EFFECT OF THE COMBINED EXTRACT OF *THUNBERGIA LAURIFOLIA* LINN. (RANG CHUET) AND *ZINGIBER OFFICINALE* (GINGER) ON PM2.5 INDUCED OXIDATION, INFLAMMATION, AND TOXICATION IN HUMAN CELLS LINE

CHATTIP SUNTHRARAK

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ผลของสารสกัดรวมของรางจืดและขิงต่อพีเอ็ม 2.5 เหนี่ยวนำให้เกิดออกซิเดชัน การอักเสบ และความเป็นพิษในเซลล์ของมนุษย์

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คำสำคัญ : รางจืด/ขิง/ฝุ่นละอองขนาดเล็ก PM2.5/สารต้านอนุมูลอิสระ/การอับเสบ

งานวิจัยนี้มุ่งประเมินปฏิสัมพันธ์ของสารสกัดรวมจากใบรางจืด (*Thunbergia laurifolia* Linn.) และขิง (*Zingiber officinale*) ต่อกิจกรรมต้านอนุมูลอิสระทั้งระดับเคมีและระดับเซลล์ อีกทั้งประเมินประสิทธิภาพของสารสกัดรวมเหล่านี้ในการต้านอนุมูลอิสระและลดการอักเสบภายหลังการสัมผัสฝุ่นละอองขนาดเล็ก PM2.5 สารสกัดรางจืด (RWE) เตรียมโดยอบแห้งด้วยวิธีสุญญากาศและสกัดด้วยน้ำ สารสกัดขิง (GEE) เตรียมโดยสกัดด้วยเอทานอล นำสารสกัดทั้งสองชนิดมาผสมรวมกันในอัตราส่วนที่แตกต่างกัน ผลการทดสอบพบว่า สารสกัดรวมแสดงฤทธิ์ต้านอนุมูลอิสระแบบเสริมฤทธิ์ในอัตราส่วน 5:1 (v/v) เมื่อวัดด้วยวิธี DPPH และ FRAP ในระดับเซลล์ พบว่า การใช้ RWE หรือ GEE เพียงอย่างเดียว รวมไปถึงสารสกัดรวม ล้วนมีผลป้องกันเซลล์และกำจัดอนุมูลอิสระภายในเซลล์ (ROS) จาก H_2O_2 ในเซลล์ตับ (HepG2) อย่างไรก็ตาม สารสกัดรวมในอัตราส่วน 1:1 ที่ความเข้มข้น 50 และ 100 µg/ml ไม่แสดงฤทธิ์ต้านอนุมูลอิสระที่เหนือกว่าสารสกัดเดี่ยว และไม่แสดงฤทธิ์ต้านอนุมูลอิสระแบบเสริมฤทธิ์ นอกจากนี้ สารสกัดรวมยังเพิ่มการแสดงออกของยีนต้านอนุมูลอิสระ เช่น HO-1, SOD, CAT และ NQO1 ได้มากกว่าสารสกัดเดี่ยว และแสดงฤทธิ์ต้านอนุมูลอิสระแบบเสริมฤทธิ์เมื่อใช้สารสกัดรวม

นอกจากนี้ งานวิจัยนี้มุ่งประเมินผลของสารสกัดรางจืด(RWE) ขิง(GEE) และสารสกัดรวมของทั้งสองชนิด (CRGE) ต่อความเครียดออกซิเดชันในเซลล์ที่ถูกกระตุ้นโดยฝุ่นละอองขนาดเล็ก PM2.5 (Standard Reference Material® 2786, NIST) ผลการศึกษาพบว่า สารสกัดรวม (CRGE) มีประสิทธิภาพในการลดความเป็นพิษของเซลล์และการสร้างอนุมูลอิสระภายในเซลล์ที่เกิดจาก PM2.5 ได้อย่างมีนัยสำคัญ นอกจากนี้ ยังช่วยเพิ่มการแสดงออกของยีนต้านอนุมูลอิสระ เช่น HO-1, SOD และ CAT แม้ว่าเซลล์จะเผชิญกับความเครียดจาก PM2.5 ในเซลล์เยื่อบุปอดของมนุษย์ (A549) และลดการแสดงออกของยีนที่บ่งชี้การอักเสบ ในเซลล์ตับ (HepG2) พบว่าการแสดงออกของยีนต้านอนุมูลอิสระถูกควบคุมโดยสารสกัดรวม อย่างไรก็ตาม ผลลัพธ์นี้ขึ้นอยู่กับอัตราส่วนของสารสกัดรวมที่ใช้ ในเซลล์มาโครฟาจของมนุษย์ (THP-1) ที่สัมผัสกับ PM2.5 ประสิทธิภาพการต้านการอักเสบของสารสกัดรวมถูกประเมินโดยวัดระดับไนตริกออกไซด์ (NO) และการแสดงออกของ Interleukin-6

(IL-6) ซึ่งเป็นเครื่องหมายการอักเสบ ผลการศึกษาพบว่า สารสกัดรวมในอัตราส่วน 1:10 (v/v) ที่มีปริมาณ GEE สูง สามารถลดระดับ NO และ IL-6 ได้อย่างมีนัยสำคัญ เมื่อเทียบกับการใช้ GEE เพียงอย่างเดียว การเปลี่ยนแปลงทางชีวโมเลกุลที่เกิดจากการสัมผัสกับ PM2.5 และผลการป้องกันของสารสกัดรวมในเซลล์ A549 ถูกวิเคราะห์โดยใช้เทคนิค Synchrotron Fourier Transform Infrared (SY-FTIR) spectroscopy และการวิเคราะห์องค์ประกอบหลัก (PCA)

สรุปได้ว่า สารสกัดรวมของ RWE และ GEE มีคุณสมบัติต้านอนุมูลอิสระ ปรับการแสดงออกของยีนต้านอนุมูลอิสระ และสามารถลดการอักเสบ จึงมีศักยภาพที่จะนำไปพัฒนาเป็นส่วนผสมในผลิตภัณฑ์เพื่อสุขภาพและเสริมอาหาร

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา ศิรทิพย์

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CHATTIP SUNTHRARAK : EFFECT OF THE COMBINED EXTRACT OF *THUNBERGIA LAURIFOLIA* LINN. (RANG CHUET) AND *ZINGIBER OFFICINALE* (GINGER) ON PM2.5 INDUCED OXIDATION, INFLAMMATION, AND TOXICATION IN HUMAN CELLS LINE. THESIS ADVISOR : ASSOC. PROF. RATCHADAPORN OONSIVILAI, Ph.D., 133 PP.

Keyword: *THUNBERGIA LAURIFOLIA* LINN./RANG CHUET/*ZINGIBER OFFICINALE* (GINGER)/ PM2.5/ANTIOXIDANT/INFLAMMATION

This study investigated the interaction between the combined extracts of *Thunbergia laurifolia* Linn. (Rang Chuet) leaves and *Zingiber officinale* (Ginger) on their antioxidant activities, both at the chemical and cellular levels. Additionally the study explored the potential of these combined extracts to provide protection against oxidative stress and inflammation caused by exposure to PM2.5 fine dust particles.

Rang Chuet water extract (RWE) was prepared using oven vacuum drying followed by water extraction. Ginger ethanol extract (GEE) was also prepared. These extracts were then combined in various ratios. The combined extract exhibited a synergistic effect in DPPH and FRAP assays at a ratio of 5:1 (v/v), indicating enhanced antioxidant activity compared to the individual extracts.

Cellular studies using Human hepatoma (HepG2) cell models demonstrated a cytoprotective effect and intracellular ROS scavenging activity from H_2O_2 for both RWE and GEE alone, as well as their combined extracts. However, the combined extract at a 1:1 ratio and concentrations of 50 and 100 µg/ml did not show greater activity than the single extracts, nor did it exhibit a synergistic effect. Interestingly, the combined extracts demonstrated a more potent upregulation of antioxidant gene expression compared to the single extracts. Genes such as HO-1, SOD, CAT, and NQO1, all exhibited a synergistic effect on their expression when treated with the combined extracts.

To further assess the impact of these extracts on PM2.5-induced cellular oxidative stress, the study employed human epithelial lung (A549) cells and HepG2 cells exposed to PM2.5 (Standard Reference Material® 2786, NIST). Pre-treatment

with the combined extract (CRGE) significantly reduced PM2.5-induced cell toxicity and intracellular ROS production. Additionally, combined extracts upregulated the expression of antioxidant genes (HO-1, SOD, and CAT) even in cells experiencing oxidative stress from PM2.5. Furthermore, the combined extract reduced the expression of inflammatory marker genes in the A549 cells model. Notably, the expression of antioxidant genes in HepG2 cells was also regulated by the combined extracts, although the effect was dependent on the specific ratio of the combined extract.

The anti-inflammatory properties of the combined extracts were evaluated in human macrophage THP-1 cells exposed to PM2.5. Nitric Oxide (NO) levels and the expression of the proinflammatory marker Interleukin-6 (IL-6) were measured. The results showed that the combined extract at a ratio of 1:10 (v/v), containing a higher concentration of GEE, significantly reduced NO and IL-6 levels compared to using GEE alone. Biomolecular changes caused by PM2.5 exposure, and the protective effect of the combined extract in A549 cells, were further analyzed using Synchrotron Fourier Transform Infrared (SY-FTIR) spectroscopy and Principal Component Analysis (PCA).

In conclusion, the combined extract of RWE and GEE demonstrates potent antioxidant activity, modulates the expression of antioxidant genes, and exhibits anti-inflammatory effects. These findings suggest its potential application as a functional ingredient in food products to combat the detrimental effects of PM2.5 exposure.

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Student's Signature ศิรกิพย์
Advisor's Signature

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6.3 PCA of FTIR spectral ranges 3,000–2,800 cm^{-1} and 1,800-1,200 cm^{-1} giving PCA score plot (A). PCA loading plot show the biomarker differences over a spectral range of samples are identified by the PC1 (B) and PC2(C) loading plots..... 121

LIST OF ABBREVIATIONS

A549 cell	=	Adenocarcinoma human alveolar basal epithelial cells
ABTS•+	=	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt radical cation
ABTS	=	2,2’-azinobis (3-ethylbenzothiazoline-6-sulphonic acid
AOC	=	Antioxidant capacity
APS	=	Ammonium persulfate
ARE	=	Antioxidant response element
AsA	=	Ascorbic acid
CAA	=	Cellular antioxidant activity
CAT	=	Catalase
CAT	=	Gene encoding catalase (CAT)
CI	=	combination index
CRGE	=	Combined Rang chute and ginger extract
DCF	=	2’,7’-dichlorofluorescin
DCFH-DA	=	2’,7’-dichlorofluorescin diacetate
DMEM	=	Dulbecco’s modified eagle medium
DMRT	=	Duncan’s multiple range test
DPPH•	=	α, α-diphenyl-β-picrylhydrazyl free radical
E	=	The expected value
EDTA	=	Ethylenediamine tetra acetic acid
FBS	=	Fetal bovine serum
Ferrozine	=	3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate
FICC	=	Ferrous ion chelating capacity
FRAP	=	Ferric reducing antioxidant power
GPx	=	Glutathione peroxidase
GAPDH	=	Gene encoding glyceraldehyde 3-phosphate dehydrogenase

LIST OF ABBREVIATIONS (Continued)

Ferrozine	=	3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p′-disulfonic acid monosodium salt hydrate
GEE	=	Ginger ethanol extract
GPx1	=	Gene encoding glutathione peroxidase (GPx)
h	=	Hour
H_2O_2	=	Hydrogen peroxide
HAT	=	Hydrogen atom transfer
HepG2	=	Human hepatocellular carcinoma cell line
HO-1	=	Heme oxygenase
HPLC	=	High performance liquid chromatography
IC_{50}	=	50% Inhibitory concentration
IL-6	=	Gene encoding Interleukin 6
IL-8	=	Gene encoding Interleukin 8
Ahr	=	Gene encoding Air and elytroid 2-related factor 2
		Nrf2, Gene encoding Nuclear factor erythroid 2-related factor 2
M	=	Molar
min	=	Minute
mM	=	Millimolar
MTT	=	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
MW	=	Microwave
NEAAs	=	Non-essential amino acids
NQO1	=	Gene encoding NAD(P)H Quinone Dehydrogenase 1
O	=	The observed value
•OH	=	Hydroxyl radical
O•-	=	Superoxide anion radical
Keap1	=	Kelch-like ECH associated-protein 1
Nrf2	=	Nuclear factor erythroid 2-related factor 2
mL	=	Milliliter
MTT	=	3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide

LIST OF ABBREVIATIONS (Continued)

PCA	=	Principal Component Analysis
ROS	=	Reactive oxygen species
RWE	=	Rang chute water extract
s	=	Second
SOD	=	Superoxide dismutase
SOD1	=	Gene encoding superoxide dismutase (SOD)
SY-FTIR	=	Synchrotron - Fourier transform infrared spectroscopy
THP-1 cell	=	Human leukemia monocytic cell line
Trolox	=	(±)-6-hydroxy-2,5,7,8-tetramethylchromane-2- carboxylic acid
TPTZ	=	2,4,6-tris(2-pyridyl)-s-triazine
µM	=	micromolar
v/v	=	volume/volume
µL	=	Microliter
°C	=	Degree centigrade

CHAPTER I
INTRODUCTION

1.1 Background and Significance of the Study

The World Health Organization reports that annually, 7 million deaths occur due to air pollution exposure. Surprisingly, over 91% of the global population resides in areas where air pollution levels exceed WHO standards. Particularly concerning are particles smaller than 2.5 μm (PM2.5), which can deeply infiltrate the respiratory system, enter the bloodstream, and spread throughout the body (Hu et al., 2017; Shim et al., 2021). Chronic exposure to PM2.5 is associated with respiratory infections, inflammation, and a range of health problems affecting not only the respiratory system but also the central nervous system, cardiovascular health, liver function, blood quality, skin health, and reproductive organs due to the diverse toxic substances present in PM2.5(Fu et al., 2020; Hu et al., 2017; Spangenberg & Green, 2017; Veerappan, Sankareswaran, & Palanisamy, 2019; M.-X. Xu et al., 2019; F. Zhang et al., 2018).

The illnesses caused by PM2.5 are complex and influenced by several factors, with exposure potentially triggering various mechanisms. Previous investigations have confirmed that PM2.5 can prompt diseases through oxidative damage, inflammatory reactions, cell autophagy, and apoptosis (Bekki et al., 2016; Deng et al., 2013; Hu et al., 2017). One significant pathway through which PM2.5 exerts cellular toxicity is by heightening oxidative stress, characterized by increased reactive oxygen species (ROS) levels. Although studies have explored inflammation- and oxidative stress-induced cytotoxicity, research on biomolecular alterations caused by PM2.5 and its impact on remains limited.

The utilization of dietary polyphenolic compounds could offer an innovative approach to mitigating the health effects of PM2.5, potentially serving as a novel therapeutic or preventive strategy to combat the health risks posed by air pollution. Rang Chuet (*Thunbergia laurifolia* L.), a member of the Acanthaceae family found across Southeast Asia, is utilized in Thai traditional medicine as an antidote against

toxins. Studies have highlighted its antioxidant, anti-inflammatory, antimicrobial, antidiabetic, antipyretic, and anti-hyaluronidase properties. Interestingly, detoxifying effect of Rang Chuet, from several previous studies demonstrated that, can detoxify chemical toxins, drug poisoning and heavy metals. Ratchadaporn Oonsivilai and Ferruzzi (2008) reported that Rang Chuet has a potential for detoxifying effect by phase II enzyme induce. However, the effect of Rung Chuet against PM2.5 induced injury and how they effect on the lung and liver cell model has been limited and remains to be studied and elucidated.

Interestingly, the combination of bioactive compounds within plant extracts and range of natural products generates synergistic outcomes, amplifying the potency of these plant extracts. (Karimi, Ghanbarzadeh, Hamishehkar, Keivani, et al., 2015). Enhancing the activity of Rang Chuet extract through the synergistic abilities of natural products is a viable option, given that Rang Chuet contains phytochemical compounds with antioxidant activity. Additionally, ginger remarkable antioxidant and anti-inflammatory attributes stand out as crucial components. This section focuses on ginger root extract, its potent antioxidative qualities and its role as a natural anti-inflammatory agent. This study collectively elucidates the synergistic interactions of antioxidant activities between Rang Chuet leaf and ginger root extracts, aiming to be applied in the field of natural product-based therapeutics. Hence, the synergistic antioxidant activity of the optimized mixture can more effective or at a low dose of plant extracts.

Polyphenolic compounds and flavonoids display diverse antioxidant properties, employing five distinct mechanisms. The phenolic hydroxyl groups found in polyphenolic compounds function as hydrogen donors, directly neutralizing various active radicals like $\cdot O^{2-}$, H_2O_2, $\cdot OH$, $ROO\cdot$, $1O_2$. Moreover, these compounds inhibit enzyme activities responsible for generating radicals, chelate metal ions triggering free radical production, and enhance antioxidant enzyme activity or the expression of antioxidant proteins. Additionally, when combined with other substances, polyphenolic compounds generate synergistic antioxidant effects (Lobo, Patil, Phatak, & Chandra, 2010; Lv et al., 2021; Meister, 1988). Therefore, plant polyphenols or flavonoid represent natural antioxidants with advantages such as higher antioxidant activity and lower toxicity. To prove the antioxidant activities of plant extracts, it is

necessary to employ various suitable assays that accurately represent their activity in term of chemical antioxidative assays and cellular model.

Cellular models, Cellular Antioxidant Activity (CAA) reflects a capability of antioxidant to mitigate intracellular oxidative stress. This assay proves particularly valuable for investigating the bioavailability of food antioxidants (López-Alarcón & Denicola, 2013). Moreover, oxidative stress-related genes, inflammation and detoxification marker help to explain suppress inflammatory and oxidative damage, thereby alleviating PM2.5-induced cellular damages from plant extracts (Bekki et al., 2016; Ge et al., 2020). For inflammatory effect from PM2.5 treatment, it increase inflammatory cytokines production, specifically *IL-6* and *IL-8* (Zhai, Wang, Sun, & Xin, 2022). The screening technique examinations have widely employed nitric oxide (NO) productions (Ozleyen, Yilmaz, & Tumer, 2021). Although the Griess reagent system is a recognized method for quantifying NO production in cells, its usage in the context of THP-1 cells exposed to PM2.5 remains limited.

Synchrotron radiation-based Fourier transform infrared (SY-FTIR) spectroscopy has already been demonstrated in various cell research studies, providing a crucial role in the precise characterization of the biochemical composition within cellular systems. (Caliskan et al., 2021; Hengpratom et al., 2018; H. Liu et al., 2018). This technique reveals molecular details associated with their macromolecular composition, as seen through changes in absorbance bands present in the mid-IR range of 4,000–400 cm^{-1}, a region known as the molecular fingerprint area of the IR spectrum. Therefore, changes on the structure of lung cell with exposed PM2.5 were explored and discriminate biomolecular change by using SY-FTIR spectroscopy.

1.2 Research objectives

1.2.1 To investigate the antioxidant activity of *Thunbergia laurifolia* Linn. (Rang Chuet) leaves, *Zingiber officinale* (Ginger) and their combined extracts at both chemical and cellular levels and to evaluate the interactions among them.

1.2.2 To determine the effects of Rang Chuet, ginger, and their combined extracts on PM2.5-induced cellular oxidative stress in A549 and HepG2 cell models.

1.2.3 To determine the anti-inflammatory activities of Rang Chuet and its combined extracts against PM2.5-induced inflammation in THP-1 macrophage cell models.

1.2.4 To explore and distinguish biomolecular changes in A549 cells exposed to PM2.5 and the protective effects of pretreatment with combined extracts against PM2.5 using SY-FTIR.

1.3 Research hypotheses

The combined extracts of Rang Chuet and ginger would have synergistic effects on chemical and cellular antioxidant activity. When interaction occurs, the combined extracts exhibit higher chemical and cellular antioxidant activity. Moreover, when exposed to PM2.5, the combined extracts showed greater cellular antioxidative and anti-inflammatory effects than Rang Chuet and ginger extracts alone and were potent in regulating antioxidant gene expression.

1.4 Scope of the study

1.4.1 Rang Chuet and ginger extracts, along with various combined extracts, were evaluated for their chemical and cellular antioxidant activities, including antioxidant gene expression, and the interactions among them were investigated.

1.4.2 The effects of Rang Chuet, ginger, and their combined extracts on PM2.5-induced cellular oxidative stress in A549 and HepG2 cell models were determined by assessing cell viability, cytotoxicity, intracellular ROS generation, and measuring antioxidative mRNA expression.

1.4.3 The anti-inflammatory activity, in terms of PM2.5-induced inflammation in THP-1 macrophages cell, was measured by assessing inflammatory markers.

1.4.4 Biomolecular changes in activated A549 cells will be evaluated using SY-FTIR spectroscopy.

1.5 Expected results

The combined extracts of Rang Chuet and ginger are likely to exhibit synergistic effects on chemical and cellular antioxidative activity, as well as antioxidative gene

expression. When these extracts interact, they exert high chemical and cellular antioxidative activity. Moreover, when exposed to PM2.5, the combined extracts demonstrated cellular antioxidative, anti-inflammatory, and detoxifying properties.

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CHAPTER II

LITERATURE REVIEW

2.1 PM2.5 human health effects

Air pollution is a complex mixture of particulate matter (PM), gases, organic compounds, and metals found indoors and outdoors, with PM posing significant health hazards. PM is classified into three main size groups: ultra-fine PM (UFPM, < 0.1 μM), fine PM (PM2.5, < 2.5 μM), and coarse PM (PM10, < 10 and > 2.5 μM). PM2.5 is generated from diverse sources such as diesel exhaust, forest fires, industrial processes. Secondary sources of PM2.5 occur when gases emitted by power plants, industries, and vehicles interact with the atmosphere. PM2.5 comprises a range of materials, including organic chemicals like polycyclic aromatic hydrocarbons (PAHs) and inorganic chemicals like sulfates, nitrates, and metals (Bekki et al., 2016; C.-W. Liu et al., 2018). PM2.5 can persist in the air for long durations, increasing the chances of inhalation and deposition in the lungs. Additionally, these particles can cross the gas-blood barrier and enter the bloodstream. The pathogenicity of PM depends on factors such as size, composition, source, solubility, and their ability to generate reactive oxygen species (ROS) (Zou, Jin, Su, Li, & Zhu, 2016).

In terms of its influence on the human respiratory system, PM2.5, recognized as an environmental toxicant from laboratory and animal investigations, has notable effects. Research shows that PM2.5 can impair, reduce, and disrupt rat tracheal cilia, which results in infections and weakened general immune defenses. This susceptibility to secondary infections is due to the impact of PM2.5 on alveolar macrophages. Furthermore, exposure of human epithelial cells (A549) to PM2.5 demonstrates an elevated oxidative potential, triggering increased production of ROS within the cells. This exposure also leads to higher levels of cell death and DNA damage in human respiratory cells (Veerappan, Sankareswaran, & Palanisamy, 2019).

In terms of its effects on neuroinflammation, prolonged exposure to environmental toxicants has been associated with the onset of neuroinflammation

and related brain conditions. Research indicates a close connection between long-term exposure to PM2.5 and neuroinflammation in humans (Calderón-Garcidueñas et al., 2015). Studies by Spangenberg and Green (2017) suggest that PM2.5 exposure heightens inflammation in the brain. Mechanisms by which PM 2.5 triggers neuroinflammation in Alzheimer's disease have been explored. Reports from B.-R. Wang et al. (2018) indicates that PM2.5 exposure amplifies neuronal damage caused by oligomeric amyloid beta and initiates the activation of the NLRP3 inflammasome in an Alzheimer's disease model. Moreover, findings by L. Wang et al. (2020) demonstrate that exposure to PM2.5 speeds up the development of Alzheimer's disease-related abnormalities in both transgenic AD mice and normal rodents, implying a relationship between PM2.5 exposure and brain pathologies. Moreover, exposure of pregnant mice to PM2.5 can hold the development of the cerebral cortex in their mice by influencing cell apoptosis (T. Zhang et al., 2018).

In the context of skin disorders, evidence linking elevated PM2.5 air pollution to oxidative stress, a predominant mechanism associated with PM2.5. Exposure of immortalized human keratinocyte (HaCaT) cells to PM2.5 led to oxidative damage and apoptosis, causing adverse health effects. Researchers found that PM2.5 induces oxidative stress and mitochondria-dependent apoptosis, resulting in skin irritation and damage (Hu et al., 2017).

In the context of Nonalcoholic fatty liver disease (NAFLD), extended exposure to PM2.5 initiates oxidative stress and inflammation, leading to chronic hepatic injury linked to dyslipidemia. The study substantiated the presence of heightened insulin resistance, glucose intolerance, peripheral inflammation, and histopathological changes in mice exposed to PM2.5. Inhalation of PM2.5 induced liver oxidative stress and inflammation, prompting abnormal liver function and lipid accumulation. In laboratory conditions, pyrrolidine dithiocarbamate (PDTC) and N-acetyl-L-cysteine (NAC) effectively mitigated oxidative stress and inflammation, indicating disruption of normal lipid metabolism due to PM2.5-induced liver cell damage. These findings suggest a novel approach for preventing and treating diseases arising from pollution (M.-X. Xu et al., 2019).

Investigations into PM2.5 effects on the human respiratory system encompassed *in vitro* and *in vivo* studies, revealing oxidative and inflammatory

injuries. Evidence also points to PM2.5 intensify neuroinflammation in the brain, skin disorders, as well as liver oxidative stress.

2.2 The association of PM2.5 with cellular responses to oxidative stress and inflammation

PM2.5 demonstrates oxidative characteristics, impacting inflammatory cells via oxidation mechanisms. The correlation between inflammation and oxidative stress is tightly linked to respiratory disorders. Macrophages, key defenders against PM2.5 exhibit various responses when exposed, encompassing inflammation, oxidative stress, and cell death. Exposure to PM2.5 enhance the expression of inflammatory genes such as interleukin-1 (*IL-1*) and cyclooxygenase-2 (*COX-2*), as well as oxidative stress-related genes like heme oxygenase-1 (*HO-1*) (Bekki et al., 2016). RAW 254.7 macrophage cells exposed to PM2.5 exhibited increased release of monocyte chemoattractant protein-1 (*MCP-1*), tumor necrosis factor-α (*TNF-α*), and *IL-6*, accompanied by enhanced mRNA expression of inducible nitric oxide synthase (*iNOS*) and *TNF-α,* initiating an inflammatory response. Molecular analysis revealed heightened expression of toll-like receptor 4 (*TLR4*), nuclear factor kappa-B (NF-B), and *COX-2* in PM2.5-stimulated RAW 254.7 cells (Fu et al., 2020). Upon triggering inflammatory responses, PM2.5 disrupts cytokine production, leading to increased lung damage and altered pulmonary function. PM2.5, easily inhaled into the respiratory tract, accumulates in lung alveoli, causing both structural damage and impairment of lung function (C.-W. Liu et al., 2018).

In studies involving epithelial cells, PM2.5 absorption by human bronchial epithelial (BEAS-2B) cells induced cellular inflammation through oxidative stress, mitochondrial dysfunction, and initiation of mitophagy. Following PM2.5 exposure, researchers investigated mitochondrial oxidative stress and inflammatory reactions. PM2.5 activated antioxidant enzymes such as *SOD* and *GPx*, reducing GSH, an essential antioxidant responsible for neutralizing excess ROS. Within 24 h of PM2.5 treatment, observed lipid peroxidation and founded the production of inflammatory cytokines, particularly *IL-6* and *IL-8* (Zhai, Wang, Sun, & Xin, 2022). Moreover, when human lung epithelial cells (A549) absorbed PM2.5, it resulted in observed oxidative

damage. This damage manifested in alterations in the activities of enzymes such as superoxide dismutase (*SOD*) and catalase (*CAT*). Additionally, the presence of PM2.5 induced autophagy, as evidenced by increased mRNA expression of autophagy-related proteins Atg5 and Beclin1. These findings suggest that PM2.5-induced oxidative stress likely plays a crucial role in triggering autophagy within A549 cells. (Deng et al., 2013). Furthermore, it induced oxidative stress, as evidenced by reduced levels of HO-1. The levels of nuclear factor erythroid 2-related factor 2 (Nrf2) significantly decreased in the cytosol and increased in the nucleus of PM2.5-treated cells. Consequently, Nrf2 may play a critical role as a transcription factor in A549 cells for detoxifying PM2.5, thereby activating the protective Kelch-like ECH-associated protein 1/Nrf2 (Shim et al., 2021).

Moreover, PM2.5 exposure in human bronchial epithelial cells (16HBE) led to an increased production of reactive oxygen species (ROS) and a reduction in mitochondrial gene expression. Conversely, PM2.5 increased the mRNA levels of NADPH oxidase and inflammatory cytokines (Jin et al., 2016). The impact of PM2.5 exposure on hepatotoxicity revealed that PM2.5 incubation led to a significant reduction in Nrf2 expression, along with a decrease in various antioxidants, resulting in anti-inflammatory degradation and TBK1/NF-B activation (Ge et al., 2020).

This literature review explores the impact of ROS-induced inflammation mediated by PM2.5 exposure in macrophages, lung epithelial cells, and hepatic cells. Exposure to PM2.5 causes a rise in ROS levels, which in turn heightens the oxidative stress response, leading to damage in DNA, proteins, lipids, and various cellular and molecular components.

2.3 Preventing and treating PM2.5-induced oxidation and inflammation by food and functional foods

To reduce the ROS-mediated toxicity caused by PM2.5 exposure have focused on identifying natural antioxidants with free radical scavenging capabilities and other beneficial properties. Polyphenols demonstrated ability to offer protection and prevention in various physiological processes by activating multiple signaling pathways. One area of study involves exploring the potential of dietary polyphenolic compounds to modulate the pathology induced by PM2.5. Numerous studies have

explored the utility of flavonoids in both preventing and treating PM2.5-related lung diseases. Therefore, it is essential to understand how natural plant extracts influence PM2.5.

Firstly, Biochanin A (BCA), an O-methylated isoflavone categorized within the flavonoid phytochemical group, demonstrated protective effects in a rat model exposed to PM2.5. BCA effectively inhibited PM2.5-induced apoptosis and the release of proinflammatory markers, including *TNF-α, IL-2, IL-6,* and *IL-8*, as assessed by ELISA. These protective actions by increased levels of antioxidant enzymes and decreased levels of malondialdehyde (MDA), lactate dehydrogenase (LDH), and alkaline phosphatase (AKP) (Xue et al., 2020). Secondly, pretreatment with morin, a natural antioxidant its ability to scavenge free radicals, significantly mitigated PM2.5-induced toxicity and prevented alterations in miRNA expression triggered by PM2.5 exposure. (Veerappan et al., 2019). Thirdly, research on the therapeutic potential of total flavonoids (TF) from Loquat Leaf in a PM2.5-induced nonalcoholic fatty liver disease (NAFLD) model suggests that TF mitigates PM2.5-induced NAFLD by modulating the IRs-1/Akt and CYP2E1/JNK pathways.(Jian et al., 2018). Fourthly, research utilizing Juglanin, a flavonoid, demonstrated its ability to enhance Nrf2 activation and promote SIKE upregulation, effectively mitigating PM2.5-induced liver damage. These findings suggest that Nrf2 regulates SIKE to suppress inflammatory and oxidative damage, thereby alleviating PM2.5-induced liver damage (Ge et al., 2020). Furthermore, both Vitamin C and quercetin have been found to inhibit respiratory oxidative damage in human bronchial epithelial cells (16HBE) exposed to PM2.5 (Jin et al., 2016). Vitamin D was shown to protect cells from particle-induced injury by inducing autophagy in a Nrf2-dependent manner (Tao et al., 2019). These findings suggest that a new class of phytochemicals could potentially be developed as therapeutic agents for diseases caused by PM2.5 in the future.

Numerous functional food ingredients, extending beyond the protective effects of phytochemicals against PM2.5-related health issues. The utilization of omega-3 fatty acids (O3FA) has proven effective in averting the onset of intracranial atherosclerosis (ICA) and cerebral vessel inflammation. The inhalation of PM2.5 can trigger ICA, have shown that omega-3 fatty acids can ameliorate this effect, (Guan et al., 2018). In addition, pre-treatment of rats with Chitosan oligosaccharides (COS) has

proven to be an effective in blocking the PM2.5-induced elevation of LDH, IL-8, and TNF-α levels in the bronchoalveolar region. (Zhao, Xu, Wang, Yi, & Wu, 2018). Furthermore, Ma Xing Shi Gan Decoction (MXD), a traditional Chinese medicine recommended for treating upper respiratory tract infections, shows protecting rats from PM2.5-induced acute lung injury. This protection may be attributed to its ability to modulate the HMGB1/TLR4/NFκB pathway and reduce inflammatory responses (Fei et al., 2019).

The efficacy of phytochemical compounds in protecting against and preventing the effects of PM2.5, which is known for inducing oxidative stress and inflammation.

2.4 Oxidative stress and antioxidants

Oxidative stress significantly affects numerous chronic diseases such as aging, atherosclerosis, diabetes, hypertension, Alzheimer's disease, kidney disease, and cancer (Roberts & Sindhu, 2009). The consumption of plant-based foods may help mitigate oxidative damage (Batool, Khan, Sajid, Ali, & Zahra, 2019). Polyphenols, a specific class of phytochemical compounds, are specialized plant metabolites that contribute to human health by mitigating the pathological effects of oxidative stress (H. Zhang & Tsao, 2016) Consequently, multiple studies have aimed to evaluate the health benefits associated with antioxidant activity.

2.4.1 Oxidative stress

Oxidative stress occurs due to an imbalance between the generation of free radicals and the capacity of body to neutralize them with antioxidants. Free radicals can arise from internal and external origins. Internally, they result from enzymatic reactions in processes like the respiratory chain, phagocytosis, and prostaglandin synthesis, as well as nonenzymatic reactions involving oxygen and organic compounds, and ionizing reactions. One of the external sources of free radicals is PM2.5 (Lobo, Patil, Phatak, & Chandra, 2010). Oxidative stress leading to ROS accumulation that led to the oxidation of lipids and proteins, causing structural and functional alterations. Free radicals interact with DNA, resulting in strand breaks, base modifications, and DNA-protein cross-linking, primarily due to hydroxide-induced DNA damage.

Reactive oxygen species (ROS) are generated from molecular oxygen involvement in redox reactions, dividing into non-radical and radical types. Non-radical ROS encompass hydrogen peroxide (H_2O_2), organic hydroperoxides (ROOH), singlet molecular oxygen ($_1O^2$), and ozone (O^3), while radical species, containing at least one unpaired electron, include the superoxide anion radical ($O^{2\cdot-}$), the hydroxyl radical (·OH), the peroxyl radical (ROO·), and the alkoxyl radical (RO·). The antioxidant defense system plays a critical role in mitigating oxidative stress.

2.4.2 Antioxidants

Antioxidants within defense mechanisms function at varying levels. Initially, preventive antioxidants inhibit the formation of free radicals. Then, there are antioxidants that scavenge active radicals, disrupting both the initiation and progression of chain reactions. Radical-scavenging antioxidants include Vitamin C, uric acid, bilirubin, albumin, and thiols, which are hydrophilic, whereas Vitamin E acts as a lipophilic antioxidant. Moreover, there are repair and *de novo* antioxidants, such as DNA repair systems, proteinases, proteases, and peptidases. These constituents, present in the cytosol and mitochondria of mammalian cells, identify, degrade, and eliminate proteins modified by oxidation to prevent the accumulation of oxidized proteins. (Lobo et al., 2010).

Antioxidants can be categorized into two groups: enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants work by converting superoxide into hydrogen peroxide, and further reducing it to water. This detoxification process is facilitated by several enzymes. A network of antioxidant enzymes, including superoxide dismutase (*SOD*s) that break down superoxide anions, and catalase that breaks down hydrogen peroxide into water and oxygen, safeguards cells from oxidative stress. Glutathione, glutathione reductase, glutathione peroxidases, and glutathione S-transferases are part of the glutathione system, involved in breaking down hydrogen peroxide and organic hydroperoxides. While antioxidants like glutathione, ubiquinol, and uric acid are produced in the body during normal metabolism, the body cannot synthesize micronutrient antioxidants and must obtain them through diet. Scientific data indicates that plant-based foods play a protective role against various chronic diseases.

Nonenzymatic antioxidants include Ascorbic acid (Vitamin C), which acts as a reducing agent to neutralize reactive oxygen species (ROS) such as hydrogen peroxide. Glutathione, another nonenzymatic antioxidant, maintains its reduced state in cells through the action of the enzyme glutathione reductase. Glutathione not only reduces other metabolites but also interacts directly with oxidants (Meister, 1988). Tocopherols and tocotrienols (Vitamin E) prevent membrane oxidation by reacting with lipid radicals generated during the chain reaction of lipid peroxidation, effectively eliminating the radical intermediates and halting the reaction.

Polyphenolic compounds come in various structures and can be classified in multiple ways. These substances exhibit varying antioxidant properties, and five distinct mechanisms of action can be categorized as shown in Figure 2.1: (a) the phenolic hydroxyl groups of polyphenolic compounds serve as hydrogen donors, directly engaging with and reducing the activities of $\cdot O^{2-}$, H_2O_2, ·OH, ROO·, $_1O^2$, and other active radicals; (b) polyphenolic compounds reduce the production of radicals by inhibiting the enzyme activities responsible for generating free radicals; (c) these compounds chelate metal ions known to induce free radical production, thereby reducing the generation of free radicals; (d) polyphenolic compounds enhance antioxidant enzyme activity or the expression of antioxidant proteins, thereby inhibiting oxidation reactions; and (e) polyphenolic compounds generate synergistic antioxidant effects when combined with other substances. Plant polyphenols represent natural antioxidants with advantages such as higher antioxidant activity, lower toxicity, and other benefits when compared to traditional synthetic chemical drugs.

2.4.3 ROS with redox signaling pathways.

To assess the molecular interactions between particular ROS molecules and specific targets within redox signaling pathways. The primary way reactive oxygen species (ROS) exert their biological effects in redox regulation is through thiol-based modifications of target proteins. Redox signaling influences protein function, resulting in alterations in signaling outcomes, enzyme activity, gene transcription, and the integrity of cellular membranes and genomes. Presented here are several prototypical of signaling targets.

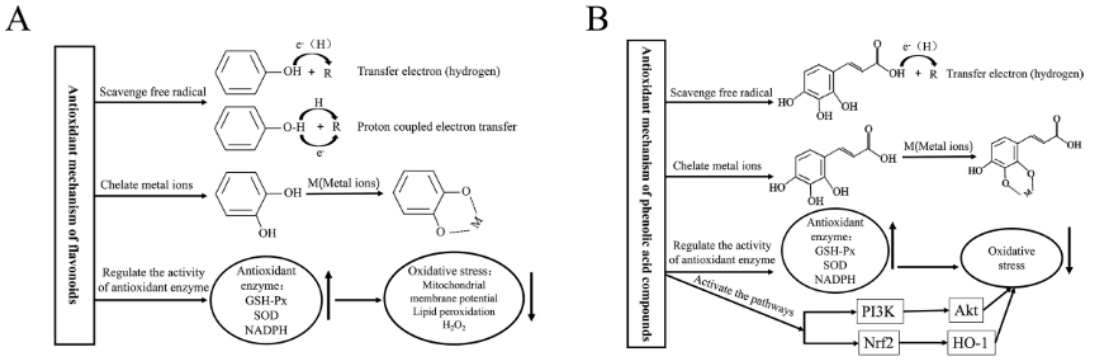

Figure 2.1 The antioxidant mechanisms of polyphenols (A) Flavonoids antioxidant mechanisms (A) and Phenolic acid antioxidant mechanisms(B).

Source : (Lv et al., 2021).

The NRF2–KEAP1 pathway and its role in antioxidant response: The NRF2–KEAP1 system serves as a sensor-effector mechanism reliant on thiol chemistry, reacting to oxidative threats and contributing to eukaryotic redox balance (Sies & Jones, 2020). The NF-**K**B pathway, crucial for inflammation regulation, undergoes regulation involving various molecules. Given its association with inflammation and H_2O_2 production, NF-**K**B is also subject to redox control. Depending on conditions, H_2O_2 can regulate or hinder NF-**K**B activity. (Oliveira-Marques, Marinho, Cyrne, & Antunes, 2009). The cellular effects of H_2O_2 based on its concentration levels. Within the physiological range, H_2O_2 is linked with typical neural development processes, including cell proliferation. Intermediate concentrations are associated with adaptation and limited apoptosis. Elevated levels of H_2O_2 induce oxidative stress, ultimately resulting in necrosis, as shown in Figure 2.2.

Redox signaling is essential for maintaining equilibrium across multiple levels, ranging from molecular to organismic levels. This awareness has led to the development of a novel discipline called redox medicine, which seeks to explore the effectiveness of targeting oxidant production and detoxification.

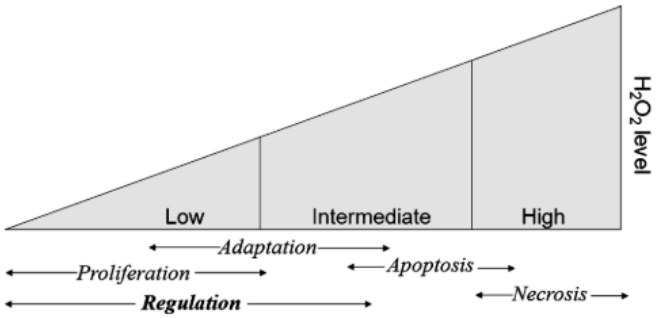

Figure 2.2 A metabolic pathway that depends on the presence of H_2O_2. The concentration of H_2O_2 affects how it works within cells. Concentration is divided in high (mM range) to Low (nM to µM range)

Source : (Oliveira-Marques, Marinho, Cyrne, & Antunes, 2009).

2.5 Antioxidant activity assays

Antioxidant activity is primarily defined as the ability of substances to shield biological systems from oxidation processes triggered by reactive oxygen species (ROS). To detect this activity, various antioxidant assays are employed for verification.

2.5.1 Chemical antioxidant activity assay

Chemical antioxidant assays or assays utilizing non-biological substrates serve as rapid and effective evaluation tools, encompassing functions like scavenging free radicals, reducing power, and metal chelation. Nonetheless, they might not fully capture the intricate antioxidant interactions within food matrices (Rawson, Patras, Oomah, Campos-Vega, & Hossain, 2013). Principal assays observing reactions through molecular probes can be divided into two categories based on their mechanisms: Hydrogen Atom Transfer (HAT)-based assays, gauging an antioxidant's ability to capture free radicals through hydrogen donation, and Single Electron Transfer (SET)-based assays, depending on capability of compound for one-electron transfer reactions with radical species. As phytochemical compounds may act independently or in combination through various mechanisms, multiple assays covering different antioxidant mechanisms are necessary.

Popular assays include the Radical/ROS scavenging method, specifically the 1,1-Diphenyl-2-Picrylhydrazyl (DPPH•) Stable free radical scavenging assay. This

assay was designed to take advantage of a stable free radical with an odd, unpaired valence electron, which is studied for its antioxidant activity. DPPH• can be stabilized by accepting an electron or hydrogen radical from an antioxidant molecule, such as a sulfhydryl group. The DPPH• assay has also been shown to involve both HAT and SET mechanisms. Another assay is the radical cation scavenging activity, where the potential antioxidant activity is evaluated by the decolorization of the ABTS•+ cation radical solution through the donation of an electron or hydrogen atom by the antioxidant compound.

Non-radical redox potential-based methods, such as the FRAP (Ferric Reducing Antioxidant Power) assay, are based on single-electron transfer reactions where an antioxidant reduces ferric to ferrous ions. When the ferric-tripyridyltriazine (Fe3+TPTZ) complex is reduced to the ferrous form, the complex absorbs light at 593 nm, producing an intense blue color (Rawson et al., 2013). In contrast to previous SET-based techniques, the FRAP test is conducted under acidic conditions (pH 3.6) to maintain iron solubility and, more importantly, to promote electron transfer. As a result, the redox potential is altered, which changes the primary reaction mechanism (Shahidi & Zhong, 2015).

Metal chelation capacity is the measure of an antioxidant's ability to bind to ferrous ions. The two most common sources of ferrous ions are ferrous sulfate and ferrozine. When antioxidants are added, a reduction in absorbance at 485 nm (for ferrous sulfate) or 562 nm (for ferrozine) indicates the formation of a complex between the metal and the antioxidant. This allows the spectrophotometric measurement of the metal chelation capacity of the antioxidant. Typically, ethylenediaminetetraacetic acid (EDTA) equivalents are used to express the metal chelation capacity of antioxidants. EDTA is a synthetic metal chelator commonly found in various dietary and pharmaceutical applications. However, due to its regulatory status as a synthetic chemical, there is a growing need to replace it with natural metal-chelating antioxidants. Researchers have explored numerous antioxidants and extracts for their metal chelation capacity (Shahidi & Zhong, 2015).

Total phenolic content (TPC) plays a significant role in assessing the total antioxidant capacity (TAC) and is widely employed in the evaluation of antioxidant extracts, including extracts. The Folin–Ciocalteu assay relies on the

alkaline reduction of the Folin–Ciocalteu reagent by phenolic compounds. This reagent contains phosphomolybdic /phosphotungstic acid complexes that, upon reduction, create a blue chromophore with a maximum absorption at 765 nm. It is generally understood that the molybdenum center in these complexes is the site of reduction, where the Mo^{6+} ion is reduced to Mo^{5+} by accepting an electron from the phenolic antioxidant. Therefore, the Folin–Ciocalteu assay is an SET-based assay associated with the reducing activity of phenolic antioxidants. Gallic acid is commonly used as a reference standard, and TPC results are usually expressed as equivalents of gallic acid, necessitating standardized reporting of results (Shahidi & Zhong, 2015).

In conclusion, no single assay can fully represent the antioxidant capacity of the compounds of interest. The most effective evaluation system involves employing various methods with different reaction mechanisms to obtain highly biologically relevant assays that can guide further studies. Furthermore, combining chemical assays with different mechanisms can be valuable in understanding the primary mechanisms of antioxidants.

2.5.2 Cellular antioxidant activity (CAA) assays

Cell-based assays are gaining popularity due to their enhanced biological relevance. The Cellular Antioxidant Activity (CAA) index reflects an antioxidant capability to mitigate intracellular oxidative stress. It goes beyond assessing the reduction potential of an antioxidant and its ability to scavenge free radicals; the CAA index also considers factors such as membrane permeability, cellular uptake, distribution, and metabolism. This assay proves particularly valuable for investigating the bioavailability of food antioxidants (López-Alarcón & Denicola, 2013). The CAA assay has been employed with HepG2 cells to explore various pure phytochemicals commonly found in food, including compounds like quercetin, gallic acid, caffeic acid, and plant extracts. However, it's important to note that the results of this assay have shown variability in some cell studies.

The CAA assay employs a fluorescent probe known as 2′,7′-dichlorofluorescin diacetate (DCFH-DA) to monitor the inhibition of peroxyl radical-induced oxidation within the cell. DCFH-DA is chosen in its ester form because it is nonionic and non-polar, allowing it to efficiently cross the cell membrane. The fundamental principle

of the CAA assay is depicted in Figure 2. Once inside the cell, DCFH-DA is deacetylated by endogenous cellular esterases, transforming it into the more oxidizable DCFH form. When a free-radical generator is introduced into the system, it triggers the formation of peroxyl radicals, alongside other reactive oxygen species (ROS) generated as part of normal cellular processes. If an antioxidant is introduced and can enter the cell, it competes with these radicals and quenches them through various mechanisms, preventing the oxidation of DCFH into the fluorescent 2′,7′-dichlorofluorescein (DCF). The antioxidant effectively scavenges the ROS, leading to a lesser extent of DCFH oxidation, resulting in reduced intracellular DCF fluorescence. Therefore, a lower DCF fluorescence level indicates a higher CAA.

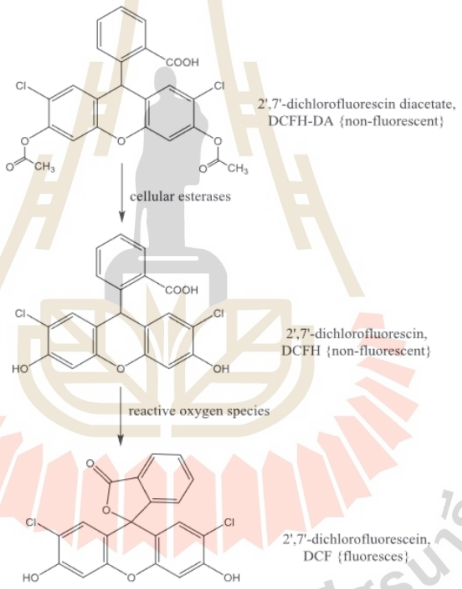

Figure 2.3 Principle of cellular antioxidant activity (CAA) assay.
Source : Kellett, Greenspan, and Pegg (2018).

2.6 Inflammation

2.6.1 Chronic Inflammation

Chronic inflammation involves the infiltration of macrophages and lymphocytes into inflamed regions. While macrophages primarily function as phagocytes to eliminate dead tissue cells, they also have the capacity to produce free radicals such as NO and O2, which may harm healthy cells. Additionally, both

macrophages and fibroblasts release cytokines like interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α), along with NO radicals, regulating cellular proliferation and inflammation. T-cells are involved in specific antigen clearance. Inflammatory mediators, such as histamine, prostaglandins, and interleukins, released by activated cells, coordinate the inflammatory response by increasing vascular permeability and attracting phagocytes and lymphocytes to infection sites (Owusu-Apenten, 2010).

2.6.2 Modulation of inflammatory mediators by nutraceuticals

Substantial evidence indicating that nutraceuticals can modulate a variety of inflammatory mediators, including cytokines, as well as the production and action of second messengers like cGMP, cAMP, and protein kinases. Additionally, they influence the expression of transcription factors such as AP1 and NF-kB, and the expression of key proinflammatory molecules, including NOS2, COX2, interleukin 1β (IL1β), TNF-α, neuropeptides (Gurnell, 2003).

A comprehensive understanding of inflammation and its associated adverse effects provides the development of therapeutic targets derived from natural sources to combat chronic inflammation-related diseases caused by PM2.5 exposure. An increasing body of research focuses on natural compounds from herbal extracts and their active components, as evidenced by reviews (Arulselvan et al., 2016; Hong et al., 2020; Lim JS, 2020; Yu, Lim, Lee, Choi, & Kim, 2021).

2.7 Thunbergia Laurifolia leaves and their bioactivity

Thunbergia laurifolia Lindl. (Acanthaceae), commonly known as Rang Chuet (RC) in Thailand, has a history of being used to detoxify toxins and has been the subject of research for the past decade. This research has aimed to explore the potential of Rang Chuet in alleviating the effects of exposure to PM2.5.

2.7.1 Thunbergia Laurifolia Leaves

Botanical Description: *Thunbergia laurifolia* Lindl. belongs to the Acanthaceae family and is native to the Indomalaya region. It is commonly known as "Babbler's Bill" in English. In Thailand, this plant goes by various names, including Rang Chuet, Yaw Kaew, Kob Sha Nang, and Gum Lung Chang Puak (Aritajat, Wutteerapol, & Saenphet, 2004). It is a climbing plant with smooth, opposite leaves

along the stem. The leaves are shrub-like, with small oblong or ovate shapes, measuring 8-10 cm in length and 4-5 cm in width. They have a broad base, narrowing to a pointed tip and often exhibit scalloped lobes near the base (see Figure 2.5). The characteristic features of T. *laurifolia* flowers are trumpet-shaped, and the seed pod has a cone-shaped structure, measuring 1 cm in length, with a round base. The plant exhibits three types of flowers: white, yellow, and purple. Purple varieties are reported to contain beneficial compounds, particularly in the stem, root, and leaves (Ratchadaporn Oonsivilai, 2007).

Figure 2.4 Characteristic of Rang Chuet leaves in Thailand.

2.7.2 Thai Traditional Medicine Properties

In Thai traditional medicine, Rang Chuet (RC), is highly regarded for its properties as an antidote against various poisonous agents. It is commonly consumed in the form of herbal tea. Different parts of the plant have been employed for various medicinal purposes. For instance, aqueous extracts from fresh leaves, dried leaves, dried roots, and bark have been used as antidotes for insecticides, ethyl alcohol, arsenic, and strychnine poisoning. Additionally, the dried root has been utilized as an anti-inflammatory and antipyretic agent. The leaves of this plant are the primary part used, although it is not yet confirmed whether the substance has detoxification properties. Furthermore, it is worth noting that the National List of Major Medicines will soon include a drug developed from RC herbs in the ophthalmic section of the hospital (Chuthaputti, 2010).

2.7.3 Chemical Composition and Active Compounds

The chemical composition of T. *laurifolia* leaves includes carbohydrates, ash, fiber, protein, and fat. On a dry basis, the weight percentages are 46.01, 18.79, 16.82, 16.70, and 1.68%, respectively (proximate analysis) (Jaiboon, Boonyanuphap,

Suwansri, Ratanatraiwong, & Hansawasdi, 2010). The phytochemistry of the leaves can be categorized into five groups: Sterols, such as beta-sitosterol, stigmasterol, and alphaspinastermkol. Phenolics, including rosmarinic, apigenin, caffeic acid, gallic acid, and protocatechuic (Chuthaputti, 2010; R. Oonsivilai, Cheng, Bomser, Ferruzzi, & Ningsanond, 2007) Carotenoids, like lutein, unclassified steroids (Chuthaputti, 2010). Glycosides, such as 8-epi-grandifloric acid, 3'-O-β-glucopyranosyl-stilbericoside, grandifloric acid, benzyl β-glucopyranoside, benzyl β-(2'-O-β-glucopyranosyl)-glucopyranoside, 6-C glucopyranosyl apigenin, 6,8-di-C-glucopyranosyl apigenin, (E)-2-hexenyl-β-glucopyranoside, and hexanol β-glucopyranoside (Chan, Lim, Chong, Tan, & Wong, 2010; Chuthaputti, 2010) . T. laurifolia leaves possess functional properties that are associated with human health benefits.

2.7.4 Biological effects of RC leaves

Antioxidant Effect: It was observed that the aqueous extraction of RC leaves had a higher total phenolic content (TPC) compared to ethanol and acetone extracts. Moreover, the aqueous extract exhibited the highest activity in free radical scavenging (DPPH) with the lowest EC_{50} value and FRAP when compared to ethanol and acetone extracts (Chan et al., 2012; Ratchadaporn Oonsivilai & Ferruzzi, 2008). Furthermore, Chan et al. (2012) reported that developing leaves had the highest TPC of 513 mg GAE/100 g, followed by young and mature leaves with values of 407 and 298 mg GAE/100 g, respectively.

Detoxification of RC Leaves Extracts: Research has explored the detoxification properties of RC leaf extracts both *in vitro* and *in vivo*, demonstrating their potential as antidotes for various toxins, including chemical toxins, insecticides, drugs, arsenic, strychnine, alcohol, and heavy metals. Given that PM2.5 contains heavy metals such as Cd, Fe, and Pd, RC leaf extract has been found to have the ability to detoxify heavy metals. Studies have shown that the extract can serve as a preventive agent, especially for RAW 264.7 macrophage cells treated with cadmium (Cd) and chlorpyrifos (CP), which are organophosphate compounds. The extract increased cell viability significantly when added before exposure to the toxin (Lipopolysaccharide), indicating its protective effects on the cells. This effect, however, was not observed when the RC extract was applied after toxin exposure or in combination with the toxin (Junsi, Siripongvutikorn, Yupanqui, & Usawakesmanee, 2017).

Additionally, human embryonic kidney (HEK293) and human liver (HepG2) cells were used to investigate the anti-Cd toxicity and cell viability in treatment groups with combined, pre-, and post-treatments. In both pre- and post-treatments, RC extracts protected and enhanced cell recovery. Moreover, RC leaf extracts demonstrated the ability to protect against Cd-induced oxidative stress in cells, likely due to their antioxidant properties, as evidenced by their low cytotoxicity, increased catalase (CAT) and glutathione peroxidase (GPx) activities, and reduced malondialdehyde (MDA) levels in both cell types (Junsi, Takahashi Yupanqui, Usawakesmanee, Slusarenko, & Siripongvutikorn, 2020).

Detoxification of Lead (Pb) and Paraquat (PQ): RC leaf extracts have been found to have a significant impact on lead acetate (PbAc)-exposed Swiss Albino rats. RC leaf extract can reverse lead-induced renotoxicity by modulating cell signaling pathways, decreasing kidney tissue alterations and apoptosis, and downregulating inflammatory markers. This indicates that RC has potential as a dietary supplement for Pb detoxification in the kidney, with antioxidant and anti-inflammatory properties (Nasiruddin Rana et al., 2020). Additionally, research by Phyu and Tangpong (2013) demonstrated that the aqueous extract of RC leaves can substantially prevent Pb-induced neurotoxicity in a dose-dependent manner. Furthermore, RC leaf extracts showed concentration-dependent increases in phenolic content and exhibited antioxidant and neuroprotective properties, protecting against lead-induced acetylcholinesterase dysfunction. The lungs are the primary target organ of Paraquat (PQ) poisoning, and RC leaf extract has been shown to have a detoxifying effect on this insecticide toxin. Oxidative stress and inflammation are key factors in PQ-induced pulmonary injury, and RC leaf extract has been effective in inhibiting PQ-induced increases in MDA levels, NOX, IL-1, and TNF expressions. Moreover, RC leaf extract has ameliorated lung pathology induced by RC leaf extract has been shown to ameliorate PQ-induced kidney injury by modulating oxidative stress, particularly by inhibiting NOX expression, including NOX-1 and NOX-4 (Palipoch, Punsawad, Koomhin, Na-Ek, et al., 2022; Palipoch, Punsawad, Koomhin, & Poonsawat, 2022).

In the context of Methomyl (MT) toxicity, post-treatment with RC leaf extract significantly increased the viability of RAW 264.7 murine macrophage cells

treated with MT. However, combined or pre-treatments did not have the same effect. Additionally, post-treatment with RC leaf extract reduced the production of nitric oxide, increased cell proliferation, and demonstrated lower cytotoxicity, even at low levels (Junsi et al., 2017). Various studies have reported the hepatoprotective activity of aqueous RC leaf extract. Rats treated with ethanolic extract have been found to be protected against hepatic disorders induced by ethanol (Ratchadaporn Oonsivilai, 2007).

Numerous studies have explored the detoxification and hepatoprotective effects of RC leaf extracts, but the exact mechanisms of how these extracts modulate metabolic detoxification pathways are not yet fully understood. Additionally, the reduction of PM2.5 toxicity by RC leaf extract is still under investigation.

2.8 The antioxidant activity and anti-Inflammatory potential of ginger root extract

Ginger, *Zingiber officinale*, is a rhizomatous plant that has been valued for centuries not only for its culinary uses but also for its medicinal properties. Ginger is a non-toxic natural antioxidant compound with a biological function, including antimicrobial, anti-inflammatory, antioxidant, immunomodulatory, and anti-carcinogenic properties. The powdered rhizome of ginger contains a composition of 3-6% fatty oil, 9% protein, 60-70% carbohydrates, 3-8% crude fiber, approximately 8% ash, 9-12% water, and 2-3% volatile oil. Among its active components, gingerols, with gingerol [5-hydroxy-1-(4-hydroxy-3-methoxy phenyl) decan-3-one] as the most abundant constituent in the gingerol series, have been identified as major contributors to these beneficial properties. Among its potential health benefits, ginger remarkable antioxidant and anti-inflammatory attributes stand out as crucial components. This section is focus on ginger root extract, shedding light on its potent antioxidative qualities and its role as a natural anti-inflammatory agent.

2.8.1 Antioxidant Activity of Ginger Root Extract

The antioxidative prowess of ginger root extract is largely attributed to the presence of bioactive compounds, prominently including gingerol, shogaol, zingerone, and paradol. These constituents collectively confer ginger with the capacity to neutralize harmful reactive oxygen species (ROS), which play a pivotal

role in cellular oxidative stress and are implicated in the development of various chronic diseases (Kumar, 2013).

Gingerol, a prominent polyphenolic compound in ginger, has emerged as a primary player in its antioxidant activity. Gingerol's effectiveness as a free radical scavenger has been well-documented, as it efficiently mitigates oxidative stress by quenching ROS and thwarting their damaging effects (Ballester et al., 2022). Moreover, ginger root extract stimulates the activity of endogenous antioxidant enzymes, such as superoxide dismutase (*SOD*) and catalase, thereby enhancing the overall cellular antioxidant capacity. This augmentation of the body innate antioxidant defenses is vital in maintaining cellular integrity and mitigating oxidative damage. Interestingly, antioxidant capabilities of ginger extend beyond ROS scavenging to modulate inflammatory pathways. Oxidative stress and inflammation are inherently intertwined, and ginger's potential to address both facets positions it as a comprehensive therapeutic agent.

2.8.2 Anti-Inflammatory Properties of Ginger Root Extract

Anti-inflammatory effects of ginger root extract are underscored by its ability to resist the production and release of pro-inflammatory mediators, including prostaglandins and cytokines. Prostaglandins and cytokines play a pivotal role in promoting inflammation, pain, and tissue damage. The inhibition of cyclooxygenase (COX) enzymes, which are central in the biosynthesis of pro-inflammatory prostaglandins, is a key mechanism through which ginger root extract exerts its anti-inflammatory influence. Furthermore, ginger root extract has been shown to downregulate the expression of pro-inflammatory cytokines, notably tumor necrosis factor-alpha (*TNF-**α***) and interleukin-6 (*IL-6*). These cytokines are principal instigators of inflammation, and their suppression by ginger extract significantly contributes to its anti-inflammatory effects.

Several studies have confirmed the anti-inflammatory action of ginger. The compounds 6-gingerol and 6-paradol have demonstrated strong anti-inflammatory activity by suppressing the production of *TNF-**α***. The *TNF-**α*** gene activation triggers the release of proinflammatory cytokines, leading to the activation of the transcriptional factor NF-**K**B. This cascade results in inflammation and related

diseases. Ginger possesses significant NF-**K**B inhibitory properties, thereby suppressing the expression of key inflammatory molecules, including COX2, 5-LOX, and iNOS, likely through the downregulation of NF-**K**B activation. Ginger acts as an anti-inflammatory agent by blocking the activation of NF-**K**B through the suppression of the proinflammatory cytokine *TNF-α*, as demonstrated in Figure 2.6.

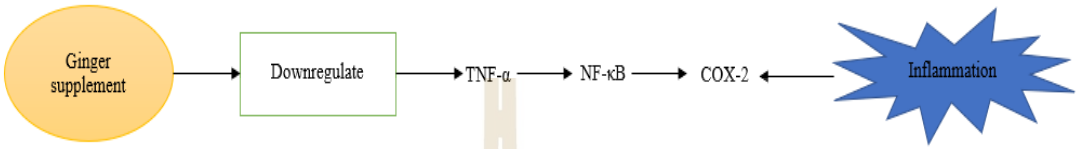

Figure 2.5 Ginger inhibits COX-2 by suppressing NF- κB activity via TNF–α.
Source: Modify from Kumar (2013).

Furthermore, ginger rhizome, ginger callus, and callus elicited by yeast extract, glycine, and salicylic acid were evaluated for their effect on pro-inflammatory (*TNF-α, IL-1,* and *IL-6*) and anti-inflammatory (*IL-10* and *TGF-β*) cytokines *in vitro*, suggesting the potential inclusion of ginger callus in nutraceutical formulations to protect against inflammatory diseases (Ali, El-Nour, & Yagi, 2018). These findings collectively underscore the therapeutic potential of natural compounds in managing inflammation-related diseases and offer a promising avenue for further research and development.

2.9 Interaction of Bioactive Compounds

2.9.1 The synergistic interactions of antioxidant activities

Plants typically contain phytochemical compounds that possess individual bioactivity. However, when these bioactive constituents from plant extracts and various natural products combine, they often produce synergistic effects. The concept of synergism in antioxidant activity encompasses several aspects, as discussed by Guleria (2017): (1) synergistic interactions between antioxidant components from various natural products, (2) synergism between antioxidant components in different solvent fractions of the same plant extract or a

mixture of different herbal preparations, and (3) synergism between synthetic antioxidants and natural products.

The first aspect involves synergistic interactions between antioxidant components from different natural products. For instance, the combination of resveratrol with other phenolic compounds, such as gallic acid and caffeic acid, has demonstrated substantial synergistic effects, with an increase in antioxidant activity of up to 137.8% (Skroza, Generalić Mekinić, Svilović, Šimat, & Katalinić, 2015).

Moreover, synergistic antioxidant effects were explored *in vitro* by identifying antioxidant components from Astragalus membranaceus (AME) and Paeonia lactiflora (PL). Eight combination extracts (E1-E8) were created using a bioactivity-guided fractionation approach. Notably, an increase in the total phenolic/flavonoid content was correlated with enhanced antioxidant activity, especially regarding the total flavonoid content and ferric-reducing capacity. Furthermore, these synergistic products displayed significant cryoprotection against H_2O_2-induced oxidative damage in MRC-5 cells by inhibiting the decline of superoxide dismutase (*SOD*), glutathione peroxidase (*GSH-Px*), and catalase (*CAT*) activities (X. Xu et al., 2014).

Furthermore, the combination of *Potentilla fruticosa* L. Leaves (PFE) with green tea polyphenols (GTP) in various oxidation systems was investigated. The analysis of isobolograms revealed that the PFE and GTP combination exhibited extensive synergism. Notably, a 3:1 (PFE: GTP) ratio displayed the most significant synergistic effect. Chemical analysis of seven phenolic compounds in PFE, GTP, and their mixtures indicated that the chemical composition did not change after the combination. This finding provides a theoretical basis for the effective utilization of combined extracts in various applications, such as compounded teas, dietary supplements, and substitutes for synthetic antioxidants (Liu, Luo, Jia, Wang, & Li, 2016).

Importantly, Lv et al. (2021) elaborated on six generally accepted mechanisms for the observed synergistic effects: (a) Enhanced chelation of phenolic compounds with metal ions, which can induce oxidation. (b) Action through chain reactions, inhibiting lipid peroxidation by capturing peroxyl radicals. (c) Increased preservation rate of other oxidative substances. (d) Providing and maintaining the level of reducing agents through electronic transfer. (e) Coupling oxidation based on

redox potential differences. Polyphenolic compounds facilitate direct reactions between antioxidants by reducing potential differences. (f) Producing synergistic antioxidant effects of the same antioxidant molecules through structural interactions. For example, an appropriate solvent can reduce the enthalpy of dissociation of the hydroxyl O-H bond of ferulic acid glyceryl ester via **π-π** stacking, thus lowering the activation energy of the reaction between an antioxidant molecule and DPPH·. These review mechanisms collectively elucidate the synergistic interactions of antioxidant activities and application in the field of natural product-based therapeutics.

2.9.2 Calculation of the interaction

The combination index (CI) was calculated by as the following equation (1).

$$CI = \frac{C1}{Cx,1} + \frac{C2}{Cx,2} \quad (1)$$

where C1 and C2 are the concentrations of the single extracts and Cx,1 and Cx,2 are the concentrations of single extract required to achieve the same effect upon % single extract in formulation to produce the obtained effect. CI > 1 indicated synergistic effects, CI < 1 indicated the antagonistic effects, and CI = 1 indicated Additive effects (Tian et al., 2021) Then, the expected value (E) is calculated as the average of individual observed amounts for each 1 and 2 combination extracts, and the observed value (O) came through the observed amounts for combined extracts (Z. Liu, Luo, Jia, Wang, & Li, 2016).

2.10 Detoxification

Xenobiotic metabolism is a mechanism for eliminating foreign substances. When cells are exposed to foreign substances, known as xenobiotics, such as drugs, toxins, or chemicals, the body employs enzymes to remove these substances (xenobiotic-metabolizing enzymes), which divide the detoxification process into two phases. The liver, being the principal organ responsible for xenobiotic metabolism and detoxification, continually processes various substances from both the digestive tract and the rest of the body.

2.10.1 The Metabolic Pathways of Detoxification

The physiological pathways for detoxification are primarily governed by the Phase I and Phase II enzyme systems. During Phase I, numerous enzymes are engaged in breaking down these substances. This phase can be considered the "subtraction" phase, during which enzymes work to extract molecules from the substances and break them down into smaller, more manageable units. As a result of this enzymatic action, some highly toxic end products (metabolites) may remain. It is crucial to rapidly channel these toxic metabolites to Phase II, where they are converted into safer, more biocompatible forms for the body. The proper functioning of these enzymes can be impaired by heavy metals. The Phase I detoxification system, which consists of oxidation and reduction reactions, is primarily executed by the cytochrome P450 enzyme system. Efficient operation of the Phase I detoxification system demands various nutrients. These cytochrome P450 reactions generate free radicals, which have the potential to induce secondary cellular damage. Thus, it is imperative to maintain a sufficient supply of key antioxidants to prevent tissue damage. These antioxidants include reduced glutathione, superoxide dismutase, along with other nutrients like beta-carotene, vitamin E, selenium, and N-acetylcysteine (NAC). Research has demonstrated that these antioxidants aid in Phase I liver detoxification.

Phase II represents the "addition" or conjugation phase, during which new substances are added or conjugated to the toxic and beneficial metabolites generated during Phase I. This process renders these metabolites more transportable, stable, and functional for the body. Specialized enzymes are responsible for adding specific substances to form new compounds. Individual xenobiotics and metabolites typically follow specific paths. Seven major biochemical reactions comprise Phase II: glutathione conjugation, amino acid conjugation, methylation, sulfation, acetylation, glucuronidation, and sulfoxidation. Each of these reactions targets specific types of intermediates and requires specific nutrients to reach successful completion. Essentially, these reactions involve adding a molecule to the Phase I intermediates, making them less toxic and more water-soluble. The final products can then be excreted from the body either in the urine or through the bile, another product of the liver. Bile exits the body as part of solid waste, as depicted in Figure 2.7.

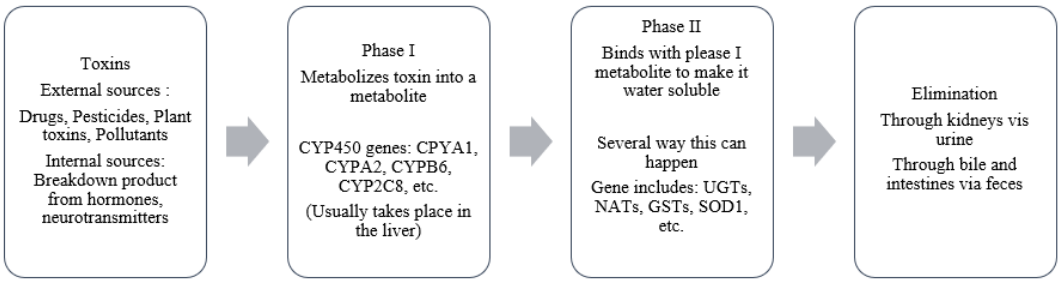

Figure 2.6 The process of detoxification and elimination in the liver.
Source: Modification from Genetic Lifehack (2015).

2.10.2 Dietary Supplements and Foods in Detoxification

Cytochrome P450 reactions, pivotal in the metabolism of xenobiotics, rely on the intricate interplay of various nutrient co-factors for their optimal functioning (Hodges & Minich, 2015). The significance of these co-factors cannot be understated, as they serve as indispensable elements in the phase I detoxification process, facilitating the conversion of a wide array of compounds into more manageable forms.

In addition to nutrient co-factors, the realm of phytonutrients has garnered attention for its potential role in enhancing phase I detoxification. Specifically, certain phytonutrients, exemplified by the indoles found in cruciferous vegetables and quercetin, have exhibited the capacity to augment the efficiency of this detoxification phase (Hodges & Minich, 2015). These findings underscore the intricate relationship between dietary components and the intricate machinery of detoxification. Nevertheless, the extent to which dietary supplements and foods can modulate detoxification processes remains a subject of ongoing exploration. Contemporary research endeavors are poised to investigate the precise mechanisms by which various dietary elements impact detoxification pathways. These investigations are underpinned by the burgeoning comprehension of the intricate interplay between detoxification functions and the formidable challenges posed by environmental factors, notably the pervasive presence of PM2.5 in modern settings. To bolster this emerging field of inquiry, it is imperative to engage with a broader array of academic sources that delve into the intricate dynamics of dietary

supplements and foods in the context of detoxification. These sources, building upon the foundations laid by Hodges and Minich (2015), offer an expanded and comprehensive perspective that contributes to the ongoing discourse in this field.

2.11 Export and discriminate biomolecular by SY-FTIR

The examination of biological samples using Fourier Transform Infrared (FTIR) spectroscopy has become highly effective. This technique reveals molecular details associated with their macromolecular composition, as seen through changes in absorbance bands present in the mid-IR range of 4,000–400 cm^{-1}, a region known as the molecular fingerprint area of the IR spectrum. These absorbance bands can be attributed to specific chemical bonds, and their presence is intimately associated with the structure and conformation of the molecules under investigation. By employing FTIR, researchers can gain a comprehensive understanding of biomolecular changes, contributing to advancements in various fields, including medicine, biology, and environmental science. Therefore, the application of FTIR microspectroscopy has already been demonstrated in various cell research studies, providing a crucial role in the precise characterization of the biochemical composition within cellular systems. (Caliskan et al., 2021; Hengpratom et al., 2018; H. Liu et al., 2018).

Moreover, the measurement of biomolecular changes can be effectively performed using Synchrotron FTIR. Synchrotron FTIR spectroscopy offers high-resolution analysis and enhanced sensitivity, enabling the characterization of molecular vibrations and functional groups in biological samples. This technique provides valuable insights into the structural and compositional alterations of biomolecules, such as proteins, lipids, and nucleic acids, under different experimental conditions.

For characteristic of biological molecules from FTIR spectra was analyzed by Principal Component Analysis (PCA). PCA was employed as a powerful statistical data-reduction approach. It transforms the original data set of variables into a new set of uncorrelated variables, known as principal components (PC)., PCA was applied to analyze the characteristics of biological molecules derived from FTIR spectra (Dunkhunthod, Thumanu, & Eumkeb, 2017). It allows for the identification of

significant wave numbers in the complex FTIR spectra, which contribute the most to the spectral variation within the sample. Multivariate analysis, such as PCA, is commonly utilized in data analysis, enabling the visualization of clustered spectra in scores plots and the observation of variables (spectral bands representing various molecular groups within the samples) in loadings plots. In this study SY-FTIR was employed to monitor and differentiate biomolecular changes induced by the potential of combined extracts against PM2.5 toxicity on A549 cells.

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CHAPTER III

CHEMICAL AND CELLULAR ANTIOXIDANT ACTIVITY OF *THUNBERGIA LAURIFORIA* LINN. (RANG CHUTE) LEAVES, ZINGIBER OFFICINALE (GINGER) AND THEIR COMBINED EXTRACTS

3.1 Abstract

Although Rang Chuet (*Thunbergia laurifolia* Lindl.) and ginger (*Zingiber officinale*) are individually known for their antioxidant properties, their combined effect remains unexplored. This study investigated the interaction between Rang Chuet water extract (RWE) and ginger ethanol extract (GEE) on their overall antioxidant activity and cellular effects. The phytochemical profile of RWE was analyzed using high-performance liquid chromatography spectrometry (HPLC). The RWE identified coumaric, caffeic, protocatechuic, rosmarinic, and gallic acid as phenolic compounds and apigenin as a flavonoid. The total phenolic content (TPC) and total flavonoid content (TFC) of RWE and GEE were determined initially. The results showed that RWE showed the highest TPC (130 ± 2.86 mg GAE/g extract) on the other hand, GEE showed the highest TFC (225 mg Quercetin E/g extract). Extensive research with chemical antioxidant activities demonstrate the Additive interaction between RWE and GEE extracts in various antioxidant assays such as the ABTS + radical cation scavenging capacity assay (ABTS), the DPPH free radical scavenging capacity assay (DPPH), the ferric reducing antioxidant power assay (FRAP), and the ferric chelating assay (FIC). Additionally, the combined extract of RWE and GEE at ratio of 5:1 (V/V) exhibited a synergistic effect in DPPH and FRAP assays. To evaluate the cytotoxicity, cytoprotective effect, and intracellular ROS scavenging, the RWE, GEE and combined extracts were exposed with H_2O_2 in HepG2 cells. However, the combined extracts have a cytoprotective effect from the toxicity of H_2O_2 and attenuated intracellular ROS generation. Moreover, from RT-qPCR gene expression, the *HO-1, SOD, CAT*, and *NQO1*, antioxidant genes expression, were induced by extract treatments and showed synergistic effects. In conclusion, a synergistic combination of

extracts might be utilized to generate nutraceutical formulations or other food items with antioxidant activity to avoid oxidative illnesses.

Keywords: Antioxidant capacity, Cellular antioxidant activity, phytochemical profile, synergistic antioxidant activity, *Thunbergia laurifolia* Lindl., *Zingiber officinale*

3.2 Introduction

Several chronic conditions, include aging, Alzheimer's disease (AD), atherosclerosis, diabetes, hypertension, kidney disease, and cancer, are adversely affected by oxidative stress (Roberts & Sindhu, 2009). Epidemiological studies indicate that the consumption of plant-based diets may help reduce oxidative damage (Batool, Khan, Sajid, Ali, & Zahra, 2019). Polyphenols, a class of phytochemical compounds, are specialized plant metabolites that benefit human health by counteracting the pathological effects of oxidative stress (Zhang & Tsao, 2016) . Therefore, numerous studies have attempted to assess the health benefits associated with antioxidant activity.

Thunbergia laurifolia Lindl. belongs to the Acanthaceae family and is found throughout India and Southeast Asia. It is referred to as Rang Chuet in Thai. Rang Chuet leaves are widely used as an antidote for poisons. This plant has antioxidant, anti-inflammatory, antimicrobial, anti-diabetic, anti-pyretic, and anti-hyaluronidase activities (Chaiyana et al., 2020; E. W. C. Chan et al., 2012; Junsi, Siripongvutikorn, Yupanqui, & Usawakesmanee, 2017; R. Oonsivilai, Cheng, Bomser, Ferruzzi, & Ningsanond, 2007; Preechasuk et al., 2020). In traditional Thai medicine, Rang Chuet is utilized. To date, scientists are interested in researching its bioactive compounds and their unique phytochemical compounds. *Zingiber officinale* (ginger) has many health benefits. Gingerol and zingerone, the main active compounds, have antioxidant effects by reducing free radical scavenging (Haniadka et al., 2013). Furthermore, ginger has anti-inflammatory properties due to a combination of 1-dehydro-[6]-gingerdione, 6-shogaol, 6-dehydroshogaol, and hexahydrocurcumin (F. Li et al., 2012).

Combining different plant extracts may result in a synergistic interaction between their various phytochemicals, hence enhancing the efficacy of plant extracts

(Karimi, Ghanbarzadeh, Hamishehkar, Keyvani, et al., 2015). Structural phytochemicals may possess similar or overlapping, which may have complementary effects in their antioxidant activities. The interaction of phytochemicals in food, plant extracts, and natural antioxidants has been reported in various studies *in vitro* and *in vivo* (Hajimehdipoor, Shahrestani, & Shekarchi, 2014; Tian et al., 2021; S. Wang, Meckling, Marcone, Kakuda, & Tsao, 2011; X. Xu et al., 2014).

This study aimed to identification of phytochemical compounds in Rang Chuet water extracts (RWE) using HPLC coupled with a DAD detector. The antioxidant properties of RWE, ginger ethanol extract (GEE), and their combined extracts were evaluated using a variety of chemical antioxidant assays, *in vitro* antioxidants and antioxidative gene expression by qRT-PCR. Additionally, combinations with synergistic effects and high antioxidant activity was selected. The results of this study lay a foundation for further elucidation of the interaction of combined RWE and GEE on antioxidant activity.

3.3 Materials and methods

3.3.1 Chemicals and reagents

1,1-diphenyl-2-picrylhydrazyl free radical (DPPH), 2,4,6-tri (2-pyridyl)-striazine (TPTZ), Ferric chloride, chloride-6-hydrate, ferrous sulfate 7-hydrate, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS), potassium persulfate, gallic acid, Ascorbic acid, Butylated hydroxytoluene (BHT), 6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteu phenol reagent, Sodium acetate ($C_2H_3NaO_2$), anhydrous sodium carbonate (Na_2CO_3), sodium nitrite ($NaNO_2$), aluminum chloride ($AlCl_3$) and sodium hydroxide (NaOH), ferrozine were sourced from Sigma Aldrich Co. (St. Louis, MO, USA). Methanol, Glacial acetic acid, and Hydrochloric acid (37% w/w) were utilized for chemical analysis. Extraction was carried out using solvents such as 95% ethanol from Mallinckrodt-Baker (Phillipsburg, NJ, USA). Acetic acid and acetonitrile solution (Mallinckrodt-Baker, Phillipsburg, NJ, USA) were certified HPLC and ACS grades for HPLC. The phenolic and flavonoid standard compounds were procured from Sigma Aldrich (St. Louis, MO, USA). For cell culture, Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were purchased from HyClone (HyClone, Logan, UT, USA). Trypsin-EDTA (ethylenediaminetetraacetic acid), L-Gln, and non-essential amino acids (NEAAs)

were sourced from Gibco (Carlsbad, CA, USA), while 3-(4,5-dimethyl-2- thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), and 2′7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, Mo, USA) for the study.

3.3.2 Plant preparation

Plant materials, Rang Chute leaves, were obtained in March 2021 from the Non-toxic Agriculture Cooperative under the Royal Initiative of His Majesty the King (NACR) The people of a neighborhood's Development Study Center in Nakhon Ratchasima Province, Thailand. A voucher specimen (Ratchadaporn 001) was prepared and kept at the School of Food Technology, Suranaree University of Technology. Then, fresh Rang Chuet leaves were hot air-dried at 60oC for 3 h. Another way, the fresh Rang Chuet leaves were also dehydrated in a microwave vacuum drier (Marchcool, Bangkok, Thailand) at 2,400 watts for 11 min, until the moisture content was below 13%. Dried Rang Chuet leaves were grounded in a blender (National, MX-T2GN, Taiwan). The Rang Chuet powder was sieved through mesh size 0.2 mm and was kept at -20°C in a vacuum package until use. Commercial ginger extract was purchased from Now Foods (Illinois, USA) and stored at -20°C until further use. The composition of commercial ginger extract was showed in appendix 1.

Preparation of extracts, the Rang Chuet powder was extracted using Oonsivilai et al. (2006) method. The 100 mg powder was extracted with 12 mL of boiling water (95 ± 2°C) in a shaking water bath (Sheldon, WS27-2 SHEL LAB, Oregon, USA) at 37°C for 15 min. Three cycles of 3 min. centrifugation at 3,000 g (Thermo IEC, Waltham, MA) was performed between extractions. Combining the filtrates and adjusting the volume to 50 mL. Five mL aliquots were transferred to 12 mL in the container and sublimated using a freeze dryer (CHRIST, Alpha 2–4 LSCplus Martin Christ Gefriertrock-nungsanlagen GmbH, Germany). The RWE was stored at -20°C until use. For the preparation of GEE, as commercial ginger extract contains ginger extract and ginger power, it was re-extracted, extraction steps were the same as Rang Chuet extraction except using 95% ethanol for solvent extraction and drying with a rotary evaporator (Büchi®, R200, New Castle, United States) instead of using a freeze dryer. The GEE was stored frozen at -20°C until use. The combined extracts of Rang Chuet and ginger with the following ratios 5:1, 3:1, 2:1, 1:1, 1:2, 1:3, and 1:5 v/v.

3.3.3 Determination of Phytochemical profile of RWE

According to Oonsivilai et al. (2007), phenolic and flavonoid compounds were determined in the RWE from both Hot air- and microwave-drying using HPLC. The analyte was analyzed using an HPLC system from the Agilent 1100 Series fitted with a diode array detector. The ZORBAX Eclipse® XDB-C18 Analytical 4.6 150 mm reversed-phase column (Agilent, US) was utilized, along with a protection column also filled with ZORBAX Eclipse® XDB-C18. The solutions were subjected to gradient elution at 35°C, with a flow rate of 1.0 mL/min and a mobile phase composed of water/acetic acid (98:2, v/v) in reservoir A and acetonitrile in reservoir B. Initially, the solvent ratio stood at 99:1 A/B, gradually shifting to 70:30 A/B over 20 minutes, followed by a 5-min linear transition back to 99:1 A/B. The process concluded with 5 min of equilibration at the initial condition, resulting in a total chromatographic run time of 30 min. The detection and tentative identification of phenolic and flavonoid compounds were identified using in-line PDA data spanning the 270–340 nm wavelength range. The RWE was weighed and dissolved in deionized (DI) water to achieve a concentration of 25 mg/mL. This process was conducted in triplicate. Subsequently, the solutions were filtered through a 0.22 µm PETP membrane prior to injection.

Reference phenolic acids standard, including caffeic acid, p-coumaric acid, ferulic acid, gallic acid, protocatechuic acid, rosmarinic acid, and sinapic acid, was employed for standardization. Additionally, quercetin and apigenin served as flavonoid standard compounds. The preparation of reference standards stock solutions involved accurately weighing and dissolving gallic, protocatechuic, caffeic, and p-coumaric acid in DI water. Ferulic acid, sinapic acid, quercetin, and apigenin were dissolved in 95% ethanol to achieve a final concentration of 500 µg/mL. These stock solutions were then diluted within a range of 1-500 µg/mL to establish calibration curves for quantifying phenolic acids and flavonoids. RWE from hot air-dried or microwave-dried leaves with high phenolic acids and flavonoid content were chosen for further analysis.

3.3.4 Determination of total phenolic and flavonoid contents

RWE and GEE were investigated.

3.3.4.1 Total phenolic content

The method was determined following the procedure outlined by Oonsivilai et al. (2007), utilizing gallic acid as the standard. In a 1.5 mL cuvette,

aliquots of 0.02 mL gallic acid standard/sample/blank were combined with 1.58 mL of DI water. Then, 0.1 mL of Folin-Ciocalteu reagent was added, and the solution was allowed to stand for 5 min. Following this, 0.3 mL of Na_2CO_3 (20% w/v) was introduced, and the mixture was stored away from light for 120 min. Absorbance measurements were conducted at 765 nm using a spectrophotometer (GENESYS, 10S Vis, Wisconsin, USA). The procedure employed gallic acid standards ranging from 0 to 500 mg/l. The results were reported as mg gallic acid equivalents (GAE)/g.

3.3.4.2 Total flavonoid content

The method was determined using quercetin as a standard. Initially, 0.1 mL of RWE and GEE were mixed with 0.3 mL of distilled water, followed by the addition of 0.03 mL of 5% $NaNO_2$. After a 5-min incubation at 25°C, 0.03 mL of 10% $AlCl_3$ was added. Following another 5-min incubation, the reaction mixture was treated with 0.2 mL of 1 mM NaOH. Subsequently, the reaction mixture was diluted to a volume of 1 mL with DI water, and the absorbance was measured at 510 nm using a spectrophotometer. The total flavonoid content was expressed as mg quercetin equivalents.

3.3.5 Determination of chemical antioxidant activities of extracts

The chemical antioxidant activities of extracts were compared to commercial standards such as BHT, ascorbic acid, and Trolox using various assays.

3.3.5.1 ABTS·+ cation radical scavenging assay

The ABTS·+ cation radical scavenging capacity (ABTS) of extracts and standard antioxidants was assessed with modifications following Ksouri et al. (2009) protocol. The ABTS+ cation radical was generated by mixing 5 mL of 14 mM ABTS solution with 5 mL of 4.9 mM potassium persulfate ($K_2S_2O_8$) solution at room temperature for 12–16 h in the absence of light. This solution was diluted with ethanol to achieve an absorbance of 0.700 ± 0.020 at 734 nm. The reaction mixture, in a final volume of 2 mL, comprised 900 µl of ABTS+ solution and 100 µl of sample extracts at different concentrations, with distilled water acting as the control. After 6 min of homogenization, the absorbance of the reaction mixture was measured at 734 nm. The ABTS+ scavenging capacity was expressed as IC_{50} (mg/mL).

3.3.5.2 DPPH· free radical scavenging assay

The DPPH free radical scavenging activity was determined following the procedure outlined by Ratchadaporn Oonsivilai and Ferruzzi (2008). Initially, a 100 µM violet solution containing 0.1 mM DPPH in methanol was prepared. Then, 0.1 mL aliquots of standard/sample/blank were transferred into a test tube. Subsequently, 1.90 mL of DPPH solution was added to the test tube, and the mixture was allowed to incubate for 15 min. The absorbance of the solution was then measured at 515 nm. The scavenging capacity of DPPH was assessed using IC_{50} (mg/mL).

3.3.5.3 Ferric-reducing antioxidant power assay

The FRAP assay, following the procedure by R. Oonsivilai et al. (2007) was conducted. The FRAP reagent was prepared by mixing 300 mM acetate buffer (pH 3.6), 10 mM TPTZ solution in 40 mM HCl, and 20 mM ferric (III) chloride solution in proportions of 10:1:1 (v/v), respectively. This reagent was freshly prepared daily and warmed to 37°C in a water bath before use. Adding 50 µl of the sample and 150 µl of DI water to 1.5 mL of the FRAP reagent, the reaction mixture was incubated for 4 minutes, and its absorbance was measured at 593 nm. Ferrous sulfate stock solution (100–1000 µM) was used to construct the standard curve, and the results were reported as mM ferrous (II)/g dry weight of the extract.

3.3.5.4 Ferric chelating assay

The Ferric chelating (FIC) assay, following the method by Junsi et al. (2017) with minor adjustments, was conducted. Initially, 50 µl of the sample, 1,000 µl of DI water, and 50 µl of 1 mM $FeCl_2$ were mixed and incubated for 5 min in the dark. Subsequently, 50 µl of 5 mM ferrozine was added, and the mixture was further incubated for 20 min. The absorbance was then measured at 562 nm. The results were reported as µg EDTA equivalents.

3.3.6 The interaction of combined extracts on chemical antioxidant activities

From the previous determination of the antioxidant activities by DPPH, ABTS, FRAP, and FIC assays, the interaction effect between the RWE and GEE is shown by the combination index (CI) as the following equation (1).

$$CI = \frac{C1}{Cx,1} + \frac{C2}{Cx,2} \quad (1)$$

where C1 and C2 are the concentrations of the single extracts and Cx,1 and Cx,2 are the concentrations of single extract required to achieve the same effect upon % single extract in formulation to produce the obtained effect x. From DPPH and ABTS assay results, the extracts that showed low IC_{50} value with high antioxidant activity would show a CI value less than 0.90, which interprets a synergistic effect. On the other hand, the CI values of extracts determined by FRAP and FIC assay showed higher than 1.10, which interprets a synergistic effect. Additionally, the CI of antioxidant activity in a range of 0.91–1.10 means a Additive effect Then, the expected value (E) is calculated as the average of individual observed amounts for each 1 and 2 combination extracts, and the observed value (O) came through the observed amounts for combined extracts (Z. Liu, Luo, Jia, Wang, & Li, 2016).

3.3.7 Cellular study of extracts

3.3.7.1 Cell culture

Human liver hepatoma (HepG2) cell lines used in this investigation were purchased from the American Type Culture Collection (ATCC® HB-8065TM, Manassas, Virginia, United States). Cells were grown in Dulbecco's modified eagle medium (DMEM) enriched with 10% (v/v) FBS, 1% (v/v) non-essential amino acid (NEAAs), 1% (v/v) L-glutamine, and 1% (v/v) penicillin-streptomycin. The cells were cultured at 37°C in a 5% CO_2 atmosphere with a 95% culture medium and replaced three times per week.

3.3.7.2 Cytotoxicity of extracts

The MTT assay was employed to assess the cytotoxicity of RWE, GEE, and CRGE on cells. Briefly, HepG2 cells were seeded at a density of 1×10^4 cells/well in 96-well plates overnight. Subsequently, they were treated with varying concentrations of extracts (ranging from 25 to 1,200 µg/mL) for 24 h. Following each treatment, the media was replaced with 0.5 mg/mL of MTT solution in 1xPBS and incubated for 3 h at 37°C. After removing the MTT solution, the formazan crystals were dissolved in 100 µl of DMSO, and the absorbance of the purple formazan solution was measured at 570 nm using a microplate reader (BMG Labtech GmbH, Ortenbreg, Germany). The percentage of cell viability was calculated using the following equation (2).

Cell viability (%) = (Abs. of the treated group/Abs. of the control group) x 100 (2)

3.3.7.3 Cytoprotective effect of all extracts

To study the cytoprotective effect of all extracts against toxic, H_2O_2 was used to apply in this study. First, preliminary the concentrations of H_2O_2 which is cytotoxic to cells, Briefly, HepG2 cells were seeded flowing the protocol decried above. For H_2O_2, cells were exposed in the range of 1-10 mM. The concentration which revealed cell viability nearly 50%, was selected to study for the next step. Next, in the cytoprotective study, cells were pretreated with RWE, GEE, and CRGE concentrations which were two concentrations for 24 h. Then, cells were washed with 1X PBS and incubated with H_2O_2 for 3 h. MTT assay was used to examine the percentage of cell viability.

3.3.7.4 Intracellular ROS scavenging by DCFH-DA assay

The DCFH-DA assay was utilized to quantify intracellular ROS production in response to toxicity. Following the seeding of HepG2 cells, they were subjected to 3 different treatments of RWE, GEE, and CRGE for 24 h. Subsequently, the media was removed, and the cells were exposed to 2 mM H_2O_2 for 30 min to induce oxidative stress. Following each treatment, the cells were stained with 20 μM DCFH-DA for 30 min at 37°C in the dark. The fluorescence intensity was measured using a fluorescence microplate reader (Thermo Scientific Varioskan, USA) at excitation /emission wavelengths of 485/535 nm.

3.3.8 Antioxidant Gene expression of by Quantitative RT-PCR

HepG2 cells were seeded at a density of $6x10^5$ cells/well in a 6-well plate. The cells were treated with both single and combined extracts and were subjected to oxidative stress using H_2O_2. Post-treatment, all cells were washed with 1×PBS. Regarding RNA Isolation and cDNA Synthesis, total RNA extraction was performed. Briefly, celllysis was conducted using Nucleospin® RNA Plus (MACHEREYNAGEL GmbH & Co. KG., Düren, Germany) following the manufacturer's protocol. The concentration of total RNA was quantified using the LVis plate and the SPECTROstar NANO system (BMG Labtech GmbH, Ortenberg, Baden-Württemberg, Germany). For cDNA synthesis, ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd., Osaka, Japan) was utilized in

accordance with the manufacturer's instructions. The resultant cDNA samples were preserved at -20℃ for future analysis.

Quantitative RT-PCR was performed using SYBR Green (2xqPCRBIO SyGreen Mix Lo-ROX, PCR BIOSYSTEMS, London, UK) protocol established by the manufacturer. cDNA was amplified by mixing 2 μl of cDNA freshly prepared reagent containing 1 μl of RNase-free DI, 2 μl of 2 nM primer, and 5 μl of SYBR Green. The PCR reaction conditions for each cycle were: 3 min at 95℃ followed by 40 cycles of 30 s at 60℃ and 1 min at 72℃. Melting curve stage at 60℃ for 1 min then 15 s at 95℃. Using QuantStudio^{MT} Design and Analysis software v.1.52. The data were analyzed using the $2^{-\Delta\Delta Ct}$ expression model. Relative fold expression of targeting gene expression was normalized to the mRNA for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The target gene primer sequences were shown in Table 3.1.

Table 3.1 The target gene primer sequences.

Target Gene	Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)
HO-1	ATGGCCTCCCTGTACCACATC	TGTTGCGCTCAATCTCCTCCT
SOD1	GCAGGTCCTCACTTTAATCCTCT	ATCGGCCACACCATCTTTGT
CAT	TGAAGATGCGGCGAGACTTT	TGGATGTAAAAAGTCCAGGAGGG
NQO1	GGATTGGACCGAGCTGGAA	AATTGCAGTGAAGATGAAGGCAAC
GAPDH	TCCAAAATCAAGTGGGGCGA	TGATGACCCTTTTGGCTCCC

HO-1 = Heme oxygenase; *SOD* = superoxide dismutase; *CAT* = catalase; *NQO1*= NAD(P)H Quinone Dehydrogenase 1; *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase.

3.3.9 The interaction of combined extract on cellular antioxidant study

From the previous determination of cellular antioxidant study including cytotoxicity, cytoprotection and intercellular ROS scavenging assays and antioxidant gene expression, the interaction effect between the RWE and GEE is shown by the combination index (CI).

3.3.10 Statistical analyses

Analysis of data was conducted using SPSS Statistics for Windows, version 23.0, NY: IBM Corp. Mean values ± standard error was calculated based on three

replicates per sample. Group comparisons were made using Student's t-test, with statistical significance set at $p<0.05$. For comparisons among multiple groups, one-way analysis of variance (ANOVA) was employed, followed by Duncan's multiple range test at a significance level of 5%.

3.4 Results and Discussion

3.4.1 Phytochemical profiling of RWE

The presence of phenolic and flavonoid compounds in RWE was determined using HPLC analysis. Table 3.2 shows the phytochemical profiles of RWE from both Hot air-dried and microwave-dried leaf extracts.

Table 3.2 The phytochemical profiles of Rang Chute extracts analyzed by HPLC.

Photo chemical	µg/g of extract									
	Gallic acid	Proto-catechuic acid	Caffeic acid	Coumaric acid	Ferulic acid	Sinapic acid	Rosmarinic acid	Quercetin	Apigenin	Total
Hot air drying	64±42^{b}	330±7.8^{b}	1027±34^{a}	162±8.0^{b}	18±0.8	ND	ND	ND	ND	1,640±10^{b}
Microwave vacuum drying	128±11^{a}	418±14^{a}	919±20^{b}	1,025±49^{a}	ND	ND	374±14	ND	107±2.9	2,976±19^{a}

Data are mean ± SD; Data in the same column with different superscripts are significantly different ($p<0.05$). ND= not detected.

RWE from microwave vacuum-dried leaves contained apigenin and several phenolic compounds, i.e., gallic acid, protocatechuic, caffeic acid, coumaric acid, and rosmarinic acid at 129 ± 13, 415 ± 31, 919 ± 39, 1,030 ± 88, and 378 ± 14 µg/g extract, respectively. Alternatively, the analysis of RWE from hot air-dried leaves revealed only phenolic compounds: gallic acid, protocatechuic acid, caffeic acid, coumaric acid, and ferulic acid. Phytochemical profiling of RWE from other scientific reports was composed of phenolic compounds, including caffeic acid, protocatechuic acid, chlorogenic acid, rosmarinic acid, and vinixin. They also have reported finding such flavonoids as apigenin, catechin, isoquercetin, quercetin, and rutin in RWE (Chuthaputti, 2010; Junsi et al., 2017; Nanthakarn, Sumet, Sophida, & Korbtham, 2020; R. Oonsivilai et al., 2007; Preechasuk et

al., 2020). This study quantified the phenolic and flavonoid compounds in RWE. However, the quantification of phytochemicals varied from the previous finding due to differences in the growth location, leafage, type of extractant, and extraction preparations (Chaiyana et al., 2020; Ratchadaporn Oonsivilai, 2006). Besides, the choice of drying methods could have influenced the phenolic and flavonoid contents. Generally, the Rang Chuet leaves were dried in an oven or sun drying. E. Chan and Lim (2006) mentioned that the total phenolic compounds of hot air-drying declined by approximately 70-80% while microwave-drying declined by approximately 38-41%. Wojdyło, Figiel, Lech, Nowicka, and Oszmiański (2014) explained that irreversible oxidative occurred during drying and exposure to thermal degradation of phenolic compounds with increased heat intensity. Involving a study on phenolic compounds in Rang Chuet, E. W. C. Chan et al. (2012) reported that microwave-dried leaves tea of Rang Chuet had total phenolic compound values at 3,050 ± 127 mg GAE/100 g RM, which was higher than hot air-dried leaves tea of Rang Chuet at 1,800 ± 57 GAE/100 g RM. Likewise, Phahom, Phoungchandang, and Kerr (2017) who studied the effect of microwave on phenolic compounds in Rang Chuet leaves extract showed higher total phenolic content and caffeic acid when applied high-microwave power.

Therefore, this research selected microwave vacuums drying technology for drying Rang Chuet leaves because it preserves phenolic compounds including other bioactive compounds that are heat sensitive. Furthermore, Therdthai and Zhou (2009) implicated that microwave drying took 85-90% shorter drying times in the drying process of mint leaves compared with hot air drying, which was a way to save energy and time in the food and pharmaceutical industries. The main phytochemical compounds in RWE from microwave-dried leaves are caffeic, coumaric, and rosmarinic acids. Caffeic acid (CA) was reported as the main chemical constituent found in Rang Chuet (Nanthakarn et al., 2020; Ruangpayungsak, Sithisarn, & Rojsanga, 2018) and showed antidote activity that might have mechanisms related to detoxification, and the effects were attributed to excellent anti-inflammatory and antioxidant activity (Ahn, Je, Kim, Park, & Kim, 2017; da Cunha et al., 2004; Gülçin et al., 2016). Moreover, the results had discovered rosmarinic, which showed a variety of health benefits; antioxidant, anti-inflammatory, anti-diabetic, and anticancer (Gülçin et al., 2016; Hossan et al., 2014; Ngo, Lau, & Chua, 2018). However, this study did not discover high rosmarinic acid content same as the 50% methanol

extraction method at ambient temperature by (Nanthakarn et al., 2020). However, the number of phytochemical compounds in RWE from microwave vacuum-dried leaves showed a higher quantity than RWE from hot air-dried leaves which was a conventional method. Selected microwave vacuum-dried method for further analysis.

3.4.2 Total phenolic and flavonoid contents of extracts

To investigate the main group of phytochemical compounds in both extracts, the total phenolic content (TPC) and total flavonoid content (TFC) of RWE and GEE are presented in Figure 3.1, which showed the significant differences between them ($p<0.05$).

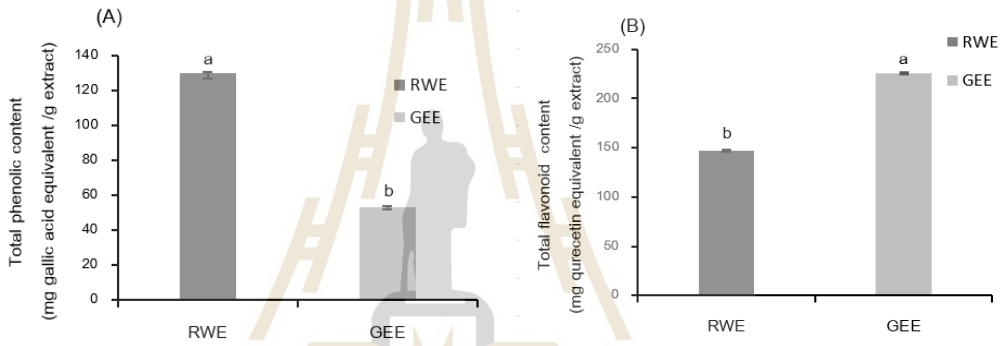

Figure 3.1 Total phenolic content (A) and total flavonoid content (B) of RWE and GEE by microwave vacuum-drying. Data are mean ± SD (n=3), a, b Different letters above error bars indicate significant mean differences ($p<0.05$) in extracts.

RWE showed high total phenolic contents at 130 ± 2.86 mg GAE/g extract. This result might have been affected by microwave vacuum drying which helped preserve phenolic compounds during the drying step. Therefore, the result showed an increase in TPC up to 20% in RWE from microwave-dried leaves. Their quantity was close to that reported by Phahom et al. (2017), who applied the microwave with blanching for drying Rang Chuet leaves. Additionally, this study showed that microwave drying took a shorter drying time than hot air drying which reduced drying times from 3 h to 11 min. Furthermore, the TPC of GEE was 53 ± 1.37 mg GAE/g extract. This result agreed with a previous study that the TPC of the ginger methanol extract was 71.1 mg GAE/g dry weight (El-Ghorab, Nauman, Anjum, Hussain, & Nadeem, 2010). However, the results showed

that the TPC of the ginger extract was 136 mg GAE/g extract in the study of Bursal, Koksal, and Gülçin (2021). In addition, the GEE showed total flavonoid content at a level of 225 mg Quercetin E/g extract, which was significantly higher than RWE (p<0.05). Furthermore, the various results from previously studied might be due to extracting methods with different solvents, which significantly affected the quantification of total flavonoid content. Notably, the ginger used in this study was from a commercial product and the preparation method was unknown. However, this work focused on the synergistic effect of both extracts.

3.4.3 Chemical antioxidant activities of RWE and GEE

The antioxidant activities of RWE and GEE were measured by DPPH, ABTS, FRAP and FIC assays, shown in Table 3.3 and 3.4.

Table 3.3 Antioxidant activities of the DPPH and ABTS assays

Samples	IC_{50} of DPPH· free radical scavenging capacity (mg extract/mL)	IC_{50} of ABTS·+ radical cation scavenging capacity (mg extract/mL)
Rang Chuet extract	1.10 ± 0.07^{c}	1.82 ± 0.01^{e}
Ginger extract	1.53 ± 0.05^{d}	1.30 ± 0.01^{d}
BHT	0.35 ± 0.02^{b}	0.08 ± 0.00^{c}
Vit C	0.08 ± 0.00^{a}	0.04 ± 0.00^{a}
Trolox	0.08 ± 0.10^{a}	0.05 ± 0.00^{b}

Data are mean ± SD; Data within the same column marked with distinct superscripts indicate significant differences (p <0.05). IC_{50}, half-maximal inhibitory concentration.

The DPPH assay, RWE, showed the IC_{50} value at 1.10 ± 0.07 mg/mL, followed by ginger at 1.53±0.05 mg/mL. DPPH· free radical scavenging capacity of RWE is related to its phenolic contents. As a result, phenolic compounds are the major components responsible for the radical scavenging capacity of Rang Chuet extract (Chaiyana et al., 2020). However, both extracts were less effective than commercial standards including Vit C, Trolox, and BHT in the case of DPPH free radical scavenging. In contrast, ABTS·+ radical cation scavenging capacity of GEE showed IC_{50} value at 1.30 ± 0.0 mg/mL, which was higher than RWE, which showed IC_{50} value at 1.82 ± 0.01 mg/mL

with significant differences (p<0.05). As ABTS assay is effective in phenolics, and flavonoids solubilized in ethanol, some bioactive compounds in RWE may not be soluble in the ethanol solvent system. Therefore, it could reduce a potential ABTS·+ radical cation scavenging capacity in RWE. However, DPPH and ABTS assays are radical or radical oxygen species scavenging methods whose dominant mechanism is single electron transfer (SET). This may also have hydrogen atom transfer at the same time. Furthermore, the antioxidant activities are determined by factors such as the structure and properties of antioxidants, solubility, and the solvent system employed. It is important to note that the scavenging capacity may vary depending on the assay method (Shahidi & Zhong, 2015).

Table 3.4 Antioxidant activities of FRAP and FIC assays.

Samples	Reducing power (mmol Fe (II) Equiv./g extract)	Ferric ion cheating (mg EDTA Equiv./g extract)
Rang Chuet extract	0.593 ± 0.03^{d}	2.857 ± 0.08^{b}
Ginger extract	0.714 ± 0.01^{e}	1.380 ± 0.03^{c}
BHT	2.42 ± 0.02^{c}	-
Vit C	8.68 ± 0.10^{a}	-
Trolox	5.76 ± 0.0.2^{b}	-
EDTA	-	16.80 ± 0.01^{a}

Data are meant ± SD; Data within the same column marked with distinct superscripts indicate significant differences (p< 0.05). - Do not measure.

The FRAP assay represents a non-radical redox potential-based method. The reducing power of GEE was shown at a level of 0.714 ± 0.01 mmol Fe (II) Equiv./g extract, which was higher than the reducing power of RWE, which was shown at a level conducted by Z. Liu et al. (2016), which indicated 0.806 mmol of Fe (II) Equiv./g (consider using the same unit as shown in Table 3.4) extract. Ferrous ions chelating activity (FIC) assay measures metal chelation capacity. The results showed that RWE showed FIC at 2.857 ± 0.08 mg EDTA equip./g extract, which was a significant difference from GEE (p<0.05). Thus, plant extracts could chelate the transition metal ions (Shahidi & Zhong, 2015). Furthermore, caffeic and protocatechuic acids were presented in RWE, and

previous reports explained that they could form complexes with ions (Genaro-Mattos, Maurício, Rettori, Alonso, & Hermes-Lima, 2015; Kakkar & Bais, 2014).

This study suggests that both RWE and GEE exhibit proficiency in chemical antioxidant activities, including hydrogen atom transfer (HAT), single electron transfer (SET), non-radical redox potential, and metal chelation capacity. However, the potency of RWE was lower compared to the typical commercial antioxidants. Nonetheless, both substances exhibit notable antioxidant capabilities. GEE, which had lower total phenolic content than RWE, showed antioxidant activities in the case of ABTS·+ radical cation scavenging capacity and reducing power than RWE. For this reason, it was expected to get an interaction in various chemical antioxidant activities when RWE and GEE were mixed to obtain outstanding antioxidant properties. Therefore, RWE and GEE mixtures as combined extracts with various ratios were applied to investigate their antioxidant activities. It might be explained that the interaction was performed under various ratios when the study was carried out.

3.4.4 The interaction of combined extracts on chemical antioxidant activity

The mixture ratios of RWE and GEE were mixed at 7 ratios of 5:1, 3:1, 2:1, 1:1, 1:2, 1:3 and 1:5 v/v. The antioxidant capacities of combined extracts using four assays such as DPPH, ABTS, FRAP, and FIC were carried out. Then, the interaction of chemical antioxidant activity with the various ratios of combined extracts was investigated, and their results are shown in Table 3.5 – 3.8. The observed (O) antioxidant capacities of the combined extracts were compared to the expected (E) value, and the combination index (CI) was calculated to classify a type of interaction.

Table 3.5 The interaction of DPPH antioxidant capacity of the CRGE.

Ratio RWE : GEE (v/v)	IC_{50}(µg/mL)		CI	Effect
	O	E		
5:1	1.064 ± 0.01*	1.212 ± 0.04	0.89	Synergistic
3:1	1.119 ± 0.10	1.244 ± 0.03	0.91	Additive
2:1	1.258 ± 0.15	1.276 ± 0.03	1.00	Additive
1:1	1.299 ± 0.10	1.341 ± 0.03	1.00	Additive
1:2	1.459 ± 0.04	1.406 ± 0.03	1.06	Additive
1:3	1.469 ± 0.03	1.438 ± 0.03	1.04	Additive
1:5	1.558 ± 0.01	1.402 ± 0.11	1.07	Additive

The values are the mean SD of three replicates (n=3). The asterisk (*) indicates that the observed value differs significantly from the expected value ($p<0.05$), T-test. O, observed value; E, expected value; CI, combination index.

Firstly, the interactions of DPPH+ free radical antioxidant activity of the combined extracts are shown in Table 3.5. Increasing the ratio of RWE in CRGE showed a low IC_{50} value. Moreover, the observed IC_{50} value (O) of ratio 5:1 was significantly different from the expected value ($p<0.05$), which demonstrated the lowest IC_{50} as compared with other ratios. Furthermore, the results showed at synergistic effect at the ratio of 5:1 while the other ratios of combined extracts showed an additive effect. In addition, no ratios showed antagonistic interaction. Yao et al. (2009) suggested that when antioxidant molecules share similar structures, their interaction may lead to a synergistic antioxidant effect. For instance, the presence of a suitable solvent could decrease the enthalpy necessary to break the hydroxyl O-H bond of ferulic acid glyceryl ester via stacking, consequently lowering the activation energy required for the reaction between an antioxidant molecule and DPPH.

Table 3.6 The interaction of ABTS·+ radical cation scavenging capacity of the CRGE.

Ratio RWE : GEE (v/v)	IC_{50}(mg/mL)		CI	Effect
	O	E		
5:1	0.184 ± 0.003	0.176 ± 0.008	1.06	Additive
3:1	0.155 ± 0.005*	0.172 ± 0.007	0.93	Additive
2:1	0.154 ± 0.003*	0.167 ± 0.006	0.95	Additive
1:1	0.150 ± 0.003*	0.158 ± 0.005	0.98	Additive
1:2	0.143 ± 0.004*	0.148 ± 0.003	0.99	Additive
1:3	0.138 ± 0.002*	0.144 ± 0.003	0.98	Additive
1:5	0.133 ± 0.002	0.154 ± 0.021	0.97	Additive

The values are the mean SD of three replicates (n=3). The asterisk (*) indicates that the observed value differs significantly from the expected value (p<0.05), T-test. O, observed value; E, expected value; CI, combination index.

Secondly, the interactions of the ABTS·+ cation scavenging capacity of the CRGE are shown in Table 3.6. The observed value in the range of ratio 3:1 to 1:3 was significantly different from expected ABTS values (p<0.05). However, the synergetic effect of ABTS·+ cation scavenging capacity of the CRGE was not found. According to Heo, Kim, Chung, and Kim (2007) research, the observed combination of two and three phenolic compounds in the ABTS assay system does not show a synergistic antioxidant effect.

Table 3.7 The interaction of reducing power of the CRGE.

Ratio RWE : GEE (v/v)	FRAP (mmol Fe (II) Equiv./g extract)		CI	Effect
	O	E		
5:1	0.714 ± 0.030*	0.644 ± 0.025	1.14	Synergistic
3:1	0.737 ± 0.016*	0.670 ± 0.023	1.14	Synergistic
2:1	0.726 ± 0.003	0.696 ± 0.021	1.09	Additive
1:1	0.750 ± 0.011	0.747 ± 0.017	1.05	Additive
1:2	0.780 ± 0.024	0.799 ± 0.013	1.04	Additive
1:3	0.783 ± 0.007*	0.824 ± 0.011	0.99	Additive
1:5	0.831 ± 0.016	0.783 ± 0.095	1.00	Additive

The values are the mean SD of three replicates (n=3). The asterisk (*) indicates that the observed value differs significantly from the expected value (p<0.05), T-test. O, observed value; E, expected value; CI, combination index.

The interactions of reducing the power of the CRGE are shown in Table 3.7. The results showed that at the ratios of 5:1, 3:1, and 1:3, the observed FRAP value showed a significant difference from expected values with significant differences (p<0.05). The increased RWE ratio at 5:1 and 3:1 showed a synergistic effect, while other ratios showed only an additive effect.

Table 3.8 The interaction of FIC of the CRGE.

Ratio RWE : GEE (v/v)	FIC (mg EDTA Equiv./g extract)		CI	Effect
	O	E		
5:1	2.633 ± 0.10	2.617 ± 0.06	1.09	Additive
3:1	2.471 ± 0.04	2.488 ± 0.02	1.10	Additive
2:1	2.328 ± 0.08	2.365 ± 0.04	1.11	Synergistic
1:1	1.872 ± 0.03	2.119 ± 0.06	1.01	Additive
1:2	1.712 ± 0.03	1.872 ± 0.02	1.03	Additive
1:3	1.446 ± 0.04	1.749 ± 0.06	0.91	Additive
1:5	1.363 ± 0.03	1.626 ± 0.04	0.90	Additive

The values are the mean SD of three replicates (n=3). The asterisk (*) indicates that the observed value differs significantly from the expected value (p<0.05), T-test. O, observed value; E, expected value; CI, combination index.

Table 3.8 shows the interactions of FIC with the CRGE. The observed values of all ratios were not significantly different (p>0.05). Furthermore, only the combined extract ratio of 2:1 showed a synergistic effect, while other ratios showed additive effect. Recently, the synergistic antioxidant effects of polyphenolic compounds by enhanced chelation of phenolic compounds with metal ions have been generally recognized (Lv et al., 2021).

In conclusion, there is an Additive interaction in RWE and GEE regarding the chemical antioxidant activity. It should be noted that the majority of the combinations showed an Additive interaction, and some ratios exhibited synergistic effects. In the DPPH

and FRAP assays, the combination at ratio of 5:1 showed the synergistic result. To obtain synergistic effects, additional ratios with GEE or other herb extracts should be performed. Additional research is required to determine the possible underlying mechanisms behind the interaction. Furthermore, their chemical antioxidant activities were compared with the cell culture approach.

3.4.5 Cellular antioxidant activities

3.4.5.1 The cytotoxicity of extracts

The cytotoxicity of extracts in HepG2 cells was thoroughly investigated as shown in Figure 3.2.

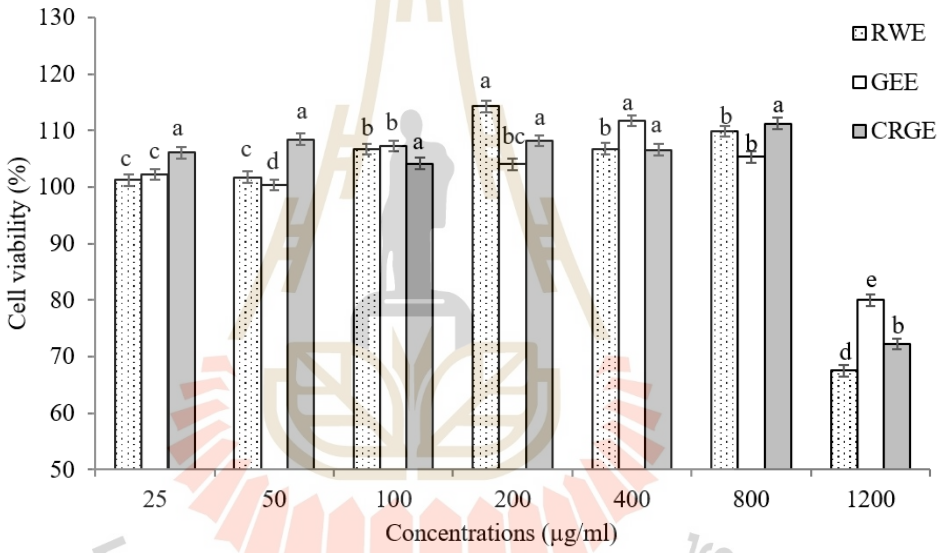

Figure 3.2 Cell viability of Rang Chuet water extract (RWE), ginger ethanol extract (GEE), and combined Rang Chuet and ginger extract (CRGE) at ratio of 1: 1 in HepG2 cells. Cells were treated in ranges of 25-1,200 µg/mL for 24 h. All values showed the mean ± SD of three independent experiments (n=3). Different lowercase letters showed significant differences in the concentration of each extract (p<0.05).

The cell viability of HepG2 cells was unaffected by RWE, GEE, and CRGE extracts at concentrations less than 800 g/mL (p>0.05). However, at a concentration of 1,200 µm of all extracts, the cell viability was significantly lower than the control

($p>0.05$). Concentrations of both single extracts (RWE and GEE) and CRGE did not stimulate cell proliferation. Additively, CRGE at concentrations of 25 and 50 µg/mL resulted in substantially higher cell viability than RWE and GEE as single extracts ($p>0.05$). There were selected to study on cytoprotective effect. However, at the concentration at 25 µg/mL of extracts did not show an effective in cytoprotective effect (data did not show). Therefore, selected concentrations of 50 and 100 µg/mL of extracts for further study.

3.4.5.2 Cytoprotective effect of extracts

Effect of extracts attenuated H_2O_2 induced cytotoxicity in HepG2 cells, H_2O_2 was used to induced oxidative damage in this study, as H_2O_2, a stable source of free radicals, is frequently employed in the development of *in vitro* oxidative stress models. The resulting preliminary concentration of 1 mM and 2mM H_2O_2 for 3 h treatment showed that the cell viability was 59% and 49%, respectively (data not showed) indicating a significant rise in the incidence of cell death. A one of the most important ROS in the redox control of biological activity is H_2O_2, However, at excessive intracellular concentrations, ROS and free radicals severely damage cells and tissues, resulting in a decrease in mitochondrial membrane potential, protein damage, and DNA fragmentation (Sies & Jones, 2020; Tian et al., 2021). The concentration at 1mM was selected. In this study, cells were pre-treated with extracts for 24 h that activated to H_2O_2 at 1 mM for 3 h to induce cell toxicity. The result showed that both low- and high-concentration of pre-treatments extracts increased the rate of cell survival, may indicating antioxidant activity and protecting cells against oxidative damage induced by H_2O_2. Furthermore, about the various extracts, it is observed that protection of RWE is greater when compared to GEE and CRGE, even pretreatment at the low or high concentration. Moreover, this not only was not significantly different from control but also effective as using NAC (N-acetyl cysteine) as a positive control to protect cell toxicity. Of note, used of combined extract at ratio of 1: 1 not showed the increasing of cell viability as expected so that should verities ratios of combined extracts for next study.

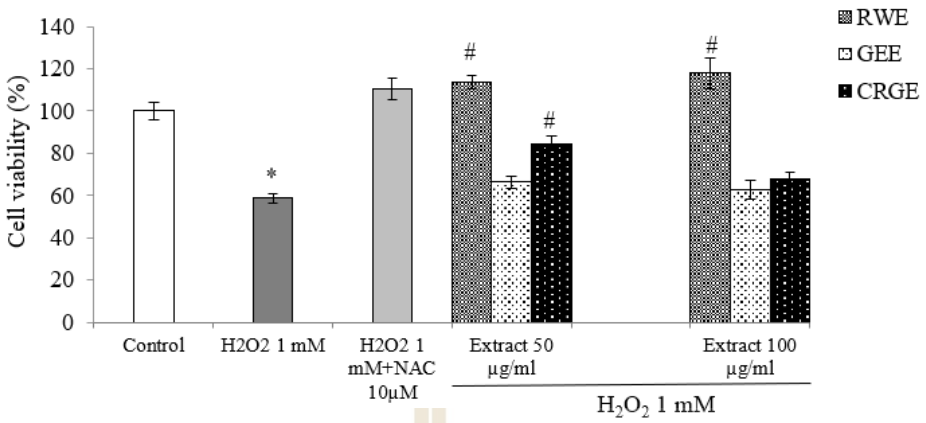

Figure 3.3 Cytoprotective of extracts from H_2O_2-induced cytotoxicity in HepG2 cell model. Cells were treated with the extracts at low and high concentrations (50 and 100 µg/mL) for 24 h then activated to H_2O_2 at 1 mM for 3 h. Control; inactivated cells, H_2O_2; activated to H_2O_2 at 1 mM for 3 h without pretreatment with extract or NAC (positive control), NAC; activated to H_2O_2 at 1 mM and co-treatment with NAC for 3 h. Extract 50 or 100 µg/mL; pretreatment with extracts at low and high concentrations for 24 h than activated to H_2O_2 at 1 mM for 3 h. *p<0.05 compared to control, #p<0.05compared to H_2O_2-activated cell toxicity.

3.4.5.3 Intracellular ROS scavenging by DCHF-DA assay

The intracellular ROS scavenging of all extracts was evaluated in H_2O_2-activated HepG2 cells. As presented in Figure 3.4.

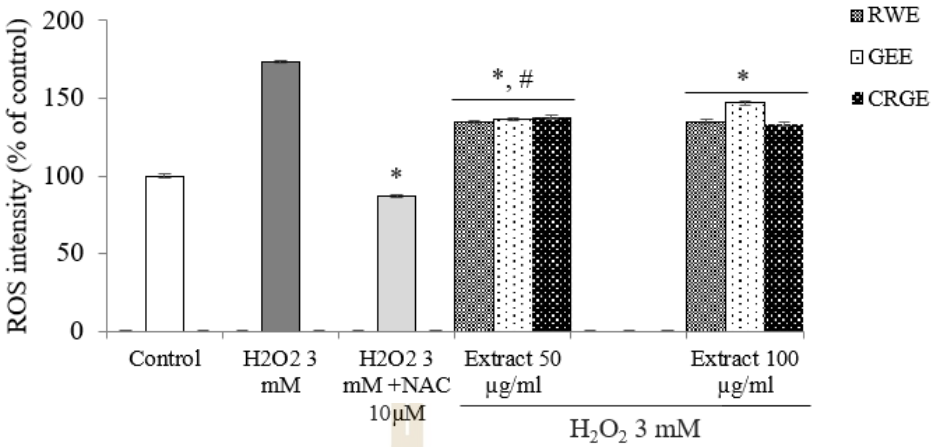

Figure 3.4 Effect of RWE, GEE, and CRGE attenuated H_2O_2-induced intracellular ROS production in HepG2 cells was determined by DCFH-DA assay. Cells were treated with the extracts at low and high concentrations (50 and 100 µg/mL) for 24 h and then activated with 3 mM H_2O_2 for 30 min. ROS intensity is expressed as a percentage of the control. Control: inactivated cells. H_2O_2: activated with H_2O_2 without the extracts; NAC: cells were activated with H_2O_2 and NAC 10 µM; Extract 50 µg/mL: cells were treated with the extracts at low concentration and then activated with H_2O_2; Extract 100 µg/mL: cells were treated with a high concentration of the extracts and then treated with H_2O_2. Values are expressed as a percent of control. ($p<0.05$). *$p<0.05$ compared to H_2O_2 induced ROS production. #$p<0.05$ between treatments.

The activation of HepG2 cells with H_2O_2 increased ROS accumulation by 1.8-fold compared to control. Cells were exposed with H_2O_2 and NAC as positive control show ROS intensity close to control. Highlight, treatment with extracts for 24 h then exposed with H_2O_2 were significantly different from activated H_2O_2 by reducing ROS intensity from 173% (without extracts) to 130-140 % of control. Pretreat extracts at the concentration of 50 µg/mL, ROS intensity was not significant both single and CRGE. These findings lead us to believe that phenolic compounds present in Rang Chuet may play an important role in protection against oxidative stress. Based on the HPLC experiment. Therefore, using 50 and 100 µg/mL of extracts can reduce ROS intensity even not much

as used of NAC. In summary, extracts (RWE, GEE, and CRGE) have cytoprotective effects from the toxic of H_2O_2 and attenuated intracellular ROS.

3.4.6 Antioxidant genes expression

To further assess whether cellular antioxidant activities of extracts to attenuated H_2O_2 activated ROS production and cell cytotoxicity, in may cause by up-regulated antioxidant enzyme genes. The expression of these antioxidant enzymes was quantitated by qRT- PCR, the enzymes namely *HO-1, SOD, CAT* and *NQO1* (Figure 3.5A-D).

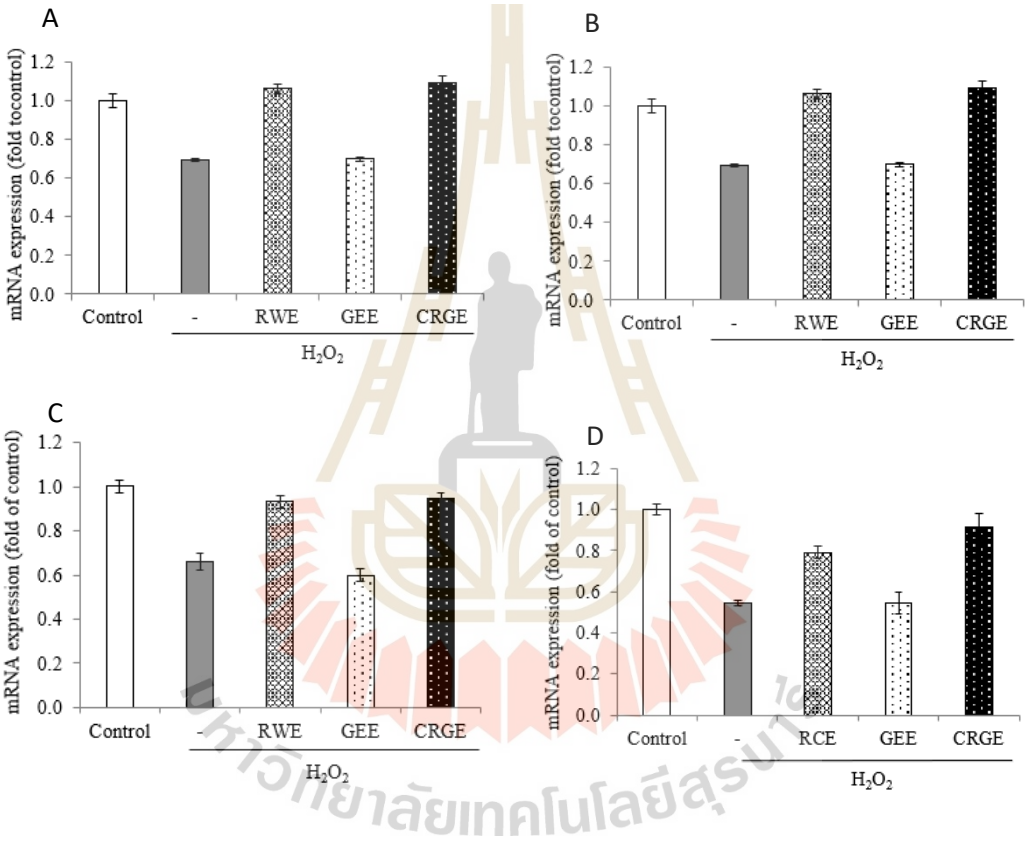

Figure 3.5 Effect of extracts on mRNA expressions of antioxidant genes. (A) *HO-1*; (B) *SOD*; (C) *CAT*; (D) *NQO1* in activated H_2O_2 in HepG2 cells. The mRNA expression was determined by real-time qRT-PCR. Data are presented as mean±SD (n=3) . Columns with different lowercase letters above bars showed significantly different from the activated H_2O_2 without treatment with extracts.

The result showed that activated H_2O_2 in HepG2 influences antioxidant enzymes expression by reducing *HO-1, SOD, CAT* and *NQO1* Importantly, reducing gene expression of *HO-1* and *NQO1*, It is common knowledge that *HO-1* and *NQO1* are two main antioxidant enzymes that play a crucial role in H_2O_2-activated antioxidant defense in hepatic cells (Ma et al., 2016). When HepG2 cells were treated with extracts significantly up-regulated *HO-1, SOD, CAT* and *NQO1* transcription even though cells were induced oxidative stress by H_2O_2 ($p< 0.05$). These results further suggested that CRGE-induced activation of antioxidant gene expression. Supporting as Rang Chuet leaves extracts have the ability to increase antioxidant enzymes such as CAT and GPx (Junsi, Takahashi Yupanqui, Usawakesmanee, Slusarenko, & Siripongvutikorn, 2020). These enzymes serve as key components of the defense mechanism against oxidative stress. This finding can be interpreted by the induction of *HO-1*, a protein responsive to various stressors (Loboda et al., 2016), in this context, the Nrf2 transcription factor acts as the primary regulator in cellular antioxidant defense responses, activating phase II antioxidant enzymes via the ARE pathway, including *HO-1* (Lever et al., 2016). The increase in Keap1 but decrease in Nrf2 suggests that, to safeguard cells from oxidative stress, Nrf2 dissociates from Keap1, translocates to the nucleus, binds to ARE, and subsequently up-regulates the expression of antioxidant enzyme genes like *HO-1* and *NQO1*, downstream targets of Nrf2 (Zhao et al., 2021). Furthermore, the combined extract pretreatment exhibits a more potent inhibition of the decline in antioxidant enzyme activity compared to individual RWE and GEE pretreatment.

3.4.7 The interaction of combined extract on cellular study

The results from the use of pre-treated combined extracts in a 1:1 ratio at a concentration of 50 µg/mL on cellular antioxidant activities (3.4.5) and antioxidative gene expressions (3.4.6) were used to calculate the combination index (CI). The combination index is shown in Tables 3.8.

Table 3.9 The interaction of CRGE on cellular study.

Cellular antioxidant activities	**CI**
Cytotoxicity	1.09±0.06
Cytoprotective effect	0.94±0.03
Intracellular ROS scavenging	1.01±0.01
Antioxidant gene expression	**CI**
OH-1	1.22±0.06
SOD	1.24±0.08
CAT	1.24±0.04
NQO1	1.37±0.10

The values are the mean SD of three replicates (n=3). CI, combination index.

The results indicate that pre-treated combined extracts in a 1:1 ratio, at a concentration of 50 µg/mL for cytoprotection and scavenging ROS from H_2O_2, did not demonstrate a synergistic effect on cellular antioxidant activities. Instead, the combined extract displayed an additive effect on cytotoxicity and intracellular ROS scavenging. Interestingly, all antioxidative gene expressions of CRGE exhibited a synergistic interaction. The CI of *SOD* was 1.24±0.08 (CI > 1.10) in CRGE at 50 µg/mL, indicating synergistic effects. These findings further corroborate the presence of a synergistic effect between RWE and GEE. Consequently, the RWE and GEE ratio of 1:1 v/v serves as an antioxidant defense against oxidative stress, with this combination exerting significant synergistic effects.

3.5 Conclusion

It can be concluded that both RWE and GEE exhibited antioxidant potential. Phytochemical profiling by HPLC revealed that RWE from the microwave-vacuum drying process comprised gallic, protocatechuic, caffeic, coumaric, and rosmarinic acids, along with the flavonoid compound apigenin. CRGE exhibited antioxidant properties through various mechanisms in four different chemical antioxidant assays. Therefore, the combination of RWE and GEE could potentially contribute to determining antioxidant capacities. CRGE can act by directly scavenging free radicals and/or modulating levels

of intracellular ROS generation and changing antioxidant genes expression through synergistic. However, the ratio of combined extracts should be carefully considered, as it may affect the level of synergism.

The synergistic effects on antioxidant activities of the combined extracts could be utilized to develop novel natural antioxidants in nutraceutical formulations, potentially reducing the required dosage of extracts while still providing valuable protection against oxidative diseases. These agents could be incorporated into various food products.

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CHAPTER IV

EFFECT OF *THUNBERGIA LAURIFORIA* LINN. (RANG CHUTE) LEAVES, GINGER AND THEIR COMBINED EXTRACTS ON PM2.5-INDUCED CELLULAR OXIDATIVE STRESS

4.1 Abstract

Epidemiological data strongly indicate that exposure to particulate matter with a diameter of less than 2.5 μm (PM2.5) causes oxidative stress and is linked to various diseases in humans. Plant extracts and various natural compounds demonstrate antioxidative properties. *Thunbergia laurifolia* Linn. (Rang Chuet) and *Zingiber officinale* (ginger) exhibit synergistic chemical and cellular antioxidative activities. However, the cellular antioxidative effects of Rang Chuet and ginger combined extracts (CRGE) against PM2.5-induced oxidative stress have not yet been established. This study assesses the toxicity of PM2.5 (Standard Reference Meterial®2786, NIST) and the cellular study of both single and combined extracts in mitigating PM2.5-induced oxidative stress. PM2.5 demonstrates cell cytotoxicity and exhibits elevated levels of oxidative potential, resulting in increased intracellular production of reactive oxygen species (ROS). This leads to higher rates of cell death and downregulation of antioxidative gene expression in Human lung epithelial (A549) cells and effect antioxidative gene regulation in Human hepatoma (HepG2) cells. The results indicate that pre-treatment with CRGE significantly reduces PM2.5-induced cell toxicity and intracellular ROS production. Furthermore, it upregulates the expression of antioxidative genes such as *OH-1, SOD*, and *CAT*, even in cells subjected to oxidative stress induced by PM2.5 in human epithelial lung cells. In conclusion, this study implicates that CRGE holds the potential to alleviate oxidative stress caused by PM2.5 and could be employed for therapeutic or protective approaches against health hazards associated with air pollution.

Keywords: Antioxidative gene expression, Cellular antioxidant activity, Oxidative stress, PM2.5 exposure, Synergistic, *Thunbergia laurifolia* Lindl.

4.2 Introduction

According to World Health Organization, 7 million deaths each year from exposure to air pollution. Surprisingly, over 91% of the world's population lives in places where air pollution levels exceed WHO guidelines. Especially, the particulate matter with a diameter of less than 2.5μm (PM2.5) can penetrate deeply into the alveolar tissue of the respiratory tract and inhaled PM2.5 can diffuse into the blood via microvasculature and then be transported throughout the body (M. Gualtieri et al., 2009; Hu et al., 2017). PM2.5 exposure leads to many diseases in the human-like respiratory tract and about 50% of them are retained in the lung parenchyma, causing respiratory infection and systemic inflammation and long-term exposure to PM2.5 affect the central nervous system, cardiovascular disease, impacts on the liver spleen and blood, skin disorder, and also reproductive system (Hu, et al., 2017: Zhang, T. et al., 2018: Veerappan, et al., 2019: Xu, et al., 2019: Fu et al., 2020).

PM2.5 comprises a complex mixture of heavy metals, transition metals, PAHs, and other hazardous compounds, with its effects influenced by diverse factors (Hu et al., 2017). Exposure to PM2.5 can activate multiple harmful pathways, including oxidative damage, inflammation, cell autophagy, and apoptosis, as evidenced by previous investigations. The principal mechanism by which PM2.5 induces cellular toxicity seems to be through heightened oxidative stress, characterized by elevated levels of reactive oxygen species (ROS) and diminished superoxide dismutase (SOD) activity. Multiple studies have reported on the inflammatory and oxidative stress-induced cytotoxicity mechanisms of PM2.5.

Recent investigations into naturally derived polyphenols reveal their potential as protective and preventative agents by influencing a multitude of physiological processes through various signaling pathways. Utilizing dietary polyphenols to modulate PM2.5-induced harm could offer a new therapeutic or preventive strategy against air pollution-related health risks (S. G. Li et al., 2015). For instance, pre-treating A549 cells with the antioxidant morin has demonstrated effective protection against PM2.5-induced toxicity and restoration of gene expression levels (Veerappan et al., 2019).

The Rang Chuet (*Thunbergia laurifolia* L.), belonging to the Acanthaceae family and widely spread throughout Southeast Asian regions, is utilized in Thai traditional medicine for its antidotal properties against poisons. This plant has been scientifically

proven to possess antioxidant, anti-inflammatory, antimicrobial, antidiabetic, antipyretic, and anti-hyaluronidase activities. Interestingly, detoxifying effect of Rang Chuet, from several previous studies demonstrated that, can detoxify chemical toxins, drug poisoning, and heavy metals. Oonsivilai, Cheng, Bomser, Ferruzzi, and Ningsanond (2008) reported that Rang Chuet has a potential for detoxifying effect by phase II enzyme induce. However, the effect of Rung Chuet against PM2.5-induced lung and liver cell models remains to be studied and elucidated.

The combination of bioactive constituents present in plant extracts and various natural products produces synergistic effects, which serve to enhance the activity of plant extracts (Karimi, et al., 2017). Therefore, the synergistic antioxidant activity of the optimized mixture can be more effective and help to lower the dose of plant extracts. Rang Chuet and ginger extracts exhibited synergy in chemical antioxidant activities (Sunthrarak & Oonsivilai, 2022). The synergistic effect on cellular antioxidant activity of the combined extracts was studied in Chapter 3, demonstrating synergism in certain ratios. To maximize their benefits, the impact of combined Rang Chuet and ginger extracts in mitigating PM2.5 exposure should also be explored.

This study aims to investigate the efficacy of Rang Chuet and its combined extracts in mitigating the adverse impacts of PM2.5 exposure, achieved by measuring cellular antioxidant activities, and changes in antioxidant, inflammatory, and detoxifying gene expression in PM2.5-exposed A549 and HepG2 cell models.

4.3 Materials and methods

4.3.1 PM2.5 preparation

Standard Reference Material (SRM 2786) served as a representation of fine PM2.5, sourced from the National Institute of Standards and Technology (NIST). This fine particulate matter was collected in Prague, Czech Republic, in 2005 and possesses an average particle diameter of about 2.8 μm. The certificate of analysis for the SRM 2786 used in this study is provided in appendix 1. To prepare a fresh stock suspension of SRM 2786, diluted it in Dulbecco's modified eagle medium (DMEM) to achieve a final concentration of 2,000 μg/mL. This suspension was then sonicated for 5 minutes with BANDELIN electronic GmbH & Co., Germany equipment and vigorously vortexed for 2 min prior to each experiment, following the protocol outlined by Xiong et al. (2015).

4.3.2 Preparation of extracts

Preparation of RWE, GEE follow Chapter 3. the combined extracts of Rang Chuet and ginger with the following ratios, 10:1 1:1, 1:10 v/v. as the result of combined extracts in narrow ranges (3:1, 2: 1, 1:1, 1:2, 1:3) in chemical antioxidant activities did not show a strongly synergistic antioxidant activity. During, cellular antioxidant activity, H_2O_2 induced cellular toxicity and ROS production the combined extracts not shown the higher on these activities than both of single extracts. Therefore, we expand range of combined extracts for next studies.

4.3.3 Oxidative stress reduction in A549 and HepG2 cells

4.3.3.1 Cell viability

Human lung epithelial (A549) cells and human hepatoma (HepG2) cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone, GA, USA) under a 5% CO_2 atmosphere at 37°C. Cells were seeded into 96-well plates at a density of $1x10^4$ cells/well in 10% fetal calf serum-DMEM and allowed to attach overnight until reaching 80–90% confluence. This process took approximately 24 h. Concurrently, PM2.5 was prepared in serum-free DMEM at concentrations ranging from 3.125 to 400 µg/mL. The PM2.5 solution was briefly shaken for 15 sec before being added to the cells, which were then exposed for 24 h. Cell viability was assessed using the MTT assay. Following treatment, the media were aspirated, and cells were washed with 1xPBS before adding 100µl of 0.5 mg/mL MTT solution in 1xPBS to each well. The plates were then incubated at 37°C, 5% CO_2 for 3 h. After removing the MTT solution, the formazan crystals were dissolved in 100 µl of DMSO, and the absorbance of the colored solution was measured at 570 nm using a microplate reader (BMG Labtech GmbH, Ortenbreg, Germany). Cell viability was calculated as a percentage relative to control cells.

4.3.3.2 Cytoprotective effect

To study the cytoprotective effect of all extracts against PM2.5-induced cell toxicity, the concentrations of PM2.5 which was cytotoxic to cells at the cell viability approximately 70%, were select for this study. Firstly, A549/ HepG2 cells were seeded flowing the protocol decried above. Next, cells were pretreated with RWE, GEE, and CRGE at the concentrations did not cytotoxic to both cells for 24 h. Then, exposed to selected PM2.5 for 24 h. after that were washed with 1X PBS. MTT assay was used to examine the percentage of cell viability.

4.3.3.3 Intracellular ROS scavenging by DCFH-DA assay

Intracellular ROS levels were assessed using the DCFH-DA assay, following the protocol of Jaiboonma et al. (2020) with modifications. Following the seeding of A549/HepG2 cells, different treatments, including RWE, GEE, and CRGE at concentrations of 50 µg/mL, were administered for 24 h, after which the media were removed. Subsequently, cells were exposed to 50 µg/mL of PM2.5 for 3 h to induce oxidative stress, based on preliminary data indicating higher ROS production with 24-h exposure (see appendix 2). After treatment, cells were stained with 20 µM DCFH-DA at 37°C for 30 min in the dark. DCFH-DA, dissolved in ethanol, was added to the cell culture, where it is converted by ROS into the fluorescent compound 2',7'-dichlorofluorescein (DCF). The total green fluorescence intensity of each well was then measured using a fluorescence multi-well plate reader (Thermo Scientific Varioskan, USA) with excitation and emission wavelengths of 485 nm and 535 nm, respectively. The results were expressed as the relative percentage of intracellular ROS.

4.3.4 Changes in gene expression

The study utilized A549 and HepG2 cells, seeded at a density of $6x10^5$ cells/well in a 6-well plate. After treatment with single and combined extracts, the cells were subjected to oxidative stress through PM2.5 conditions as described in Table 4.1. Subsequent to treatment, all cells were washed with 1×PBS and were harvested using 0.05% trypsin-EDTA.

Table 4.1 The testing condition on gene expression.

Treatments	Pre-treatments	Activation
Control	Media (24 h)	Media (24 h)
PM2.5 (Negative control)	Media (24 h)	PM2.5 (24 h)
RWE	RWE 50 µg/mL (24 h)	Media (24 h)
GEE	GEE 50 µg/mL (24 h)	Media (24 h)
RWE+PM2.5	RWE 50 µg/mL (24 h)	PM2.5 50 µg/mL (24 h)
GEE+PM2.5	GEE 50 µg/mL (24 h)	PM2.5 50 µg/mL (24 h)
RWE: GEE (1:1) + PM2.5	RWE: GEE (1:1) 50 µg/mL (24 h)	PM2.5 50 µg/mL (24 h)
RWE: GEE (1:10) + PM2.5	RWE: GEE (1:10) 50 µg/mL (24 h)	PM2.5 50 µg/mL (24 h)

For RNA Isolation and cDNA Synthesis, total RNA extraction was conducted from A549 and HepG2 cell models. Briefly, cell lysis was performed using Nucleospin®RNA Plus (MACHEREYNAGEL GmbH & Co. KG., Düren, Germany) following the manufacturer's protocol. The concentration of total RNA was determined using the LVis plate and the SPECTROstar NANO system (BMG Labtech GmbH, Ortenberg, Baden-Württemberg, Germany). For cDNA synthesis, ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd., Osaka, Japan) was utilized as per the manufacturer's instructions. The resulting cDNA samples were stored at -20°C for subsequent analysis.

Quantitative RT-PCR analysis, or qRT-PCR, was carried out using the SYBR Green protocol (2xqPCRBIO SyGreen Mix Lo-ROX, PCR BIOSYSTEMS, London, UK) following the manufacturer's guidelines. The amplification of cDNA involved mixing 2 µl of freshly prepared cDNA reagent, comprising 1 µl of RNase-free DI, 2 µl of 2 nM primer, and 5 µl of SYBR Green. PCR cycling conditions included an initial denaturation step at 95°C for 3 min, followed by 40 cycles of 30 sec at 60°C and 1 min at 72°C. Subsequently, a melting curve analysis was performed at 60°C for 1 min, followed by 15 sec at 95°C. Data analysis was conducted using QuantStudioMT Design and Analysis software v.1.52, applying the $2^{\Delta\Delta Ct}$ expression model. The relative fold expression of target genes was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The primer sequences for the target genes are detailed in Table 4.2.

4.3.5 Statistical analyses

The statistical analysis was carried out using IBM SPSS Statistics for Windows, version 23.0 (IBM Corp., NY). Results were presented as mean ± standard error. Multiple group comparisons were evaluated using one-way analysis of variance (ANOVA) followed by Duncan's multiple range test. A significance level of $p<0.05$ was considered statistically significant.

Table 4.2 The target gene primer sequences.

Target Gene	Forward primer sequence (5′ to 3′)	Reverse primer sequence (5′ to 3′)
HO-1	ATGGCCTCCCTGTACCACATC	TGTTGCGCTCAATCTCCTCCT
SOD	GCAGGTCCTCACTTTAATCCTCT	ATCGGCCACACCATCTTTGT
CAT	TGAAGATGCGGCGAGACTTT	TGGATGTAAAAAGTCCAGGAGGG
GPx	TGAAGATGCGGCGAGACTTT	TGGATGTAAAAAGTCCAGGAGGG
NQO1	GGATTGGACCGAGCTGGAA	AATTGCAGTGAAGATGAAGGCAAC
IL-6	F-CACACAGACAGCCACTCACC	R-AGTGCCTCTTTGCTGCTTTC
IL-8	F-GACTTTCGCTCTCCATCCAC	R-TGAATTCTCAGCCCTCTTCAA
Ahr	F-ATCACCTACGCCAGTCGCAAG	R-AGGCTAGCCAAACGGTCCAAC
Nrf2	CTTGGCCTCAGTGATTCTGAAGTG	CCTGAGATGGTGACAAGGGTTCTA
GAPDH	TCCAAAATCAAGTGGGGCGA	TGATGACCCTTTTGGCTCCC

HO-1 = Heme oxygenase; *SOD* = superoxide dismutase; *CAT* = catalase; *GPx* = Glutathione Peroxidase; *NQO1*= NAD(P)H quinone dehydrogenase 1; *IL-6*= Interleukin 6; *IL-8* = Interleukin 8, *Ahr* = Air and elytroid 2-related factor 2 ; *Nrf2*, NF-E2-related factor 2, *GAPDH* = glyceraldehyde-3-phosphate dehydrogenase

4.4 Results and discussion

4.4.1 Oxidative stress reduction in A549 and HepG2 cells

Cellular antioxidative activities of extracts were studied in A549 cell as human epithelial lung cell as that the main part that contact to PM2.5 toxicity and HepG2 cells from the liver organ as it is recognized it has the function of removing waste products from the body. In this part, we study cytotoxicity, cytoprotective effect, and intracellular ROS scavenging of single and combined extracts protect cell PM2.5 toxicity.

4.4.1.1 Cytotoxicity of PM2.5 exposed A549 and HepG2 cells

Cell viability both A549 and HepG2 cells exposed to PM2.5 for 24 h. PM2.5 can induce cell toxicity by decreasing cell viability in a concentration-dependent manner (Figure 4.1).

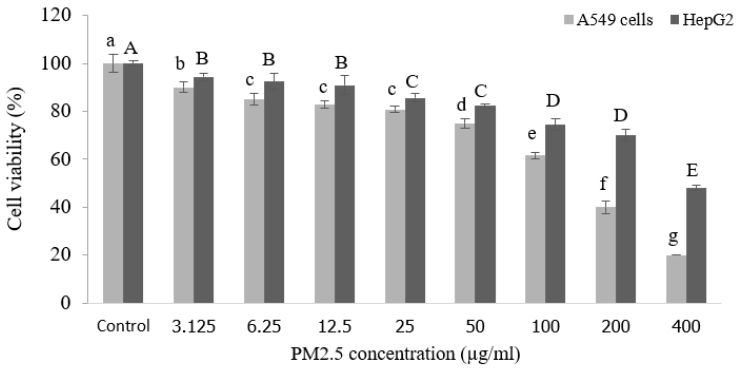


Figure 4.1 Cell viability in A549 Vs HepG2 cells. Cells were treated with PM2.5 at 3.125 -400 µg/mL for 24 h.

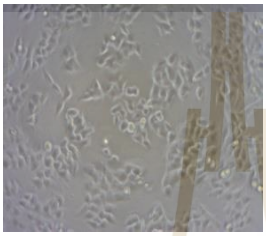
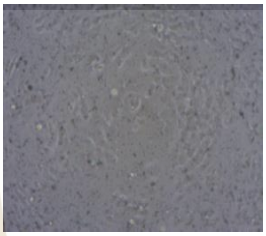
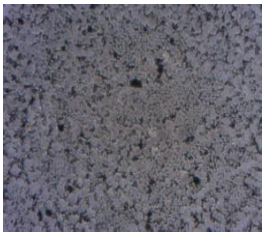
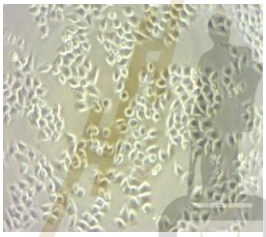
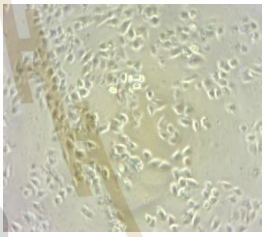
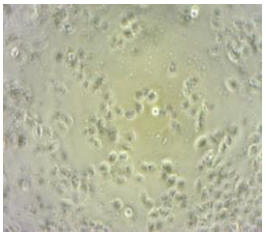
Cell viability of lung cell exposure with PM2.5 for 24 h close to the previous study at 50 µg/mL reduces cell viability to 70% (Deng et al., 2013). However, cytotoxicity of PM2.5 exposure in depend on compositions of PM2.5 by collected sources (Kouassi et al., 2010). Supporting from Deng et al. (2013) studied exposed PM2.5 at the same concentration 50 µg/mL contact time at 4 and 12 h. cell viability not significant from control without PM2.5. During, Shim et al. (2021) demonstrated that PM2.5 at 50 µg/mL for 24 h cell viability close to our study, moreover, at high concentrations 200-400 µg/mL cell viability reduce to 20%. Moreover, exposure of human bronchial epithelial (BEAS-2B) cells to PM2.5 at a concentration of 150 µg/mL for 24 h resulted in a decrease in cell viability to 76% compared to the control without PM2.5. Likewise, PM2.5 exposure also led to reduced cell viability in HepG2 cells. Previous investigations have examined the toxicity of PM2.5 on HepG2 cells, proposing a potential association between PM2.5 exposure and nonalcoholic fatty liver disease (M.-X. Xu et al., 2019).

A549 cells proved to be more sensitive to PM2.5 exposure compared to HepG2 cells. Given that HepG2 cells originate from the liver and are involved in waste removal from the body, they may exhibit resistance to toxic exposures. Therefore, specific concentrations of PM2.5 were chosen for the subsequent studies. In the A549 cell model, a concentration of 50 µg/mL of PM2.5 was used for both the cytoprotective effect and intracellular ROS studies, with a duration of 24 h for exposure. Conversely,

HepG2 cells were exposed to 100 µg/mL for the cytoprotective effect study and 50 µg/mL for the intracellular ROS study, as ROS production was not detected in dead cells.

4.4.1.2 Cell change of A549 and HepG2 cell exposed PM2.5

Refer to Figure 4.2 for the cell changes observed after exposure to PM2.5. Using a light microscope at X10 magnification.

HepG2 (X10)	W/O PM2.5	50 µg/mL	400 µg/mL
Not wash PM2.5			
Wash PM2.5			

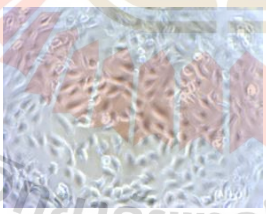
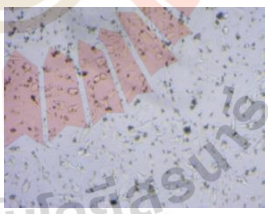
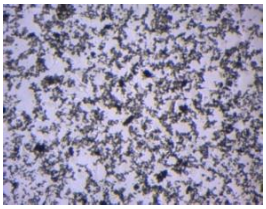
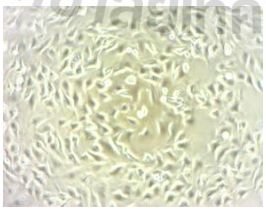
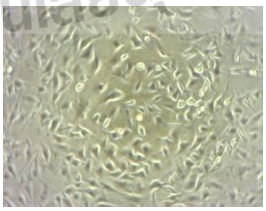
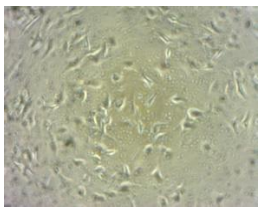
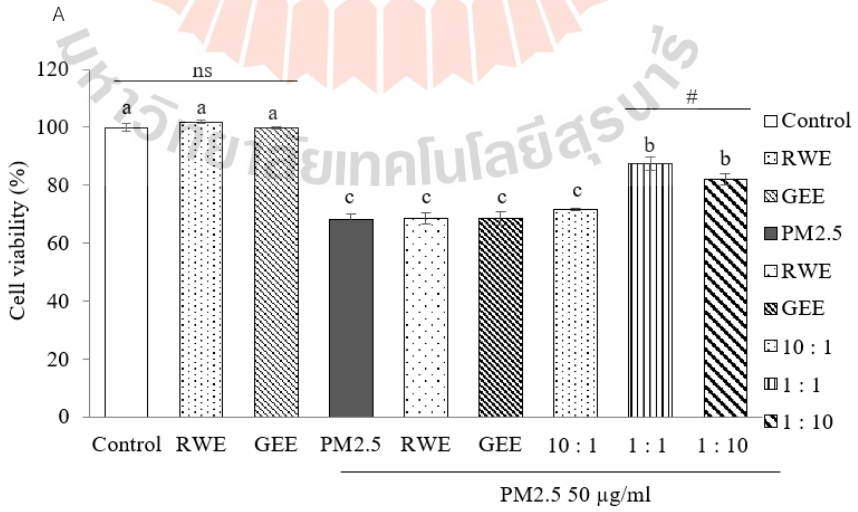
A549 (X10)	W/O PM2.5	50 µg/mL	400 µg/mL
Not wash PM2.5			
Wash PM2.5			

Figure 4.2 Cell change of A549 (A) and HepG2 (B) cells without PM2.5 exposures and after being exposed to PM2.5 at 50 and 400 µg/mL for 24 h. Using a light microscope (X10).

It was noted that PM2.5 particles spread extensively in the media before washing, particularly with higher concentrations added. Consequently, washing cell to remove the PM2.5 that suspended in the media., leading to decreased cell confluence on the plate. The highest PM2.5 concentration of 400 µg/mL, overall cell shrinkage was observed. However, at concentrations of 50 and 400 µg/mL, cells still adhered to the plate, indicating their viability. These cells exhibited greater susceptibility to higher PM2.5 doses, as evidenced by the increased internalization of particles observed in morphological examinations (M. Gualtieri et al., 2009). M. Gualtieri et al. (2009) showed at 50X a numerous cells exhibited cytoplasmic inclusions and phagocytic organelles that were exposed with PM2.5 as PM2.5 contain insoluble components (Zou, Jin, Su, Li, & Zhu, 2016). Furthermore, PM2.5 treatment at a concentration of 150 µg/mL caused evident changes in the cellular morphology of BEAS-2B cells, including the enlargement of mitochondria and endoplasmic reticulum, as well as the accumulation of autophagosomes and autolysosomes. In summary, A549 cells exposed to PM2.5 exhibited signs of penetration, damage, and destruction.

4.4.1.3 Cytoprotective effect of extracts

Cytoprotective effect of extracts attenuated cytotoxic from PM2.5 in A549 and HepG2 cells were shown in Figure 4.2.



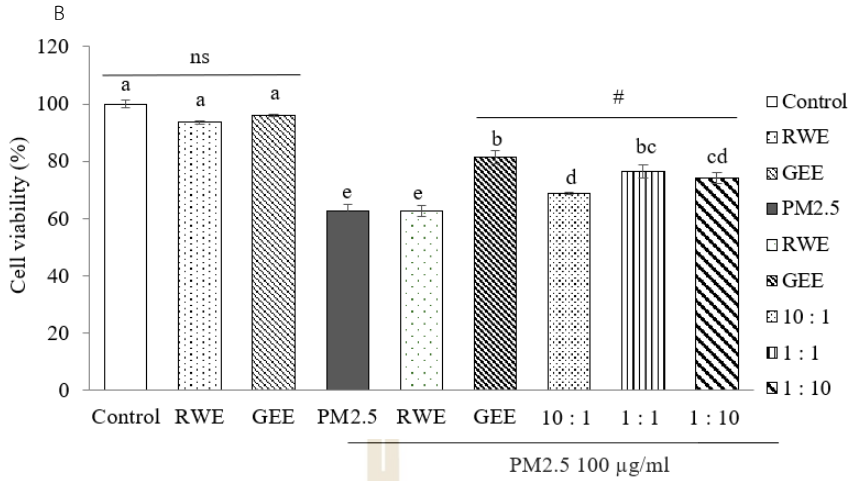


Figure 4.3 Cytoprotective effects of extracts from PM2.5-induced cell toxicity in A549 cells (A) and HepG2 cells (B). Cells were pre-treated with extracts at 50 µg/mL then exposed to PM2.5 at 50 µg/mL in A549 cells and PM2.5 at 100 µg/mL in HepG2 cells. Lowercase letter indicates significant difference compared to the control group ($p < 0.05$); ns denotes not significant from control, and # indicates significant difference compared to PM2.5 induced cell toxicity ($p < 0.05$).

In the A549 cells study, treatment to RWE, and GEE cell viability (%) was not significantly different from control. By exposure to PM2.5 at 50 µg/mL for 24 h, cell viability was decreased to 68%. The major contributor to PM2.5-mediated toxicity has been an imbalance between the antioxidant system and oxidation, which is brought on by particles and/or organic compounds and transition metals that have been coated (Zhai et al., 2022). CRGE at ratios of 1:1 and 1:10 then exposed to PM2.5 were significantly different from PM2.5 exposed without extracts by increasing cell viability (%). Highlight, increasing cell viabilities were higher than used of single extracts. These combined extracted showed effectiveness of cytoprotection from PM2.5 than using only single extracts at the same concentration.

PM2.5 at 100 µg/mL was used to induce oxidative damage in HepG2 cells. The concentrations of RWE and GEE at 50 µg/mL when compared with the control without extracts do not differ significantly ($p < 0.05$). The results show that at GEE and CRGE at 3 ratios, viability of cell protection is greater when compared to RWE and significantly different from PM2.5 induced cell toxicity without extracts. The result

showed along similar line with used of RWE: GEE at the ratio of 1 : 1 can protect HepG2 cells from H_2O_2-induced cytotoxicity (Date in Chapter 3). Using CRGE at 50 µg/mL could have a cytoprotective effect from PM2.5 exposure for 24 h. When oxidative stress occurs, the excessive production of ROS and subsequent decline in antioxidants cause the complete failure of endogenous antioxidant defense systems. Consequently, cells become unable to defend themselves against oxidative stress-induced damage. (Ma et al., 2016).

4.4.1.4 Intracellular ROS scavenging of extracts on PM2.5 induced oxidative stress

As PM2.5-induced toxic response were due to an interruption of oxidative stress, the intracellular ROS scavenging potential of all extracts was evaluated in PM2.5 induces oxidative stress. As presented in Figure 4.3.

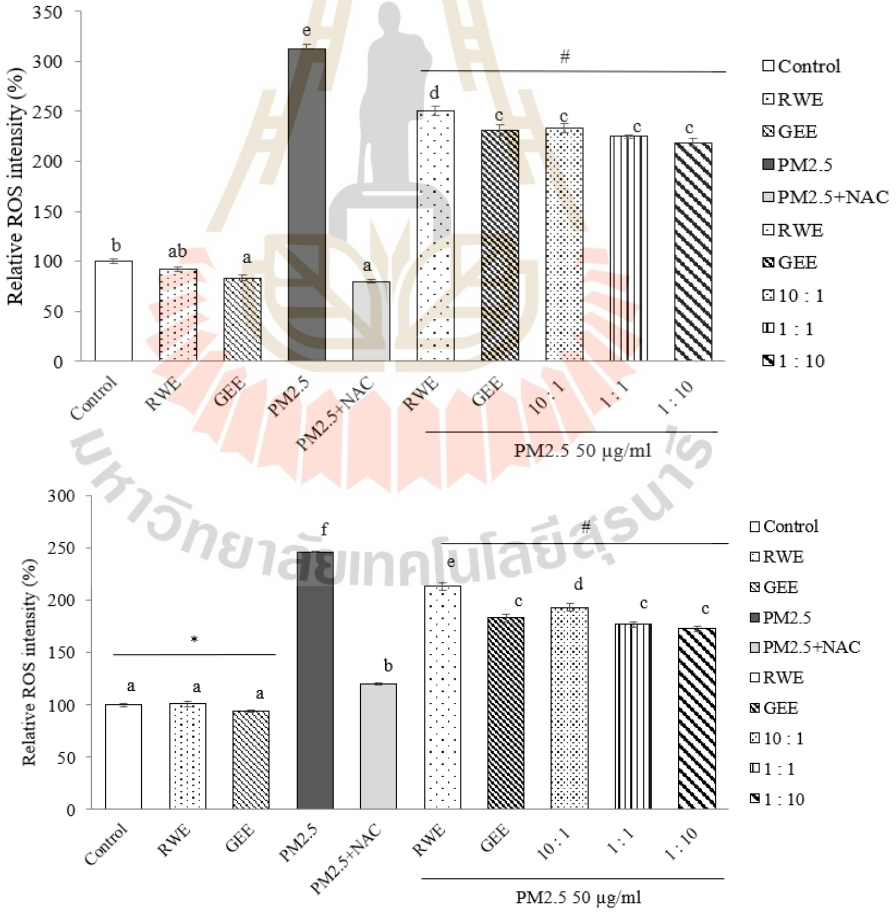


Figure 4.4 Effect of RWE, GEE, and CRGE on attenuating PM2.5-induced intracellular ROS production in A549 cells (A) and HepG2 cells (B) as determined by

DCFH-DA assay. Cells were treated with the extracts at 50 µg/mL for 24 h and then exposed to 50 µg/mL PM2.5 for 3 h. ROS intensity is expressed as a percentage of the control. *p < 0.05 compared to control, # p < 0.05 compared to PM2.5-induced ROS production.

The exposure of A549 cells with PM2.5 for 3 h increased ROS accumulation approximately 3-fold compared to inactivated A549 cells (control). During, M. Gualtieri et al. (2009) expose PM2.5 at 25 µg/mL increase ROS intensity to 190%. However, considering solubility of PM2.5 compositions such as organic solvent-extractable fraction or water-soluble fraction affect to generated ROS and cytotoxicity with difference (C.-W. Liu et al., 2018). Increasing ROS production by PM2.5 expose as it heavy metals can generate ROS ever expose at low level (Shim et al., 2021). Pre-treatment with extracts for 24 h, then exposed with PM2.5 was significantly different from exposed PM2.5 without extracts by reducing ROS intensity from 2 to 2.2-2.5 times of control. Notably, the GEE and CRGE (3 ratios) at a concentration of 50 µg/mL significantly decreased relative ROS production compared with PM2.5 exposure without extracts. However, RWE was not significantly different from the PM2.5 without extracts (p<0.05). To increase intracellular ROS scavenging of extracts might have to increase the concentrations of the extracts by more than 50 µg/mL. Remarkable, not more than 200 µg/mL refer to cell viability of extracts study.

The result showed that PM2.5 at a concentration of 50 µg/mL was exposed to HepG2 cells for 3 h (Figure 4.3B). The increased ROS accumulation was approximately 2.5-fold higher compared to inactivated HepG2 cells. As mentioned previously, PM2.5 exposure to HepG2 cells for 3 h resulted in a higher external ROS intensity compared to exposure over a longer period of 24 hours, as reported by Maurizio Gualtieri et al. (2012). Their study showed a significant increase in ROS levels after 3 hours of PM2.5 treatment, suggesting that both organic and inorganic water-soluble components of PM2.5 may induce this early response (M. Gualtieri et al., 2009). Treatment with extracts for 24 h followed by exposure to PM2.5 significantly reduced ROS intensity, ranging from 1.6 to 2.1 times that of the control. Importantly, the combination of GEE and CRGE at a concentration of 50 µg/mL significantly decreased ROS production compared to single extract (RWE) pretreatment groups. These findings

suggest that flavonoid compounds found in ginger may play a crucial role in protecting against oxidative stress. These data demonstrate that the synergistic effect of Rang Chuet and ginger extracts effectively shielded HepG2 cells from PM2.5-induced oxidative stress by enhancing the ability to scavenge or inhibit ROS production. To enhance intracellular ROS scavenging, extracts may need to be administered at concentrations exceeding 50 µg/mL, but not exceeding 200 µg/mL, as indicated in the study of extract cell viability.

4.4.2 Gene expression in A549 and HepG2 cells

4.4.2.1 Change in gene expression of A549 and HepG2 cells exposed PM2.5

Effect of PM2.5 exposure on mRNA expression was analyzed in A549 and HepG2 cells using RT-qPCR.

A549 cells, which represent epithelial lung cells in contact with PM2.5, gene expression of antioxidant enzymes (*HO-1, SOD, CAT, GPx*), as well as the phase II detoxification enzyme *NQO1* and inflammatory markers *IL-6* and *IL-8*, were studied. PM2.5 exposure at a concentration of 50 µg/mL for 24 h showed alterations in the expression of marker genes, as shown in Figures 4.4.

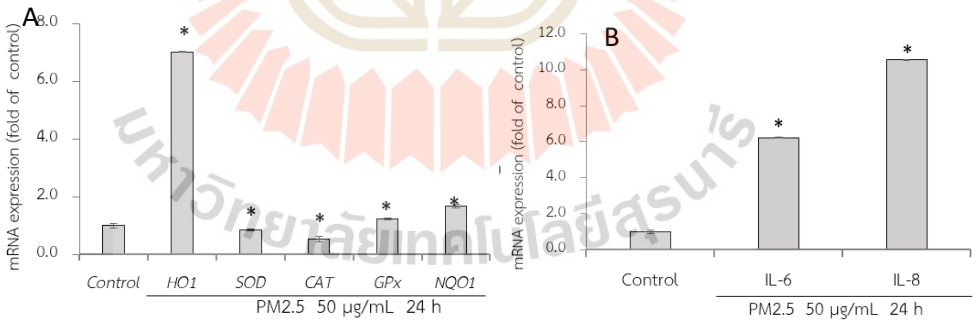


Figure 4.5 Relative mRNA expressions of antioxidant and phase II detoxification enzymes (A) and inflammatory cytokines (B) in PM2.5-exposed A549 cells for 24 h, determined by real-time qRT-PCR. Data are presented as mean ± SD (n=3). * Indicates significantly different from the control without PM2.5 exposure.

RT-qPCR analysis revealed the effects of PM2.5 exposure on A549 cells, demonstrating a significant reduction in the expression of SOD and CAT compared to the control group (without PM2.5) ($p<0.05$). Interestingly, after 24 h of PM2.5 exposure, HO-1 expression increased approximately 7-fold in the A549 cell model (Figure 4.4A). The gene expression of different antioxidant enzymes exhibited variability under PM2.5 exposure, likely due to involvement in multiple mechanisms. This variability is supported by the findings of Zhai et al. (2022), which suggested that PM2.5 exposure in BEAS-2B cells increases HO-1 expression through the activation of Nrf2 and its downstream targets, thereby significantly boosting antioxidant enzyme production. Nrf2 plays a pivotal role in cellular oxidative stress processes by interacting with antioxidant response elements (ARE) to enhance the expression of downstream genes responsible for encoding antioxidant proteins, including HO-1 and phase II detoxification enzymes (Jiang et al., 2017). This may elucidate the observed increase in NQO1, a phase II detoxification enzyme, following exposure to PM2.5.

The reduced expression of *SOD* and *CAT* observed in this study may be associated with decreased *SOD* enzyme activity during PM2.5 exposure (Hu et al., 2017). *SOD* is an antioxidant enzyme that scavenges free radicals, converting superoxide radicals into hydrogen peroxide, thereby providing cytoprotection against damage caused by toxic oxygen free radicals (Cui et al., 2018). Furthermore, our findings support the notion that PM2.5 reduces the antioxidant enzymatic activity of *SOD* and *CAT* in A549 cells following PM2.5 exposure (Deng et al., 2013). Considering these results, it is evident that PM2.5 induces the generation of reactive oxygen species (ROS), which directly interact with antioxidant enzymes such as superoxide dismutase (*SOD*), glutathione peroxidase (*GPx*), and catalase (*CAT*), resulting in reduced enzymatic activity. However, it is noteworthy that *GPx* was upregulated in this study upon exposure to PM2.5, as *GPx* primarily acts as a hydrogen peroxide scavenger by catalyzing glutathione oxidation and utilizing hydroperoxides as substrates. In conclusion, PM2.5 induces oxidative stress-related damage in A549 cells by disrupting antioxidant gene expression and compromising the antioxidant defense system.

In terms of inflammatory cytokine expression, we observed an up-regulation of inflammatory response markers, specifically *IL-6* and *IL-8* (Figure 4.6B), indicating an inflammatory response. These findings are consistent with results reported

by K. Liu, Hua, and Song (2022), where PM2.5 treatment induced the production of inflammatory cytokines in A549 cells, potentially through the activation of the TLR4/NF-**K**B/COX-2 signaling pathway. This pathway was also documented in a study involving RAW264.7 cells, macrophages exposed to PM2.5 (Fu et al., 2020). The pro-inflammatory effects can be attributed to toxic components within PM2.5, such as polycyclic aromatic hydrocarbons (PAHs) and metals (e.g., As, Pb, Fe, and Al), which have been identified as major contributors to the inflammatory response (Zou et al., 2016). Therefore, PM2.5 exposure resulted in adverse effects on antioxidant and phase II detoxification enzymes, influencing the expression of inflammatory marker genes in our study. However, it is worth considering the sources of the collected PM2.5, which may exhibit variations in toxic compositions, the duration of exposure to PM2.5, and the specific cell models used. These factors can contribute to diverse results in gene expression.

Considering HepG2 cells as liver cells, the primary site for xenobiotic metabolism and detoxification, gene expressions of antioxidant enzymes (*HO-1, SOD, CAT, GPx*) and their regulatory mechanisms (*Ahr* and *Nrf2*) were investigated. PM2.5 exposure at a concentration of 50 µg/mL for 24 h showed alterations in the expression of marker genes, as shown in Figures 4.5.

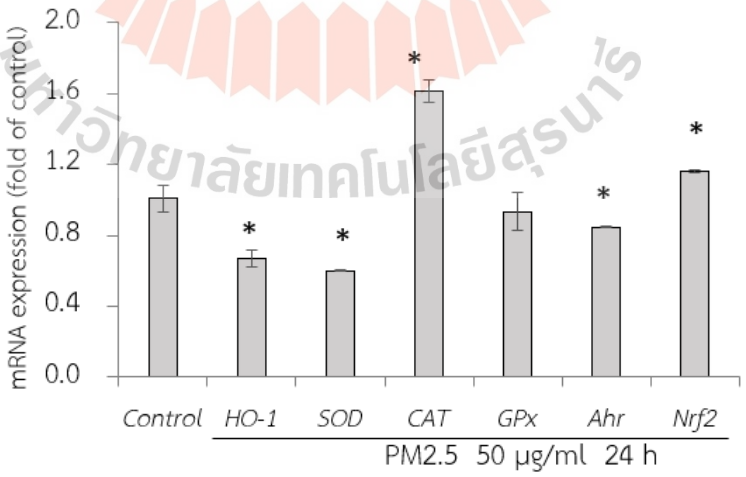


Figure 4.6 Relative mRNA expressions of antioxidant enzymes in PM2.5-exposed HepG2 cells for 24 h, determined by real-time qRT-PCR. Data are presented

as mean ± SD (n=3). * Indicates significant difference compared to the control without PM2.5 exposure.

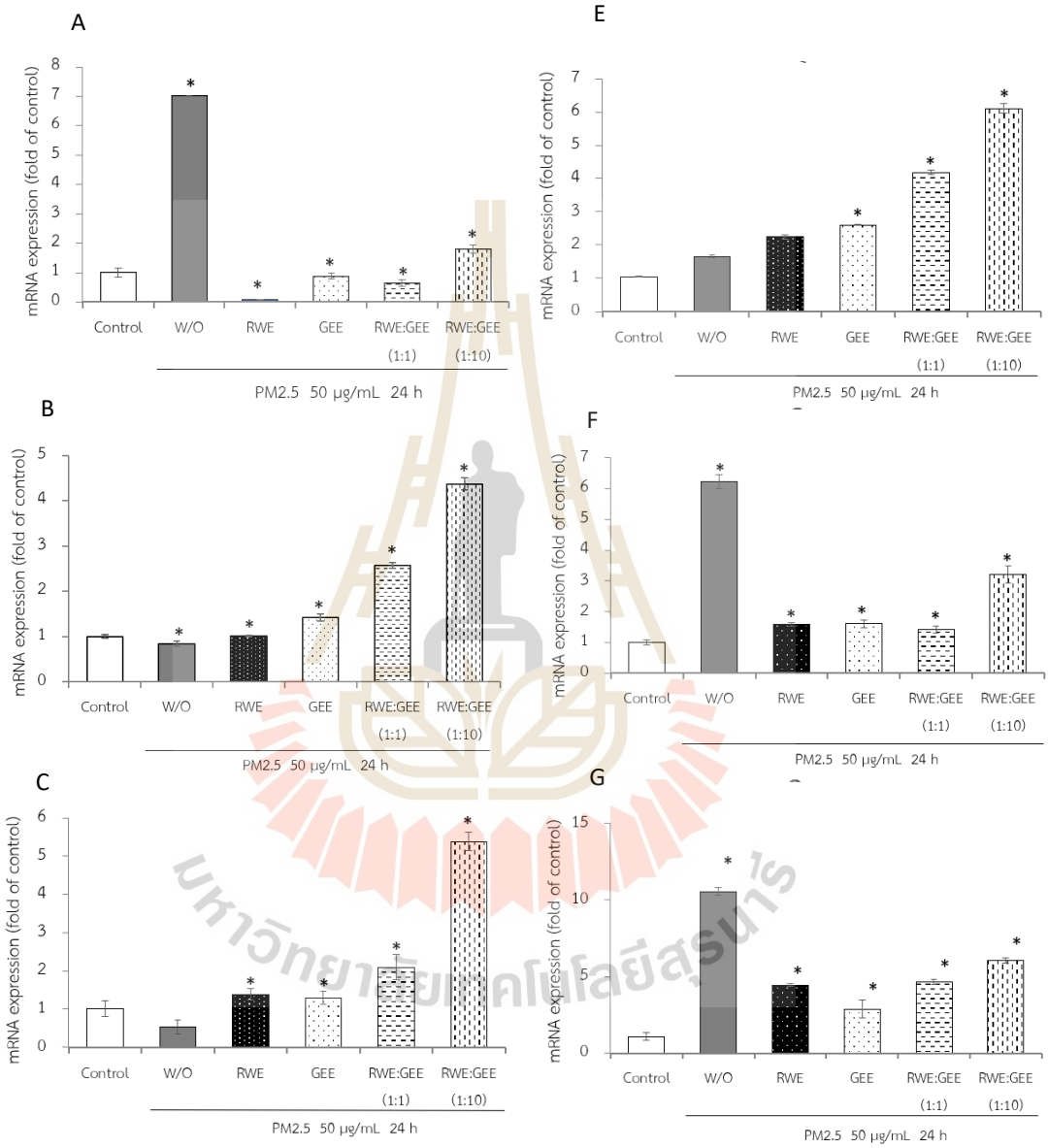
The results indicate that *GPx* expression did not change under PM2.5 exposure conditions. However, *HO-1* and *SOD* expression levels decreased, while *CAT*, *Ahr*, and *Nrf2* expressions increased. Nrf2, a critical regulator of endogenous antioxidant genes, plays a crucial role in defending against oxidative stress by translocating to the nucleus upon dissociation from Keap1, where it initiates the expression of various phase II defense enzymes to restore cellular redox homeostasis (Jiang, Wang, & Guo, 2017). This explains the observed increase in Nrf2 expression after exposure to PM2.5, involving the activation of antioxidant response elements (ARE) and the upregulation of phase II antioxidant enzymes, including *HO-1* (Lever et al., 2016). However, although Nrf2 is involved in regulating *HO-1*, our study did not observe a simultaneous increase in *HO-1* expression, possibly due to severe PM2.5 exposure suppressing *HO-1*.

Numerous studies have consistently highlighted the crucial role of the Nrf2-antioxidant response pathway in combating oxidative stress triggered by PM2.5. Nrf2 not only enhances the cellular defense system by activating antioxidant genes but also safeguards against inflammation and detoxifies environmental electrophiles like aromatic hydrocarbon quinones, crotonaldehyde, acrylamide, methylmercury, and cadmium (K. Liu et al., 2022). The disruption in the balance between oxidation and the antioxidant system caused by particulate matter, organic chemicals, and transition metals is recognized as the primary reason for PM2.5-induced toxicity (Zhai et al., 2022). This imbalance results in excessive production of reactive oxygen species (ROS) during oxidative stress, leading to antioxidant depletion and breakdown of the body's natural defense mechanisms, ultimately failing to protect cells from damage caused by oxidative stress.

4.4.2.2 Gene expression of single and combined extracts on PM2.5 exposure

The single and combined effects of Rang Chuet and ginger extracts on PM2.5-exposed A549 cells and the activities of antioxidant enzyme expressions are presented in Figure 4.6 (A-E). The study conditions included a control group, PM2.5 exposure, and four treatment conditions: pre-treatment with extracts

(RWE, GEE, RWE: GEE at a ratio of 1:1 v/v, and RWE: GEE at a ratio of 1:10 v/v) at a concentration of 50 µg/mL for 24 h, followed by exposure to PM2.5 at 50 µg/mL for an additional 24 h, after which mRNA gene expressions were determined.



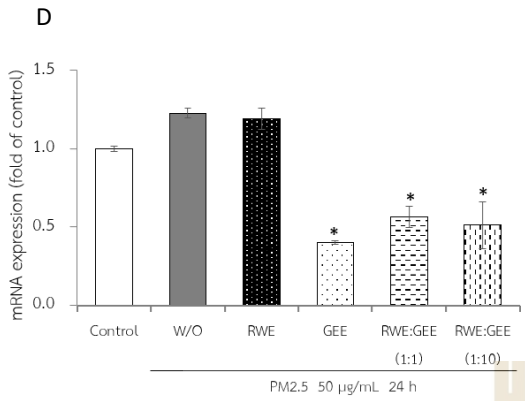


Figure 4.7 The relative mRNA expressions of antioxidant enzymes and phase II detoxification enzymes and inflammatory cytokines on pre-treatment single and combined extracts on PM2.5-exposed to A549 cells, *OH-1* (A), *SOD* (B), *CAT* (C), *GPx* (D), *NQO1* (E), *IL-6* (F), *IL-8* (G). The mRNA expression was determined by real-time qRT-PCR. Data are presented as mean ± SD (*n*=3). * Above column showed significantly different from PM2.5 exposure without pre-treatment with extracts.

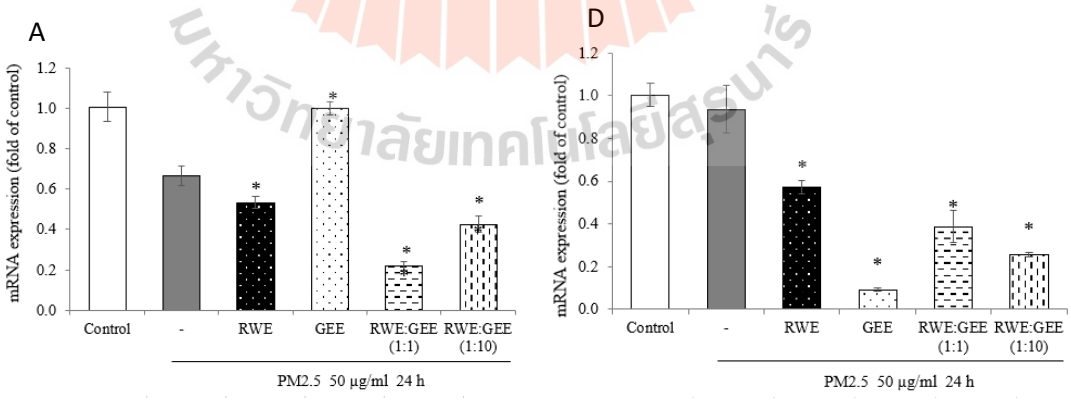
The results demonstrate that PM2.5 exposure caused a significant decline in *SOD, CAT*, and *NQO1* concentrations. These findings align with studies exploring PM2.5 impacts on A549 cells, and these effects were reversed by pretreating cells with extracts, either single or in combination, for 24 h. This can be explained by the composition of Rang Chuet, particularly rosmarinic acid (RA), which not only directly scavenges free radicals but also provides molecular protection. Evidence from Fetoni et al. (2015) suggests that RA enhances *SOD* and *HO-1* levels, indicating activation of the cell's stress response *in vivo*, and strengthens the Nrf2/HO-1 signaling pathway. Additionally, gallic acid in RWE activates Nrf2/HO-1 signaling and reduces oxidative stress (Lv et al., 2021).

Focused on combined extracts at two different ratios, these CRGE formulations surprisingly up-regulated the expression of antioxidant genes, including *HO-1, SOD, CAT, GPx,* and *NQO1,* which serve as phase II antioxidant enzymes (Figure 4.6A, B, C, E). Moreover, the ratio of RWE at 1:10 exhibited the highest level of

expression. This result may be attributed to the interaction of the extracts in the appropriate proportion. The increase in antioxidant enzyme activity has been supported by several studies. For example, the phenolic hydroxyl in tea polyphenols can interact with phenolic substances in kelp to form stable chemical complexes (Z. Li et al., 2021). Studies have shown that tea polyphenols, Pueraria flavones, and sweet potato extracts increase *SOD* activity (Liu et al., 2015). However, these studies focused on specific plants of interest and may not provide a comprehensive view of the entire mechanism that occurs. Notably, *HO-1* and *GPx* were down-regulated by all extracts.

This study reveals that the protective effect of single and combined extracts against inflammation was achieved by reducing the expression of inflammatory cytokines *IL-6* and *IL-8*. Benzo[a]pyrene (BaP), a major component of PM2.5, increases *IL-6* and *IL-8* through the activation of the EGFR-ERK1/2 signaling pathway (Ge et al., 2020). Zingerone, an active ingredient in ginger, activates the AMRK/Nrf2/HO-1 signaling pathway (K. Liu et al., 2022; Zhu et al., 2021). In conclusion, we have found that CRGE represents a valuable therapeutic target for mitigating oxidative damage to lung cells caused by PM2.5 inhalation.

The single and combined effects of Rang Chuet and ginger extracts on PM2.5-exposed HepG2 cells and the activities of antioxidant enzyme expressions are presented in Figure 4.7 (A-F).



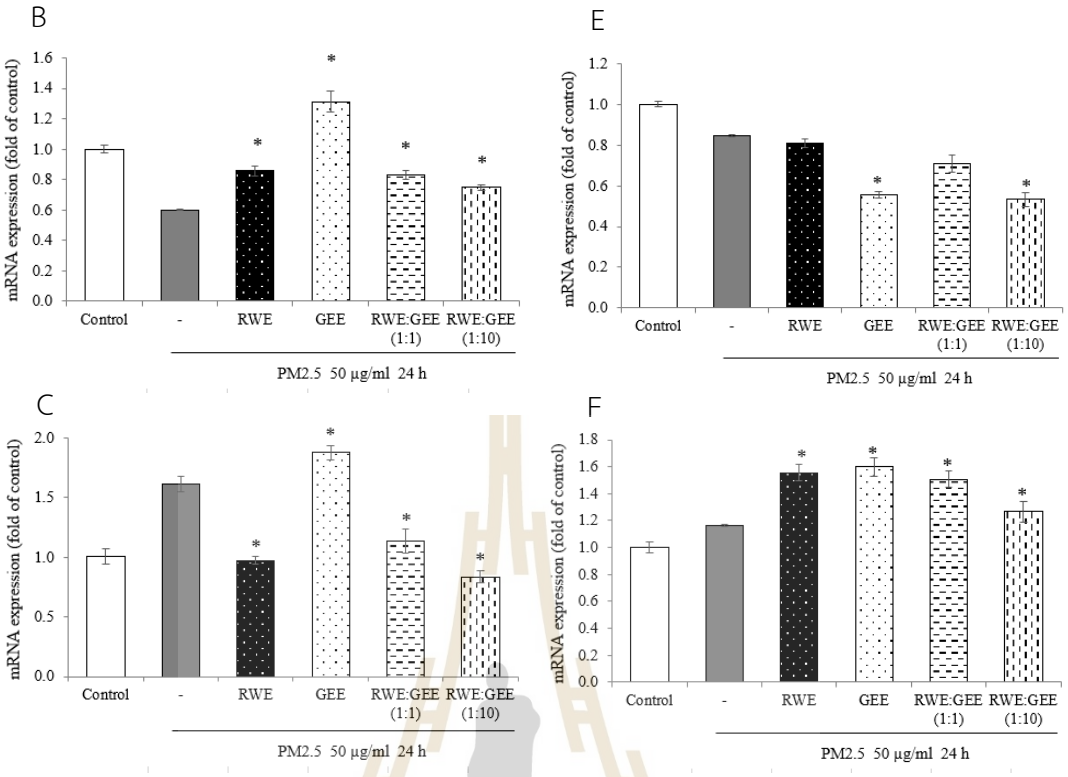


Figure 4.8 Effect of single and combined extracts on PM2.5-exposed to HepG2 cells relative mRNA expressions of antioxidant enzymes and antioxidant transcription regulators. The mRNA expression was determined by real-time qRT-PCR. Data are presented as mean ± SD (*n*=3). * Above column showed significantly different from PM2.5 exposure without pre-treatment with extracts.

The result show that HepG2 cells were treated with single extracts of RWE and GEE. RWE significantly decreased *OH-1, CAT* and *GPx* expression, elevated the activities of antioxidant enzymes Ahr compared to normal levels. Notably, exposure to 10-1000 mg of crude Rang Chuet extract (Junsi et al., 2020) resulted in increased *CAT* enzyme activity, influenced by caffeic acid, which enhances redox balance in rat liver (Olayinka, Ola, Ore, & Adeyemo, 2017). On the other hand, GEE application increased *HO-1, SOD* and *CAT* activity. The variation in antioxidant gene expression with different extracts suggests diverse underlying mechanisms. As a result, CRGE emerged as the

most potent extract, boosting CAT and SOD expression and thereby enhancing catalase and SOD activities.

4.5 Conclusion

PM2.5 causes cell cytotoxicity by inducing ROS production and regulating the expression of antioxidative genes. Excessive ROS leads to cellular oxidative damage, resulting in downregulation of gene expression related to antioxidant enzymes such as *HO-1, SOD*, and *CAT*. Therefore, PM2.5 exposure can damage airway epithelial cells (A549 cells), causing oxidative stress including toxication and lung inflammation through the release of proinflammatory cytokines. Additionally, cellular oxidative stress occurs in HepG2 cells, representing the liver organ responsible for detoxification.

Rang Chuet and ginger extracts efficiently decrease ROS production triggered by PM2.5 exposure, safeguarding A549 and HepG2 cells from PM2.5-induced toxicity while neutralizing intracellular ROS. Additionally, these combined extracts stimulate antioxidant enzyme production in response to PM2.5, diminishing oxidative stress and enhancing cellular antioxidant defenses. Nonetheless, the diverse compositions of PM2.5 introduce limitations, influencing the effectiveness of these extracts in combating PM2.5-induced oxidative stress.

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CHAPTER V

THE ANTI-INFLAMMATORY POTENTIAL OF COMBINED EXTRACTS OF *THUNBERGIA LAURIFORIA* LINN. (RANG CHUTE) LEAVES AND *ZINGIBER OFFICINALE* (GINGER) ON THP-1 MACROPHAGE CELLS EXPOSED PM2.5

5.1 Abstract

The increasing levels PM2.5 in the atmosphere have been linked to various inflammatory diseases. This study explores the anti-inflammatory effects of combined extracts of Rang Chuet water extract and ginger ethanol extracts (CRGE) on human macrophage THP-1 cells exposed to PM2.5 (Standard reference material, SRM 2786, NIST). The THP-1 cells were pre-treatment with single or combined extracts for 24 h following exposed PM2.5. The cytotoxicity, Nitric Oxide (NO) levels and pro-inflammatory mediators by modulation of *IL-6* expression were investigated. PM2.5 demonstrated a significant increase in NO levels and *IL-6* dose dependent manner. These results provide important implications that combined extracts at ratio 1: 10 v/v showed an anti-inflammatory on THP-1 cells from PM2.5 exposure. In conclusion, the combined extract of RWE and GEE can reduce inflammatory effects in THP-1 cells and be further applied as functional ingredients in functional food products.

Keywords: THP-1 cells, *IL-6*, Anti-inflammatory, PM2.5, Combined plant extracts.

5.2 Introduction

PM2.5, or particulate matter with a diameter of 2.5 micrometers or less, consists of fine particles and droplets suspended in the air. The rise in PM2.5 pollution is increasingly concerning due to its association with various harmful health effects. These tiny particles can be inhaled into the lungs, where they may interact with immune cells like macrophages. The presence of PM2.5 in the environment has been linked to

inflammation and damage to alveolar cells. When PM2.5 reaches the alveolar space, macrophages serve as the primary defense by eliminating foreign particles and initiating inflammation alongside epithelial cells. Inflammation is a critical factor in the adverse health effects resulting from particle exposure (G. Wang et al., 2020). During inflammation, macrophages release chemicals such as interleukin-6 (IL-6) and nitric oxide (NO) (Hong et al., 2020). The THP-1 cell line, which models human monocytes and macrophages, is an ideal tool for studying the impacts of PM2.5 exposure.

Nitric oxide (NO) is a molecule involved in numerous bodily processes, both normal and pathological. In macrophages, NO production is often triggered by the presence of foreign particles and pathogens as part of the immune response. Exposure to PM2.5 can cause macrophages to produce NO as a protective measure. However, NO production can have both positive and negative effects, depending on the context and duration of exposure (Bogdan, 2015). It's important to understand how PM2.5 exposure influences NO production in macrophages because excessive NO can lead to oxidative stress and inflammation, which are linked to respiratory diseases such as asthma, chronic obstructive pulmonary disease (COPD), and lung infections. High NO levels can interact with free radicals during inflammation, potentially causing damage to cell membranes and leading to tissue injury or cell death (Ncube et al., 2021). Although the Griess reagent system is a common method for measuring NO production, its application to THP-1 cells exposed to PM2.5 is limited.

Interleukin-6 (IL-6) is a multifunctional cytokine involved in immune responses, inflammation, and tissue repair. High levels of IL-6 have been associated with several inflammatory diseases (Tanaka et al., 2014). Therefore, examining how PM2.5 exposure affects IL-6 production is vital. While research on PM2.5 effects and inflammatory responses in THP-1 cells is ongoing, there is a noticeable lack of studies on plant extracts as potential anti-inflammatory agents. Plant extracts have been shown to modulate inflammation in various cell types, but their effects on PM2.5-exposed THP-1 cells remain largely unexplored.

This study aims to assess the anti-inflammatory properties of Rang Chuet and ginger combined extracts in PM2.5-exposed THP-1 cells, focusing on NO production and IL-6 expression.

5.3 Materials and methods

5.3.1 Materials

Our study employed Standard Reference Material (SRM 2786) as a representation of fine PM2.5, which was sourced from the National Institute of Standards and Technology (NIST). This fine PM was collected in Prague, Czech Republic, in 2005 and possesses an average particle diameter of around 2.8 μm. The certificate of analysis for the SRM 2786 utilized in this study is accessible online (NIST, 2013). To create a fresh stock suspension of SRM 2786 for our experiments, RPMI 1640 medium was used at a final concentration of 2 mg/mL. This suspension was then sonicated for 5 min with BANDELIN equipment (GmbH & Co., Germany) and vigorously vortexed for 2 min before each experiment, following the procedure described by Xiong et al. (2015).

5.3.2 Cell culture

Human monocytic cell line THP-1 (ATCC TIB-202) was kindly provided by Assoc. Prof. Parinya Noisa (Suranree University of Technology, Nakorn Ratchasima, Thailand. THP-1 monocytes were cultured in RPMI 1640 medium (HyClone, GA, USA) supplemented with 10% FBS and 1% penicillin–streptomycin (Solar bio, China). The cells were maintained in a humidified atmosphere with 5% carbon dioxide (CO_2) at 37°C. THP-1 monocytes were differentiated into macrophages by treating them with 10 ng/mL phorbol-12-myristate-13-acetate (PMA) for 24 h and further incubated overnight for the resting stage. (P. Li et al., 2021; Ozleyen et al., 2021).

5.3.3 Cell viability

Cell viability was assessed using the MTT assay and compared to that of untreated groups. Suspension cells were seeded in 96-well plates with a cell density of 2×10^4 cells per well after being mixed with the respective treatments. The concentrations tested ranged from 6.25 to 400 μg/mL for the individual extracts of RWE and GEE. Following the determination of the concentrations of the single extracts, we confirmed that the concentrations of the combined extracts were not cytotoxic to THP-1 cells. For the PM2.5 test, concentrations ranging from 12.5 to 400 μg/mg were used, and two concentrations that exhibited cytotoxicity to the cells were selected for further experiments.

5.3.4 Measurement of Nitric oxide production

In this study, measured the concentration of nitrite, a stable oxidized product of nitric oxide (NO), in the cell culture medium. To quantify nitric oxide production, we employed the Griess reagent, which was obtained from Promega (Madison, WI, USA). THP-1 macrophage cells were initially seeded in 96-well plates at a density of 2×10^4 cells per well. They were then exposed to various concentrations of extracts, resulting in a final volume of 50 µl per well. After this treatment, the cells were incubated for a period of 24 h. Following the incubation, the treatments were removed, and the cells were exposed to PM2.5 for an additional 24 h. Subsequently, we collected 50 µl samples of the supernatant from the treated culture medium and mixed them with 50 µl of a solution containing 1% sulfanilamide in 5% phosphoric acid (referred to as the sulfanilamide solution). This mixture was incubated at room temperature for 10 min, protect it from light. Afterward, we added 50 µL of a solution containing 0.1% N-1-naphthylethylenediamine dihydrochloride in water (referred to as the NED solution), followed by another 10 min incubation at room temperature while safeguarding it from light. The absorbance of these samples was then measured at a wavelength of 530 nm using a microplate spectrophotometer. To determine the levels of nitric oxide (NO) in each experimental sample, we utilized a standard curve ranging from 0 to 100 µM of $NaNO_2$, as detailed in Appendix 3.

5.3.5 Quantification of pro-inflammatory mediators by enzyme-linked immunosorbent assay

Inflammatory response was evaluated by quantification of IL-6 pro-inflammatory mediators. IL-6 were measured using Elabscience® Human IL-6 (Interleukin 6) ELISA Kit. Absorbance was measured according to the supplier procedure using a microplate reader (Synergy H1, BioTek, VT, USA). The concentrations of mediators were calculated as pg/mL.

5.3.6 Statistical Analysis

All experiments were replicated 3 or 5 times, and assay results are expressed as the means ± SD. The difference in mean values among at the treatments

were analyzed using the one-way analysis of variance (ANOVA) with Duncan's multiple range test. *p*-values < 0.05 were considered statistically significant.

5.4 Results and Discussion

5.4.1 Cell viability on THP-1 cells

The cell viability of RWE and GEE on THP-1 cells were shown in figure 5.1

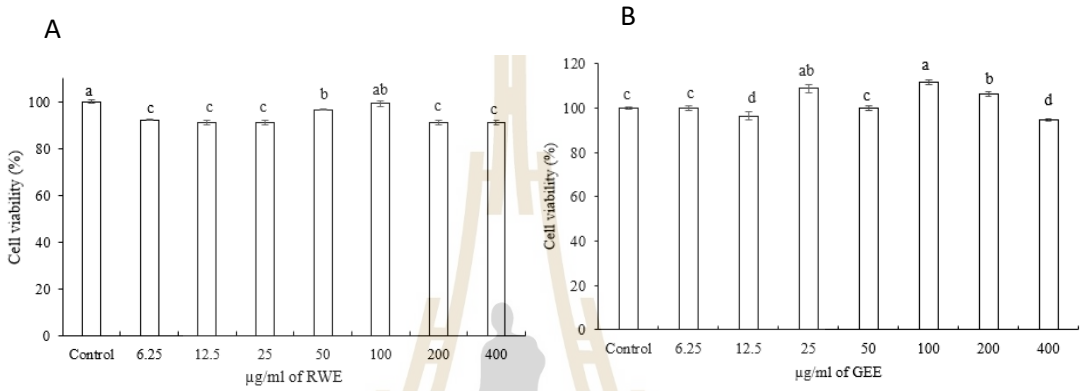

Figure 5.1 Cell viability of RWE (A) GEE (B) on THP-1 cells for 24 h. Letter over bar show significant differences at 95% (p<0.05).

The cell viability of RWE at 100 µg/mL and GEE at 50 µg/mL after 24 h of treatment exhibit a significant difference compared to the control without extracts. However, GEE at 25 and 100 µg/mL demonstrated an increase in cell viability in THP-1 cells When considering the concentration test at 50 and 100 µg/mL, based on the results from Chapter 4, the study in HepG2 and A549 cells at the same concentrations did not reveal any cytotoxic effects. Therefore, we selected 50 and 100 µg/mL of extracts to study cell viability, combining them in various ratios as presented in Table 5.1. The results indicated that the combined extracts of RWE and GEE at the ratios of 1:1 and 1:10 (which were found effective in Chapter 4) at both concentrations of 50 and 100 µg/mL did not significantly differ from the control group without extracts (Figure 5.2). Consequently, we chose these treatment conditions for further study.

Table 5.1 The treatment conditions for study cell viability.

Single extracts	Concentration (µg/mL)	Combined extracts (v/v)	Concentration (µg/mL)
RWE	50	RWE : GEE 1 : 1	50
RWE	100	RWE : GEE 1 : 1	100
GEE	50	RWE : GEE 1 : 10	50
GEE	100	RWE : GEE 1 : 10	100

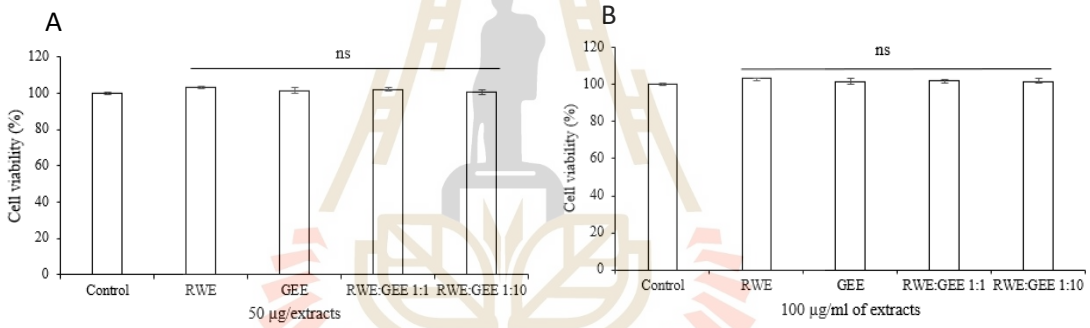

Figure 5.2 Cell viability of single and combined extracts at 50 µg/mL (A) and 100 µg/mL (B) for 24 h. ns, not significant from control (p<0.05).

The cell viability of THP-1 cells exposed PM2.5 were shown in figure 5.3. The result show that PM2.5 no statistical significance was observed relative to the control at a low concentration (12.5 µg/mL) (p>0.05). Even at the high dose (400 µg/mL), it exhibited high cytotoxicity to cells, while at the medium dose (25-200 µg/mL), it caused approximately a 10% reduction in cell viability. Therefore, we selected concentrations of PM2.5 at 50 and 100 µg/mL for further investigation, as they resulted in reduced cell viability and were significantly different from the control(p<0.05).

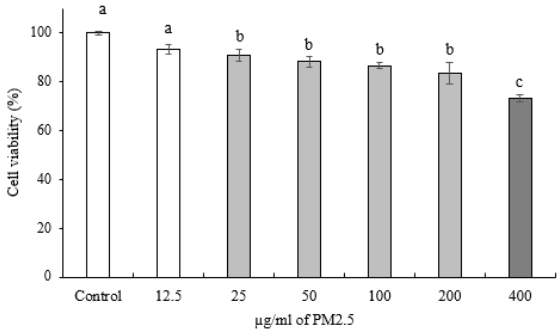

Figure 5.3 Cell viability of PM2.5 exposed to THP-1 cells for 24 h. Letter over bar show significant differences at 95% (p<0.05).

5.4.2 Inhibition of nitric oxide production in PM2.5-explosed THP-1 cells

Excessive NO levels have been implicated in cell death, inflammatory responses, and the pathogenesis of several disease states (Yu et al., 2021). In the current study, the inhibition of NO production in PM2.5-exposed THP-1 cells using both single and combined extracts with various concentrations as depicted in Figures 5.4A and B.

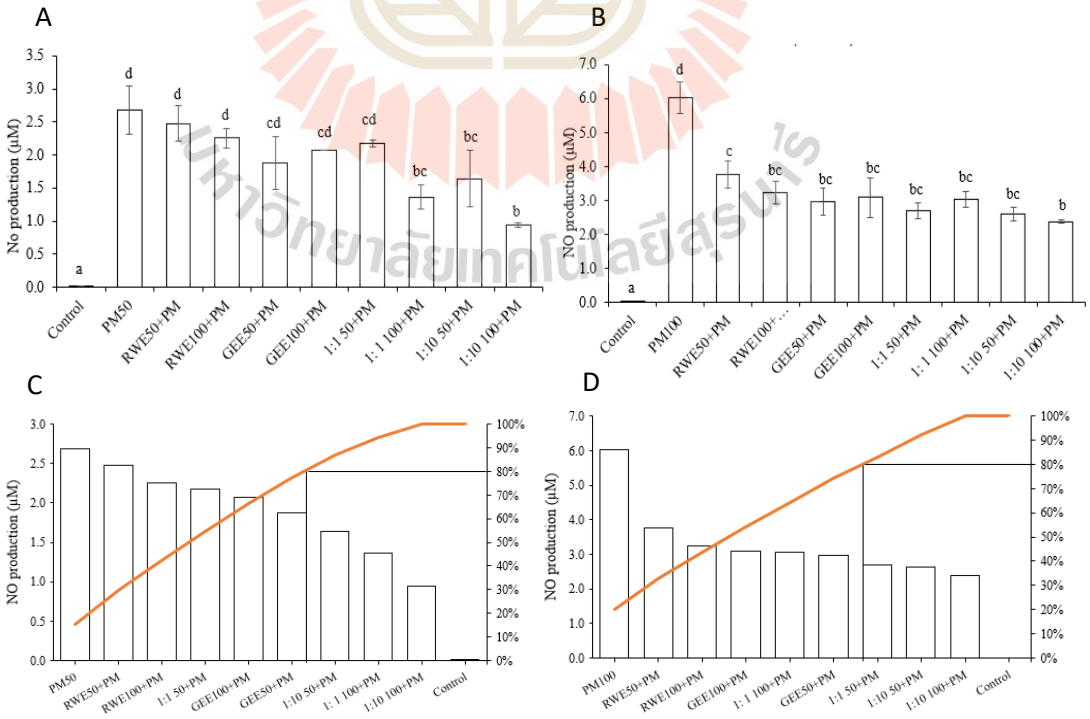

Figure 5.4 The inhibition of NO production in the PM2.5-exposured THP-1 cells, PM2.5 at 95% confidential level (p<0.05). A Pareto diagram with an 80% cut-offline of NO production in the PM2.5-exposured THP-1 cells, PM2.5 at 50 µg/mL (C) and PM2.5 at 100 µg/mL (D).

Initially, examined NO production in THP-1 cells exposed to PM2.5 for 24 h. At concentrations of 50 µg/mL and 100 µg/mL, NO levels were measured at 2.68 µM (Figure 5.3A) and 6.02 µM (Figure 5.4B), respectively. Nitrite accumulation in the cells increased in a dose-dependent manner following PM2.5 exposure. When analyzing the impact of extracts on inhibiting NO production in the presence of PM2.5 at a concentration of 50 µg/mL, a noticeable reduction trend was observed. The increase in NO production caused by PM2.5 was significantly suppressed by both single and combined extracts. However, single extracts of RWE at 50 µg/mL did not significantly reduce NO production from PM2.5 at the concentration of 50 µg/mL (Figure 5.4A). Interestingly, combined extracts exhibited potent inhibitory effects on NO production of PM2.5 exposure.

To a comparison between the results in Figures 5.4C and 5.4D, using a Pareto diagram with an 80% cut-offline, revealed that the combine extract, remained effective at concentrations below the 80% cut-off line when exposed to PM2.5 at 50 µg/mL. For exposures to PM2.5 at up to 100 µg/mL, only the use of combined extracts remained under the cut-off line, as depicted in Figure 5.4D. In this study, combined extracts significantly inhibited the production of nitrites in PM2.5-exposed THP-1 macrophage cells. Nevertheless, the concentrations of extracts, along with the ratios of combining importance factors, played a crucial role in mitigating the effects of PM2.5.

5.4.3 Effects of a combined extract on the production of PM2.5-stimulated pro inflammatory mediators in THP-1 cells

The anti-inflammatory effects of a combined extract of RWE and GEE on the production of PM2.5-stimulated proinflammatory mediators in THP-1 cells. The release of the pro-inflammatory mediator *IL-6* in the cell culture medium was measured using ELISA (Figure 5.5).

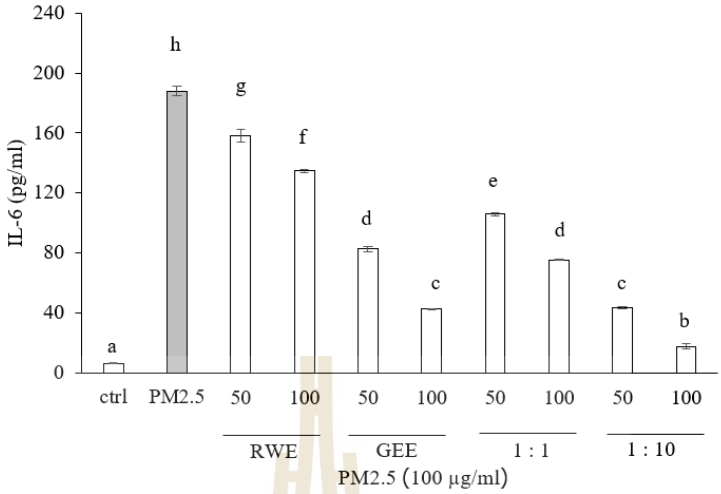

Figure 5.5 Effects of single extract (RWE and GEE) and combined extracts at the ratio of 1: 1 and 1: 10 on PM2.5-induced *IL-6* release in THP-1 cells. Cells were pretreated extracts then exposed to PM2.5 for 24 h. *IL-6* release was measured by ELISA. Data are represented as mean ± SD.

The result show that IL-6 levels were significantly elevated, showing a up to 188±3.24 pg/mL increase in cultures exposed to 100 µg/mL of PM2.5 for 24 h compared to corresponding unexposed controls (7±0.21 pg/mL) (p<0.05). When observed a significant dose-dependent decrease in *IL-6* levels with all single and combined extract tests compared to PM2.5 exposure without pre-treatments. However, for the combined extract at a 1:10 ratio, IL-6 levels showed a significant decrease at medium and high doses of extracts (50 and 100 µg/mL). Meanwhile, the single extract showed that a significant decrease was only observed in the high dose of extracts for RWE and GEE (p< 0.05).

Interestingly, the combination of extracts at a 1:10 ratio showed a more pronounced effect on all measured pro-inflammatory markers following 24 h of PM2.5 exposure compared to the 1:1 ratio. Moreover, across all markers examined, the reduction in expression with both combined extracts was notably significant, indicating secretion levels about 4-fold lower than PM2.5 exposure alone. This reduction is

attributed to the extracts ability to inhibit NO production, as IL-6 induces iNOS expression, leading to increase NO levels (Yang, Deng, Chen, Liu, & Zheng, 2021). The inhibition of IL-6 and other pro-inflammatory cytokines has been proposed as a therapeutic approach for immune-related disorders (Lim JS, 2020). RWE, rich in bioavailable phenolic compounds such as rosmarinic acid (RA), effectively inhibited the secretion of TNF-α, IL-1β, and IL-6 in a human THP-1 macrophage model (Villalva et al., 2018). Similarly, GEE, which contains gingerol, blocked the release of IL-6, TNF-α, ROS, NO, and iNOS (F. Zhang et al., 2018). Furthermore, 6-Gingerol has been shown to have anti-inflammatory and antioxidant effects in the context of sepsis-related acute lung injury by affecting the NF-kB and Nrf2 pathways (Yang et al., 2021). These results imply that a 1:10 ratio of the combined extract acts as a potent anti-inflammatory agent by suppressing inflammatory responses.

5.5 Conclusion

The combination of RWE and GEE extracts effectively mitigates inflammation in the THP-1 macrophage model. Our findings underscore the effectiveness of this combined extract, particularly at a 1:10 ratio (v/v), as a powerful anti-inflammatory agent that suppresses the pro-inflammatory response and reduces NO production. One limitation of this study is the incomplete identification of the signaling pathway associated with the anti-inflammatory effects of these extracts. Nonetheless, these results hold promise for advancing innovative approaches to treating inflammation.

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CHAPTER VI

BIOMOLECULAR CHANGES IN A549 CELLS EXPOSED TO PM2.5 AND THE PROTECTIVE EFFECT OF CRGE AGAINST PM2.5 USING SYNCHROTRON FOURIER TRANSFORM INFRARED (SY-FTIR) SPECTROSCOPY

6.1 Abstract

PM2.5 exposure results in oxidative stress, which contributes to various health conditions. The combined Rang Chuet and Ginger root extract (CRGE) has shown potential for countering PM2.5 toxicity with its antioxidative and anti-inflammatory properties. This research aims to examine the biomolecular changes triggered by PM2.5 and the protective impact of CRGE on A549 cells (human epithelial lung cells) through Synchrotron-Fourier Transform Infrared (SY-FTIR) spectroscopy. The results highlight changes in the signal intensity and integrated areas related to lipids, lipid esters, and proteins in the PM2.5-exposed A549 cells. Principal component analysis (PCA) identified three distinct clusters in the FTIR spectra, suggesting that CRGE may help alleviate the oxidative stress induced by PM2.5.

Keywords: PM2.5 exposure, oxidative Stress, CRGE (Combined Rang Chuet and ginger root extract), A549 Cell Model, SY-FTIR Spectroscopy.

6.2 Introduction

PM2.5, a type of fine particulate matter with a diameter less than 2.5 μm, is a widely recognized environmental hazard with significant implications for human health. These particles originate from diverse sources such as industrial emissions, vehicle exhaust, and natural events. Due to their small size, PM2.5 particles can

deeply penetrate the respiratory system, posing a particular threat to lung health and overall well-being. A major concern associated with PM2.5 exposure is its ability to induce oxidative stress in the human body. Oxidative stress arises from an imbalance between free radicals and antioxidants, potentially causing damage to cells, tissues, and DNA. This imbalance is a recognized precursor to various diseases, including respiratory ailments, cardiovascular disorders, and cancer.

Recent studies have pointed to the combined use of Rang Chuet and Ginger root extract (CRGE) as a promising strategy against PM2.5-induced oxidative stress. CRGE, recognized for its antioxidative and anti-inflammatory properties, has shown effectiveness in mitigating the harmful effects of PM2.5 exposure. Despite encouraging findings, further investigation into how CRGE influences biomolecular changes under PM2.5 conditions is essential.

This study aims to observe the biomolecular changes elicited by CRGE in the presence of PM2.5. By utilizing the A549 cell model, these were performed using Synchrotron Fourier Transform Infrared (SY-FTIR) spectroscopy to examine the biomolecular changes, with a particular focus on lipids, lipid esters, and proteins. The resulting data, analyzed using Principal Component Analysis (PCA), promises to offer a comprehensive understanding of how CRGE functions as a shield against oxidative stress caused by PM2.5 exposure. The contribute to our understanding of the protective potential of CRGE in mitigating the health risks associated with PM2.5 and potential interventions in air pollution-related health issues.

6.3 Materials and methods

6.3.1 Cell culture and treatments

A549 cells were employed in this study. They were initially seeded at a density of $6x10^5$ cells per well in a 6-well plate using DMEM supplemented with 10% fetal bovine serum (FBS, Hyclone, GA, USA). The cells were then incubated in a 5% CO_2 atmosphere at 37°C for 24 h. Following the initial incubation, the cells were subjected to study through three conditions: first, a control group without any treatment; second, exposure to PM2.5 without the application of any extract; and finally, a treatment condition involving a combination of Rang Chuet and Ginger root

extract (RWE) and Ginger extract (GEE) in a 1:1 ratio (v/v) at a concentration of 50 µg/mL for 24 h, followed by exposure to PM2.5 at 100 µg/mL for another 24 h. After the exposure period, all treated cells were thoroughly washed with 1×PBS and harvested using 0.05% trypsin-EDTA.

6.3.2 The measurement of biomolecular changes using Synchrotron FTIR

The biomolecular changes induced by the combined extract demonstrated protective effects on A549 cells exposed to PM2.5. The combined extracts, which exhibited synergistic cellular antioxidant activity against PM2.5, were selected for SY-FTIR investigation. Experimental conditions included a control group (without any treatments), PM2.5 exposure under cytotoxic conditions, and pretreatment with the combined extract followed by PM2.5 exposure. After treatment, A549 cells were collected and centrifuged at 3,000 x g for 4 min. Cells were then adjusted with normal saline to achieve a concentration of $1x10^6$ cells/mL. Cell suspensions were centrifuged again, and the cell pellets were washed twice with normal saline. Subsequently, the cells were diluted in 20 µl of DI water, gently resuspended, and 0.5 µl of the suspension was deposited onto an IR-transparent, 2-mm thick barium fluoride (BaF_2) optical window (Crystran Ltd.). To ensure optimal conditions for analysis, the specimens were placed under vacuum to remove excess water for 20 min and then stored in a desiccator until FT-IR measurement.

The measurements using FTIR were conducted at the Synchrotron Light Research Institute in Thailand, using a Vertex 70 FTIR spectrometer connected to a Bruker Hyperion 2000 microscope (Bruker Optics Inc., Ettlingen, Germany). FTIR spectra were acquired using synchrotron radiation as the IR source in transmission mode. The microscope was equipped with a 64x64 element MCT and FPA detector, enabling simultaneous capture of spectral data with a 36x objective. Spectra were collected over the range of 4000-600 cm^{-1} with a spectral resolution of 4 cm^{-1} and an aperture size of 10x10 µm. A total of 64 scans were averaged, and OPUS 7.5 software (Bruker Optics Ltd., Ettlingen, Germany) controlled the equipment and performed specimen measurements. Preprocessing involved second derivative transformations with the Savitzky-Golay algorithm (nine smoothing points). Spectra were further normalized using extended multiplicative signal correction (EMSC), focusing on spectral regions from 3000-2800 cm^{-1} for lipids and 1800-1200 cm^{-1} for

proteins and nucleic acids. Integration of areas with high-intensity second derivative spectra was conducted using the OPUS program. Principal component analysis (PCA) was carried out using UnscramblerX 10.4 software (CAMO Software AS., Oslo, Norway). Score plots (2D) and loading plots were used to visualize different data classes and relationships between variables in the dataset. Spectra representative evaluation was performed using Sigmaplot version 14.0 software (Systat Software Inc., UK).

Table 6.1 FTIR band assignments for functional groups.

Wave number (cm^{-1})	Band assignment
2963	Asymmetrical stretching (C-H) from methyl (-CH3) groups of phospholipids
2924	Asymmetrical stretching (C-H) from methylene (-CH2) groups of lipids
2852	Symmetrical stretching (C-H) from methylene (-CH2) groups of lipids
1659	Amide I of alpha helix proteins stretching, C=O (80%) and C-N (10%) N-H (10%)
1641	Amine I: beta-sheet
1545	Amide II alpha helix proteins stretching, N-H (60%) and C-N (40%) stretching
1234-1247	PO^{2-} asymmetric stretch of phosphodiester group in nucleic acids and phospholipids

6.4 Result and discussion

Biomolecular changes in A549 cells pre-treated with combined extracts to protect against PM2.5 exposure were assessed using Synchrotron FTIR microspectroscopy. Figure 6.1A displays the average original FTIR spectrum bands of the control group (without treatments), the PM2.5 exposure group, and the pretreated CRGE group against PM2.5 exposure in A549 cells. To further analyze the data, the raw spectrum underwent second derivative analysis in specific spectral regions: from 3,000-2,800

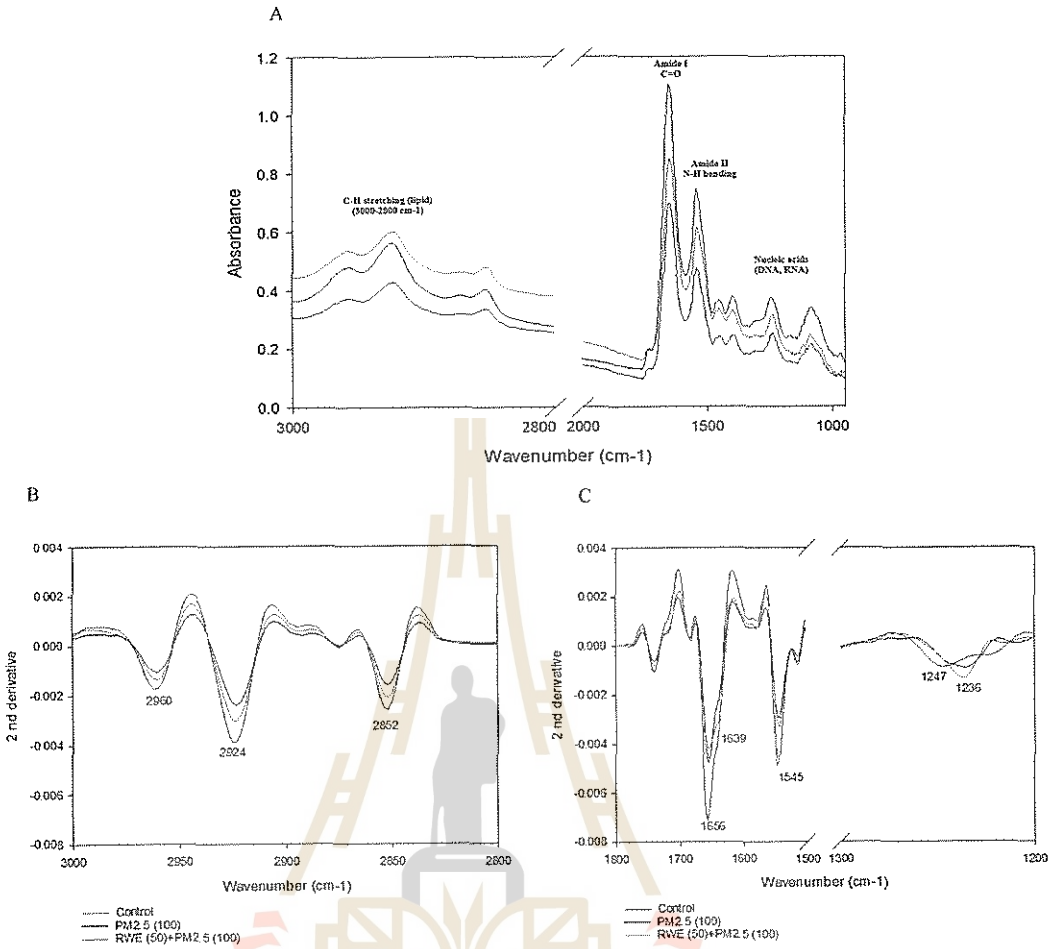

Figure 6.1 The average original SR-FTIR spectra (A) and average second derivative spectra of Control, PM2.5 exposure, and pretreatment CRGE against PM2.5 exposure in A549 cells. The data were represented in two regions: (B) lipid regions (3,000–2,800 cm^{-1}) and (C) protein and RNA, DNA, and phospholipids regions (1,800-1,200 cm^{-1}).

Subsequently, we calculated the FTIR band assignments and integrated areas of the second derivative spectra for the lipid regions and protein include nucleic acids regions of these groups, as presented in Figure 6.2A and 2B, respectively.

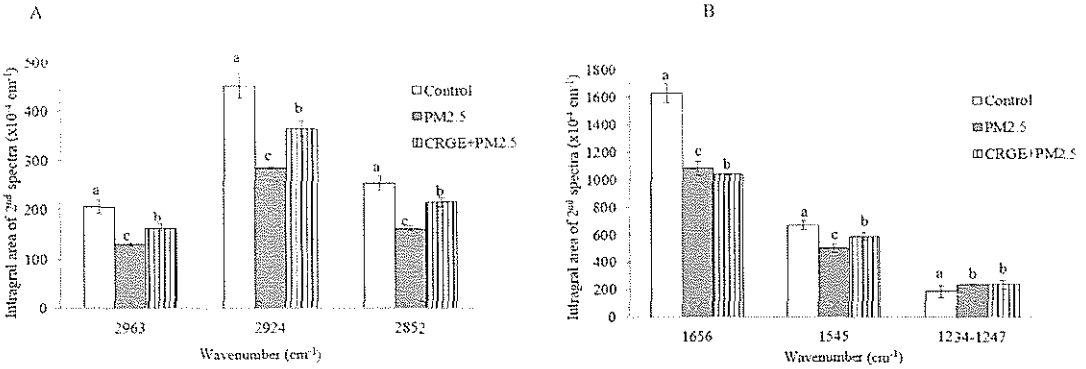

Figure 6.2 The integral area second derivative spectra of Control, PM2.5 exposure, and pretreatment CRGE against PM2.5 exposure in A549 cells. The data were represented in two regions: (A) lipid regions (3,000–2,800 cm^{-1}) and (B) protein and RNA, DNA, and phospholipids regions (1,800-1,200 cm^{-1}).

Changes in cellular lipids were observed in the lipid region (3,000–2,800 cm^{-1}). The average second derivative spectra of A549 cells under different experimental conditions revealed three distinct peaks. At 2,960 cm^{-1}, asymmetrical stretching vibrations of the CH_3 groups in the phospholipid membrane were observed, while vibrations at 2,924 cm^{-1} and 2,852 cm^{-1} were assigned to the CH_2 groups of lipids with asymmetric and symmetric stretching, respectively (Figure 6.1B). The integrated areas of the lipid regions in the second derivative spectra were calculated (Figure 6.2A). The results indicated that the relative absorbance in the lipid regions of PM2.5-exposed A549 cells was lower compared to the control group. Additionally, a significant decrease in the integrated area of the lipid region was observed in PM2.5-exposed A549 cells under different experimental conditions, suggesting potential changes in phospholipids and lipids within the cells. The interaction between particles and cell membranes is the initial step in particle internalization and cytotoxicity generation, followed by membrane system lysis (M. Gualtieri et al., 2009). Changes in the relative absorbance of the lipid region of A549 cells exposed to PM2.5 may affect the cell membrane, consistent with microscopic observations. Quartz crystal microbalance with dissipation (QCM-D) measurements have demonstrated that PM2.5 adhesion to the membrane induces membrane disruption (Zhou et al., 2016). Interestingly, pretreatment with CRGE against PM2.5

exposure resulted in the lipid region approaching levels observed in the control group in terms of integral lipid areas. This effect may be attributed to the ability of combined extract to protect against changes in the cell membrane, consistent with previous findings showing protection against cell cytotoxicity and reduction in intracellular ROS generation, which can damage cell membranes.

Changes in cellular proteins were observed within the intervals of 1,800 cm^{-1} and 1,200 cm^{-1}, indicating a high intensity of amide I-alpha helix (1,659 cm^{-1}), amide II (1,545 cm^{-1}), and RNA, DNA, and phospholipids (1,234 cm^{-1}) vibrations (Figure 6.1B). Among the three samples, the control group displayed stronger peaks at 1,656 cm^{-1} and 1,545 cm^{-1}, which were assigned to the stretching vibrations of α-helix secondary protein structure (Amide I and II) and showed the highest signal intensity compared to the other groups. These results are based on the integrated areas of the protein regions, where the protein content in control A549 cells is significantly higher than in PM2.5-exposed A549 cells and CRGE-pretreated A549 cells ($p < 0.05$, Figure 6.2B). Thus, these results reflect that PM2.5 exposure altered the cellular protein profile in all treated groups compared to the control. However, the amide II vibration was considerably higher at 1,545 cm^{-1} when pre-treated with CRGE compared to PM2.5-exposed A549 cells ($p < 0.05$), which is close to the control.

Furthermore, in the spectral region of 1,300-1,200 cm^{-1}, it corresponds to RNA, DNA, and phospholipids (Figure 6.2A). According to the data, the band centered at 1,247 cm^{-1} is present in control A549 cells, but in PM2.5-exposed cells, it shifts to 1,234 cm^{-1}. This shift is primarily attributed to the asymmetric stretch of the PO^{2-} phosphodiester group in nucleic acids and phospholipids, and it can be explained. The indications imply that nucleic acids serve as the foundation for DNA and RNA in the A cells, aligning with the MTT assay cytotoxicity assessment, which might signify DNA degradation. Additionally, these results provide additional support for the idea that the early stage of apoptosis, triggered by a cellular stimulus, can be understood by analyzing the shift in nucleic acid bands (H. Liu et al., 2018). However, the shift in RNA and DNA could have been inhibited by these phenolic and flavonoids, leading to protein accumulation.(Siriwong, Thumanu, Hengpratom, & Eumkeb, 2015).

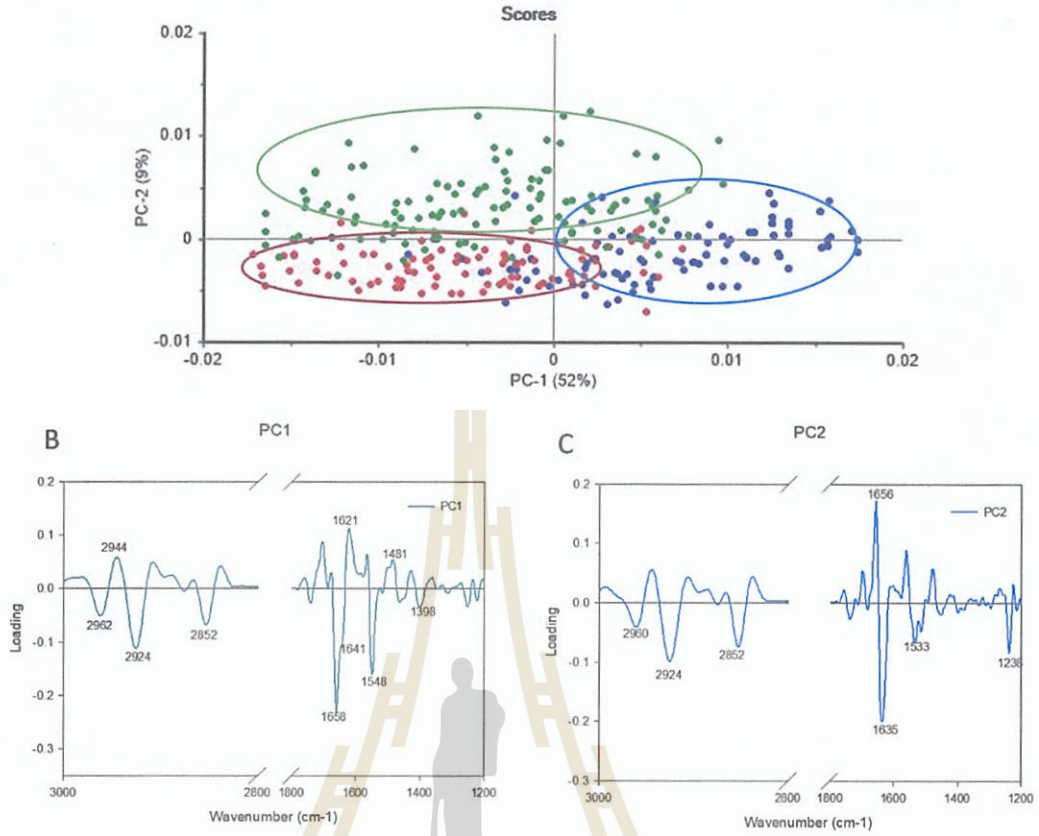

Figure 6.3 PCA of FTIR spectral ranges 3,000–2,800 cm^{-1} and 1,800-1,200 cm^{-1} giving PCA score plot (A). PCA loading plot show the biomarker differences over a spectral range of samples are identified by the PC1 (B) and PC2(C) loading plots.

Principal Component Analysis (PCA) is a valuable tool used to identify the main sources of variation in the fingerprint region, enabling the differentiation and classification of biomolecular changes in A549 cells. In this study, we employed PCA to further distinguish the data obtained from the three samples. The PCA results were generated from the second-order derivative spectra at 3,000–2,800 cm^{-1} and 1,800-1,200 cm^{-1}. The 2-dimensional PCA clustering (Figure 6.3A) clearly demonstrates a distinct separation of the control, PM2.5 exposure, and CRGE pretreatment groups before PM2.5 exposure. The PCA score plot shows that control A549 cells are well-separated from PM2.5-exposed and CRGE-pretreated A549 cells

along PC1 (accounting for 52% of the variation) and PC2 (accounting for 9% of the variation).

The PCA loading plots (Figure 6.3B) were utilized to identify the key spectral regions contributing to the clustering. In the PC1 score plot, the positive scores of the spectra from control A549 cells were clearly separated from the negative scores of the spectra from PM2.5 exposed and CRGE-pretreated cells. This separation was attributed to remarkably negative PC1 loading at 2,962, 2,924, and 2,852 cm^{-1}, associated with the C-H stretching of lipids. The positive score plot of the control indicated a higher lipid content compared to PM2.5 exposure. Additionally, PC1 loading at 1,658, 1,641, 1,548, and 1,552 cm^{-1} suggested the presence of α-helix protein structure for amide I and β-sheet protein and amide II, respectively. These findings are consistent with the results from the PC1 score plot and the 2nd derivative spectra, indicating that the control had a higher absorbance intensity than CRGE-pretreated and PM2.5 exposed cells. Consequently, PM2.5 exposure reduced the lipid region and affected the protein structure. Furthermore, the PC2 loading plot also discriminates loading spectra, as PC1 did, indicating the presence of lipids and protein regions. Additionally, PM2.5 exposure exhibited negativity at 1,238 cm^{-1}, resulting from asymmetrical stretching (PO^{2-}) mainly in nucleic acids. These results are consistent with the second derivative spectra.

These results suggest that CRGE treatment may provide protection to the cell membrane through its antioxidant properties. Inflammatory responses often trigger an excessive production of reactive oxygen species (ROS), which can lead to protein oxidation, lipid peroxidation, and damage to nucleic acids. Phenolics, in this context, play a vital role as scavengers of ROS, effectively locating and neutralizing radicals before they can inflict damage upon the cellular structure. Therefore, the phenolic-enriched extract derived from CRGE could serve as a shield for proteins, preventing their oxidation during instances of oxidative stress, while enhances the cellular protein content.

6.5 Conclusion

FTIR generates data indicating its potential for assessing the biochemical changes of CRGE pretreatment against PM2.5 cytotoxicity in A549 cells. The results suggest that the combination of RWE and GEE could influence lipids, including phospholipids, leading to cytoplasmic membrane damage. The protein structures of these treated cells showed decreased peak intensities, but they can recover when pretreated with CRGE. Furthermore, there was a release of nucleic acids along with changes in RNA and DNA. Although FTIR offers evidence of the biochemical changes facilitated by CRGE, further research is imperative to gain a more comprehensive understanding of the changes in biomolecular structure.

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CHAPTER VII

SUMMARY

Chemical antioxidant activities demonstrate Additive interactions between RWE and GEE extracts in various antioxidant assays, such as ABTS+ radical cation scavenging capacity, DPPH free radical scavenging capacity, ferric reducing antioxidant power, and ferric chelating capacity. Additionally, the combined extract of RWE and GEE at a ratio of 5:1 exhibited a synergistic effect in DPPH and FRAP assays. Moreover, the combined extracts showed a cytoprotective effect against H_2O_2 toxicity and attenuated intracellular ROS generation. Furthermore, gene expression analysis by qRT-PCR revealed that HO-1, SOD, CAT, and NQO1 antioxidant genes were induced by extract treatments, demonstrating synergistic effects. Therefore, the combination of RWE and GEE has the potential to significantly enhance antioxidant capacities. CRGE can act by directly scavenging free radicals and/or modulating intracellular ROS levels and altering the expression of antioxidant genes synergistically. However, the ratio of combined extracts should be carefully considered, as it may affect the level of synergism.

PM2.5 causes cell cytotoxicity by inducing ROS production and regulating the expression of antioxidative genes include cause toxication in human epithelial lung cells (A549). Pre-treatment with RWE, GEE, and CRGE significantly reduces PM2.5-induced cell toxicity and intracellular ROS production. Furthermore, the combined extracts upregulate the expression of antioxidative genes such as HO-1, SOD, and CAT, even in cells subjected to oxidative stress induced by PM2.5 in human epithelial lung cells. Nonetheless, the diverse compositions of PM2.5 introduce limitations that influence the effectiveness of these extracts in combating PM2.5-induced oxidative stress. The anti-inflammatory properties of the combined extracts were evaluated in human macrophage THP-1 cells exposed to PM2.5. Nitric Oxide (NO) levels and the expression of the proinflammatory marker Interleukin-6 (IL-6) were measured. The results showed that the combined extract at a ratio of 1:10 (v/v), containing a higher

concentration of GEE, significantly reduced NO and IL-6 levels compared to using GEE alone. Biomolecular changes caused by PM2.5 exposure and the protective effect of the combined extract in A549 cells were further analyzed using Synchrotron Fourier Transform Infrared (SY-FTIR) spectroscopy and Principal Component Analysis (PCA).

APPENDIX

Information about Standard Reference Material® 2786 Fine Atmospheric Particulate Matter

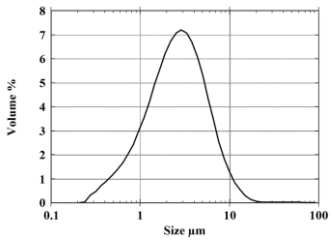

Figure A.1 Particle size distribution for SRM 2786 after 1 h. The solid line represents the volume in percentage.

Table A.1 Analyze composition in SRM 2786.

composition	Mass Fraction (µg/kg)
Inorganic constituents	167,794.9
Nitro-substituted PAHs (nitro-PAHs)	1,411.9
Sugars	592.9
Hexabromocyclododecane (HBCD) isomers	561.0
Polybrominated diphenyl ether (PBDE) congeners	10.5
Polycyclic aromatic hydrocarbons (PAHs)	7.9
Dibenzofuran (PCDF) congeners	7.3
Polychlorinated dibenzo-p-dioxin (PCDD)	6.0

Table A.2 Inorganic Constituents in SRM 2786.

Inorganic Constituents	Mass Fraction (µg/kg)
Calcium	73500 ± 1900
Iron	48900 ± 2400
Magnesium	8300
Chlorine	17390 ± 440
Sodium	14920 ± 370
Titanium	2460 ± 140
Zinc	1793 ± 88
Nickel	243 ± 4
Antimony	192.1 ± 9.4
Arsenic	36.7 ± 1.1
Lanthanum	20.72 ± 0.68
Cobalt	19.55 ± 0.96
Scandium	7.2
Thorium	5.8
Cesium	4.01 ± 0.22
Samarium	2.840 ± 0.090

Table A.3 Ginger root information.

Major component(s)	Manufacturer	Product name	Matrices	Lebel claim	Estimated level (w/w purity)
Ginger	Now Foods	Ginger Root extracts, 250 mg	Capsule	12.5 mg gingerols/capsules (498.02 mg/capsule)	2.51% total constituents

BIOGRAPHY

Ms. Chattip Sunthrarak was born on May 5, 1988, in Lampang Province, Thailand. She received a bachelor's degree in B.Sc. (Food Science and Technology) from the Faculty of Agro-Industry, Chiang Mai University, Thailand, in 2010. She was awarded the Industrial and Research Projects for Undergraduate Students (IRPUS) fund. In 2015, she earned a Master of Science in Product Development from the Faculty of Agro-Industry at Kasetsart University, Thailand, and presented orally at the 8th Thailand-Taiwan Bilateral Conference in the same year.

During her graduate studies, she worked in the food industry for 14 years in product development and quality assurance. In 2018, she received the One Research One Grant (OROG) Scholarship from Suranaree University of Technology to pursue a Doctor of Philosophy in Food Technology. She also secured research grants for graduate education development in 2022 from the National Research Council of Thailand (NRCT). During this time, she presented her research at the Food Innovation Conference (FIAC) in 2022, where she received an honorable mention in the graduate student poster competition. She also delivered an oral presentation at the Food and Applied Bioscience 2022 conference and published her research titled "Chemical Antioxidant Activity of Thunbergia Laurifolia Linn. (Rang Chuet) Leaves and its Combined Extracts" in the Food and Applied Bioscience Journal, Volume 10 (1), pages 13-29.