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Research article

Anti-Inflammatory and Antioxidant Activities of Crude Protein Extracts from *Etlingera pavieana* Rhizomes Grown at Different Cultivation Sites in Thailand

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Abstract: This study investigated the anti-inflammatory and antioxidant activities of crude protein extracts derived from *Etlingera pavieana* rhizomes cultivated in three eastern provinces in Thailand: Rayong, Chanthaburi, and Trat. Crude proteins were extracted using Tris-HCl buffer, and protein profiles were evaluated by SDS-PAGE. Anti-inflammatory activity was assessed via nitric oxide (NO) inhibition in lipopolysaccharide-stimulated RAW 264.7 macrophages, while antioxidant activity was assessed using DPPH radical scavenging, metal-chelating, and reducing power assays. All the crude protein extracts exhibited dose-dependent biological activities, with no significant cytotoxicity observed. The extract from Trat rhizomes showed the highest NO inhibition, DPPH scavenging activity, and reducing power, while the Rayong sample exhibited the strongest metal chelation. The findings demonstrate that the cultivation site affects the bioactivity of crude protein extracts, underscoring the potential application of *E. pavieana* rhizome proteins as functional ingredients in food or nutraceutical products.

Keywords: *Etlingera pavieana*; crude protein extract; antioxidant activity; anti-inflammatory activity; cultivation site

1. Introduction

Plant-based protein extraction has attracted significant scientific interest in recent years due to the growing global demand for alternative protein sources in the food industry. This demand is driven not only by increasing consumer preference for sustainable and health-promoting ingredients, but also by environmental and health concerns associated with animal protein production. Plant proteins offer distinct advantages over animal proteins, including higher dietary fiber content and the presence of bioactive compounds with potential health benefits [1]. Furthermore, plant proteins are considered more sustainable and lower in fat, making them ideal for use in functional foods and

nutraceuticals [2]. Plants of the Zingiberaceae family are traditionally valued for their culinary and medicinal uses. However, accumulating evidence indicates that crude protein extracts from the rhizomes of various Zingiberaceae species also exhibit biological activities, highlighting them as potential sources of bioactive compounds [3, 4, 5]. *Etlingera pavieana* (Pierre ex Gagnep.) R.M. Sm., a member of the Zingiberaceae family, is indigenous to eastern Thailand. Its rhizomes are commonly used in the regional cuisine and herbal remedies and are currently cultivated as intercrops in fruit orchards [6, 7]. Previous studies have found that *E. pavieana* rhizomes exhibit several biological properties, including antioxidant, anti-inflammatory, antimicrobial, and anticancer effects [8, 9, 10]. These findings signify the significant potential of *E. pavieana* rhizomes as functional ingredients in health-promoting food and supplements. However, the bioactivity of protein extracts derived from *E. pavieana* rhizomes remains largely unexplored.

Cultivation location can influence the phytochemical composition and biological activities of plants. Hao et al. [11] found that mulberry cultivated in Guangxi, China, exhibited higher antioxidant activity than those grown in Guangdong and Chongqing. Similarly, Khumaida et al. [12] conducted a comparative study on *Curcuma zedoaria* rhizomes from various cities in Indonesia and found that specimens grown in Pakem, Yogyakarta, had the highest phenolic content. Yahyaoui et al. [13] also reported significant regional variations in antioxidant activity and phenolic content in hawthorn fruits in Tunisia. These findings demonstrate the influence of geographic origin on the functional properties of plant-derived extracts. The present study compared the antioxidant and anti-inflammatory activities of crude protein extracts obtained from *E. pavieana* rhizomes cultivated in three provinces in eastern Thailand: Rayong, Chanthaburi, and Trat. The anti-inflammatory activity of the extracts was assessed by measuring the inhibition of nitric oxide (NO) in lipopolysaccharide (LPS)-stimulated macrophages, which are key effector cells in the innate immune response. Antioxidant activity was evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging, metal-chelating, and reducing power assays. The findings of this study are expected to advance the scientific understanding of *E. pavieana* as a source of bioactive proteins and confirm its economic and health value in the development of functional foods and nutraceuticals.

2. Materials and Methods

2.1 Materials

Acrylamide, aminoguanidine, Coomassie Brilliant Blue R-250, DPPH, Dulbecco's Modified Eagle Medium (DMEM), EDTA, ferrozine, fetal bovine serum (FBS), LPS from *Escherichia coli* O111:B4, N-(1-naphthyl) ethylenediamine dihydrochloride, potassium ferricyanide, sulfanilamide, sodium carbonate, and thiazolyl blue tetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used were of analytical grade.

2.2 Methods

2.2.1 Protein Extraction

Fresh *E. pavieana* rhizomes were harvested in October 2023 at the stage of commercial maturity, which local farmers typically define as approximately one year after planting. Samples were collected from agricultural plantations in Chanthaburi, Rayong, and Trat. The rhizomes were thoroughly washed with tap water, sliced into smaller pieces, and then dried completely at 55 °C. The dried samples were ground using a mechanical grinder and stored at 4 °C until used for protein extraction. Protein extraction was performed as described by Deesrisak et al. [14], with slight modifications. Briefly, crude protein was extracted by mixing 30 g of rhizome with 450 mL of 10 mM Tris-HCl buffer (pH 7.0) and shaking the mixture at room temperature for 24 h. The resulting extract was filtered, and the protein amount was examined using the Bradford assay.

2.2.2 Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

SDS-PAGE consisted of a 12.5% separating gel and a 4% stacking gel. Each sample, consisting of 20 µg protein mixed with 6× bromophenol blue loading dye, was denatured by heating at 95°C for 5 minutes and then loaded into a gel. A pre-stained molecular weight protein marker was applied to the reference lane. SDS-PAGE was performed at a constant voltage of 120 V. Subsequently, the gel was stained with Coomassie Brilliant Blue R-

250, followed by destaining until clear protein bands became visible. The molecular weights of the protein bands were estimated by comparison with the standard protein marker.

2.2.3 Cell Viability Assay

Macrophage RAW 264.7 cells were obtained from the ATCC (Rockville, MD, USA). Cell viability was assessed using the MTT assay, as described by Srisook et al. [15]. RAW 264.7 cells (1.5×10^5 cells/well) were seeded into 24-well plates. The cells were then treated with protein extracts and LPS (1 μg/mL) for 24 hours. After treatment, the medium was removed and replaced with DMEM containing 0.1 mg/mL MTT, followed by incubation for 2 hours. Formazan crystals were solubilized in DMSO, and the absorbance was measured at 550 nm using a microplate reader (Versamax, USA). Cell viability was calculated as follows:

$$\% \text{ Cell viability} = (A_{treated}/A_{control}) \times 100$$

2.2.4 Nitric Oxide Inhibition Assay

No inhibition by the protein extracts was assessed using the Griess reaction, as described by Srisook et al. [15]. RAW 264.7 macrophages were seeded as described above and treated with protein extracts and LPS (1 μg/mL) for 24 h. Nitrite concentrations were determined using a sodium nitrite standard curve (3.12–50 μM), with aminoguanidine serving as the positive control. NO inhibition was calculated as follows:

$$\% \text{ NO inhibition} = 100 \times [(\text{Nitrite concentration of treated well/LPS control}) \times 100]$$

2.2.5 DPPH Radical Scavenging Assay

DPPH radical scavenging activity was evaluated as described by Pechroj et al. [16], with slight modifications. Protein extracts at various concentrations were dissolved in 10 mM Tris-HCl buffer (pH 7.0) and mixed with a 0.2 mM DPPH solution prepared in methanol in a microplate. For the assay, 50 μL of each extract was combined with 100 μL of the DPPH solution and incubated at room temperature in the dark for 30 min. Absorbance was then measured at 517 nm. Ascorbic acid was used as the positive control. The percentage of DPPH scavenging activity was calculated as follows:

$$\% \text{ DPPH scavenging} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

2.2.6 Reducing Power Assay

The reducing power of the extracts was determined following the method described by Uthairat et al. [17]. Briefly, protein extract (50 μg/mL) was mixed with 1% potassium ferricyanide. The mixture was incubated for 30 minutes, then 10% TCA was added, and it was centrifuged at 3000 × g for 10 minutes. The supernatant was combined with distilled water and 0.1% ferric chloride. The absorbance was measured at 700 nm. Reducing power was expressed as gallic acid equivalents (GAE) per gram of extract, based on a gallic acid standard curve.

2.2.7 Metal-Chelating Activity

Metal-chelating activity was evaluated as described by Uthairat et al. [17]. The reaction mixture consisted of 10 μL of 2 mM ferrous sulfate, 200 μL of protein extract or EDTA (positive control), and 20 μL of 5 mM ferrozine. The absorbance was measured at 562 nm using a microplate reader. Chelating activity was calculated as follows:

$$\% \text{ Chelating activity} = [(A_{control} - A_{sample})/A_{control}] \times 100$$

2.2.8 Statistical Analysis

All data are expressed as the mean ± standard deviation (n=3). Statistical analysis was performed using Minitab 18. One-way ANOVA was performed, followed by Tukey's post hoc test to determine significant differences between groups at $p < 0.05$.

3. Results and Discussion

3.1 SDS-PAGE Analysis of Crude Protein Extracts from E. pavieana Rhizomes

The protein profiles of the crude extracts derived from *E. pavieana* rhizomes collected from Rayong, Chanthaburi, and Trat provinces were evaluated by SDS-PAGE. Protein bands were observed across a molecular weight range of approximately 16–175 kDa in all samples (Figure 1). Notably, for each extract,

prominent bands appeared at approximately 25, 35, 40, and 51 kDa, indicating the presence of major protein constituents that may have been conserved among rhizomes from different cultivation regions.

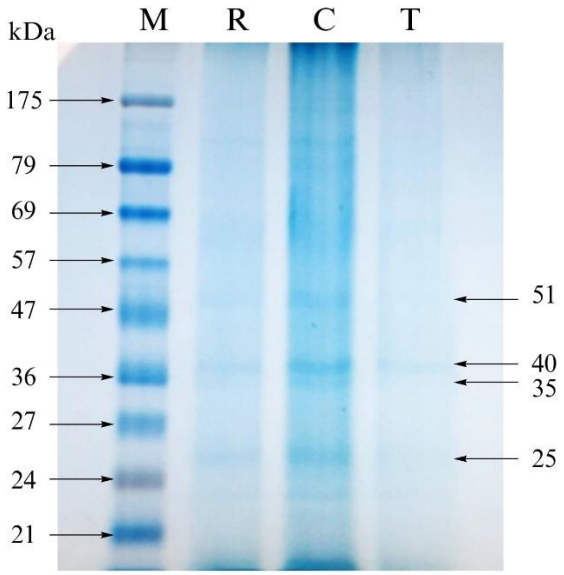

Figure 1. SDS-PAGE profiles of crude protein extracts from Etlingera pavieana rhizomes collected from the three provinces. Lane M: molecular weight marker; Lane R: Rayong; Lane C: Chanthaburi; Lane T: Trat

3.2 Nitric Oxide (NO) Inhibitory Effect of Crude Protein Extracts from E. pavieana Rhizomes

The cytotoxicity of crude protein extracts derived from *E. pavieana* rhizomes collected from the Chanthaburi, Rayong, and Trat provinces was evaluated in RAW 264.7 macrophages. At concentrations of 0.04–0.16 μg/mL, the extracts showed no statistically significant cytotoxicity compared to the untreated controls. Although slight reductions in cell viability were observed at concentrations of 0.31–1.25 μg/mL, all viability values remained above 85% (Figure 2). Therefore, concentrations ranging from 0.04 to 1.25 μg/mL were used for further NO inhibition assays. NO is an inflammatory mediator synthesized from the amino acid L-arginine by nitric oxide synthase (NOS). This reaction produces NO and L-citrulline, requiring cofactors such as NADPH, FAD, FMN, heme, calmodulin, and tetrahydrobiopterin [18]. In inflammation triggered by infection or foreign stimuli, immune cells, such as macrophages, are activated and induce the expression of inducible NOS (iNOS), which triggers excessive NO production. NO reacts with superoxide anions ($O_2^{\bullet-}$) to form peroxynitrite ($ONOO^-$), which kills microbes but also causes tissue damage, thereby intensifying the inflammatory response. The prolonged overproduction of NO is associated with chronic inflammation and the pathogenesis of various diseases, such as rheumatoid arthritis, inflammatory bowel disease, diabetes, and neurodegenerative conditions, such as Alzheimer's disease [19, 20]. Thus, the absence of NO inhibition serves as an indicator of anti-inflammatory potential.

The anti-inflammatory activity of the crude protein extracts from *E. pavieana* rhizomes was assessed by measuring the accumulation of nitrite, a stable end-product of NO oxidation, in the culture supernatant. Untreated control cells and cells stimulated with LPS alone yielded nitrite levels of 0.03 ± 0.02 and 12.85 ± 0.15 μM, respectively. Treatment with crude protein extracts (0.04–1.25 μg/mL) significantly reduced NO production in LPS-stimulated RAW 264.7 macrophages in a concentration-dependent manner (Figure 3). Therefore, the observed reduction in NO production was not caused by the cytotoxic effects of the extracts, but rather by their NO-inhibitory properties. The half maximal inhibitory concentration (IC_{50}) values for NO inhibition indicated that the extract from Trat samples exhibited the most potent inhibitory effect, followed by those from Rayong and Chanthaburi (Table 1). Aminoguanidine (50 μM), which was used as a positive control, inhibited NO production by 61% compared with the LPS-only group. To the best of our knowledge, this is the first report demonstrating the anti-inflammatory potential of crude protein extracts from *E. pavieana* rhizomes. Chantaranothai et al. [3] investigated the NO inhibitory activity of crude protein extracts from 15 species of

Zingiberaceae rhizomes and identified only four species that showed inhibitory activity: *Curcuma aeruginosa* Roxb., *Curcuma aromatica*, *Hedychium coronarium*, and *Zingiber ottensii* Valeton. The present study demonstrated that the crude protein extracts from *E. pavieana* also possess strong NO inhibitory activity. Notably, the IC_{50} values observed here were approximately 38- to 100-fold lower than those previously reported for the species. However, differences in the extraction methods between the two studies may have contributed to the discrepancy.

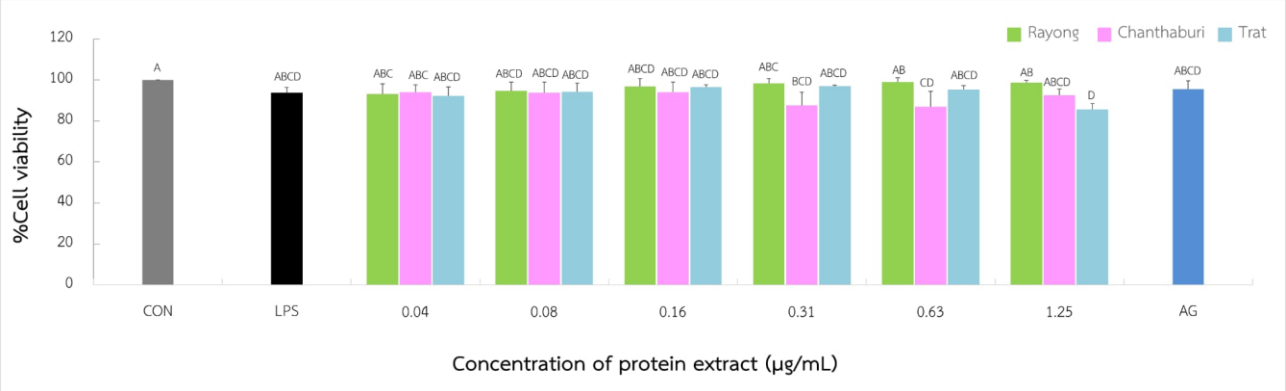

Figure 2. Effect of crude protein extracts from *Etlingera pavieana* rhizomes on the viability. RAW 264.7 macrophages were treated for 24 h with the indicated extract concentrations in the presence of LPS (1 µg/mL). Cell viability was assessed using the MTT assay. CON, control; LPS, lipopolysaccharide; AG, aminoguanidine. Different superscript letters (A–F) indicate statistically significant differences between groups at $p < 0.05$.

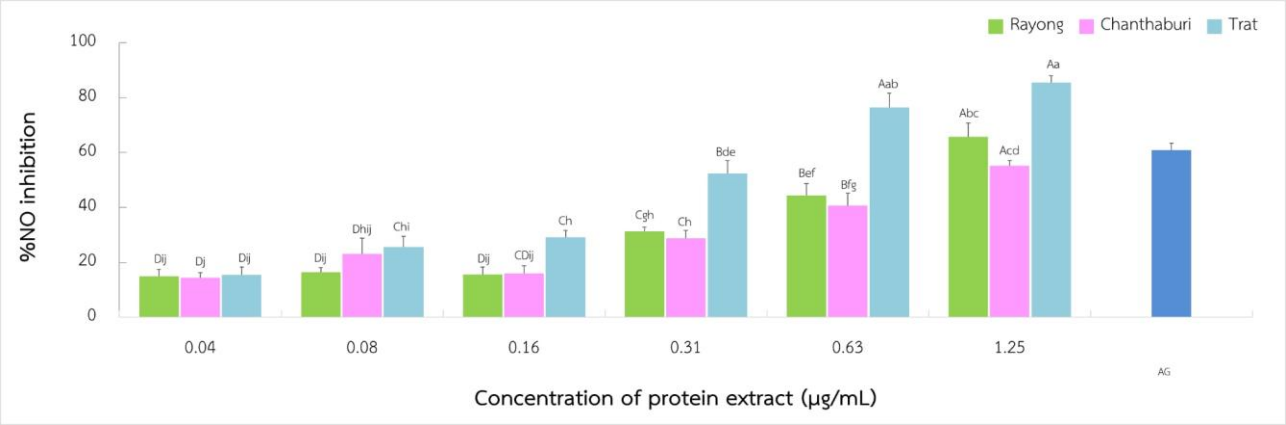

Figure 3. Inhibitory effects of crude protein extracts from *Etlingera pavieana* rhizomes on nitric oxide (NO) production in lipopolysaccharide-stimulated RAW 264.7 macrophages. Cells were stimulated with 1 µg/mL LPS and co-treated with various concentrations of the extract. The nitrite content of the culture supernatant was measured using the Griess reagent. Different uppercase letters (A–E) indicate statistically significant differences among concentrations within the same province, whereas different lowercase letters (a–g) indicate significant differences between provinces at the same concentration. Statistical significance was set at $p < 0.05$. AG, aminoguanidine.

Table 1. IC_{50} values for nitric oxide inhibition in lipopolysaccharide-stimulated RAW 264.7 macrophages treated with crude protein extracts from *Etlingera pavieana* rhizomes collected from three different cultivation locations

Province	IC_{50} value (μg/mL)
Rayong	0.84 ± 0.08^{b}
Chanthaburi	1.03 ± 0.07^{c}
Trat	0.46 ± 3.41^{a}

Different superscript letters (a, b, c) indicate statistically significant differences between groups at $p < 0.05$. IC_{50}, half maximal inhibitory concentration.

3.3 Antioxidant Activity of Crude Protein Extracts from E. pavieana Rhizomes

The antioxidant activity of the crude protein extracts from *E. pavieana* rhizomes was determined using the DPPH radical scavenging assay. All extracts exhibited concentration-dependent scavenging activity (Figure 4), with half-maximal effective concentration (EC_{50}) values ranging from 7.31 ± 0.11 to 11.52 ± 0.16 μg/mL. Based on EC_{50} values, extracts from Trat and Rayong exhibited significantly greater scavenging activity than those from Chanthaburi. In this study, the DPPH radical scavenging activities of the extracts from Rayong and Trat were notably higher than those from Chanthaburi (Table 2). The antioxidant capacity of the crude protein extracts was greater than that of ascorbic acid, a widely known reference antioxidant. These findings are consistent with previous studies on the DPPH radical scavenging activities of crude protein extracts from other Zingiberaceae rhizomes, which report variations in antioxidant potency across plant species [3, 4]. However, among the 15 species evaluated by Chantaranothai et al. [3], three species, K*aempferia galanga* Linn., *Alpinia galanga* (Linn.) Swartz and Boesenbergia pandurata Roxb. did not exhibit detectable DPPH radical scavenging activity. This lack of activity may be attributed to either the absence of active protein components or their low activity. The DPPH radical scavenging activity of plant-derived proteins or peptides is primarily attributed to specific amino acid functional groups, such as the hydroxyl groups (−OH) in tyrosine and serine; thiol groups (-SH) in cysteine; aromatic rings in tyrosine, tryptophan, and phenylalanine; and amino groups in lysine and arginine. These functional groups can donate hydrogen atoms or electrons to DPPH radicals, reducing them from their purple-colored radical form to the yellow-colored reduced form (DPPH-H) [21]. However, since the DPPH solution was prepared in methanol, we cannot exclude the possibility that methanol-sensitive proteins partially precipitated, or that co-extracted phytochemicals contributed to the observed antioxidant activity. Therefore, the results should be interpreted as reflecting the overall radical scavenging capacity of the extracts rather than protein-specific activity.

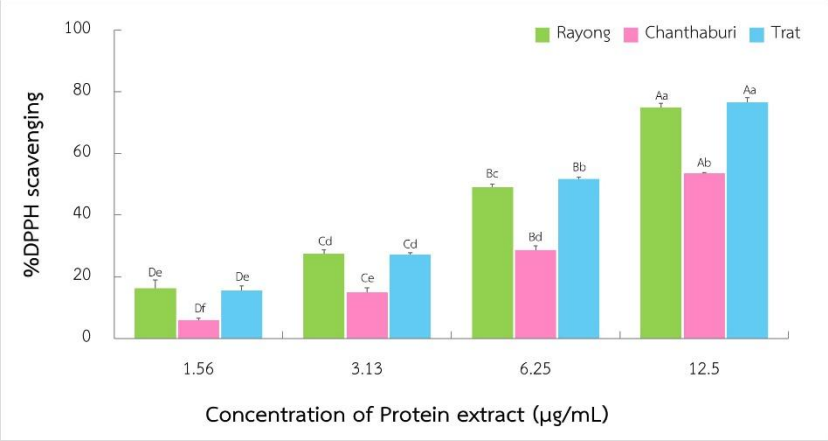

Figure 4. DPPH radical scavenging activity (%) of different concentrations of crude protein extracts from *Etlingera pavieana* rhizomes collected from Rayong, Chanthaburi, and Trat provinces. Different uppercase letters (A–E) indicate significant differences among concentrations within the same province, while different lowercase letters (a–g) indicate significant differences between provinces at the same concentration ($p < 0.05$).

Table 2. DPPH radical scavenging activity (EC_{50}) of the crude protein extracts from *Etlingera pavieana* rhizomes collected from the three provinces and the positive control (ascorbic acid)

Province	EC_{50} for DPPH scavenging (μg/mL)
Rayong	7.53 ± 0.19^{a}
Chanthaburi	11.52 ± 0.16^{b}
Trat	7.31 ± 0.11^{a}
Ascorbic acid	15.22 ± 0.77

Different superscript letters (a, b) indicate statistically significant differences between the groups at $p < 0.05$.

Crude protein extracts from *E. pavieana* rhizomes collected in Rayong, Chanthaburi, and Trat provinces showed significant variation in reducing power ($p < 0.05$). The extract from Trat rhizomes demonstrated the highest reducing activity, followed by those from Rayong and Chanthaburi (Table 3). The redox activity involving the electron donation of proteins and peptides is primarily attributed to the presence of specific functional groups capable of donating electrons that facilitate the reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}). Functional groups, such as hydroxyl, thiol, and peptide bonds, are crucial in that they underlie the strong antioxidant potential of protein-based compounds [21, 22]. Furthermore, the crude protein extracts may also contain trace amounts of naturally occurring phenolic compounds in *E. pavieana* rhizomes, which could further enhance their DPPH radical scavenging activity and reducing power by donating electrons or neutralizing free radicals, thereby mitigating oxidative damage in cells.

Table 3. Reducing power of crude protein extracts from *Etlingera pavieana* rhizomes collected from the three provinces.

Province	Reducing power (mg GAE/g extract)
Rayong	205.05 ± 4.28^{b}
Chanthaburi	153.24 ± 1.59^{c}
Trat	363.70 ± 3.41^{a}

Different superscript letters (a, b, c) indicate statistically significant differences between the groups at $p < 0.05$.

The metal-chelating activity of proteins is a key antioxidant mechanism, particularly in inhibiting iron-catalyzed oxidative reactions, such as the Fenton and Haber–Weiss reactions, which generate highly reactive hydroxyl radicals. In this study, various concentrations (0.39–6.25 μg/mL) of crude protein extracts from E. pavieana rhizomes were evaluated for their ability to chelate ferrous ions, showing significant concentration-dependent increases in metal chelation activity ($p < 0.05$). The Rayong extract exhibited the highest chelating effect (6.25 μg/mL), although no significant differences were observed between the Trat and Chanthaburi extracts (Figure 5). The chelating ability of these protein extracts stems from specific amino acid residues that can coordinate metal ions, particularly those with functional groups, such as the imidazole group in histidine, the thiol group in cysteine, and carboxyl groups in aspartic and glutamic acids. These groups serve as electron-donating sites, facilitating stable interactions with metal ions and thereby preventing the catalytic formation of reactive oxygen species [23-24]. Although minor variations were observed among the three extracts, the metal-chelating capacities were comparable overall, indicating the limited impact of geographic origin on this metal-chelating property.

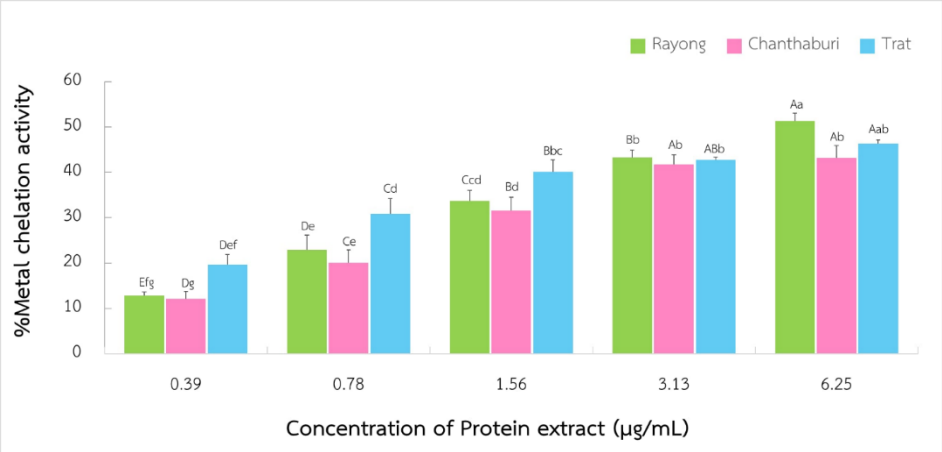

Figure 5. Metal chelating activity of crude protein extracts from *Etlingera pavieana* rhizomes collected from the Rayong, Chanthaburi, and Trat provinces. Different capital letters (A–E) indicate statistically significant differences among concentrations within the same province, while different lowercase letters (a–g) indicate significant differences between provinces at the same concentration ($p < 0.05$).

The differences in the anti-inflammatory and antioxidant activities of the crude protein extracts derived from *E. pavieana* rhizomes collected from Rayong, Chanthaburi, and Trat may be attributed to environmental, agronomic, and biochemical factors, as well as the harvesting stage. As demonstrated in previous studies, variations across cultivation sites can significantly affect the phytochemical profiles and bioactive compound contents of medicinal plants [11, 13]. Soil composition, microclimate, and sunlight exposure, among other factors, influence not only the yield but also the molecular structure and functionality of plant proteins and associated metabolites [12]. For protein extracts, the composition and abundance of amino acid residues with antioxidant or metal-chelating functional groups—such as hydroxyl, thiol, and carboxyl groups found in tyrosine, cysteine, and aspartic acid—may vary depending on the growing conditions. These residues are critical for radical scavenging and reducing power [21, 23]. Furthermore, despite the standardization of protein extraction procedures, trace levels of co-extracted secondary metabolites, such as phenolics or flavonoids, which synergistically enhance antioxidant effects, may differ between samples due to regional variations in metabolic pathways [3, 4]. These minor components may contribute to the variability observed in the DPPH scavenging, reducing power, and nitric oxide inhibition assays. Taken together, these results demonstrate the impact of environmental and agronomic factors on the biological activity of plant protein extracts. Understanding these variations is crucial for optimizing cultivation site selection and standardizing the quality of plant-based functional ingredients. Nevertheless, future studies employing protease digestion or protein depletion experiments will be required to confirm that the observed effects are specifically attributable to proteins.

4. Conclusions

In summary, the findings of this study demonstrate that crude protein extracts from *E. pavieana* rhizomes collected from three different cultivation sites exhibited variations in anti-inflammatory and antioxidant activities. Trat extracts showed the highest NO inhibitory activity and reducing power, while Rayong extracts exhibited the strongest metal-chelating capacity. These results contribute to the growing body of research on plant-derived protein extracts, particularly within the Zingiberaceae family, and highlight the impact of cultivation site on bioactivity. This study also highlights the potential of *E. pavieana* protein extracts as functional ingredients for health-promoting applications. Further studies should focus on identifying specific bioactive peptides and elucidating their mechanisms of action.

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Conflicts of Interest: "The authors declare no conflict of interest."

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