Souza et al., 2020). In human lymphocytes, only citral at 800 µg/mL has cytotoxicity to cells by reducing cell viability to 75.69%. Although citral exhibited DNA damage at 100 and 25 µg/mL and above, demonstrated by increased nuclear area and the number of diffused nuclei (Sinha, Jothiramajayam, Ghosh, & Mukherjee, 2014).

2.5.6 Geraniol

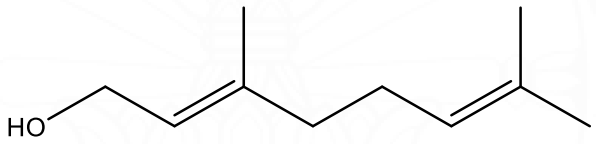

Figure 2.12 Chemical structure of geraniol

Biological activities:

Antibacterial activity: The antibacterial activity determined by disc diffusion assay reveals that geraniol has good activity against the Gram-positive bacteria (*S. aureus* and *S. faecalis*) in dose 8 µl/disc *(*Mangalagiri et al., *2021)*. The micro broth dilution technique showed MIC values of 312.5 µg/mL on *B. cereus*, *S. epidermidis*, *S. aureus*, *S. pyogenes*, *Serratia marcescens*, and *P. aeruginosa* but showed less activity on *P. acne* with MIC 625 µg/mL (Dangol et al., 2023).

Anti-inflammation activity: pre-treatment of geraniol at a concentration of 40 and 80 µmol L−1 inhibition the expression of iNOS and COX-2 and downregulated the production of TNF-**α** and IL-6 in mouse chondrocytes. Moreover, it can potentially inhibit the PI3K/Akt/NF-**K**B and MAPK signaling pathways in mouse OA chondrocytes, alleviating the inflammation and degeneration of chondrocytes (Wu, Wang, Fu, Lin, & Yu, 2020).

Antipyretic activity: It has not been reported.

Anti-nociceptive activity: Geraniol has antinociceptive activity, especially in pain related to inflammation, by reducing the number of writhes induced by acetic acid at concentrations of 12.5, 25, or 50 mg/kg intraplanar injection (i.p.) with 76.52%, 68.9%, and 90.5% inhibition and at concentrations of 50 or 200 mg/kg p.o. with 43.2% and 55.3% inhibition, respectively. Geraniol also had antinociceptive activity, but only in the second phase of the formalin test. The Effect is equal to morphine at 10 mg/kg (i.p.) (La Rocca et al., 2017).

Cytotoxicity: Geraniol at doses 25, 50, 100, 200, 400, and 800 µg/mL does not have cytotoxicity in human lymphocyte cells. Additionally, it does not show DNA damage at all concentrations (Sinha et al., 2014). Toxigenic geraniol causes embryo coagulation, yolk sac edema, slow pigmentation, and tail deformity. The lethal concentration of 50% (LC_{50}) is 31.3 µg/mL (Singulani et al., 2018).

2.5.7 Elemol

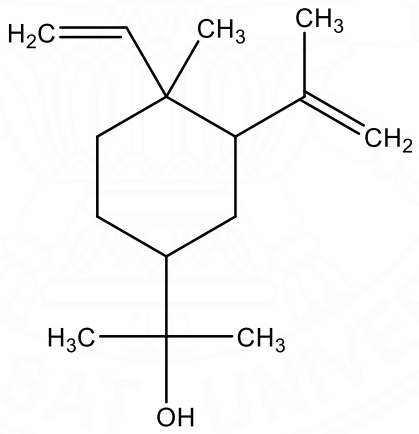

Figure 2.13 Chemical structure of elemol

Biological activities:

Antibacterial activity: It has not been reported.

Anti-inflammation activity: The expression of IL-6 and I**K**B**α** was decreased following treatment with 2, 5, and 10% elemol topical on skin lesions of Atopic dermatitis induced by 2,4-dinitrochlorobenzene (DNCB) in rats. The expression of TNF-**α** and IL-1β mRNA was decreased following treatment with elemol in a dose-

dependent manner. Applied 10% elemol can reduce serum IgE levels (resulting in reduced newly inflammatory molecules and cytokines) measured using a mouse IgE ELISA Ready-Set-Go kit (Yang et al., 2015).

Antipyretic activity: It has not been reported.

Anti-nociceptive activity: It has not been reported.

Cytotoxicity: It does not have cytotoxicity on genotoxicity, reproductive toxicity, local respiratory toxicity, phototoxicity/photo allergenicity, or skin sensitization (Api et al., 2017).

2.5.8 α - Eudesmol

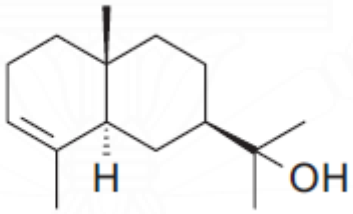

Figure 2.14 Chemical structure of α - Eudesmol

Biological activities:

Antibacterial activity: It has not been reported.

Anti-inflammation activity: It has not been reported.

Antipyretic activity: It has not been reported.

Anti-nociceptive activity: It has the effects of reducing the neurogenic inflammation in the trigemino-vascular system by blocking the Ca2+ channel, inhibiting the evoked CGRP and substance P (neuropeptides that relate to nociceptive stimulations) (Asakura et al., 2000).

Cytotoxicity: It has cytotoxicity with an IC_{50} of 9.71 ± 1.56, 10.60 ± 1.33, 5.38 ± 1.10, and 12.01 ± 1.53 μg/mL on human hepatocellular carcinoma (HepG2), human chronic myelocytic leukaemia (K562), mouse melanoma (B16-F10), and human lymphocyte cells, respectively (Bomfim et al., 2013).

2.5.9 β-Eudesmol

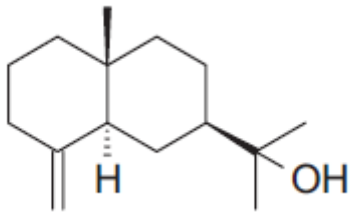

Figure 2.15 Chemical structure of β-Eudesmol

Biological activities:

Antibacterial activity: It has not been reported.

Anti-inflammation activity: β-Eudesmol in 5–100 μM can downgrade the level of ROS production in human lung A549 cells. (Sghaier, 2016)

Antipyretic activity: It has not been reported.

Anti-nociceptive activity: It has not been reported.

Cytotoxicity: It has cytotoxicity with an IC_{50} of 24.57 ± 2.75, 22.50 ± 1.04, 16.51 ± 1.21, and 19.80 ± 1.09 μg/mL on human hepatocellular carcinoma (HepG2), human chronic myelocytic leukemia (K562), mouse melanoma (B16-F10), and Human lymphocyte cells, respectively (Bomfim et al., 2013).

CHAPTER 3
RESEARCH METHODOLOGY

3.1 List of instruments, chemicals and reagents

The scientific instruments of this study are shown in Table 3.1. The chemicals and reagents that were used in this study are shown in Table 3.2.

Table 3.1 A list of scientific instruments of this study.

Description	Model	Source
Karl Fischer	CA-310	Nittoseiko Analytech, Japan
Centrifuge	Universal 320R	Hittich, Germany
CO_2 incubator	3111	Thermo scientific, USA
Microplate reader	Varioskan LUX	Thermo scientific, USA
Biosafety cabinet class II	Logic+	Lab Conco, USA
Incubator	IN 110	Memmert, Germany
Water bath	WNB14	Memmert, Germany
Vortex	G560E	Scientific industry, USA

Table 3.2 A list of chemicals and reagents of this study.

Name	Source
3-(4,5-dimethyl-2-thiazolyl)-2-5, diphenyl-2H-tetrazolium bromide (MTT)	TCI, Japan
Ethanol 95% (commercial grade)	RCI LabScan, Thailand
Ethanol HPLC grade	RCI LabScan, Thailand
Acetonitrile	RCI LabScan, Thailand
Brain Heart Infusion broth (BHI broth)	Difco, USA
Brain Heart Infusion agar (BHI agar)	Difco, USA
Chloroform	RCI Labscan, Thailand
Dulbecco' modified eagle medium (DMEM)	Gibco, USA

Table 3.2 A list of chemicals and reagents of this study. (Cont.)

Name	Source
Dimethylsulfoxide (DMSO)	RCI Labscan, Thailand
Hexane	RCI Labscan, Thailand
Hydrochloric acid (HCl) fuming 37%	Merck, Germany
IL-6 ELISA Kit	R&D Systems, USA
Lipopolysaccharide (LPS), from *Escherichia coli*	Sigma, USA
Mueller Hinton Broth (MHB)	Difco, USA
Methanol HPLC grade	RCI LabScan, Thailand
Nutrient Agar (NA)	Difco, USA
Phosphate-buffered saline (PBS)	Gibco, USA
Resazurin sodium salt	Sigma-Aldrich, USA
Sodium carbonate (Na_2CO_3)	Sigma-Aldrich, USA
Sodium phosphate dibasic	Sigma-Aldrich, USA
Sodium phosphate monobasic monohydrate	Sigma-Aldrich, USA
TNF-α ELISA Kit	R&D Systems, USA
PGE-2 ELISA Kit	Cayman, USA
0.4%Trypan blue stain	Gibco, USA
Trypsin-EDTA	Gibco, USA

3.2 Plant materials

Zingiber montanum was obtained from Chachoengsao Province, while *Z. zerumbet* was obtained from Pathum Thani Province, Thailand. The plant specimens were taxonomically identified by a specialist and subsequently deposited at the Sirindhorn Herbarium, Department of Agriculture, Bangkok, Thailand. *C. nardus* was obtained from Pathum Thani Province, Thailand. Then, it was sent to the Herbarium of Mahidol University, Mahidol University, Nakhon Pathom, Thailand, to identify the plant's taxonomy. *C. citratus* was collected from Ang Thong Province, Thailand. The

plants were identified and stored at the Thai Traditional Medicine Herbarium, Department of Thai Traditional and Alternative Medicine, in Bangkok, Thailand.

3.3 Quality controls

All plant materials were evaluated for quality, guided by the Thai Herbal Pharmacopeia (THP) (Thai Herbal Pharmacopoeia, 2024).

3.3.1 Determination of water

Water content analysis is performed in plants according to the herbal standards specified by THP. The titrimetric determination of water depends on the quantitative reaction between water and an anhydrous solution of sulfur dioxide and iodine, which are dissolved in pyridine and methanol. Hence, samples containing volatile oil can be applied in this test.

The Karl Fischer moisture meter can accurately measure plants' water content. For the first step, deionized water was dispensed into a titration flask using either a dropper bottle or a micro syringe. This water was then titrated to establish the water standard factor. After determining the factor, the plant materials were ground into a fine powder and weighed at approximately 1-2 grams. Before testing, the Karl Fischer Meter was pre-heated at 105°C for 2 minutes to eliminate any moisture present. Following the pre-heating process, the samples were inserted into the machine and placed into burning at 105 °C until a stable weight was achieved. The water content in the plants was determined using titration with an iodine solution, and the percentage of water content was automatically calculated.

Table 3.3 The criteria for accepting the water content of plant materials

Plants	% Water content
Zingiber zerumbet (L.) Smith.	Not more than 13.0 % w/w
Zingiber montanum (Koening) Link ex Dietr.)	Not more than 11.0 % w/w
Cymbopogon nardus Rendle.	Not Defined
Cymbopogon citratus (DC.)	Not Defined

3.3.2 Total ash

Each plant's powder was precisely measured to 1 gram and placed in a crucible. Firstly, it was burned at 450 ° C until it was carbon-free. It will then be permitted to cool within the desiccator. After that, it was weighed, and the percentage of total ash was calculated using the formula below. The total ash will be evaluated against the standards outlined in the THP, as presented in Table 3.4.

$$\%\ \text{Total ash} = \left(\frac{\text{Weight of sample after burned (g)}}{\text{Weight of sample before burned (g)}}\right) \times 100$$

Table 3.4 The criteria for accepting the total ash content of plant materials

Plants	% Total ash
Zingiber zerumbet (L.) Smith.	Not more than 10 % w/w
Zingiber montanum (Koening) Link ex Dietr.)	Not more than 9 % w/w
Cymbopogon nardus Rendle.	Not Defined
Cymbopogon citratus (DC.)	Not Defined

3.3.3 Acid insoluble ash

The total ash of each plant was boiled with 25 mL of 10% hydrochloric acid for 5 minutes. Then, it was filtered through ashless filter paper and washed with hot deionized water until the filtrate was neutral. Then, the filter paper was collected and brought to ignite at 500 °C in a crucible until it reached a constant weight. The weight was recorded and calculated using the formula below. The sample

values were compared with the standard criteria for each plant in THP, as shown in Table 3.5.

$$\%\ \text{Acid insoluble ash} = \left(\frac{\text{Weight of acid insoluble ash (g)}}{\text{Weight of samples (g)}}\right) \times 100$$

Table 3.5 The acceptance criteria for acid-insoluble ash of plant materials

Plants	% Acid insoluble ash
Zingiber zerumbet (L.) Smith.	Not more than 5 % w/w
Zingiber montanum (Koening) Link ex Dietr.)	Not more than 3 % w/w
Cymbopogon nardus Rendle.	Not Defined
Cymbopogon citratus (DC.)	Not Defined

3.3.4 Extractive value

3.3.4.1 Ethanol soluble extractive value

The coarse powder of plant materials was weighed accurately at 5 g and macerated with 100 mL of ethanol in a closed flask. The flask was shaken frequently during the first 6 hours and allowed to stand for 18 hours. After that, the extract was filtered through a 0.45 µm filter paper, and 20 mL of the solution was transferred to an evaporating dish and evaporated in a hot air oven at 105 °C until a stable weight. The percentage of ethanol-soluble extractive values was calculated and compared to the THP standard. The standard criteria of each plant are shown in Table 3.6.

$$\%\ \text{Ethanol soluble extractive} = \left(\frac{\text{weight of extract (g)}}{\text{weight of plant powder (g)}}\right) \times 100$$

Table 3.6 The acceptance criteria for ethanol-soluble extractive values of plant materials

Plants	% Ethanol soluble extractive value
Zingiber zerumbet (L.) Smith.	Not less than 2 % w/w
Zingiber montanum (Koening) Link ex Dietr.)	Not Defined
Cymbopogon nardus Rendle.	Not Defined
Cymbopogon citratus (DC.)	Not Defined

3.3.4.2 Water soluble extractive value

The water-soluble extractive value was assessed using a similar method to the ethanol-soluble extractive, with the only modification being the replacement of ethanol with chloroform and water. The extractive value was calculated using the prescribed formula and then compared to the standard provided in the THP, as indicated in Table 3. 7.

$$\%\text{Water soluble extractive} = \left(\frac{\text{weight of extract (g)}}{\text{weight of plant powder (g)}}\right) \times 100$$

Table 3.7 The acceptance criteria for water-soluble extractive values of plant materials

Plants	% Water soluble extractive value
Zingiber zerumbet (L.) Smith.	Not less than 9 % w/w
Zingiber montanum (Koening) Link ex Dietr.)	Not Defined
Cymbopogon nardus Rendle.	Not Defined
Cymbopogon citratus (DC.)	Not Defined

3.3.4.3 Hexane soluble extractive value

The hexane-soluble extractive value was determined using the same procedure as the ethanol-soluble extractive, with the only difference being the replacement of ethanol with hexane. This approach is employed to determine the extractable potency of *Zingiber montanum*. The value was calculated with the

following formula and then compared to the THP standard, which must be equal to or greater than 3% w/w.

$$\%\ \text{Hexane soluble extractive} = \left(\frac{\text{weight of extract (g)}}{\text{weight of plant powder (g)}}\right) \times 100$$

3.3.4.4 Chloroform soluble extractive value

The chloroform-soluble extractive value was determined using the same procedure as the hexane-soluble extractive, with the only difference being a replacement of ethanol with chloroform. This approach was used to determine the extractive activity of *Zingiber montanum*. The value was determined by applying the following formula and then compared to the standard in THP, which must be equal to or more than 5% w/w.

$$\%\ \text{Chloroform soluble extractive} = \left(\frac{\text{weight of extract (g)}}{\text{weight of plant powder (g)}}\right) \times 100$$

3.3.5 Heavy metal contamination (Kalagbor & Opusunju, 2015)

The concentration of heavy metals in accordance with the THP must remain within the specified limits, as excessive intake can pose significant health risks. The heavy metal solution was prepared by boiling the total ash with 25 mL of 10% hydrochloric acid for 5 minutes. The resulting mixture was filtered through ashless filter paper, rinsed with hot deionized water, and subsequently stored in a centrifuge tube. The concentrations of arsenic and other heavy metals were then determined using atomic absorption spectrometry (AAS) and compared against the permissible limits specified for each plant in the THP (Thai Herbal Pharmacopoeia, 2024), as presented in Table 3.8.

Table 3.8 The acceptance criteria for heavy metal contamination of plant materials

Heavy metal	Criteria
Lead (Pb)	Not more than 4 ppm
Arsenic (As)	Not more than 0.3 ppm
Cadmium (Cd)	Not more than 10 ppm

3.4 Preparation of plant extracts

The rhizomes of *Z. montanum*, *Z. zerumbet*, *C. nardus*, and *C. citratus*. will be cleaned and dried at 45 °C in a hot air oven until dry. Then, they will be weighed equally and pounded into small pieces. The plants will be separated into 2 groups. First, the Tri-Phon-That remedy is the same as the book. Second, the Tri-Phon-That remedy will be applied to *C. citratus* instead of *C. nardus*. The remedy and its plant ingredients will be extracted by decoction and maceration.

For the decoction, the botanical specimens will undergo a process of boiling in water for 15 minutes. Subsequently, heat all the refined solutions until only one portion remains, and desiccate it using a freeze dryer.

An additional part of the plant components will be macerated using 95% ethanol. The plant materials will undergo maceration for 72 hours, after which they will be filtered and concentrated using a rotary evaporator. The residue will undergo two more cycles of maceration and will be subjected to drying at 45 °C in a hot air oven until its constant weight.

The extracts will be evaluated for yield and kept at a temperature of -20℃ until the activity is tested.

$$\%\text{Yield} = \left(\frac{\text{weight of extract (g)}}{\text{weight of plant powder (g)}}\right) \times 100$$

3.5 Antibacterial activities

3.5.1 Tested microorganisms

In the present study, Tri-Phon-That remedy and plant ingredient extracts were investigated for antibacterial activity against five gram-negative bacteria, including *Shigella dysenteriae* DMST15110, *Salmonella* Typhi DMST22842, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC700603, and *Pseudomonas aeruginosa* ATCC9027, and three gram-positive bacteria, including *Staphylococcus aureus* ATCC25923, *Streptococcus pyogenes* ATCC19615, and *Streptococcus pneumoniae* ATCC49619.

S. aureus, *S. dysenteriae*, *S.* Typhi, *E. coli*, *P. aeruginosa*, and *K. pneumoniae* were subcultured on nutrient agar (NA), while *S. pyogenes* was subcultured on brain heart infusion (BHI) agar and incubated at 37 ºC for 18-24 h. However, in anaerobic conditions, *S. pneumoniae* was cultured on BHI agar and incubated at 37 ºC for 18-24 h.

3.5.2 Determination of minimum inhibitory concentration (Sarker, Nahar, & Kumarasamy, 2007)

The broth microdilution method was employed to evaluate the antibacterial activity of the extracts against various bacterial strains following their cultivation under optimal conditions. The maximum concentration tested for both ethanolic and aqueous extracts was 5 mg/mL. Ethanolic extracts were dissolved in dimethyl sulfoxide (DMSO), while aqueous extracts were dissolved in deionized (DI) water and subsequently sterilized using a 0.22 µm syringe filter. Serial two-fold dilutions of each extract were prepared accordingly.

For *S. pneumoniae* and *S. pyogenes*, dilutions were performed in brain heart infusion (BHI) broth. In contrast, Mueller-Hinton broth was used for *S. aureus*, *S. dysenteriae*, *S.* Typhi, *E. coli*, *P. aeruginosa*, and *K. pneumoniae*. Each dilution (50 µL) was dispensed into a 96-well microtiter plate. Bacterial suspensions were standardized to a turbidity equivalent to 0.5 McFarland and subsequently diluted 1:100 with the corresponding broth media before being added to the wells (50 µL per

well). Plates were incubated at 37°C for 24 hours in a shaker incubator. For *S. pneumoniae* and *S. pyogenes*, incubation was conducted under anaerobic conditions at the same temperature and duration. Following incubation, 10 µL of resazurin solution was added to each well, and plates were further incubated for 2 hours. Bacterial growth was assessed by observing the color change of resazurin from blue to pink. The minimum inhibitory concentration (MIC) was defined as the lowest concentration of extract at which no color change was observed. All experiments were conducted in triplicate.

3.5.3 Determination of minimum bactericidal concentration (Elshikh et al., 2016)

After determining the MIC, the minimum bactericidal concentration testing was performed. The sample solution that does not change the resazurin color was pipetted onto the NA and BHI agar at a volume of 3 µL per well. The plate was incubated under the appropriate conditions for each of the bacteria, and then the growth of the bacteria was observed. The lowest concentration with no bacterial colony was defined as the minimum bactericidal concentration (MBC). The experiment was performed in triplicate.

3.6 Anti-inflammatory activities

3.6.1 Cell culture

The anti-inflammatory activities were performed using a murine macrophage cell line, RAW264.7 cells, ATCC TIB-71. Cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 10,000 units/mL of penicillin, and 10,000 µg/mL of streptomycin (complete DMEM) at 37 °C with 5% CO2 control. Cells were trypsinized using 0.25% trypsin for 10 min every 4-5 days.

3.6.2 Cell viability assay on RAW264.7 cells

Prior to the assessment of anti-inflammatory activity, the cytotoxicity of the samples on RAW264.7 cells was evaluated using the MTT assay (Kumar, Nagarajan, & Uchil, 2018). Initially, RAW264.7 cells were seeded into 96-well plates at

a density of 1 × 10^5 cells/well (100 µL/well) and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. Following incubation, the ethanolic extract was dissolved in dimethyl sulfoxide (DMSO) and adjusted to a 50 mg/mL concentration. In contrast, the aqueous extract was dissolved in deionized (DI) water, adjusted to 10 mg/mL, and sterilized through a 0.22 µm syringe filter. Both extracts were subsequently diluted using two-fold serial dilutions in complete DMEM.

The culture medium in each well was replaced with 100 µL of fresh medium, followed by the addition of 100 µL of the respective diluted sample solutions. The plates were incubated again for 24 hours under the same conditions. After incubation, the supernatant was removed, and 100 µL of 0.5 mg/mL MTT solution was added to each well. The plates were incubated for an additional 2 hours at 37°C with 5% CO_2. The MTT solution was then removed, and 100 µL of DMSO was added to each well to dissolve the formazan crystals. The absorbance was measured at 570 nm using a microplate reader. The cell viability was calculated using the specified formula. A survival rate exceeding 70% was considered non-cytotoxic and, therefore, suitable for further evaluation of anti-inflammatory activity (International Conference on Harmonisation, 2003).

$$\%\text{Survival} = \left(\frac{\text{OD sample}}{\text{OD control}}\right) \times 100$$

3.6.3 Nitric oxide inhibitory effects from RAW 264.7 cells (Makchuchit, Rattarom, & Itharat, 2017).

RAW264.7 cells were seeded into 96-well plates at a density of 1 × 10^5 cells/well and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. Following incubation, the culture medium was removed and replaced with fresh medium containing lipopolysaccharide (LPS) at a final concentration of 10 ng/mL (100 µL/well) to induce nitric oxide production. The extracts of the Tri-Phon-That remedy and its individual components were prepared using the same procedure as described for the cell viability assay. The prepared extract solutions (100 µL/well) were added to the respective wells and incubated for an additional 24 hours under the same conditions.

After incubation, 100 µL of the supernatant from each well was transferred to a new 96-well plate for the detection of nitric oxide production using the Griess reagent (100 µL/well). The absorbance was measured at 570 nm using a microplate reader. The percentage inhibition of nitric oxide production at each concentration was calculated using the designated formula, and the half-maximal inhibitory concentration (IC_{50}) values were determined using GraphPad Prism version 5. All experiments were performed in triplicate, and results were expressed as the mean ± standard deviation (SD).

$$\%\text{Inhibition} = \left(\frac{(\text{OD control} - \text{OD sample})}{\text{OD control}} \right) \times 100$$

3.6.4 Assay for tumor necrosis factor-alpha (TNF-α), interleukin 6 (IL-6), and prostaglandin E_2 (PGE_2) inhibitory effect (Panthong et al., 2020)

RAW 264.7 cells were seeded into 96-well plates at a density of 1 × 10^5 cells/well in a volume of 100 µL/well and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. Following incubation, the culture supernatant was discarded and replaced with 100 µL/well of lipopolysaccharide (LPS) solution at a concentration of 1 µg/mL to induce prostaglandin E2 (PGE_2) production and 10 ng/mL to induce tumor necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6) production. The extracts were prepared using the same procedure described in the nitric oxide inhibition assay. Various concentrations of the extract solutions (100 µL/well) were added to the wells and incubated under the same conditions for 24 hours. After incubation, the supernatants were collected to quantify PGE_2, TNF-α, and IL-6 levels using enzyme-linked immunosorbent assay (ELISA) kits.

For the detection of PGE_2, a competitive ELISA method was employed. First, 50 µL of the collected supernatant was added to the wells of the PGE_2 ELISA plate, followed by 50 µL/well of tracer. Subsequently, 50 µL/well of the PGE_2 -specific antibody was added, and the plate was incubated at 4°C for 18 hours. After incubation, the wells were washed five times with the provided wash buffer to remove unbound components. Ellman's reagent (200 µL/well) was then added, and

the plate was incubated at room temperature for 60 to 90 minutes. The optical density was measured at 420 nm, and the percentage of inhibition was calculated using the specified formula.

$$\%\text{Inhibition} = \left(\frac{(\text{OD sample} - \text{OD control with LPS})}{\text{OD control without LPS} - \text{OD control with LPS}} \right) \times 100$$

The sandwich ELISA technique was employed to detect the production of interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α). Initially, 96-well plates were coated with 100 µL/well of capture antibody and incubated overnight at 4°C. The plates were then washed four times with the provided wash buffer and blocked with reagent diluent for 1 hour at room temperature. After blocking, the plates were rewashed, and 100 µL/well of the collected supernatant was added and incubated at room temperature for 2 hours. Subsequently, the plates were rewashed, and 100 µL/well of detection antibody was added and incubated for another 2 hours at room temperature. The plates were then washed, and 100 µL/well of streptavidin–horseradish peroxidase (HRP) conjugate was added, followed by incubation for 20 minutes at room temperature. After washing the plates four more times, 100 µL/well of tetramethylbenzidine (TMB) substrate was added and incubated for 20 minutes. The reaction was terminated by the addition of 1 M sulfuric acid (H_2SO_4). Absorbance was measured at 450 nm using a microplate reader, and the percentage of inhibition was calculated using the specified formula.

$$\%\text{Inhibition} = \left(\frac{\text{Cytokine concentration control} - \text{Cytokine concentration sample}}{\text{Cytokine concentration control}} \right) \times 100$$

The IC_{50} was calculated using the GraphPad Prism 5 program. The experiment was performed in triplicate, and the results were expressed as mean ± standard deviation.

3.7 Cell viability assay on Human keratinocyte cells (HaCaT cells)

Cytotoxicity on the HaCaT cell line was tested using an MTT assay (Kumar et al., 2018). Initially, HaCaT cells were seeded into 96-well plates at a density of 20,000 cells/well in a volume of 100 µL/well and incubated at 37°C in a 5% CO_2 atmosphere for 24 hours. Following incubation, the ethanolic extract was dissolved in dimethyl sulfoxide (DMSO) and adjusted to a concentration of 50 mg/mL, whereas the aqueous extract was dissolved in deionized water, adjusted to 10 mg/mL, and filtered through a 0.22 µm sterile syringe filter. The sample solutions were subsequently prepared using 2-fold serial dilutions with complete DMEM. The culture medium was replaced with 100 µL/well of fresh DMEM, followed by the addition of 100 µL/well of the prepared sample solutions at various concentrations. The plates were then incubated under the same conditions for another 24 hours. After incubation, the supernatant was removed and replaced with 100 µL/well of MTT solution (0.5 mg/mL), followed by incubation at 37°C with 5% CO_2 for 2 hours. Finally, the MTT solution was discarded and replaced with 100 µL/well of DMSO to dissolve the resulting formazan crystals. The optical density was measured at 570 nm using a microplate reader. Cell viability was calculated using the designated formula. A survival rate greater than 70% was considered indicative of non-cytotoxicity.

$$\%\text{Survival} = \left(\frac{\text{OD sample}}{\text{OD control}}\right) \times 100$$

3.8 Analyzed the chemical compounds by High-Performance Liquid Chromatography (HPLC)

Tri-Phon-That and its ingredient extracts were analyzed for chemical content using HPLC. The selection of chemical substances for examination was based on previous studies (Khemawoot et al., 2016; Chavan & Dey, 2023). The chemical compounds, including zerumbone, compound D, and DMPBD, were used as chemical markers of Tri-Phon-That and its ingredient extracts. The samples were injected into a

C18 reverse-phase column (Shimadzu Shim-pack GIST 5 µm C18, 100 Å analytical column, 150 × 4.60 mm), and UV detection was performed at 260 nm. Agilent ChemStation software was used to analyze the area under the curve.

3.8.1 Validation method (International Council for Harmonisation [ICH], 2005)

3.8.1.1 Linearity

Standard compounds were prepared in the methanol solution. Zerumbone was prepared at concentrations of 12.5, 25, 50, 100, 200, 400, and 800 µg/mL. Compound D was created at concentrations of 12.5, 25, 50, 100, 200, 400, and 600 µg/mL. DMPBD was prepared at 25, 50, 100, 200, and 400 µg/mL. All solutions were filtered through a 0.45 µm nylon syringe filter, injected into the HPLC column, and analyzed to construct calibration curves. The linearity of each curve was confirmed by a coefficient of determination (r^2) greater than 0.999.

3.8.1.2 Accuracy

The sample extracts were prepared at a concentration of 2 mg/mL, and then zerumbone and compound D at concentrations of 50, 100, 200, and 400 µg/mL were spiked into the sample extracts to assess recovery. The mixture was filtered through a 0.45 µm nylon syringe filter and injected into the HPLC column. The accuracy of the method was evaluated by calculating the percent recovery and relative standard deviation (%RSD) of the spiked compounds. The experiment was conducted in triplicate and repeated three times.

$$\% \text{ Recovery} = (\text{amount detected} / \text{amount spiked}) \times 100$$

3.8.1.3 Precision

Precision was assessed by evaluating the %RSD of replicate injections. Mixtures of zerumbone, compound D, and sample extracts were prepared at concentrations of 50, 100, 200, and 400 µg/mL. Intra-day and inter-day precision

were evaluated by analyzing the samples on the same day and across three different days, respectively. Each test was performed in triplicate and repeated three times.

3.8.1.4 Limit of Detection (LOD) and Limit of Quantification (LOQ)

The LOD and LOQ were calculated using standard deviation and slope-based formulas. The LOD represented the lowest amount of analyte detectable, while the LOQ indicated the minimum quantifiable amount with acceptable precision and accuracy.

$$LOD = 3.3\sigma/S$$

$$LOQ = 10\sigma/S$$

σ is the standard deviation of the y-intercepts of the regression lines

S is the slope of the calibration curve.

3.8.2 Quantification of the chemical compounds

Sample extracts were prepared in methanol at a concentration of 10 mg/mL and filtered through a 0.45 µm nylon syringe filter. A volume of 10 µL of each sample was injected into the HPLC column. The mobile phase composition is detailed in Table 3.9, and the flow rate was set at 1 mL/min. Chemical compounds were identified by comparing retention times and quantified based on peak areas relative to the standard calibration curves.

Table 3.9 The mobile phase ratio of HPLC method of Tri-Phon-That remedy.

	Mobile phase ratio (%)	
Time (Minutes)	Acetonitrile	0.1% phosphoric acid
0.00	10	90
20.00	50	50
40.00	60	40
50.00	90	10
50.10	90	10
55.10	10	90
60.00	10	90

3.9 Stability testing under accelerated conditions

Extracts that demonstrated the highest anti-inflammatory activity were subjected to accelerated stability testing. Samples were stored in stability chambers at 40 ± 2°C and 75 ± 5% relative humidity for six months, following ICH guidelines (2003). Chemical constituents and nitric oxide inhibitory activity were analyzed on days 0, 30, 60, 90, 120, 150, and 180.

3.10 Statistical analysis

All experiments were performed in triplicate. Data were expressed as mean ± standard error of the mean (SEM). IC_{50} values were calculated using GraphPad Prism version 5. Statistical comparisons between groups were performed using appropriate parametric or non-parametric tests, with statistical significance considered at $p < 0.05$.

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Plant material and quality control

All plant materials were taxonomically identified by a qualified taxonomist and subsequently deposited in the plant herbarium, including Sirindhorn Herbarium, Department of Agriculture, Bangkok, Thailand, Herbarium of Mahidol University, Mahidol University, Nakhon Pathom, Thailand, and Thai Traditional Medicine Herbarium, Department of Thai Traditional and Alternative Medicine, Bangkok, Thailand, as shown in Table 4.1. The identification and testing of plant materials were performed following the guidelines of the THP (Thai Herbal Pharmacopoeia, 2024). Although the physicochemical properties of *C. nardus* and *C. citratus* are not specified in the standard guidelines of the THP, both plants were analyzed for their physicochemical characteristics to provide reference data on their chemical and physical properties. All samples met the established standard criteria following quality control procedures, as presented in Table 4.2 – 4.3.

Table 4.1 The description and voucher specimen number of plant materials

Plant	Plant Herbarium	voucher specimen number
Zingiber zerumbet (L.) Sm.	Sirindhorn Herbarium	BK No. 085176
Zingiber montanum (J.Koenig) Link ex A. Dietr.	Sirindhorn Herbarium	BK No. 085337
Cymbopogon nardus (L.) Rendle	Herbarium of Mahidol University	PBM 006473
Cymbopogon citratus (DC.) Stapf	Thai Traditional Medicine Herbarium	TTM No. 0006791

Table 4.2 The physicochemical and standard criteria of *Z. zerumbet* and *Z. montanum*

Parameters	*Z. zerumbet*			*Z. montanum*		
	Standard criteria	Results	Conclusion	Standard criteria	Results	Conclusion
Water content (% w/w)	< 11.0	5.00±1.02	Pass	< 13.0	11.96±1.37	Pass
Total Ash (% w/w)	< 10	8.67±0.13	Pass	< 9	5.79±0.06	Pass
Acid-insoluble ash (% w/w)	< 5	3.96±0.13	Pass	< 3	1.65±0.00	Pass
Extractive value (% w/w)						
- Ethanol soluble	> 2	4.22±0.19	Pass	Not defined	-	-
- Water soluble	> 9	16.24±0.88	Pass	Not defined	-	-
- Hexane soluble	Not defined	-	-	> 3	11.48±0.83	Pass
- Chloroform soluble	Not defined	-	-	> 5	16.87±0.16	Pass
Heavy metal (ppm)						
- Lead	< 4	0.33±0.11	Pass	< 4	0.08±0.25	Pass
- Arsenic	< 0.3	Undetectable	Pass	< 0.3	Undetectable	Pass
- Cadmium	< 10	0.05±0.00	Pass	< 10	0.07±0.20	Pass

Table 4.3 The physicochemical and standard criteria of *C. nardus* and *C. citratus*

Parameters	*C. nardus*			*C. citratus*		
	Standard criteria	Results	Conclusion	Standard criteria	Results	Conclusion
Water content (% w/w)	Not defined	8.71±0.24	-	Not defined	10.90±0.39	-
Total Ash (% w/w)	Not defined	7.73±0.33	-	Not defined	7.40±0.02	-
Acid-insoluble ash (% w/w)	Not defined	5.21±1.38	-	Not defined	5.17±0.08	-
Extractive value (% w/w)						
- Ethanol soluble	Not defined	5.97±0.22	-	Not defined	5.78±0.25	-
- Water soluble	Not defined	9.26±0.15	-	Not defined	7.54±0.17	-
Heavy metal (ppm)						
- Lead	< 4	0.13±0.04	Pass	< 4	Undetectable	Pass
- Arsenic	< 0.3	Undetectable	Pass	< 0.3	Undetectable	Pass
- Cadmium	< 10	0.06±0.02	Pass	< 10	0.08±0.02	Pass

4.2 Plant extraction

The plant powders were weighed and mixed to formulate the Tri-Phon-That remedy. Two types of remedy were prepared: one following the original formulation and the other in which *C. citratus* was used as a substitute for *C. nardus.* The ratio of plant ingredients in the remedy extracts is shown in Table 4.4.

Table 4.4 The ratio of plant ingredients in the Tri-Phon-That remedy extracts.

Plants	Ratio	
	Tri-Phon-That (*C. nardus*-based formulation)	Tri-Phon-That (*C. citratus*-based formulation)
Z. zerumbet	1	1
Z. montanum	1	1
C. nardus	1	-
C. citratus	-	1

Subsequently, the Tri-Phon-That remedy and its plant components were extracted using two conventional methods: decoction and maceration. The aqueous (water-based) extracts yielded a higher percentage than the ethanolic extracts, indicating greater solubility of the active constituents in water. Details of the extraction solvents, extract codes, and percentage yields for the remedy and its plant ingredients are presented in Table 4.5.

Table 4.5 Solvents, extract codes, and percentage yields of the prepared extracts.

Sample	Solvent	Code	Yield of extract (% w/w)
Tri-Phon-That	Ethanol	TPECN	9.06
(*C. nardus*-based formulation)	Water	TPWCN	13.74
Tri-Phon-That	Ethanol	TPECC	8.68
(*C. citratus*-based formulation)	Water	TPWCC	14.98
Z. zerumbet	Ethanol	ZZE	7.77
	Water	ZZW	14.05
Z. montanum	Ethanol	ZME	10.60
	Water	ZMW	18.73
C. nardus	Ethanol	CNE	7.64
	Water	CNW	9.13
C. citratus	Ethanol	CCE	6.21
	Water	CCW	11.42

4.3 Antibacterial activities of Tri-Phon-That remedy and plant ingredient extracts

The results of the antibacterial activity showed that the Tri-Phon-That remedy extract and its herbal components exhibited greater inhibitory effects against Gram-positive bacteria (MIC = 39–2500 µg/mL) compared to Gram-negative bacteria (MIC > 5000 µg/mL). Additionally, the ethanol extract demonstrated better antibacterial activity, as indicated by the MIC and MBC values, compared to the water extract, as shown in Table 4.6 and Table 4.7. Notably, the extracts exhibited potent antibacterial activity against *S. pyogenes*, with MIC values ranging from 39 to 312.5 µg/mL. Among the tested extracts, CNE demonstrated the highest potency against *S. pyogenes*, with an MIC of 78 µg/mL, followed by TPECN, TPECC, ZZE, CCE, and ZME. The MBC values of these extracts ranged from 156 to 625 µg/mL, indicating their bactericidal potential. In addition, the extracts also showed activity against

S. pneumoniae, with MIC values ranging from 39 to 625 µg/mL. TPECN exhibited the strongest effect, with an MIC of 39 µg/mL and an MBC of 156 µg/mL. Furthermore, the MBC/MIC values of *S. pyogenes* and *S. pneumoniae* showed that the TPECN, TPECC, ZZE, CCE, and ZME extracts have a bactericidal effect, as their MBC/MIC ratios were ≤4, similar to the positive drugs of each bactericidal (Klimek et al., 2021; Makade et al., 2024). Conversely, the ethanol extract did not exhibit antibacterial activity against *S. aureus*. Furthermore, the antibacterial efficacy of the Tri-Phon-That remedy and its ingredients remained less effective than that of the standard antibiotic, as substantially higher concentrations were required to achieve comparable effects. Additionally, the formulation lacked specificity against the other tested bacterial strains.

Table 4.6 The MIC and MBC (µg/mL) of Tri-Phon-That remedy and ingredient extracts against gram-positive bacteria.

Sample	*S. aureus*			*S. pyogenes*			*S. pneumoniae*		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
TPECN	2500	>5000	ND	156	156	1	39	156	4
TPWCN	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
TPECC	5000	>5000	ND	156	156	1	78	156	2
TPWCC	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
ZZE	625	>5000	ND	156	156	1	78	78	1
ZZW	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
ZME	>5000	>5000	ND	312.5	625	2	625	1250	2
ZMW	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
CNE	2500	>5000	ND	78	156	2	78	78	1
CNW	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
CCE	5000	>5000	ND	156	156	1	78	156	2
CCW	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
Ampicillin	3.125	6.25	2	-	-	-	-	-	-
Amoxycillin	-	-	-	0.076	0.48	6.32	0.019	0.019	1

ND means Not Determined.

Table 4.7 The MIC and MBC (µg/mL) of Tri-Phon-That remedy and ingredient extracts against gram-negative bacteria.

Sample	*S.* Typhi			*E. coli*			*K. pneumoniae*			*P. aeruginosa*			*S. dysenteriae*		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
TPECN	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
TPWCN	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
TPECC	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
TPWCC	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
ZZE	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
ZZW	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
ZME	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
ZMW	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
CNE	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
CNW	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
CCE	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
CCW	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND	>5000	>5000	ND
Norfloxacin	0.095	25	263.15	0.027	0.054	2	1.093	1.093	1	0.27	0.54	2	0.034	0.034	1

ND means Not Determined.

4.4 Anti-inflammatory activities

4.4.1 Cell viability assay

The MTT assay was performed to evaluate the cytotoxicity of the extracts and to determine non-cytotoxic concentration ranges; cell survival should not be below 70% (International Organization for Standardization, 2009). RAW264.7 cells were treated with various concentrations of the extracts (6.25–100 µg/mL). The ethanolic extracts demonstrated a dose-dependent reduction in cell viability, indicating potential cytotoxic effects at higher concentrations. In contrast, the aqueous extracts showed no cytotoxicity at any tested concentrations in this study. Specifically, TPECN and TPECC were non-toxic at concentrations below 50 µg/mL, as shown in Figure 4.1. ZZE was non-toxic at 12.5 µg/mL, while ZME and CNE demonstrated non-cytotoxicity up to 50 µg/mL. CCE exhibited no cytotoxic effects at all tested concentrations, as presented in Figure 4.2.

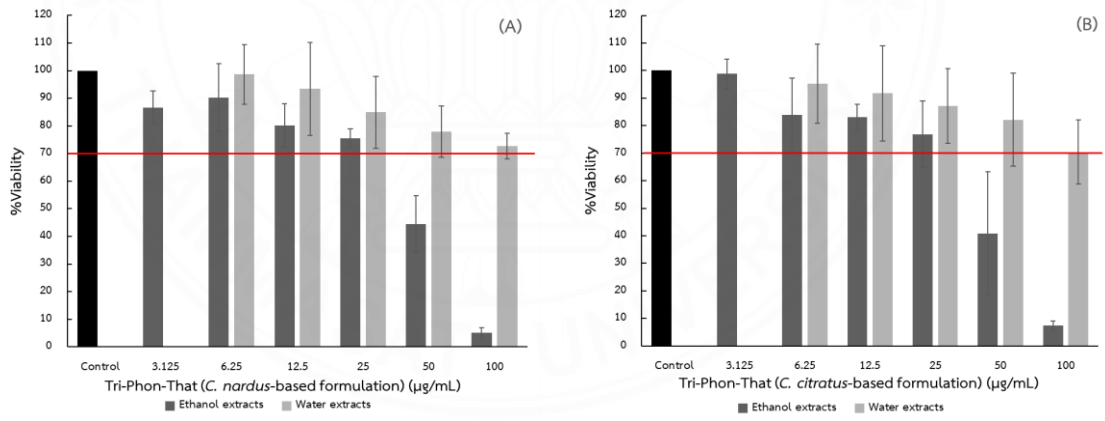

Figure 4.1 Effect of (A) Tri-Phon-That (*C. nardus*-based formulation) and (B) Tri-Phon-That (*C. citratus*-based formulation) on the viability of RAW264.7 cells.

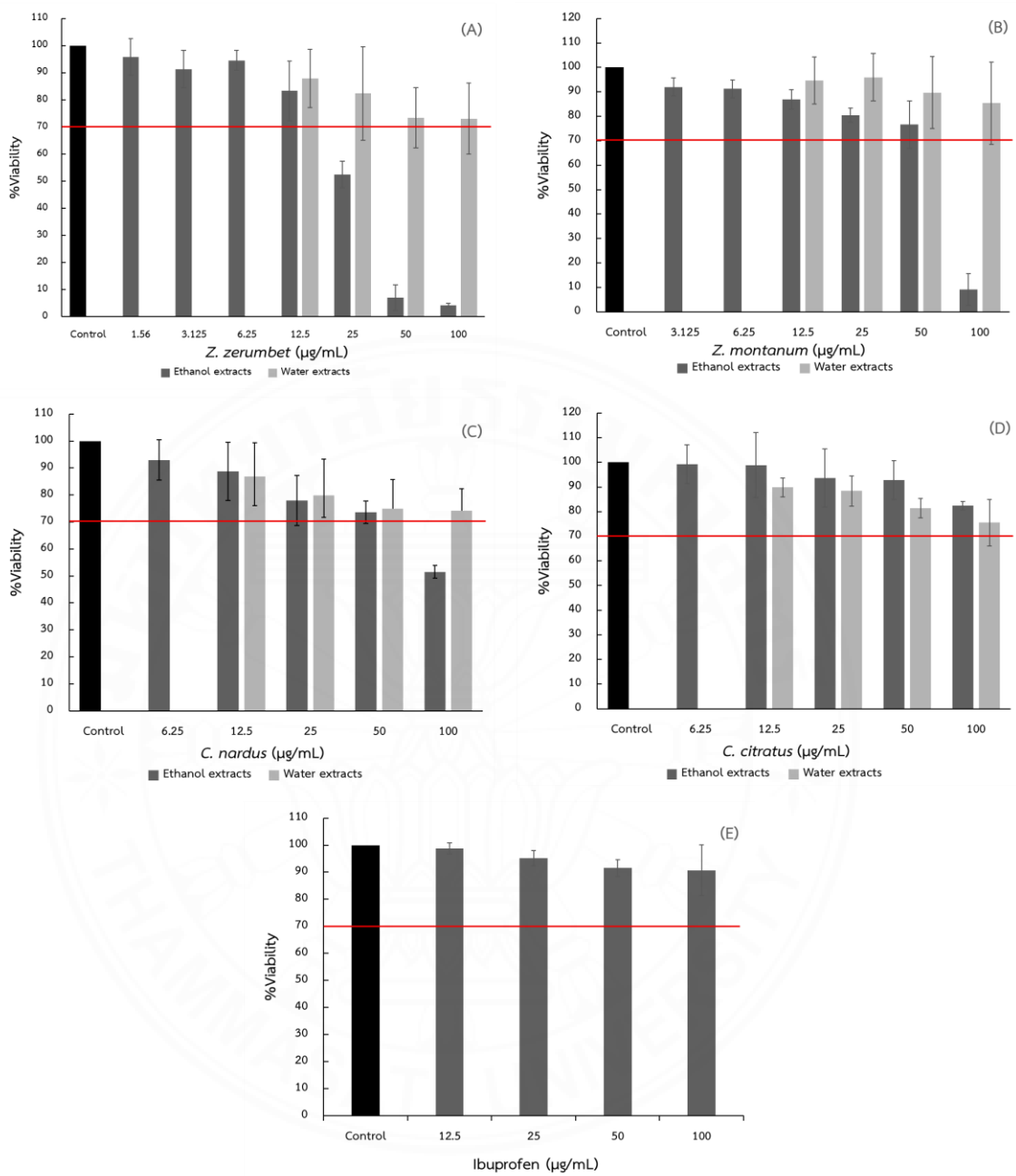

Figure 4.2 Effect of (A) *Z. zerumbet*, (B) *Z. montanum*, (C) *C. nardus*, (D) *C. citratus* extracts, and (E) ibuprofen on the viability of RAW264.7 cells.

4.4.2 Nitric oxide inhibitory effects

Following the determination of non-cytotoxic concentrations using the MTT assay, the extracts were further evaluated for their anti-inflammatory activity by assessing their ability to inhibit NO production. Inflammation was induced in RAW264.7 cells using LPS, and NO production was quantified by measuring sodium nitrite ($NaNO_2$) levels. The percentage of NO inhibition was then calculated.

The results demonstrated that the TPECN and TPECC extracts significantly suppressed NO production in a dose-dependent manner, with significant effects observed at concentrations of 12.5 and 25 µg/mL. Although TPWCN and TPWCC extracts exhibited some reduction in NO levels, the effects were not statistically significant. Interestingly, these aqueous extracts showed a trend toward increased NO production at higher doses, as illustrated in Figure 4.3.

When individual plant extracts were analyzed, the ethanolic extracts (ZZE, ZME, CNE, and CCE) exhibited a dose-dependent inhibition of NO production. ZZE significantly reduced NO levels at concentrations of 6.25 and 12.5 µg/mL, while ZME showed significant effects at concentrations above 25 and 50 µg/mL. CNE and CCE demonstrated significant inhibitory effects at doses exceeding 50 µg/mL, as shown in Figure 4.4.

The overall inhibitory activity of all samples is summarized in Table 4.8. The results are presented as percentage inhibition and IC_{50} values. Among the tested samples, ZZE exhibited the strongest activity, with an IC_{50} of 1.79 ± 0.21 µg/mL, followed by TPECN and TPECC, which had IC_{50} values of 5.35 ± 0.77 µg/mL and 7.02 ± 0.61 µg/mL, respectively. ZME, CNE, and CCE showed IC_{50} values of 18.66 ± 2.08, 33.73 ± 4.41, and 37.65 ± 4.92 µg/mL, respectively. All of these extracts exhibited stronger NO inhibitory effects than the reference drug, ibuprofen, which had an IC_{50} of 89.63 ± 7.21 µg/mL.

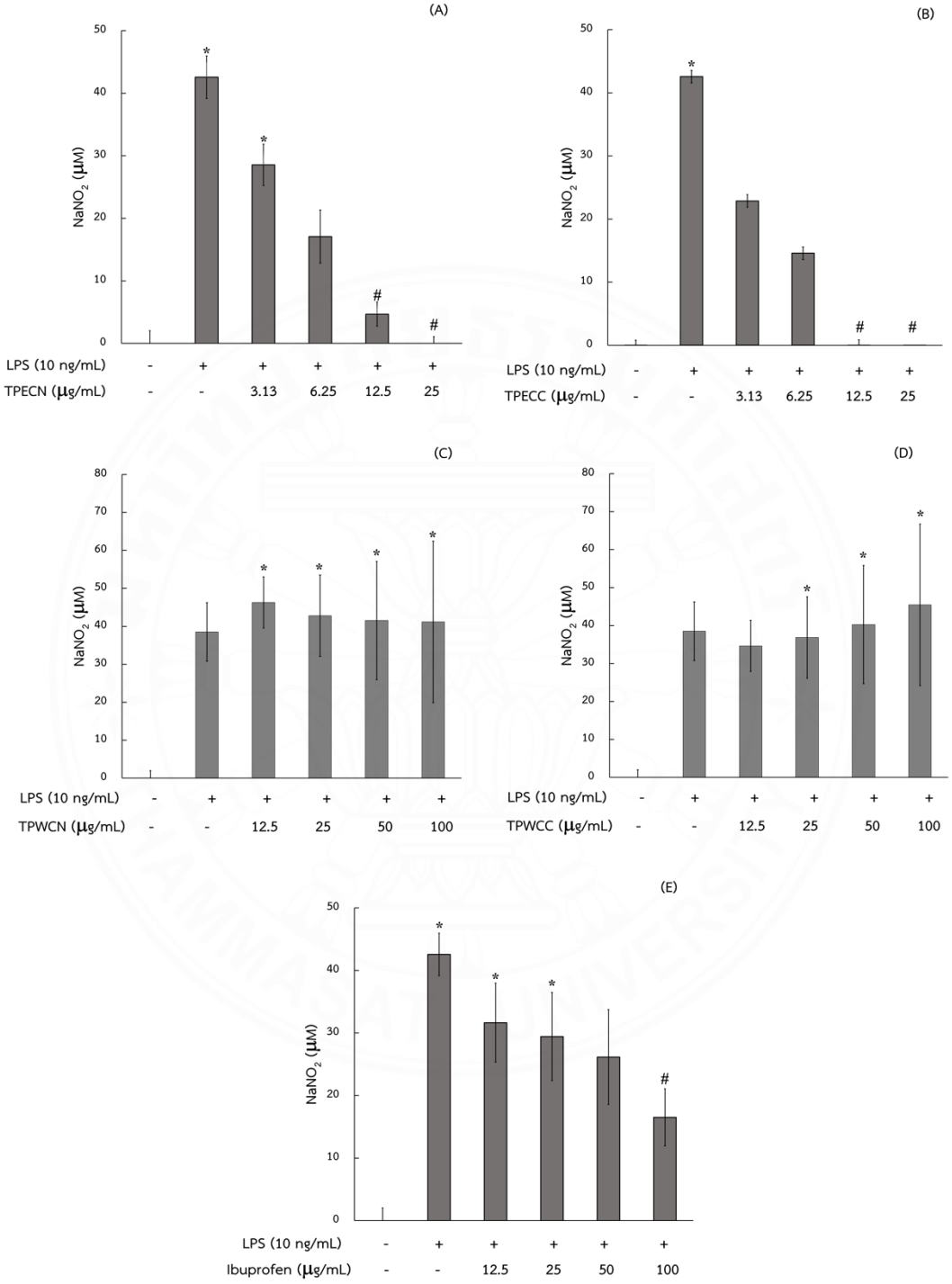

Figure 4.3 Effect of (A) TPECN, (B) TPECC, (C) TPWCN, (D) TPWCC, and (E) Ibuprofen on NO production in RAW264.7 cells. *Significant effect ($p < 0.05$) when compared to the untreated control, #Significant effect ($p < 0.05$) when compared to LPS-stimulating cells by the Kruskal-Wallis H test.

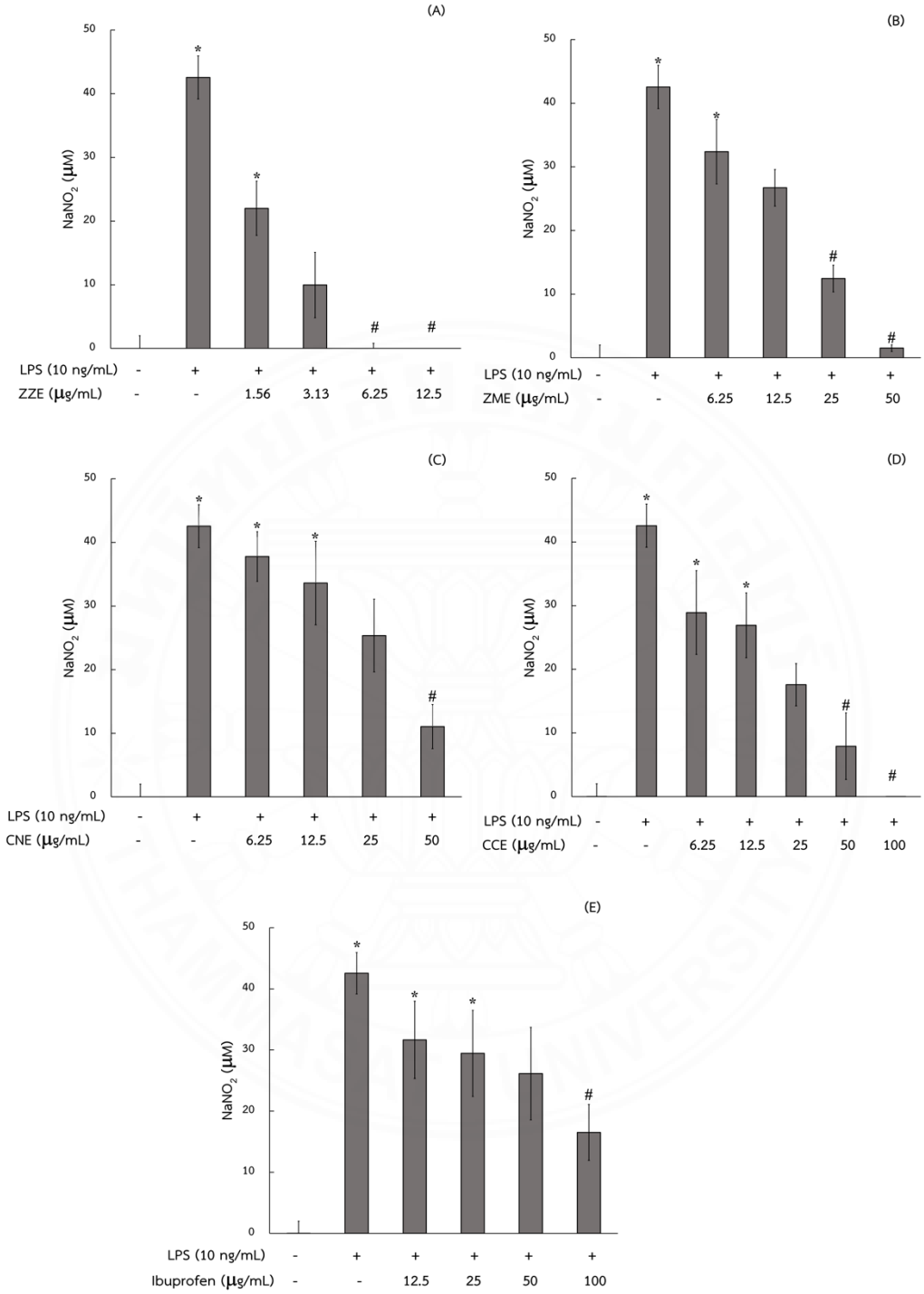

Figure 4.4 Effect of (A) ZZE, (B) ZME, (C) CNE, (D) CCE, and (E) Ibuprofen on NO production in RAW264.7 cells. *Significant effect ($p < 0.05$) when compared to the untreated control, #Significant effect ($p < 0.05$) when compared to LPs stimulating cells by the Kruskal-Wallis H test.

Table 4.8 The effect of Tri-Phon-That remedy and ingredient extracts on nitric oxide production in LPS-stimulated RAW264.7 cells.

Sample	Inhibition of nitric oxide production at various concentrations (%)							IC_{50}
	100 µg/mL	50 µg/mL	25 µg/mL	12.5 µg/mL	6.25 µg/mL	3.125 µg/mL	1.56 µg/mL	(µg/mL)
TPECN	Toxic	Toxic	97.78 ± 1.92	86.95 ± 3.99	57.67 ± 6.92	30.02 ± 2.98	Not tested	5.35 ± 0.77
TPWCN	-30.62 ± 7.85	-13.30 ± 12.77	-1.98 ± 17.83	7.23 ± 24.51	13.85 ± 25.76	Not tested	Not tested	>100
TPECC	Toxic	Toxic	96.47 ± 1.44	80.14 ± 3.79	45.04 ± 3.99	25.25 ± 6.62	Not tested	7.02 ± 0.61
TPWCC	-16.43 ± 13.66	-2.64 ± 19.20	6.43 ± 25.06	12.32 ± 26.74	14.88 ± 27.46	Not tested	Not tested	>100
ZZE	Toxic	Toxic	Toxic	100.63 ± 0.80	96.25 ± 1.20	73.63 ± 7.43	46.04 ±3.72	1.79 ± 0.21
ZME	Toxic	92.11 ± 1.66	65.94 ± 3.60	32.92 ± 8.15	18.00 ± 8.93	Not tested	Not tested	18.66 ± 2.08
CNE	Toxic	70.52 ± 4.85	36.10 ± 7.43	16.08 ± 7.31	7.98 ± 6.67	Not tested	Not tested	33.73 ± 4.41
CCE	88.24 ± 2.27	60.26 ± 8.12	35.85 ± 1.26	8.43 ± 5.67	Not tested	Not tested	Not tested	37.65 ± 4.92
Ibuprofen	57.47 ± 4.24	31.77 ± 6.57	24.01 ± 3.30	21.08 ± 3.65	Not tested	Not tested	Not tested	89.63 ± 7.21

4.4.3 Inhibitory effects of Tri-Phon-That remedy and its component extracts on IL-6, PGE_2, and TNF-α production in LPS-stimulated RAW264.7 cells

The anti-inflammatory activity of the remedy extracts (TPECN, TPECC, TPWCN, and TPWCC) was initially assessed by evaluating their ability to inhibit the production of pro-inflammatory cytokines, including IL-6, PGE_2, and TNF-α. Extracts that demonstrated a significant reduction in these cytokine levels were subsequently selected for further testing to evaluate the anti-inflammatory properties of their herbal components.

4.4.3.1 IL-6 Inhibition by Tri-Phon-That remedy and its component extracts

IL-6 levels were released following LPS stimulation compared to the untreated control group. The ethanolic extracts of the remedies, TPECN and TPECC, reduced IL-6 production in a dose-dependent manner. TPECN showed a significant reduction at a concentration of 25 µg/mL, whereas TPECC did not show differences from the LPS-stimulated group. In contrast, the aqueous extracts (TPWCN and TPWCC) did not affect to decrease IL-6 levels, as shown in Figure 4.5. The inhibitory effects of the plant ingredient extracts are shown in Figure 4.6. ZZE reduced IL-6 production at concentrations ranging from 3.125 µg/mL above and showed a significant in 25 µg/mL. ZME, CNE, and CCE showed significant effects at 50 µg/mL. Statistical analyses were performed using the Kruskal–Wallis test. The percent inhibition and IC_{50} values for each extract are presented in Table 4.9. The ethanolic extracts exhibited inhibitory activity, while the aqueous extracts showed no effect, as indicated by IC_{50} values greater than 100 µg/mL. The IC_{50} values for TPECN, TPECC, and ZZE were 7.81 ± 0.31, 10.46 ± 0.08, and 3.51 ± 0.23 µg/mL, respectively—comparable to that of the positive control, ibuprofen (IC_{50} = 16.63 ± 0.48 µg/mL). ZME CNE and CCE had IC_{50} values of 23.03 ± 4.83, 37.11 ± 16.13, and 47.29 ± 0.64 µg/mL, respectively.

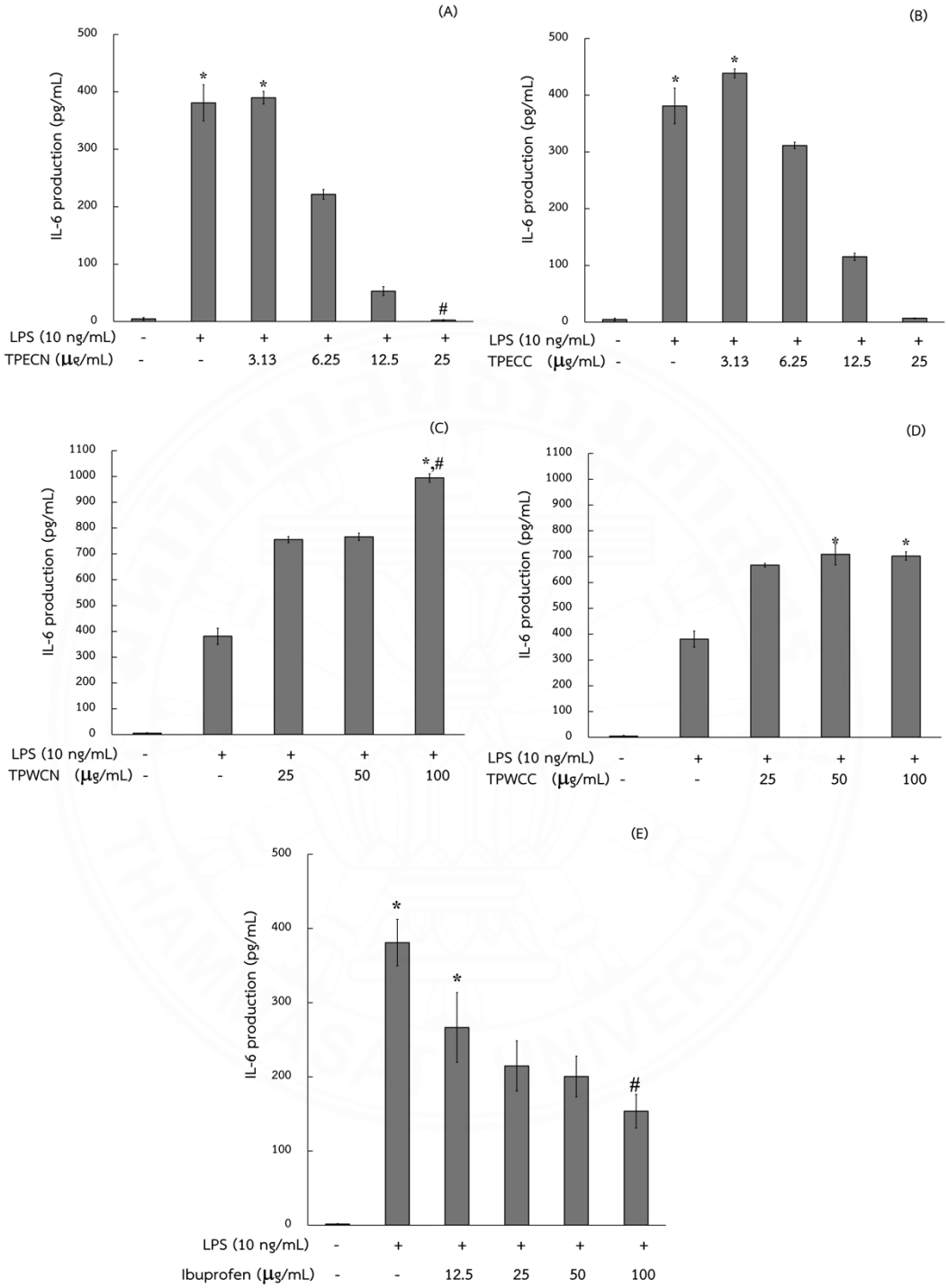

Figure 4.5 Effect of (A) TPECN, (B) TPECC, (C) TPWCN, (D) TPWCC, and (E) ibuprofen on IL-6 production in RAW264.7 cells. *$p < 0.05$ vs. untreated control; $^{\#}p < 0.05$ vs. LPS-stimulated group by the Kruskal-Wallis H test.

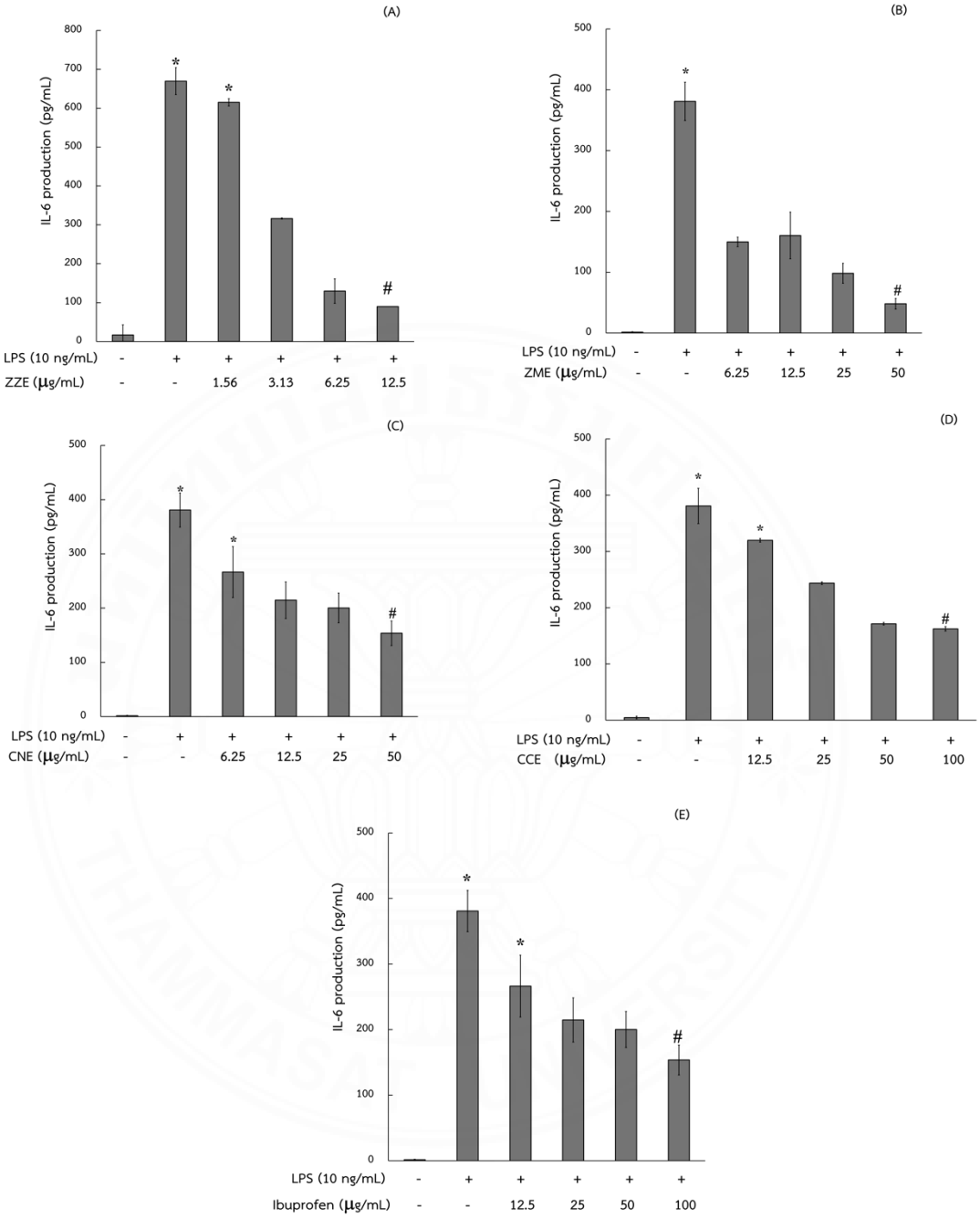

Figure 4.6 Effect of (A) ZZE, (B) ZME, (C) CNE, (D) CCE extracts, and (E) ibuprofen on IL-6 production in RAW264.7 cells. *$p < 0.05$ vs. untreated control; $^{\#}p < 0.05$ vs. LPS-stimulated group by the Kruskal-Wallis H test.

Table 4.9 The inhibitory effect of Tri-Phon-That remedy and ingredient extracts on IL-6 in LPS-stimulated RAW264.7 cells

Samples	Inhibition of IL-6 production at various concentrations (%)							IC_{50} (µg/mL)
	100 µg/mL	50 µg/mL	25 µg/mL	12.5 µg/mL	6.25 µg/mL	3.125 µg/mL	1.56 µg/mL	
TPECN	Toxic	Toxic	99.29 ± 0.16	85.19 ± 1.55	38.26 ± 1.70	-8.67 ± 2.17	Not tested	7.81 ± 0.31
TPWCN	-177.35 ± 3.25	-113.69 ± 2.79	-110.75 ± 2.32	Not tested	Not tested	Not tested	Not tested	>100
TPECC	Toxic	Toxic	98.05 ± 0.16	67.84 ± 1.24	13.17 ± 1.08	-22.31 ± 1.55	Not tested	10.46 ± 0.08
TPWCC	-95.88 ± 3.25	-97.58 ± 8.06	-85.97 ± 1.39	Not tested	Not tested	Not tested	Not tested	>100
ZZE	Toxic	Toxic	Toxic	84.75 ± 0.42	77.86 ± 4.38	46.26 ± 1.28	-4.54 ± 3.99	3.51 ± 0.23
ZME	Toxic	77.42 ± 2.87	53.96 ± 5.47	24.77 ± 12.77	-23.46 ± 8.86	Not tested	Not tested	23.03 ± 4.83
CNE	Toxic	57.16 ± 6.35	44.14 ± 7.67	40.12 ± 9.42	25.71 ± 13.14	Not tested	Not tested	37.11 ± 16.13
CCE	54.68 ± 0.77	52.20 ± 0.47	32.06 ± 0.47	10.84 ± 0.62	Not tested	Not tested	Not tested	47.29 ± 0.64
Ibuprofen	66.33 ± 2.24	61.33 ± 2.83	57.48 ± 2.04	45.45 ± 0.42	Not tested	Not tested	Not tested	16.63 ± 0.48

4.4.3.2 PGE_2 Inhibition by Tri-Phon-That remedy and its component extracts

The remedy extracts significantly reduced PGE_2 production at a dose of 25 µg/mL (TPECN and TPECC), exhibiting dose-dependent inhibition. In contrast, the aqueous extracts (TPWCN and TPWCC) showed no reduction in PGE_2 levels, as illustrated in Figure 4.7. The inhibitory effects of the extracts are presented in Figure 4.8, where a dose-dependent reduction in PGE_2 is observed, with ZME showing a significant effect only at 50 µg/mL. Statistical significance was determined using the Kruskal-Wallis H tests.

Table 4.10 shows the percent inhibition and IC_{50} values for each extract. TPECC exhibited the most potent effect, with an IC_{50} value of 17.50 ± 0.03 µg/mL, followed by TPECN (IC_{50} = 24.75±0.33 µg/mL) and ZME (IC_{50} = 20.51±0.05 µg/mL). However, all extracts showed less inhibition than the positive control (ibuprofen), which had an IC_{50} value of 6.81±0.09 µg/mL.

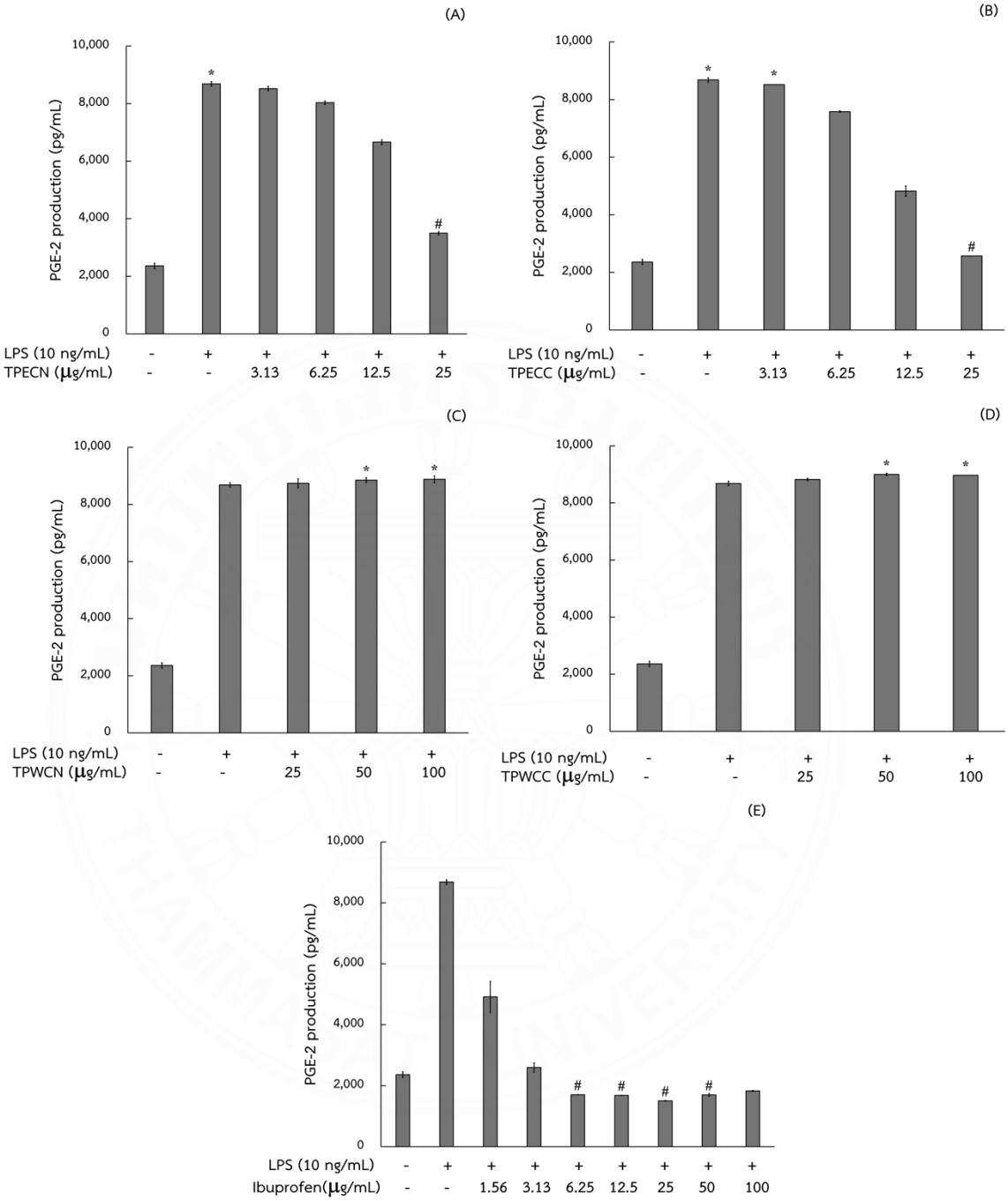

Figure 4.7 Effect of (A) TPECN, (B) TPECC, (C) TPWCN, (D) TPWCC, and (E) Ibuprofen extracts on PGE-2 production in RAW264.7 cells. *$p < 0.05$ vs. untreated control; $^{\#}p < 0.05$ vs. LPS-stimulated group by the Kruskal-Wallis H test.

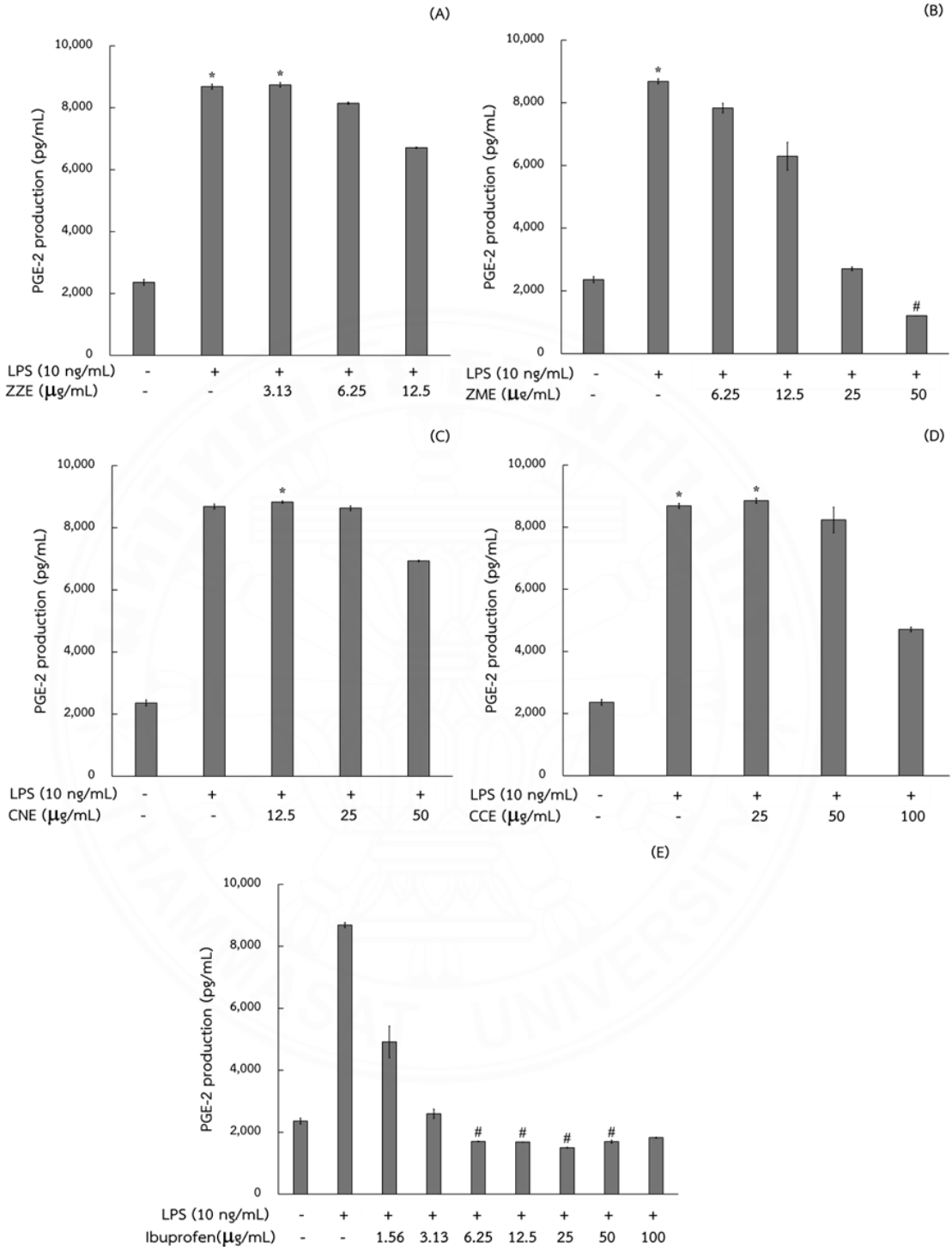

Figure 4.8 Effect of (A) ZZE, (B) ZME, (C) CNE, (D) CCE, and (E) Ibuprofen on PGE-2 production in LPS-stimulated RAW264.7 cells. *$p < 0.05$ vs. untreated control; $^{\#}p < 0.05$ vs. LPS-stimulated group by the Kruskal-Wallis H test.

Table 4.10 The inhibitory effect of Tri-Phon-That remedy and ingredient extracts on PGE_2 in LPS-stimulated RAW264.7 cells

Samples	Inhibition of PGE_2 production at various concentrations (%)							IC_{50} (µg/mL)
	100 µg/mL	50 µg/mL	25 µg/mL	12.5 µg/mL	6.25 µg/mL	3.125 µg/mL	1.56 µg/mL	
TPECN	Toxic	Toxic	51.11 ± 1.45	9.28 ± 0.19	2.40 ± 0.37	0.54 ± 0.49	Not tested	24.75 ± 0.328
TPWCN	-0.65 ± 0.09	-0.56 ± 0.04	-0.21 ± 0.80	Not tested	Not tested	Not tested	Not tested	>100
TPECC	Toxic	Toxic	87.26 ± 5.089	25.97 ± 0.96	4.36 ± 0.11	0.55 ± 0.23	Not tested	17.50 ± 0.03
TPWCC	-0.94 ± 0.32	-1.04 ± 0.46	-0.47 ± 0.16	Not tested	Not tested	Not tested	Not tested	>100
ZZE	Toxic	Toxic	Toxic	9.01 ± 0.19	1.94 ± 0.27	-0.19 ± 0.01	Not tested	>12.5
ZME	Toxic	111.94 ± 0.05	80.20 ± 2.14	11.80 ± 2.66	3.23 ± 0.71	Not tested	Not tested	20.51 ± 0.05
CNE	Toxic	7.72 ± 0.37	0.19 ± 0.01	-0.48 ± 0.43	Not tested	Not tested	Not tested	>50
CCE	27.50 ± 0.46	1.72 ± 1.42	-0.56 ± 0.04	Not tested	Not tested	Not tested	Not tested	>100
Ibuprofen	62.71 ± 0.71	69.82 ± 2.79	65.69 ± 1.09	66.15 ± 6.34	62.36 ± 0.11	61.09 ± 0.27	28.75 ± 3.61	6.81 ± 0.09

4.4.3.3 TNF-α inhibition by Tri-Phon-That remedy and its component extracts

TPECN and TPECC extracts demonstrated the inhibition of TNF-α levels in a dose-dependent manner. They showed significant reducing effects at a 25 µg/mL concentration compared to the LPS-stimulating cells. The TPWCN and TPWCC did not show these effects, as shown in Figure 4.9. The %inhibition is 37.88 ± 3.56 µg/mL for TPECN and 39.94 ± 0.45 µg/mL for TPECC. The aqueous extracts did not show any concentration effects on TNF-α levels. However, all the extracts have an IC_{50} of more than 25 and 100 µg/mL, as shown in Table 4.11.

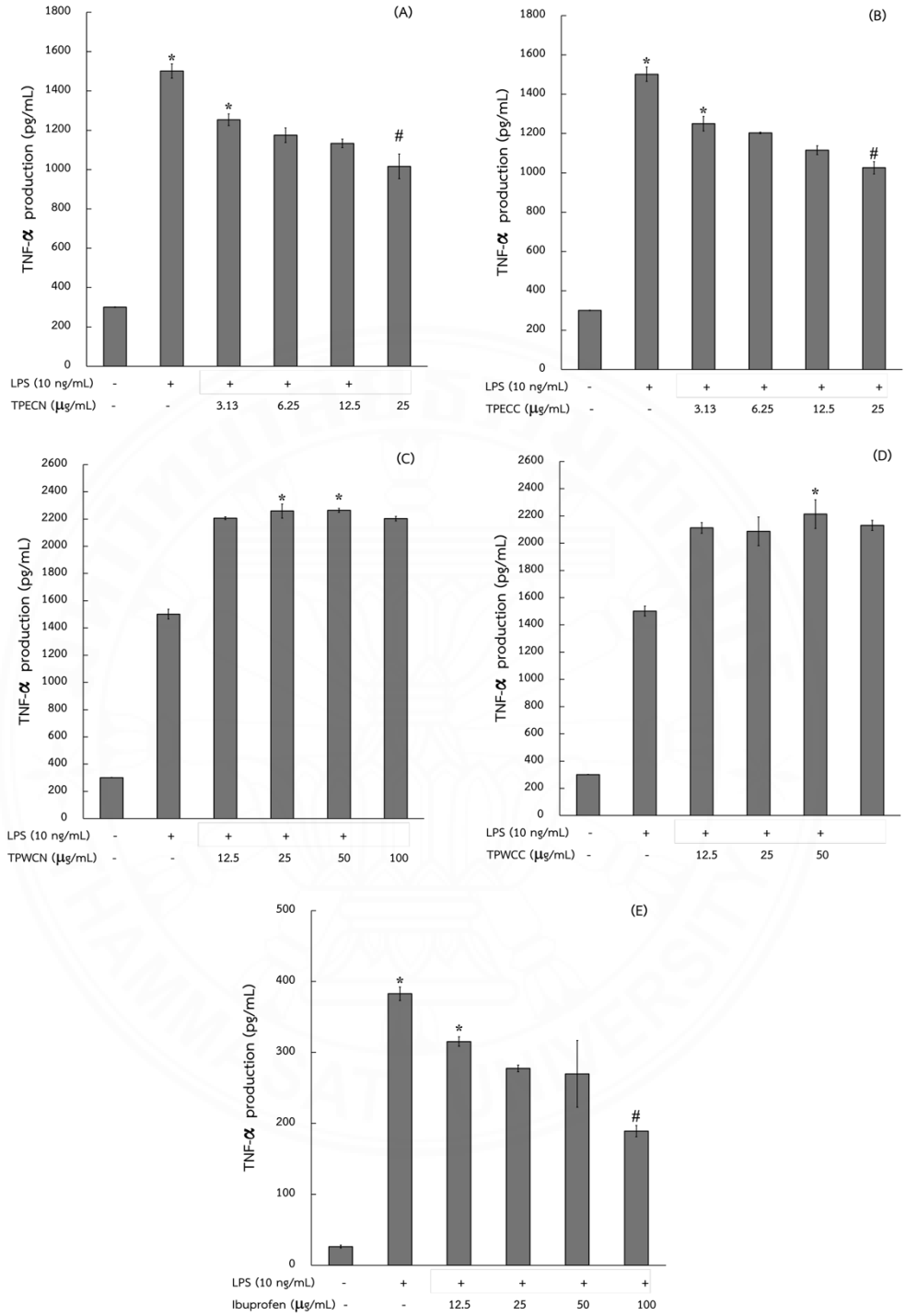

Figure 4.9 Effect of (A) TPECN, (B) TPECC, (C) TPWCN, and (D) TPWCC extracts on TNF-α production in LPS-stimulated RAW264.7 cells. *$p < 0.05$ vs. untreated control; $^{\#}p < 0.05$ vs. LPS-stimulated group by the Kruskal-Wallis H test.

Table 4.11 The inhibitory effect of Tri-Phon-That remedy and ingredient extracts on TNF-**α** production in LPS-stimulated RAW264.7 cells

Sample	Inhibition of TNF-**α** levels at various concentrations (μg/mL)						IC_{50} (μg/mL)
	100 μg/mL	50 μg/mL	25 μg/mL	12.5 μg/mL	6.25 μg/mL	3.125 μg/mL	
TPECN	Toxic	Toxic	37.88 ± 3.56	30.56 ± 0.14	27.56 ± 0.48	20.72 ± 0.07	> 25
TPWCN	-60.67 ± 3.20	-64.85 ± 1.97	-63.31 ± 0.38	-60.78 ± 2.91	Not tested	Not tested	> 25
TPECC	Toxic	Toxic	39.94 ± 0.45	30.78 ± 1.96	23.92 ± 1.29	21.25 ± 0.38	> 100
TPWCC	-52.99 ± 0.81	-57.87 ± 2.01	-47.29 ± 2.11	-51.36 ± 0.71	Not tested	Not tested	> 100
Ibuprofen	54.30 ± 0.67	31.52 ± 15.21	29.53 ± 0.78	18.89 ± 0.42	Not tested	Not tested	89.42 ± 10.37

4.5 Cytotoxicity of Tri-Phon-That remedy and ingredient extracts on human keratinocyte cell line (HaCaT)

The cytotoxicity on HaCaT cells was performed to determine the safe concentration range of Tri-Phon-That remedy and ingredient extracts on keratinocyte cells. Cell viability was used as an indicator of toxicity, with extracts considered cytotoxic if cell survival dropped below 70% (International Organization for Standardization, 2009). The result revealed that all the aqueous extracts were less toxic than the ethanol extracts. TPECN and TPECC exhibited cytotoxicity at concentrations above 25 µg/mL and 50 µg/mL, respectively. They demonstrated cell viability greater than 70% at 30.81 ± 12.55 and 60.41 ± 11.40 µg/mL, respectively. Meanwhile, TPWCN and TPWCC showed no cytotoxic effects on HaCaT cells. ZZE was the most cytotoxic among the plant ingredient extracts, with effects observed at concentrations above 12.5 µg/mL and demonstrating the safe doses at 18.40 ± 3.44 µg/mL. ZME and CNE exhibit cytotoxicity at a concentration of 100 µg/mL, with safe doses at 68.30 ± 3.90 and 71.79 ± 22.75 µg/mL, respectively. While CCE exhibited no cytotoxicity, as shown in Figure 4.10.

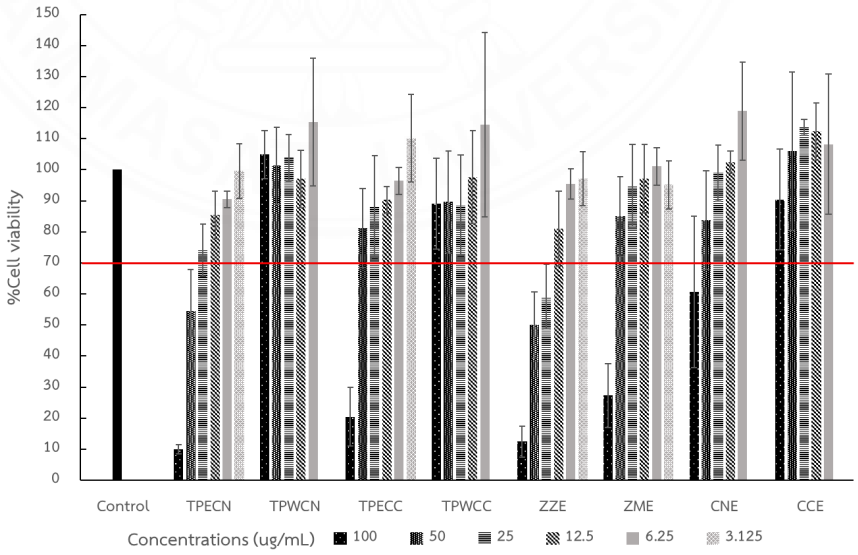

Figure 4.10 The effect of Tri-Phon-That and ingredient extracts on the viability of HaCaT cells.

4.6 Chemical Analysis of Tri-Phon-That Remedy and Its Ingredient Extracts

4.6.1 Validation of HPLC method

The HPLC method was validated to evaluate the system's suitability and reliability for analyzing chemical compositions. Two standard compounds were used in this study: Compound D and zerumbone. Compound D and zerumbone were quantified using calibration curves established in this experiment. The correlation coefficients (R^2), along with the limits of detection (LOD) and limits of quantification (LOQ) for each compound, are summarized in Table 4.12. The standard curves and spectra are shown in Figures 4.11–4.12. Chromatographic retention times were 15.37 minutes for Compound D and 38.11 minutes for zerumbone, as illustrated in Figure 4.13.

Table 4.12 Linear ranges, regression equation, coefficient of determination (R2), LOD, and LOQ of calibration curves of standard compounds.

Parameters/ Compounds	Linear range (µg/mL)	Regression equation	R^2	LOD (µg/mL)	LOQ (µg/mL)
Compound D	12.5 - 600	y = 45.889x +34.442	0.9995	1.58	4.80
Zerumbone	12.5 - 800	y = 19.221x + 7.982	0.9996	4.04	12.24

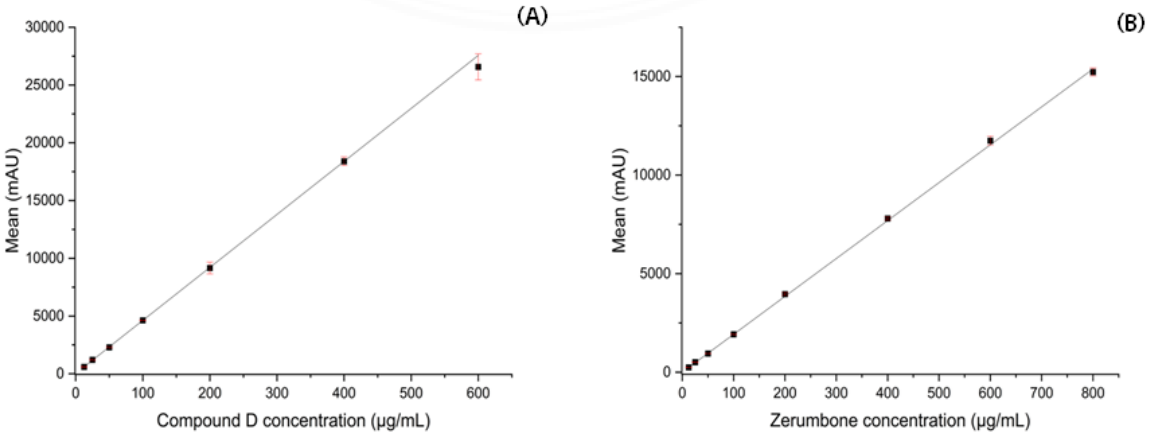

Figure 4.11 The standard curves of (A) Compound D, (B) zerumbone, and (C) DMPBD

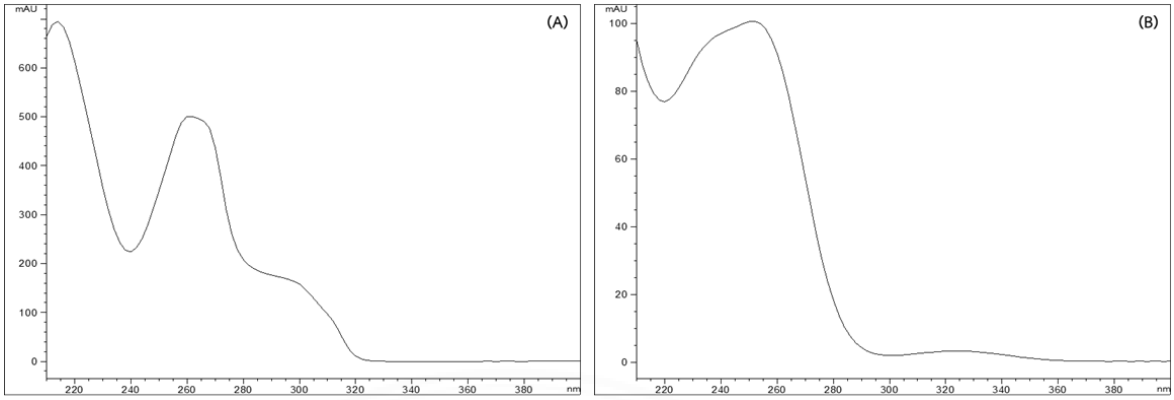

Figure 4.12 The spectra of (A) Compound D, and (B) zerumbone at UV detection 260 nm.

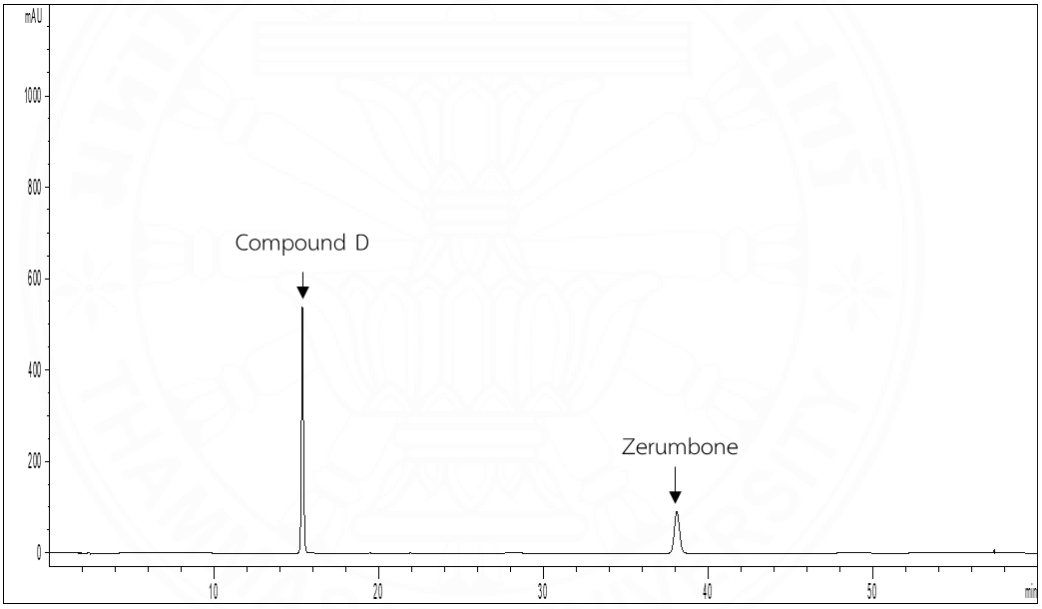

Figure 4.13 HPLC chromatograms of 100 µg/mL Compound D and zerumbone at UV detection 260 nm.

The precision is assessed by intra-day and inter-day with %RSD values, as shown in Table 4.13. Precision was assessed through both intra-day and inter-day measurements for compound D and zerumbone at four concentration levels (50, 100, 200, and 400 µg/mL). The relative standard deviation (%RSD) values for intra-day precision ranged from 1.05% to 1.81%, while inter-day %RSD values were between 1.07% and 1.61%. According to the United States Pharmacopeia (USP) guidelines, precision is considered acceptable if %RSD does not exceed 2% for quantitative

methods involving active pharmaceutical ingredients (International Council for Harmonisation [ICH], 2005). Therefore, the %RSD values obtained in this study demonstrate acceptable intra-day and inter-day precision levels for both analytes.

Table 4.13 Intra-day and inter-day precision of compound D and zerumbone.

Compound	Concentration (µg/mL)	Inter-day (n=9)	%RSD	Intra-day (n=3)	%RSD
Compound D	400	388.17 ± 6.07	1.41	384.62 ± 5.41	1.41
	200	201.51 ± 2.91	1.61	200.00 ± 3.21	1.61
	100	101.26 ± 1.92	1.08	102.09 ± 1.10	1.08
	50	51.42 ± 0.92	1.37	50.79 ± 0.70	1.37
Zerumbone	400	405.69 ± 5.48	1.35	411.70 ± 2.48	0.60
	200	203.42 ± 2.18	1.07	201.94 ± 2.35	1.16
	100	102.23 ± 1.42	1.39	102.27 ± 1.08	1.05
	50	50.34 ± 0.68	1.36	49.75 ± 0.90	1.81

The accuracy was presented as the percentage recovery, along with %RSD values, in Table 4.14. The acceptable range of the %RSD value is below 2%. The accuracy was evaluated through each compound's percentage recovery at four spike levels. The recovery rates for Compound D ranged from 96.16% to 104.18%, with %RSD values between 0.95% and 1.73%. For zerumbone, recovery rates ranged from 99.51% to 103.22%, with %RSD values between 0.83% and 1.30%. The observed recovery values are generally within the acceptable range of 80-120 % for standard analytical methods, with minor deviations considered acceptable based on sample concentration. Given these slight variations and the consistently low %RSD values, the method demonstrates acceptable accuracy.

Table 4.14 Accuracy validation for compound D and zerumbone.

Compound	Spike volume (µg/mL)	%Recovery			Mean ± SD	%RSD
		N1	N2	N3		
Compound D	400	96.16	96.74	98.24	97.04 ± 1.07	1.11
	200	100.00	100.43	101.83	100.76 ± 0.96	0.95
	100	102.09	99.25	102.45	101.26 ± 1.76	1.73
	50	101.59	102.72	104.18	102.83 ± 1.30	1.26
Zerumbone	400	102.93	100.44	100.90	101.42 ± 1.32	1.30
	200	100.97	102.64	101.53	101.71 ± 0.85	0.83
	100	102.27	103.22	101.20	102.22 ± 1.01	0.99
	50	99.51	101.33	101.20	100.68 ± 1.02	1.01

4.6.2 The quantification of chemical compounds in the Tri-Phon-That remedy extract and plant ingredients

Following method validation, HPLC analysis was performed to quantify the standard compounds in the Tri-Phon-That remedy extract and its individual herbal components, using UV detection at 260 nm. Compound D and zerumbone were identified by comparing their retention times and UV spectra with validated standards established in this study. DMPBD was identified based on its spectral characteristics by comparison with data from a reference database. Quantification of DMPBD was performed using a pre-established standard curve derived from a compound library, as illustrated in Figure 4.14. A previous study established a standard curve for DMPBD within the concentration range of 25–400 µg/mL. The resulting regression equation was $y = 31.255x - 34.468$, with a coefficient of determination (R^2) of 0.9996, indicating excellent linearity and reliability for quantitative analysis. All three standard compounds were detected in TPECN, TPWCN, TPECC, and TPWCC extracts, as shown in Figures 4.15-4.16.

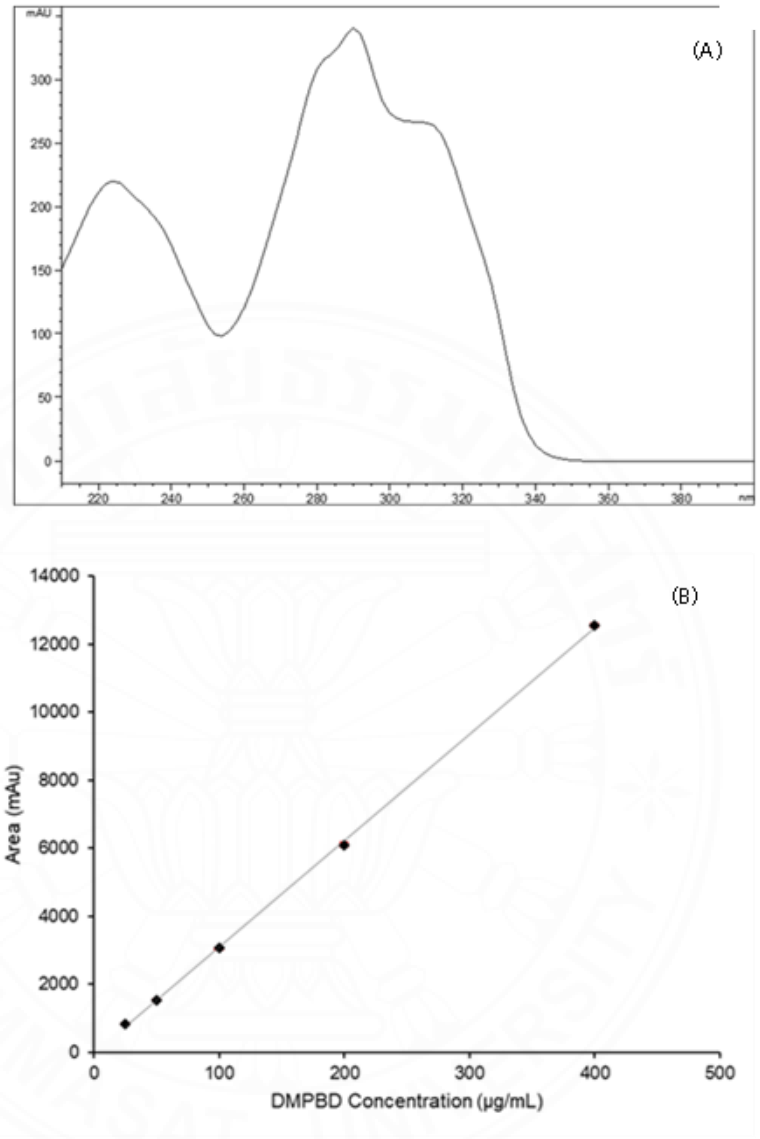

Figure 4.14 The (A) spectra and (B) standard curve of DMPBD at UV detection 260 nm.

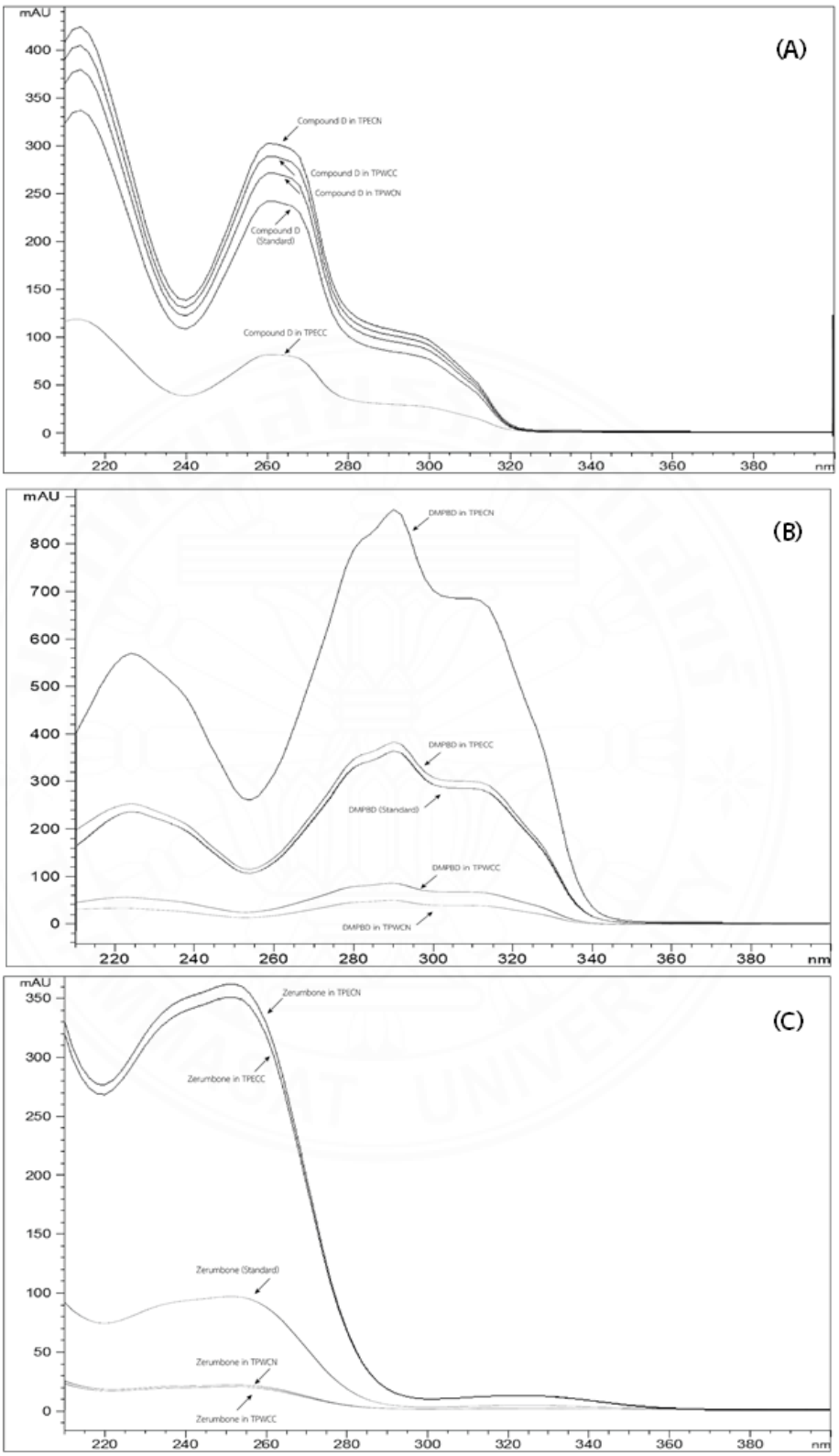

Figure 4.15 The UV spectra of (A) compound D, (B) DMPBD, and (C) zerumbone in the TPECN, TPWCN, TPECC, and TPWCC extracts at UV 260 nm.

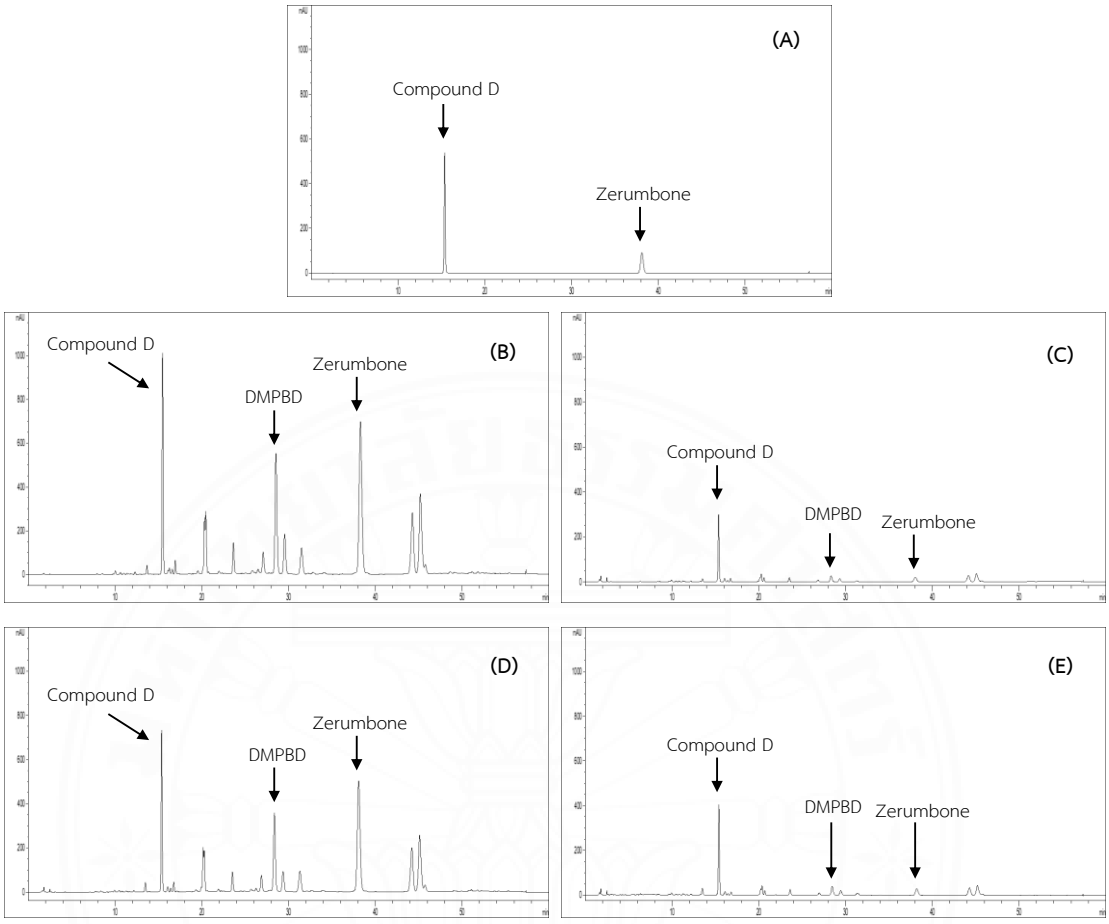

Figure 4.16 The HPLC chromatogram of (A) compound D and zerumbone, (B) TPECN, (C) TPWCN, (D) TPECC, (E) TPWCC, detected at UV at 260 nm.

The chemical analysis of the herbal extracts used in the remedy showed that both ethanolic and aqueous extracts of *Z. montanum* and *Z. zerumbet* contained their main marker compounds. In *Z. montanum*, compound D and DMPBD were found to be the major components, while zerumbone was mainly found in *Z. zerumbet*. These compounds are known to have anti-inflammatory properties, which may support the use of these herbs in treating inflammation, as shown in Figure 4.17-4.18.

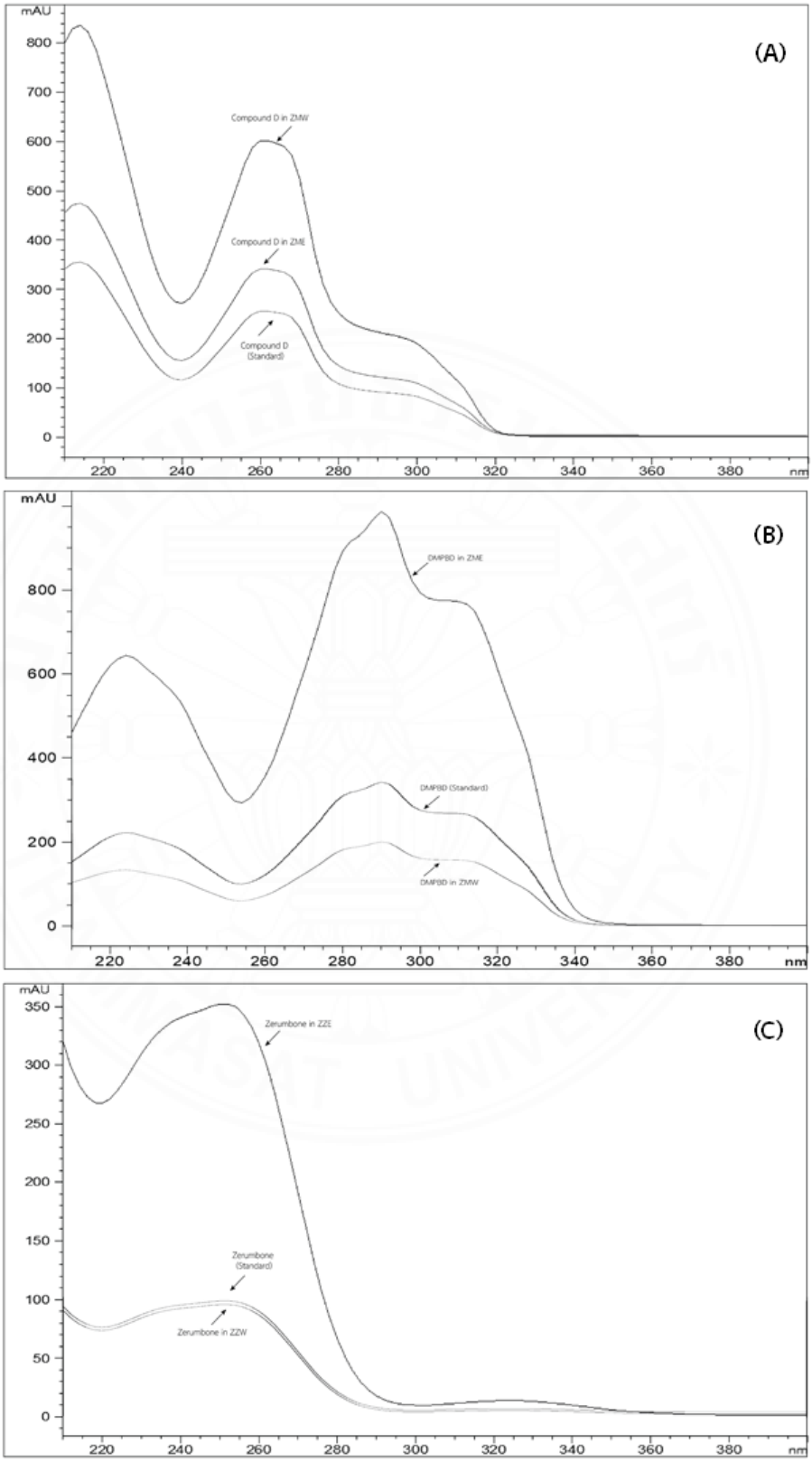

Figure 4.17 The UV spectra of (A) compound D, (B) DMPBD, and (C) zerumbone in the ZME, ZZE, ZMW, and ZZW extracts at UV 260 nm.

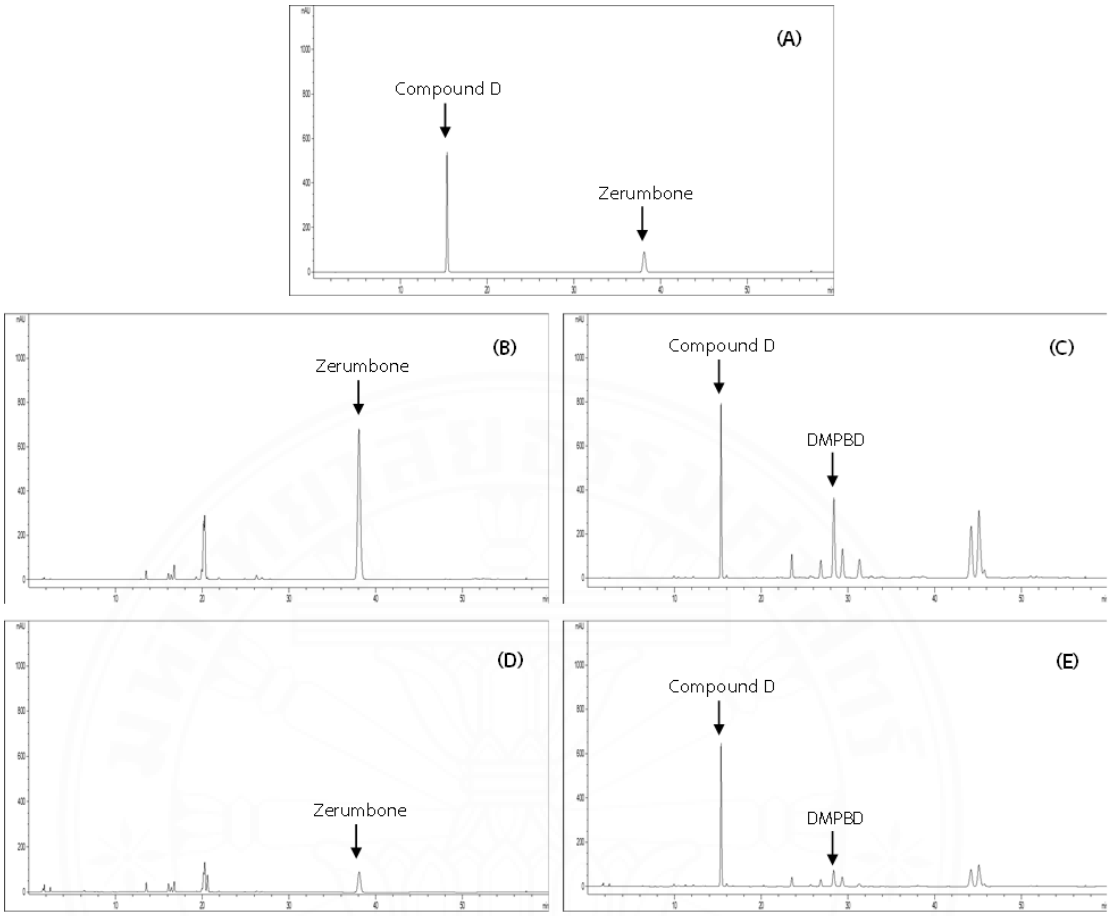

Figure 4.18 The HPLC chromatogram of compound D and zerumbone in (A) Standard compounds, (B) ZZE, (C) ZME, (D) ZZW, and (E) ZMW extracts detected at UV at 260 nm.

In addition, other compounds commonly found in *C. nardus* and *C. citratus* were examined, including geraniol, (±)-citronellal, β-Citronellal, and β-Eudesmol. These compounds were not detectable at a wavelength of 260 nm but showed clear chromatographic peaks at 215 nm, as illustrated in Figure 4.19. The retention times were 24.330 minutes for geraniol and 27.024 minutes for β-Eitronellal. For (±)citronellal, three distinct peaks were observed at 19.454, 27.701, and 35.118 minutes, suggesting the presence of isomeric forms. β-Eudesmol showed two peaks under UV detection at 27.837 and 40.721 minutes. Based on the chromatographic profile, β-Eudesmol appears to be the main compound present in the roots of *C. nardus* and *C. citratus*, with a marked retention time around 27.80 minutes, as shown

in Figure 4.20. However, due to its relatively low concentration in the extract, this compound was not quantified in this study.

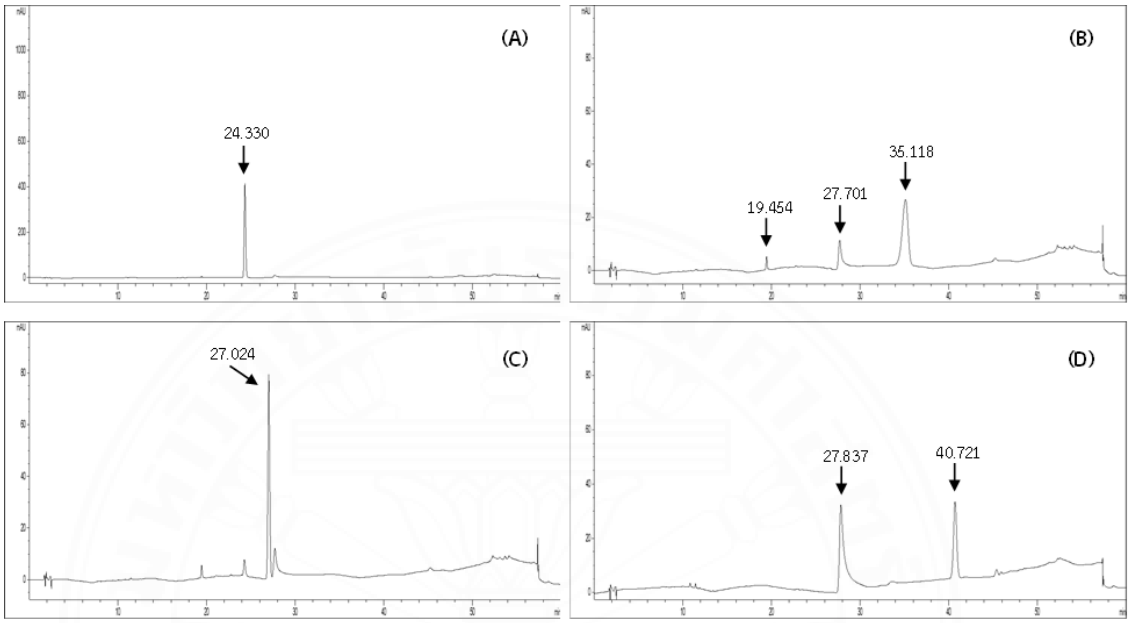

Figure 4.19 The retention time of the chromatogram of (A) Geraniol, (B) (±) Citronellal, (C) β-Citronellal, and (D) β-Eudesmol. UV detection at 215 nm.

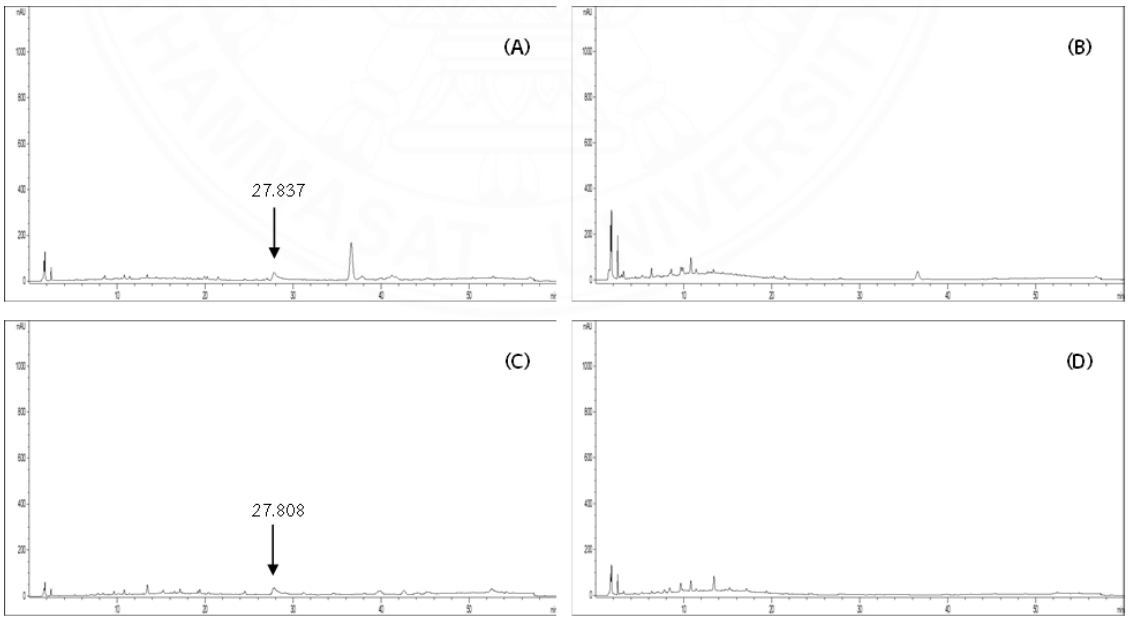

Figure 4.20 The retention time of the chromatogram of (A) CNE, (B) CNW, (C) CCE, and (D) CCW extracts. UV detection at 215 nm.

Compound D, zerumbone, and DMPBD were quantified by comparing them to the standard curve and presented as a percentage by weight of each compound. The results showed that ethanolic extracts contained higher levels of these standard compounds than the aqueous extracts. Among the ethanolic extract of remedies TPECN and TPECC), zerumbone was the highest content, followed by DMPBD and compound D. Furthermore, the findings indicated that compound D and DMPBD were identified in large amounts in the ZME, with a concentration of 6.97 ± 0.28 and 8.58 ± 0.24 % w/w, respectively. Zerumbone was shown in the highest content in the ZZE (36.05 ± 1.67% w/w), as shown in Table 4.15.

Table 4.15 Amount of standard compounds in Tri-Phon-That and ingredient extracts.

Samples	% w/w of standard compounds		
	Compound D	DMPBD	Zerumbone
TPECN	3.16 ± 0.17	4.6 ± 0.30	13.31 ± 0.87
TPWCN	0.56 ± 0.05	0.14 ± 0.01	0.23 ± 0.01
TPECC	2.38 ± 0.02	3.05 ± 0.08	10.06 ± 0.31
TPWCC	0.69 ± 0.03	0.17 ± 0.01	0.30 ± 0.02
ZZE	ND	ND	36.05 ± 1.67
ZZW	ND	ND	1.07 ± 0.02
ZME	6.97 ± 0.28	8.58 ± 0.24	ND
ZMW	1.11 ± 0.08	0.33 ± 0.02	ND
CNE	ND	ND	ND
CCE	ND	ND	ND

ND means Not Detectable.

4.7 Stability testing

After testing the anti-inflammatory activities, the two extracts that exhibited the highest anti-inflammatory effect, including TPECN and TPECC, were selected for stability testing. Both extracts were stored in a stability chamber for six

months. During this duration, their inhibitory effects on NO and IL-6 production were monitored, and their chemical constituents were quantified. The results showed a gradual decline in the inhibitory activity against NO and IL-6, indicating a reduction in the anti-inflammatory activity of the extracts over the accelerated condition period. The result of TPECN is shown in Table 4.16 and Figure 4.21, and TPECC is shown in Table 4.17 and Figure 4.22. After 180 days of stability testing, the IC_{50} values for NO inhibition were 18.88 ± 5.52 µg/mL for TPECN and 15.78 ± 7.70 µg/mL for TPECC. For IL-6 inhibition, the IC_{50} values were 19.69 ± 1.40 and 21.78 ± 0.70 µg/mL, respectively. Statistical analysis revealed a significant reduction in NO inhibition by TPECN since day 120, and TPECC was significant since day 90 compared to day 0. For IL-6 inhibition, significant reductions were observed from day 120 toward TPECN remedy, statistically significant differences from day 90, 150, and 180 for TPECC, indicating the impact of accelerated storage on bioactivity.

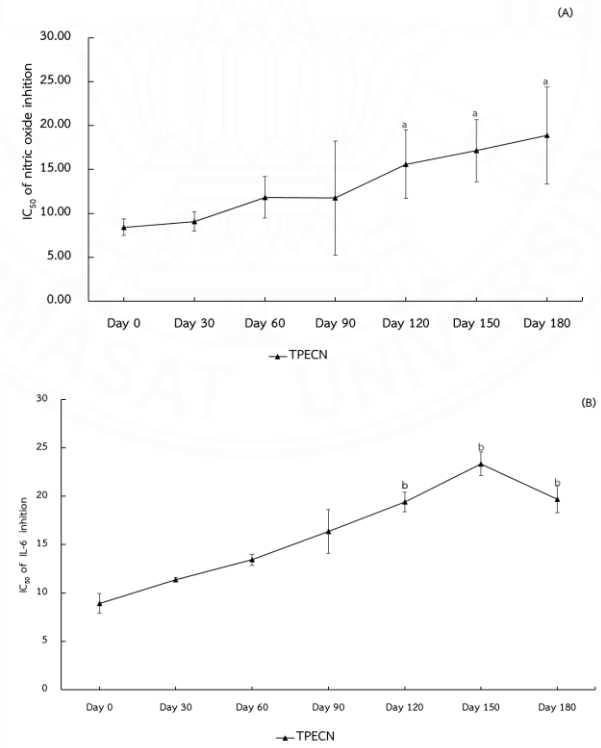

Figure 4.21 The IC_{50} of stability testing of (A) nitric oxide inhibition, (B) IL-6 inhibition of TPECN extracts. [b] Significant differences ($p < 0.05$) were observed when compared to Day 0, as tested by the Kruskal-Wallis H test.

Table 4.16 The inhibitory activity against Nitric oxide and IL-6 of TPECN remedy after stability testing.

Day	Conc. (µg/mL)	%Survival	Nitric oxide		IL-6	
			%Inhibition	IC_{50} (µg/mL)	%Inhibition	IC_{50} (µg/mL)
Day0	25	75.10 ± 5.22	97.81 ± 1.50	8.41 ± 0.93	92.52 ± 0.99	8.93 ± 1.02
	12.5	87.45 ± 6.46	78.16 ± 2.02		70.15 ± 1.60	
	6.25	100.15 ± 11.77	42.40 ± 0.16		34.85 ± 10.27	
	3.125	90.24 ± 8.56	22.63 ± 2.41		24.25 ± 11.50	
Day30	25	71.98 ± 0.67	94.58 ± 3.24	9.08 ± 1.08	87.22 ± 0.92	11.39 ± 0.22
	12.5	91.16 ± 5.03	70.37 ± 7.95		53.27 ± 0.13	
	6.25	96.04 ± 8.68	30.39 ± 3.38		24.92 ± 5.39	
	3.125	94.22 ± 3.05	8.65 ± 1.97		-1.83 ± 10.11	
Day60	25	79.70 ± 5.36	86.68 ± 9.04	11.83 ± 2.36	71.76 ± 0.43	13.43 ± 0.58
	12.5	89.00 ± 5.95	58.90 ± 14.78		46.70 ± 1.89	
	6.25	94.08 ± 3.71	27.85 ± 10.27		26.24 ± 1.43	
	3.125	96.53 ± 3.01	15.39 ± 7.11		21.83 ± 4.85	
Day90	25	77.92 ± 3.24	83.72 ± 13.81	11.75 ± 6.48	73.20 ± 1.61	16.37 ± 2.27
	12.5	87.76 ± 4.52	51.33 ± 16.97		45.38 ± 0.96	
	6.25	96.31 ± 3.42	31.43 ± 17.30		31.80 ± 1.33	
	3.125	93.82 ± 4.70	12.14 ± 1.91		13.95 ± 5.63	
Day120	25	81.74 ± 2.50	73.94 ± 4.71	15.60 ± 3.89[a]	64.32 ± 4.71	19.42 ± 1.03[b]
	12.5	94.78 ± 0.83	40.86 ± 11.57		44.96 ± 1.78	
	6.25	100.61 ± 7.01	21.65 ± 7.06		39.04 ± 8.21	
	3.125	92.31 ± 2.37	11.55 ± 2.87		-10.71 ± 4.83	
Day150	25	80.09 ± 6.50	71.19 ± 9.96	17.13 ± 3.55[a]	55.70 ± 3.57	23.37 ± 1.23[b]
	12.5	93.91 ± 5.32	36.57 ± 10.29		13.32 ± 5.67	
	6.25	100.62 ± 5.55	18.11 ± 7.14		1.54 ± 1.47	
	3.125	92.58 ± 11.49	11.07 ± 1.73		-10.47 ± 14.80	

Table 4.17 The inhibitory activity against Nitric oxide and IL-6 of TPECN remedy after stability testing.(Cont.)

Day	Conc. (µg/mL)	%Survival		Nitric oxide		
		%Inhibition	%Inhibition	IC_{50} (µg/mL)	IC_{50} (µg/mL)	
Day180	25	80.86 ± 4.45	63.41 ± 11.43	18.88 ± 5.52[a]	61.46 ± 0.21	19.69 ± 1.40[b]
	12.5	90.32 ± 7.29	35.24 ± 10.00		38.91 ± 4.28	
	6.25	93.33 ± 3.39	17.18 ± 3.01		27.88 ± 3.92	
	3.125	96.08 ± 7.63	10.55 ± 1.21		16.48 ± 4.07	

Note: The result represents mean ± SD from three experiments. [a, b]Significant differences ($p < 0.05$) were observed when compared to Day 0, as tested by the Kruskal-Wallis H test.

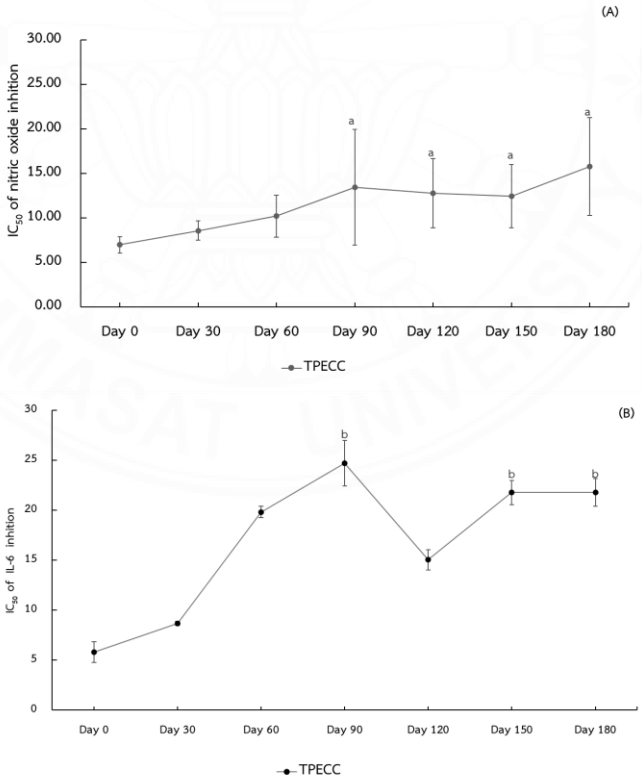

Figure 4.22 The IC_{50} of stability testing of (A) nitric oxide inhibition, (B) IL-6 inhibition of TPECC extracts. [a, b] Significant differences ($p < 0.05$) were observed when compared to Day 0, as tested by the Kruskal-Wallis H test.

Table 4.18 The inhibitory activity against Nitric oxide and IL-6 of TPECC remedy after stability testing.

Day	Conc. (µg/mL)	%Survival	Nitric oxide		IL-6	
			%Inhibition	IC_{50} (µg/mL)	%Inhibition	IC_{50} (µg/mL)
Day0	25	96.49 ± 2.89	99.66 ± 3.09	6.97 ± 1.07	97.86 ± 0.02	5.80 ± 0.28
	12.5	101.63 ± 11.46	88.67 ± 4.34		84.87 ± 1.12	
	6.25	92.50 ± 33.96	58.00 ± 8.41		52.56 ± 2.18	
	3.125	84.24 ± 43.92	34.03 ± 4.43		37.30 ± 6.30	
Day30	25	97.83 ± 11.32	97.38 ± 3.16	8.56 ± 2.22	92.18 ± 0.11	8.67 ± 0.98
	12.5	104.09 ± 14.06	73.27 ± 0.95		63.16 ± 0.87	
	6.25	98.74 ± 7.19	44.07 ± 6.10		42.05 ± 3.38	
	3.125	104.03 ± 16.77	23.40 ± 4.76		33.95 ± 0.41	
Day60	25	96.33 ± 4.41	92.51 ± 6.36	10.21 ± 1.92	63.66 ± 3.44	19.82 ± 1.85
	12.5	104.27 ± 10.86	70.01 ± 17.60		31.37 ± 2.16	
	6.25	106.74 ± 10.83	41.42 ± 11.42		14.77 ± 4.94	
	3.125	102.29 ± 15.40	24.23 ± 12.06		2.23 ± 0.45	
Day90	25	100.58 ± 15.40	89.02 ± 10.72	13.44 ± 4.44[a]	50.89 ± 0.76	24.68 ± 0.31[b]
	12.5	100.04 ± 9.71	58.22 ± 17.59		21.30 ± 0.25	
	6.25	107.99 ± 14.63	28.15 ± 11.46		12.12 ± 2.33	
	3.125	102.86 ± 17.30	12.98 ± 5.24		5.68 ± 0.13	
Day120	25	96.13 ± 1.17	89.45 ± 3.01	12.77 ± 4.25[a]	62.98 ± 3.05	15.03 ± 1.46
	12.5	100.59 ± 11.31	61.71 ± 11.04		41.48 ± 4.36	
	6.25	93.12 ± 4.40	35.76 ± 9.45		19.09 ± 3.07	
	3.125	93.66 ± 8.74	19.75 ± 2.55		12.86 ± 1.67	
Day150	25	93.26 ± 2.91	87.20 ± 4.17	12.46 ± 1.51[a]	61.42 ± 2.37	21.75 ± 1.31[b]
	12.5	97.41 ± 0.52	54.21 ± 7.76		26.48 ± 6.30	
	6.25	94.54 ± 5.52	26.00 ± 8.85		19.56 ± 3.17	
	3.125	95.89 ± 3.83	15.26 ± 8.77		14.62 ± 1.88	
Day180	25	90.54 ± 3.55	80.81 ± 9.51	15.78 ± 7.70[a]	53.91 ± 1.43	21.78 ± 0.70[b]
	12.5	99.20 ± 3.23	47.01 ± 13.99		28.74 ± 0.37	

Table 4.18 The inhibitory activity against Nitric oxide and IL-6 of TPECC remedy after stability testing.

Day	Conc. (µg/mL)	%Survival	Nitric oxide		IL-6	
			%Inhibition	IC_{50} (µg/mL)	%Inhibition	IC_{50} (µg/mL)
	6.25	92.79 ± 3.40	20.57 ± 10.73		11.08 ± 1.61	
	3.125	95.56 ± 5.70	4.71 ± 2.50		8.03 ± 2.69	

Note: The result represents mean ± SD from three experiments. [a, b] Significant differences ($p < 0.05$) were observed when compared to Day 0, as tested by the Kruskal-Wallis H test.

Furthermore, the concentrations of the three standard compounds exhibited a consistent decreasing trend over 180 days under accelerated storage conditions. The analysis was performed using HPLC, and the results were expressed as the percentage of the remaining amount (mean ± SD) compared to the initial concentration on day 0. Both of the remedy extracts, a significant reduction of DMPBD and zerumbone content starting from day 120 onwards. On the other hand, compound D in the extracts demonstrated greater stability; it observed a significant reduction only at day 30 in TPECC extracts. These findings are presented in Table 4.18 and Figure 4.23, highlighting the differences in chemical stability between the two formulations.

Table 4.19 The percentage remaining of the standard compound in Tri-Phon-That extracts after stability testing

Sample	Day	Compound D	DMPBD	Zerumbone
TPECN	D0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	D30	105.15 ± 6.52	73.35 ± 5.02	82.82 ± 5.90
	D60	96.19 ± 6.62	40.79 ± 5.36	52.03 ± 8.17
	D90	97.50 ± 6.27	35.49 ± 0.93	49.66 ± 1.33
	D120	96.30 ± 4.83	22.69 ± 2.52[b]	33.59 ± 2.39[c]
	D150	94.31 ± 6.50	16.73 ± 2.19[b]	26.26 ± 5.17[c]
	D180	94.09 ± 7.35	16.44 ± 2.64[b]	26.51 ± 3.22[c]
TPECC	D0	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	D30	115.08 ± 6.93[a]	69.06 ± 5.77	83.40 ± 6.95
	D60	108.14 ± 5.82	43.17 ± 1.79	63.16 ± 3.44
	D90	106.47 ± 1.21	29.22 ± 0.15	50.38 ± 2.78
	D120	106.66 ± 3.07	18.21 ± 0.69[b]	37.32 ± 4.17[c]
	D150	103.13 ± 3.43	12.97 ± 1.17[b]	29.97 ± 1.23[c]
	D180	99.96 ± 2.00	9.13 ± 0.88[b]	24.81 ± 0.80[c]

Note: The result represents mean ± SD from three experiments. [a, b, c]Significant differences ($p < 0.05$) observed when compared to Day 0, tested by the Kruskal-Wallis H test.

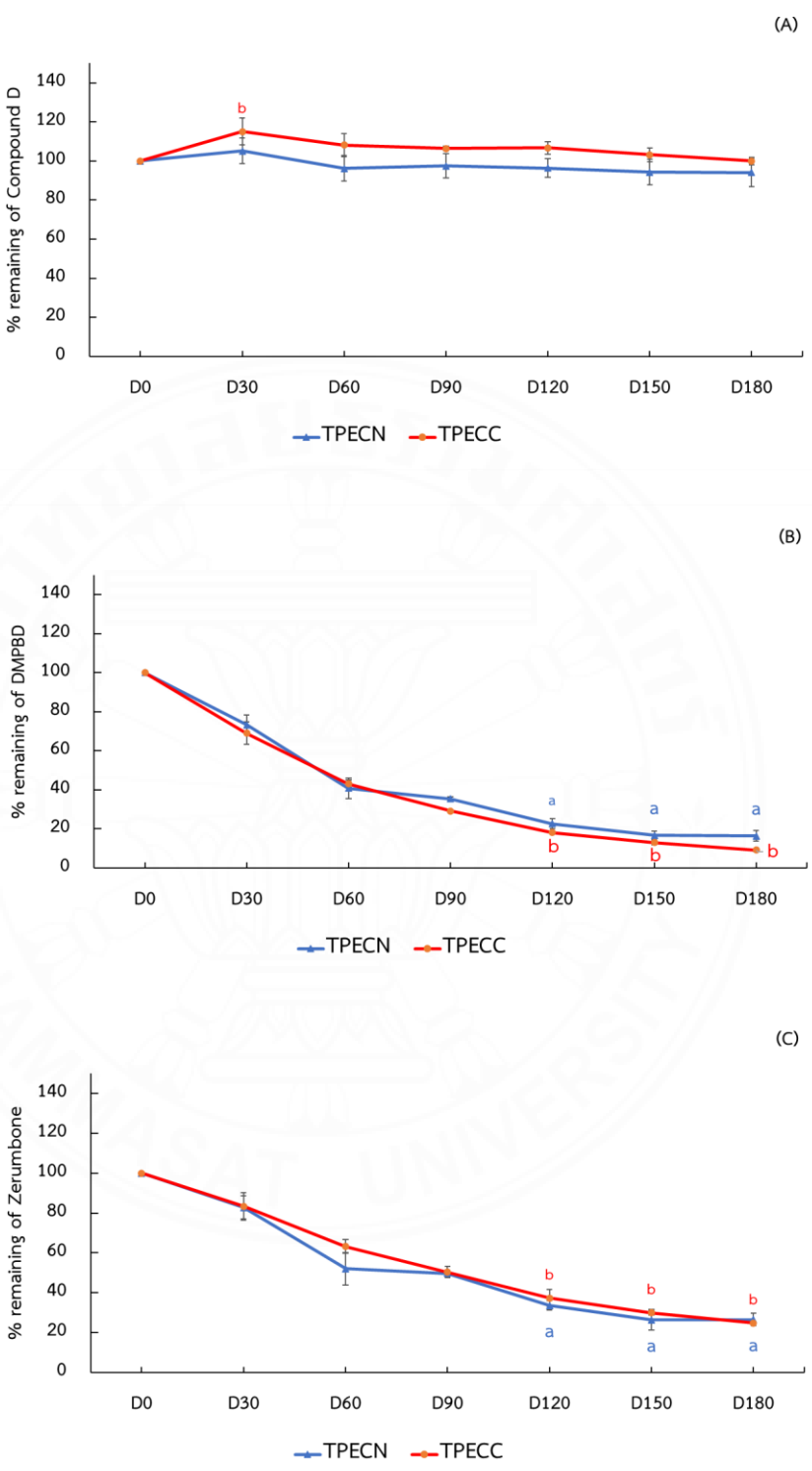

Figure 4.23 The percentage remaining of (A) compound D, (B) DMPBD, and (C) zerumbone in TPECN and TPECC extracts after stability testing. [a,b] Significant differences ($p < 0.05$) were observed when compared to Day 0, tested by the Kruskal-Wallis H test.

4.8 Comparison of bioactivity and chemical constituents of the two types of Tri-Phon-That remedy, using *C. nardus* and *C. citratus*.

Tri-Phon- That remedy consists of a root *Z. zerumbet, Z. montanum*, and *C. nardus*, which are used to reduce fever and inflammation. From the literature review, they found the anti-inflammatory and anti-nociceptive effects on the animal model and reported no cytotoxicity (Deeoun et al., 2014). However, it might be irritating and slightly interrupt the development of the cells due to the essential oil from *C. nardus* (Piasecki et al., 2021; European Food Safety Authority et al., 2024). Two types of remedy were prepared in this study: one following the original formulation and the other in which *C. citratus* was used as a substitute for *C. nardus*. The cytotoxicity results of the extracts on human keratinocyte (HaCat) cells in this study support the previous findings indicating that *C. nardus* exhibits higher cytotoxic effects than *C. citratus*. This finding can be attributed to differences in the concentration thresholds considered safe for cells. The CNE exhibited a cell-safe concentration of 71.79 ± 22.75 µg/mL, whereas the CCE remained non-toxic even at concentrations exceeding 100 µg/mL. Consequently, the TPECN formulation displayed significant cytotoxicity, with cell-safe concentrations of 30.81 ± 12.55 µg/mL and 60.41 ± 11.40 µg/mL. These findings suggest that *C. citratus* may be more suitable for topical formulations where cytocompatibility is a key consideration.

Moreover, this study compared the anti-inflammatory and anti-bacterial effects of the conventional herbal remedy with an alternative formulation in which *C. citratus* was substituted for *C. nardus* to evaluate their potential interchangeability. Overall, the ethanolic extracts expressed greater potential than aqueous extracts in both anti-inflammatory and anti-bacterial activity. The antibacterial effects of TPECN and TPECC were comparable, with both formulations effectively inhibiting the growth of gram-positive bacteria. However, neither extract showed inhibitory effects against gram-negative bacteria at the tested concentrations. Notably, *S. pyogenes* and *S. pneumoniae* were sensitive to both TPECN and TPECC, with similar levels of efficacy,

as shown in Table 4.19. Other bacterial strains included in this study did not respond to treatment with either extract, indicating a selective antibacterial ability.

Table 4.20 The comparison of the MIC and MBC of TPECN and TPECC

Samples	*S. pyogenes*		*S. pneumoniae*	
	MIC (µg/mL)	MBC (µg/mL)	MIC (µg/mL)	MBC (µg/mL)
TPECN	156	156	39	156
TPECC	156	156	78	156

The anti-inflammatory properties of the tested extracts were evaluated based on their ability to inhibit inflammatory mediators, including nitric oxide, PGE_2, IL-6, and TNF-α, which are known to play an important role in the pathogenesis of inflammatory responses (Chen et al., 2018; Furman et al., 2019). The results revealed that both Tri-Phon-That remedy extracts, including TPECN and TPECC, exerted a notable anti-inflammatory effect. TPECN demonstrated significantly stronger inhibitory activity against NO production, as reflected by its significantly lower IC_{50} values compared to TPECC. On the other hand, the activities on PGE2 and IL-6 TPECC inhibition did not show a difference between the 2 groups. Neither extract affects TNF-α production at the tested concentration, as shown in Table 4.20.

Table 4.21 The IC_{50} (µg/mL) of TPECN and TPECC on inflammatory mediators.

Samples/ Activities	Nitric oxide inhibition	PGE_2 inhibition	IL-6 inhibition	TNF-α inhibition
TPECN	5.35 ± 0.77	24.75 ± 0.328	7.81 ± 0.31	> 100
TPECC	7.02 ± 0.61*	17.50 ± 0.03	10.46 ± 0.08	> 100

*Significant differences ($p < 0.05$) were observed when compared to each other, as determined by the Kruskal-Wallis H test.

Based on statistical analysis, TPECN demonstrated a greater overall anti-inflammatory effect, but TPECC still expressed comparable efficacy. Despite the significant differences in the NO secretion inhibitory activities of the two remedies, both formulations exhibited better activity than the standard drug, Ibuprofen, which has an IC_{50} of 89.63 ± 7.21 µg/mL. These findings suggest that *C. citratus* may replace *C. nardus*, but it remains a promising alternative in specific applications.

4.9 Discussion

The Tri-Phon-That remedy is a traditional Thai medicinal (TTM) remedy with historical documentation, where it has long been recognized for its therapeutic effects in alleviating fever, swelling, and musculoskeletal pain. It consists of three herbal ingredients: *Zingiber zerumbet, Zingiber montanum, and Cymbopogon nardus* (Foundation for the Promotion of Thai Traditional Medicine, 2005). Experimental research has demonstrated the analgesic effects of this remedy in rodent models involving inflammation-induced paw edema, indicating its potential as a pain-relieving agent (Deeoun *et al.*, 2014). Additionally, clinical investigations have also investigated its effectiveness in relieving lower back pain (Jarukitsakul et al., 2024). However, the precise pharmacological mechanisms underlying its anti-inflammatory and analgesic effects remain unclear. To address this knowledge gap, the present study evaluated the inhibitory effects of Tri-Phon-That remedy on key inflammatory mediators, including nitric oxide, PGE_2, IL-6, and TNF-**α**, which are critically involved in the inflammatory response (Chen et al., 2018; Furman et al., 2019). Furthermore, antibacterial activity was tested to explore the potential of the remedy in managing infections associated with febrile conditions (El-Radhi, 2018). A comparative study was also conducted between the original formulation containing *C. nardus* and a modified version using *C. citratus*, a substitution proposed due to concerns over cytotoxic and potential skin-irritating effects associated with *C. nardus* (Piasecki et al., 2021; European Food Safety Authority et al., 2024).

Extracts were prepared using both 95% ethanol and water. The antibacterial activity, as evaluated by minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC), demonstrated that the extracts were more effective against gram-positive than gram-negative bacteria. This can be explained by the structural differences in bacterial cell walls. Gram-negative bacteria possess an outer membrane composed of lipopolysaccharides, which limits the penetration of antimicrobial agents, whereas gram-positive bacteria lack this barrier, allowing greater access to bioactive compounds (Cock, 2008; Koohsari et al, 2015; Silhavy, Kahne, & Walker, 2010). Interestingly, the antibacterial effects of *Z. montanum*, *C. nardus*, and *C. citratus* extracts against *S. aureus* differ from previous studies (Hammer, Carson, & Riley, 1999; Wei & Wee, 2013; Verma et al., 2018; Siddique, Pendry, & Rahman, 2019; Hassan et al., 2023). It may also be attributed to the differences in plant species, geographical environment, or harvesting season (Ncube et al., 2012).

The present results further confirm that ZZE and ZME possess potent anti-inflammatory effects, notably through the suppression of NO, IL-6, and PGE_2. These findings are consistent with prior research indicating that these herbal extracts exert anti-inflammatory responses via the downregulation of NF-**K**B signalling and suppression of iNOS and COX-2 expression (Han, Kim, Jeong, Lee, & Seo, 2005; Chien, Huang, Lee, Tsai, & Wang, 2008; Kaewchoothong et al., 2012; Park et al., 2015; Khemawoot et al., 2016; Chien et al., 2016; Haque et al., 2019; Rawat et al., 2023). Furthermore, TPECN and TPECC extracts demonstrate good efficacy in inhibiting NO and IL-6, which are endogenous mediators of acute fever. These substances are rapidly secreted during the initial phase after exposure to various inflammatory agents (Sharma et al., 2007; Menzel et al., 2021). Therefore, the Tri-Phon-That extract is suitable for acute fever or acute inflammation. The evidence of phytochemical analysis confirmed that the major active constituents, including compound D, DMPBD, and zerumbone, were present in high concentrations in the ethanolic extracts (ZME, ZZE) and the combined remedies (TPECN, TPECC), whereas their presence in aqueous extracts contained substantially lower levels of these active compounds. Although *C. nardus* and *C. citratus* have previously been reported to contain anti-inflammatory

constituents, such as citronellal, geraniol, citral, nerol, and elemol (Phuneerub, 2014; Lawal et al., 2017; Lulekal et al., 2019; Jayaganesh et al., 2020). Only a small amount of β-Eudesmol was detected in the current study's extract under the given analytical conditions. Moreover, β-Eudesmol has not been well-characterized for its role in cytokine inhibition, leaving its pharmacological significance uncertain.

All the results revealed that ethanolic extracts have more potential anti-inflammatory and antibacterial activity than their aqueous extracts. This difference is likely due to the greater solubility of low-polarity bioactive compounds, leading to higher dissolution in ethanol compared to water. Previous research on *Z. montanum* extraction using 40% ethanol, a medium to low polarity solvent, yielded the highest amount of Compound D DMPBD (Tangyuenyongwatana & Gritsanapan, 2009). Additionally, zerumbone is known to extract well in organic solvents but poorly in water, thus contributing to the superior efficacy observed in the ethanol fraction (Kesharwani & Bhat, 2020). Conversely, we found that the aqueous extract of the Tri-Phon-That remedy not only failed to exhibit anti-inflammatory activity but also increased the production of NO, IL-6, PGE_2, and TNF-α. This might be due to the aqueous extract acting as an immunostimulant, leading to the activation of inflammatory mediators. This is similar to previous research on the effects of polysaccharides derived from *Ganoderma lucidum*, which are highly soluble in water (Ren, Zhang, & Zhang, 2021; Song et al., 2021). While both *C. nardus* and *C. citratus* have mild effects, the combination of plant ingredients in Tri-Phon-That extracts demonstrated enhanced biological activity compared to individual plant extracts, supporting previous findings that polyherbal formulations can show synergistic therapeutic effects (Zhao et al., 2020). This aligns with the principles of Thai Traditional medicine (TTM) in formulating remedies, which involve primary, secondary, and complementary herbs that help alleviate various symptoms (Foundation for the Promotion of Thai Traditional Medicine, 2005). The primary goal in alleviating fever is to reduce the heat from the fire element. This is accomplished by administering bitter-tasting herbs or flavorless herbs that promote diuretic effects, thereby reducing heat by expelling and balancing the water element. Furthermore, spicy-tasting herbs are

incorporated to enhance the circulation of the wind element, helping to reduce fever-related symptoms such as fatigue, nausea, vomiting, dizziness, and muscle pain (Lumlerdkij et al., 2020; Prommee et al., 2021; Thiantongin & Pisaipan, 2023). Both *C. nardus* and *C. citratus* contain citral as their primary active compound (Lawal et al., 2017; Lulekal et al., 2019; Kaur et al., 2021), which has diuretic properties (Ossibi et al., 2019). From the perspective of TTM principles, this may help reduce body heat and improve the flavor possesses a bitter and astringent taste from *Z. montanum* and *Z. zerumbet*, making them easy to consume. However, past research has not found a clear, consistent correlation between increased diuresis and a decrease in body temperature. Therefore, this study recommends that the Tri-Phon-That remedy is well-suited for reducing mild fevers, especially when accompanied by wind element affects symptoms. According to TTM preparation processes, 28 methods of drug preparation are specified (Foundation for the Promotion of Thai Traditional Medicine, 2005). This research simulated drug preparation by decoction (boiling in water) and maceration in alcohol for oral administration. The results of this study found that the aqueous extracts had no anti-inflammatory activity, nor did they inhibit bacteria that could cause fever. Conversely, the ethanol extracts exhibited good efficacy. However, alcohol consumption is contraindicated in the treatment of febrile patients in TTM, as stipulated in the Tak-ka-si-la scripture (Foundation for the Promotion of Thai Traditional Medicine, 2016). This suggests that the alcohol-based preparation may not be suitable for fever reduction as effectively as desired. Therefore, if this remedy is to be utilized, other preparation methods should be considered, such as pulverization with a medicinal vehicle or distillation with alcohol, where the vapor is consumed, which would result in a reduced alcohol content. Alternatively, the extract could be processed using an alcohol extraction method and subsequently formulated into a capsule dosage form. In addition, under accelerated storage conditions, a noticeable decline in antinflammatory activity was observed in TPECN and TPECC, particularly in their ability to inhibit NO and IL-6. This reduction correlated with the degradation of active compounds, as revealed through quantitative HPLC analysis. Zerumbone and DMPBD demonstrated significant instability, with a marked decrease after day 120.

These results align with previous studies recommending cold storage at -20°C to preserve the bioactivity of zerumbone and maintain its bioactivity (Kesharwani & Bhat, 2020). Similarly, the extract of *Z. montanum* has been shown to degrade rapidly when exposed to high temperature at 50°C or UV light, further supporting the recommendation that these extracts be stored in cool, dark conditions to preserve their chemical integrity (Janpim et al., 2013).

Based on the objective of comparing the efficacy of Tri-Phon-That containing *C. nardus* and *C. citratus* due to safety concerns associated with *C. nardus*, this study demonstrates that CNE extract exhibits higher toxicity than CCE extract. The safe concentration for CNE extract was determined to be 71.79 ± 22.75 µg/mL, whereas CCE extract showed no toxicity even at a concentration of 100 µg/mL. Furthermore, the TPECN demonstrated significantly higher cytotoxicity to HaCat cells compared to the TPECC. These findings highlight the potential interest in using *C. citratus* as a substitute. Considering the antibacterial and anti-inflammatory effects, *C. citratus* has comparable activities to *C. nardus* in the Tri-Phon-That remedy. Although, particularly due to its comparatively lower efficacy on NO production. However, it has comparable IL-6 inhibition and also a stronger effect on PGE_2 inhibition and better activity than the standard drug (ibuprofen), suggesting that it may serve as a substitute for specific therapeutic effects.

CHAPTER 5
CONCLUSIONS AND RECOMMENDATIONS

The Tri-Phon-That remedy, a traditional Thai formulation, has demonstrated promising therapeutic effects, particularly in alleviating pain and inflammation. The remedy consists of three principal herbal components: *Z. zerumbet*, *Z. montanum*, and *C. nardus*. This study revealed that a better understanding of the anti-inflammatory and antibacterial effects of this formulation and plant ingredient extracts is needed. Additionally, the study compared the efficacy of the original remedy containing *C. nardus* with a modified version using *C. citratus* as a substitute. The findings from the current study highlight the greater biological activities of the ethanolic extracts of the Tri-Phon-That remedy, with more potent anti-inflammatory and antibacterial effects than aqueous extracts. The key bioactive compounds, including compound D, DMPBD, and zerumbone, were found to be more abundant in the ethanolic extracts. The study also confirmed that the TPECN remedy has anti-inflammatory efficacy comparable to the TPECC remedy, which is linked to the inhibition of pro-inflammatory mediators, as evidenced by reduced levels of NO, IL-6, and PGE_2. This suggests that *C. citratus* may be used as a substitute for *C. nardus*. However, stability testing revealed that the active compounds, particularly zerumbone and DMPBD, exhibited instability under accelerated storage conditions, significantly reducing their concentrations. This instability suggests that the extracts should be stored at low temperatures and away from light to maintain their bioactivity.

Our current study indicates a need for further scientific inquiry into the phytochemistry and biological activities of *C. nardus* and *C. citratus*, as their precise chemical constituents are largely unknown. We recommend Gas Chromatography-Mass Spectrometry (GC-MS) analysis to identify potential bioactive compounds, which will enable subsequent quantitative analyses to determine their concentrations. Furthermore, investigating formulations excluding *C. nardus* and comparing with the original remedy containing it will clarify its therapeutic contribution and could streamline herbal sourcing and application if it proves dispensable. To advance their

potential clinical application, investigating the immunomodulatory effects of aqueous extracts is crucial. This should involve analyzing the extracts' mechanisms of action at the cellular level using various cell lines, such as K562, J779 macrophage, or cutaneous squamous cell carcinoma, alongside studying the *in vivo* immune response in animal models. Additionally, long-term stability studies and safety and efficacy trials in clinical models should be conducted to confirm compound stability, product efficacy, and sustained anti-inflammatory activity.

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APPENDIX

Table A1 Concentration (µg/mL) of compound D spike in the extracts

Samples (mg/mL)	Compound D	Mean ± SD		
	Spike (µg/mL)	N1	N2	N3
TPECN 2mg	0	69.75 ± 0.20	68.05 ± 3.63	60.67 ± 0.24
TPECN 2mg	400	454.37 ± 5.40	454.99 ± 9.35	453.62 ± 5.70
TPECN 2mg	200	269.74 ± 3.39	268.92 ± 3.60	264.34 ± 3.23
TPECN 2mg	100	171.84 ± 1.27	167.29 ± 2.16	163.12 ± 1.42
TPECN 2mg	50	120.54 ± 0.80	119.41 ± 3.37	112.76 ± 1.13

Table A2 Concentration (µg/mL) of zerumbone spike in the extracts

Samples (mg/mL)	Zerumbone	Mean ± SD		
	Spike (µg/mL)	N1	N2	N3
TPECN 2mg	0	309.29 ± 0.87	295.54 ± 21.03	268.16 ± 0.09
TPECN 2mg	400	720.99 ± 2.66	700.77 ± 22.89	668.55 ± 5.22
TPECN 2mg	200	511.23 ± 3.06	500.34 ± 22.56	471.67 ± 1.50
TPECN 2mg	100	411.56 ± 1.01	398.61 ± 22.55	369.68 ± 1.03
TPECN 2mg	50	359.04 ± 0.91	345.50 ± 20.94	318.94 ± 0.16

BIOGRAPHY

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Publications

Chitkrachang, N., Panthong, S., Ploysombun, S., Choosrichom, S., Ngamkham, N., Kwanchian, D., & Sriyom, R. (2023). A comparison of antibacterial activity against acne-inducing bacteria, anti-inflammatory activity, and total phenolic content of fresh and dried leaves of *Thunbergia laurifolia* Lindl extracts. *Journal of Thai Traditional and Alternative Medicine*, *21*(2), 356-366.

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