Journal of Food Health and Bioenvironmental Science
Journal homepage : http://jfhb.dusit.ac.th/

Comparison of Extraction Techniques for 1'-Acetoxychavicol Acetate from Dried *Alpinia galanga* (L.) Willd. Rhizomes

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Article info

Article history:
Received : 30 May 2024
Revised : 3 July 2024
Accepted : 11 July 2024

Keywords:
1'-Acetoxychavicol acetate, *Alpinia galanga* (L.) Willd, Extraction, HPTLC

Abstract

Alpinia galanga (L.) Willd., a member of the Zingiberaceae family, contains various biologically active compounds. Among these, 1'-acetoxychavicol acetate (ACA) is a major volatile component in the galangal rhizome, exhibiting numerous pharmacological properties. Notably, ACA is absent in galangal essential oil obtained through steam distillation. This study aimed to determine the best extraction technique for ACA from galangal rhizome powder (GRP). GRP was subjected to four extraction techniques (maceration extraction (ME), ultrasonic-assisted extraction (UAE), reflux extraction (RE) and Soxhlet extraction (SE) and the resulting crude extracts (CEs) were analyzed for ACA content using high-performance thin-layer chromatography (HPTLC). The ACA content was expressed as a percentage of weight per CE and GRP. The study found that the extraction technique significantly influenced the yield of CEs and the quantity of ACA per CE and GRP. The SE technique yielded the highest amount of CEs (19.15 ± 0.66%). However, the RE technique provided the highest ACA content per CE (41.77 ± 4.58%) and per GRP (3.79 ± 0.57%), even though it did not yield the highest CE (9.05 ± 0.44%). The technique that produced the highest CEs resulted in more impurities than the technique that yielded the highest ACA content. Therefore, it was concluded that RE is the best technique to achieve the highest percentage of ACA per CE and per GRP.

Introduction

Kha, scientifically known as *Alpinia galanga* (L.) Willd., is highly valued in Thai traditional medicine for its diverse therapeutic properties. This rhizomatous herb is celebrated for its effectiveness in treating various ailments. In traditional practices, Kha is often used to address digestive issues such as bloating, indigestion and nausea. Its warming properties make it an effective remedy for colds, coughs and respiratory congestion (Jiratchariyakul & Mahady, 2005). Additionally, Kha is renowned for its analgesic properties, frequently applied

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topically to alleviate pain and inflammation related to arthritis and muscle strains. It also promotes skin health due to its antimicrobial properties, which are beneficial for treating skin infections and aiding wound healing (Thongchai et al., 2013). Over the centuries, Kha has established itself as a fundamental element of Thai traditional medicine, providing a holistic approach to wellness and healing (GlobinMed, 2018).

The common name of *Alpinia galanga* (L.) Willd. is galangal. The galangal rhizome contains a variety of chemical constituents that contribute to its medicinal and culinary properties. The major constituents include volatile oils rich in sesquiterpenes such as α-pinene, β-pinene, camphene, sabinene, myrcene and limonene (Van et al., 2021; Nampoothiri et al, 2016). These volatile oils are responsible for the characteristic aroma and flavor of galangal. Additionally, the galangal rhizome contains phenolic compounds including flavonoids such as quercetin, kaempferol, and their derivatives, as well as phenolic acids like gallic acid and caffeic acid (Aljobair, 2022). Moreover, galangal contains diarylheptanoids such as 7-(4-hydroxyl-3-methoxyphenyl)-1-phenyl-4-en-3-heptanone, (R)-5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-1- phenyl-3-heptanone, alpinoid A, officinaruminane A, and alpinin A (Zhang et al, 2016). Other constituents present in galangal rhizome include alkaloids, tannins, and carbohydrates (Yan et al., 2014, Khairullah et al., 2020; Eram et al., 2019; Patwekar et al., 2022). The galangal rhizome possesses a wide array of pharmacological properties, making it a valuable medicinal plant. Its rhizome contains bioactive compounds, which contribute to its diverse pharmacological effects. Studies have shown that its rhizome exhibits antioxidant properties, scavenging free radicals and reducing oxidative stress, thereby potentially preventing various chronic diseases (Aljobair, 2022; Al-Snafi, 2014). Moreover, it displays anti-inflammatory activity by inhibiting the production of inflammatory mediators like cytokines and prostaglandins, suggesting its usefulness in treating inflammatory conditions such as arthritis (Verma et al., 2011; Verma & Sharma, 2022). Galangal also demonstrates antimicrobial activity against a range of pathogens, including bacteria, fungi, and viruses, indicating its potential in combating infections (Aljobair, 2022; Chouni & Paul, 2018). Additionally, it has been found to possess antidiabetic properties by modulating glucose metabolism and enhancing insulin sensitivity (Verma et al., 2011; Verma & Sharma, 2022). Furthermore, galangal rhizome has been investigated for its neuroprotective, hepatoprotective and anticancer activities, highlighting its potential as a versatile therapeutic agent in traditional and modern medicine (Chouni & Paul, 2018; Sing & Negi, 2022).

ACA, the pungent principal of galangal rhizomes, is one of the major volatile components. ACA has garnered significant attention due to its potential pharmacological properties and health benefits. It possesses anti-inflammatory, antioxidant, antimicrobial, and anticancer activities, making it a promising candidate for various medicinal applications (Yang & Eilerman, 1999; Kojima-Yuasa & Matsui-Yuasa, 2020). ACA has been studied extensively for its role in inhibiting inflammatory mediators such as cyclooxygenase and lipoxygenase enzymes, thus suggesting its potential in treating inflammatory conditions (Kojima-Yuasa & Matsui-Yuasa, 2020). Additionally, ACA's antioxidant properties help in scavenging free radicals, which can reduce oxidative stress and prevent cellular damage (Kojima-Yuasa & Matsui-Yuasa, 2020; Chouni & Paul, 2018). Furthermore, its antimicrobial activity makes it effective against various pathogens (Chouni & Paul, 2018), while its anticancer properties have shown promise in inhibiting the growth of cancer cells (Kojima-Yuasa & Matsui-Yuasa, 2020). Overall, ACA represents a valuable bioactive compound in galangal rhizome with potential therapeutic implications in treating a range of health conditions (Kojima-Yuasa & Matsui-Yuasa, 2020; Yang & Eilerman, 1999). ACA is not stable in aqueous solutions and undergoes hydrolysis/isomerization reactions (Yang & Eilerman, 1999). The degradation of ACA from galangal can be influenced by the extraction technique. During extraction, it is important to avoid using strong acidic or basic solvents, as these can hydrolyze ACA or cause other undesirable reactions. High extraction temperatures can accelerate the breakdown of ACA, especially through isomerization and hydrolysis processes. Extended extraction times, even at mild temperatures, can gradually degrade ACA. Finding effective extraction techniques that reduce processing time can help minimize this problem (Kojima-Yuasa & Matsui-Yuasa, 2020). As a result, ACA is absent in galangal essential oil obtained by steam distillation (Yang & Eilerman, 1999). To achieve high ACA content from galangal rhizome powder (GRP), the correct extraction technique is essential.

This study aimed to compare four extraction techniques, namely maceration extraction (ME), ultrasonic-assisted extraction (UAE), reflux extraction

(RE) and Soxhlet extraction (SE), to determine the highest ACA content from GRP. ME is one of the oldest and simplest extraction techniques used in various fields such as food science, pharmacy and chemistry to extract flavors, active compounds, or compounds from plant materials (Chibuye et al, 2023). In this method, plant material, either in coarse or as a powder, is soaked in a solvent (such as water, alcohol, or oil) for an extended period. Prolonged soaking causes the cell walls to break down, releasing bioactive components into the solvent. The resulting solution can then be filtered through a filter press to separate the plant material and recover the bioactive compounds. The efficiency factors of ME encompass several variables crucial to optimizing the process. These factors include the choice of solvent, which significantly influences extraction efficacy through its polarity and solvent power; the solvent-to-solid ratio, which determines the extent of contact and dissolution; temperature, which affects solubility and diffusion kinetics; the duration of maceration, which is pivotal for thorough extraction; the particle size of the plant material, which impacts the surface area and contact; agitation, which enhances solute diffusion but must be balanced to avoid degradation; pH levels, which alter solubility and interactions; the presence of co-solvents or additives, which can modify solvent properties; and the nature of the material itself, considering factors like cell structure and composition (Azwanida, 2015; Patel et al, 2019). By meticulously controlling these parameters, practitioners can enhance the efficiency and yield of ME for diverse applications in various fields. Advantages of this technique include its simplicity, lack of heat involvement, suitability for heat-sensitive compounds, low installation costs and low maintenance costs (Azwanida, 2015; Patel et al, 2019; Patra et al, 2022). Disadvantages include long extraction times, suboptimal yields, potential degradation of bioactive compounds, and fermentation risks (Azwanida, 2015; Patel et al, 2019). UAE is a technique used to enhance the extraction of bioactive compounds from plant materials by utilizing ultrasonic waves (Patel et al, 2019). In this method, the plant material is placed in a solvent, and ultrasonic waves are applied to the mixture. The ultrasonic waves create cavitation bubbles in the solvent. These bubbles rapidly expand and collapse, generating localized high temperatures and pressures. This phenomenon helps to disrupt cell walls and increase the mass transfer of compounds from the solid matrix into the solvent (Azwanida, 2015; Patel et al, 2019). As a result, extraction efficiency is improved and the process can be completed more rapidly compared to traditional extraction methods. The efficiency of UAE is influenced by several factors. The factors include the choice of solvent which plays a significant role, as it affects the solubility of target compounds and their interaction with the matrix; ultrasonic power, frequency and duration of treatment directly impacts extraction efficiency by influencing the cavitation phenomenon, which enhances mass transfer and disrupts cell walls; the particle size of the sample affects the surface area and accessibility of the compounds to the solvent, thus influencing extraction efficiency; temperature also plays a crucial role, as it affects solvent viscosity and diffusion rates; the presence of co-solvents or additives, as well as pH adjustments, can further optimize extraction efficiency by modifying solvent properties and enhancing target compound solubility (Azwanida, 2015). By carefully controlling these factors, researchers can maximize the efficiency of UAE for various applications in various fields. UAE offers several advantages, including shorter extraction times, higher extraction yields and reduced solvent consumption. Additionally, it is considered a green and environmentally friendly extraction technique due to its reduced energy consumption and solvent usage (Azwanida, 2015; Patel et al, 2019). The disadvantage of this technique includes high initial investment and the potential for product degradation (Azwanida, 2015). RE is a technique used to extract compounds from plant material by using a heated solvent (Chibuye et al, 2023). The solvent is vaporized and then condensed back onto the plant material, continuously extracting the desired compounds. This process is repeated until the desired amount of extraction is achieved (Azwanida, 2015). The efficiency factors of RE encompass several key elements. The elements include the choice of solvent which plays a pivotal role, as it determines the solubility of target compounds and their interaction with the matrix being extracted; the reflux ratio, which refers to the ratio of condensed solvent that is returned to the extraction vessel, which influences the efficiency by maintaining a steady concentration gradient for extraction; temperature control is crucial, as it impacts both solubility and reaction kinetics; optimal temperatures can enhance extraction rates without causing thermal degradation; the duration of RE affects the extent of extraction, with longer extraction times typically leading to higher yields but may also risk over-extraction or degradation of sensitive compounds; the design of the reflux apparatus, including

factors such as reflux condenser efficiency and agitation mechanisms, can also impact extraction efficiency by ensuring efficient heat transfer and maximizing contact between solvent and sample (Azwanida, 2015; Chibuye et al, 2023). Advantages of this technique include a very efficient method to extract compounds from a plant material and used to extract a wide variety of compounds from a variety of plant materials as well as being a relatively simple technique (Azwanida, 2015, Patra et al, 2022). Disadvantages of this technique include a time-consuming process especially for large plant materials, requiring specialized equipment (such as a reflux condenser and a heating mantle), it is unsuitable for thermolabile compounds, and dangerous if not done properly (Azwanida, 2015). SE is a combination of both, percolation and maceration techniques (Azwanida, 2015; Patra et al, 2022). It has been one of the most widely used extraction methods which is still used extensively. The extraction is carried out in a special apparatus known as the Soxhlet apparatus. Apparatus consists of an extraction chamber connected to a vapor duct and siphon tube which extends down to the joint where a round bottom flak can be attached. A thimble of filter paper or a cotton plug is placed in the extraction chamber to prevent the blockage of the siphon tube when powdered plant material is added. The powdered plant material is packed in the extraction chamber, and a condenser and a round bottom flask are attached to the Soxhlet apparatus at their respective positions. The solvent is then added from the top which enters the extraction chamber. Once the solvent level rises above the siphon bend, it flows into the flask through the siphon tube. The flask is heated using a water bath or heating mantle, causing the solvent to boil. Its vapors travel through a vapor duct to a condenser, where they condense and fall as droplets onto the plant material in the extraction chamber (percolation). The extraction chamber gradually fills with solvent, allowing the powdered plant material to remain in contact with the solvent (maceration). When the solvent level reaches above the siphon tube, it flows back to the round bottom flask through the siphon tube, and the cycle continues. Each time the extraction chamber receives fresh solvent, preventing saturation with solutes. This process, known as continuous extraction or Soxhlet extraction, ensures exhaustive extraction of the plant material (Azwanida, 2015). The efficiency of SE, a widely used method for extracting active compounds from plant materials, is influenced by several factors. These include the choice of solvent, which should have a high solubility for the target compounds and a low boiling point to facilitate evaporation during the extraction process; the duration and number of extraction cycles performed, which should have longer extraction times and multiple cycles generally resulting in higher yields; the size of the plant material and its surface area exposed to the solvent also play crucial roles, as smaller particle sizes and increased surface area lead to more efficient extraction; the temperature of the extraction process, which should be carefully controlled to optimize the solubility and prevent degradation of the target compounds; the design and condition of the Soxhlet apparatus, including the size and packing of the extraction thimble, can impact efficiency by influencing solvent flow rates and extraction kinetics (Azwanida, 2015; Chibuye et al, 2023). Overall, careful consideration of these factors is essential for maximizing the efficiency of SE. Advantages of this technique include the increase in mass transfer rate due to the use of high temperatures and recycling of fresh solvent (Chibuye et al, 2023). Disadvantages of this technique include a time-consuming process especially for large samples, requiring specialized equipment (such as a Soxhlet apparatus and a heating mantle), it is unsuitable for thermolabile compounds and dangerous if not done properly (Azwanida, 2015; Patra et al, 2022; Patel et al, 2019).

HPTLC operates on the same principles as traditional thin-layer chromatography, utilizing a stationary phase coated on a flat surface, typically glass, as a thin layer (Reich & Schibli, 2007). However, HPTLC enhances performance through advancements in both stationary phase technology and instrumentation. It offers higher resolution, increased sensitivity and faster analysis times compared to conventional TLC (Reich & Schibli, 2007). The principle involves the separation of compounds based on their differential partitioning between the stationary and mobile phases. As the mobile phase travels through the stationary phase, compounds are separated according to their affinity for the stationary phase, with more strongly interacting compounds moving more slowly than those with weaker interactions (Reich & Schibli, 2007; Ramu & Chittela, 2018). Detection techniques such as UV-Vis spectroscopy or fluorescence allow for visualization and quantification of separated compounds (Reich & Schibli, 2007). HPTLC offers several advantages over other chromatographic techniques, including its simplicity, cost-effectiveness and versatility (Reich & Schibli, 2007; Ramu & Chittela, 2018). One of its primary advantages is its ability to

handle multiple samples simultaneously, leading to high throughput analysis. Moreover, HPTLC requires minimal sample preparation and can separate a wide range of compounds with high resolution, deeming it suitable for various applications in pharmaceuticals, food chemistry and environmental analysis (Reich & Schibli, 2007). Additionally, HPTLC provides rapid analysis, with results typically available within minutes. However, HPTLC also has its limitations. It may lack the sensitivity and detection limits of other chromatographic techniques like high-performance liquid chromatography (HPLC) or gas chromatography (Reich & Schibli, 2007; Ramu & Chittela, 2018). Additionally, quantification can be challenging due to issues such as non-uniformity in spot application and detection, which can affect reproducibility and accuracy. Furthermore, the availability of standardized procedures and stationary phases may be limited compared to more established chromatographic methods, requiring careful optimization for specific applications. Despite these drawbacks, the simplicity, speed and versatility of HPTLC make it a valuable tool in analytical chemistry (Reich & Schibli, 2007). HPTLC densitometric technique is simple, economical and requires minimum sample cleanup. It was therefore selected in this study for the quantitative determination of ACA from GRP.

Materials and methods

1. Plant materials

The galangal rhizomes used in this research were harvested in July 2023, when they were 8 months old. These rhizomes were sourced from a farm in Samut Prakan, Thailand (coordinates: 13°32'01.0"N 100°48'59.8"E). The plant was identified and authenticated as *Alpinia galanga* (L.) Willd. by Dr. Rumrada Meeboonya, a botanist from the College of Allied Health Sciences, Suan Sunandha Rajabhat University. The freshly harvested rhizomes underwent a thorough cleaning process involving three washes with deionized water, followed by slicing and drying in an oven maintained at temperatures ranging from 40 to 50°C. Subsequently, the cleaned and dried rhizomes were finely ground using the cutting mill SM 100 (Retsch®, Germany). The resulting galangal rhizome powder (GRP) was then stored under ambient conditions in a dark and dry environment until further use.

2. Chemical and reagents

1'-Acetoxychavicol acetate reference standard, which is D, L-1'-acetoxychavicol acetate (minimum 98.0%, CAS 52946-22-2, 53890-21-4), was obtained from LKT Laboratories Inc., USA. Absolute ethanol was purchased from Merck, Germany.

3. Extraction techniques

Four extraction techniques-ME, UAE, RE, SE-have been employed to extract ACA from GRP. All extraction techniques utilized absolute ethanol (99.8%) as the solvent for extraction to avoid hydrolysis reactions. Three replications of extraction were performed in this study.

3.1 Maceration extraction

100 g of GRP was extracted in 1,000 mL of ethanol using the maceration method. The extraction process was controlled using an orbital shaker (Stuart® model SSL1, UK) and carried out for 6 hr with continuous shaking at 210 rpm. After extraction, the resulting mixture was filtered using Whatman no.1 filter paper and a vacuum suction pump. The ethanol extract was then collected and evaporated to dryness using a vacuum rotary evaporator (Buchi Rotavapor® model R-210, Switzerland) at 40°C. The results were expressed as %(w/w) yields of the crude extracts (CEs). Additionally, each CE underwent quantification analysis for ACA using HPTLC.

3.2 Ultrasonic-assisted extraction

100 g of GRP was immersed in 1000 mL of ethanol and the mixture was sonicated using an ultrasonic bath (Cole-Parmer® model 08895-74, China) for 6 hr. The resulting extract was filtered, collected and evaporated to dryness. Finally, the %(w/w) yields of the CEs were calculated before analysis, following a quantification analysis for ACA using HPTLC.

3.3 Reflux extraction

100 g of GRP was extracted in 1000 mL of ethanol using the RE technique. The extraction apparatus was heated on a heating mantle (Glas-Col® Cat. No. 100B TM117, USA) at 78°C for 6 hr. After extraction, the resulting mixture was filtered, collected and evaporated to dryness. Finally, the % (w/w) yields of the CEs were calculated before analysis, following a quantification analysis for ACA using HPTLC.

3.4 Soxhlet extraction

100 g of GRP was inserted in the thimble while 1000 mL of ethanol was placed in the flask of the Soxhlet apparatus. The temperature of the process was at 78°C and the extraction time was set for 6 hr After extraction, the resulting mixture was filtered, collected and evaporated to dryness. Finally, the %(w/w) yields of the CEs were calculated before analysis, following a quantification analysis for ACA using HPTLC.

4. Preparation of sample solution

The CEs from ME (50 mg) was accurately weighed

and dissolved with 25 mL ethanol in a 25 mL volumetric flask. The concentration of ME solution was 2 mg/mL.

The CEs from UAE (20 mg) was accurately weighed and dissolved with 10 mL ethanol in a 10 mL volumetric flask. The concentration of the UAE solution was 2 mg/mL.

The CEs from RE (100 mg) was accurately weighed and dissolved with 10 mL ethanol in a 10 mL volumetric flask. The concentration of RE solution was 10 mg/mL.

The CEs from SE (60 mg) was accurately weighed and dissolved with 10 mL ethanol in a 10 mL volumetric flask. The concentration of SE solution was 6 mg/mL.

Due to the varying solubility of each extract, it was necessary to prepare solutions at different concentrations to ensure clear, sediment-free samples. Consequently, the authors prepared four CE samples at the following concentrations: 2 mg/mL for ME, 2 mg/mL for UAE, 10 mg/mL for RE and 6 mg/mL for SE.

5. Chromatography

Performing HPTLC on aluminum-backed plates (10 × 10 cm) coated with 0.2 mm layers of silica gel 60 F254 (Merck, Germany). Before use, the plates were prewashed with methanol and then activated at 70°C for 60 min. Samples were applied onto the plate using a Linomat V sample applicator (CAMAG®, Switzerland) equipped with a microliter syringe, with a bandwidth of 6 mm. The plates underwent linear ascending development over a distance of approximately 80 mm using a mobile phase of dichloromethane-ethyl acetate (8:2 v/v) in a twin trough glass chamber, which was previously saturated with mobile phase vapors for 10 min at 25°C. Subsequently, the dried plate was scanned at the wavelength of 219 nm (λ_{max} of ACA) using a TLC scanner 3 with winCATS 1.4.4.6337 software (CAMAG®, Switzerland).

6. Preparation of ACA calibration curve

To create the calibration curve, a standard stock solution of ACA at a concentration of 10 mg/mL was meticulously prepared by dissolving 50 mg of accurately weighed ACA in ethanol and diluting it to 5 mL in a volumetric flask. Working standard ACA solutions spanning concentrations of 600, 800, 1,000, 1,200 and 1,400 µg/mL (equivalent to 6,000, 8,000, 10,000, 12,000 and 14,000 ng, respectively) were then generated by diluting the stock solution accordingly. Each of these solutions, in volumes of 10 µL, was meticulously applied in triplicate onto the HPTLC plate. Subsequently, the plates were subjected to development and scanning procedures as previously outlined. The calibration curve was then meticulously constructed by plotting the average peak areas against the corresponding quantities, and the regression equation for ACA was calculated accordingly.

7. Quantification of ACA

Volumes of suitable diluted solutions of each extract (ME 10 µL, UAE 10 µL, RE 2 µL, SE 8 µL) were applied in triplicate onto an HPTLC plate alongside a reference standard. The plate was developed under the predetermined conditions described above and scanned at 219 nm. Peak areas were recorded and calculated the ACA content in each extract using the provided calibration curve. The results are expressed as %(w/w) of ACA content per CE and GRP.

The linearity range of ACA in this study is between 6,000 and 14,000 ng per band. Consequently, it is necessary to load sample solutions with ACA quantities within this specified range, resulting in variations in the loading of sample solutions.

8. Statistical analysis

The experiments were conducted independently three times and the results were analyzed and presented as the mean±standard deviation (SD). Specifically, the aim was to investigate the differences between extraction techniques in terms of CE and ACA specificity. Data analysis was performed using Microsoft Excel software within the Microsoft 365 App. Statistical analysis employed analysis of variance (ANOVA) for multiple measurements, with significance determined by a *P*-value of less than 0.05.

Results and discussion

This study compared four techniques for extracting ACA from GRP. The procedures involved the conversion of fresh galangal rhizome into dried powder, followed by extraction using four different techniques: ME, UAE, RE and SE. Ethanol was employed as the solvent for all extraction techniques and the extraction process took 6 hr. The %yield of crude extracts (CEs) was calculated for each technique. Statistical analyses were utilized to compare the quantities of CEs obtained through different techniques. The CEs from each extraction technique were further analyzed for the content of ACA, using HPTLC. The %ACA content was calculated as per CE, as well as per GRP. The study aimed to compare the quantities of ACA obtained per CE and GRP for each technique using statistical analyses.

1. Preparation of GRP

Fresh plants contain a large and variable amount of water. Therefore, the percentage of extracts or active substances is usually compared to dry plants, which is more consistent and reliable. Thus, preparing GRP involved dehydrating fresh rhizomes. Moreover, distilling volatile oil containing ACA from fresh plants using water distillation can lead to the hydrolysis of ACA, an ester, further reducing its quantity, as mentioned in the introduction. During this process, the application of elevated temperatures prompts various chemical reactions, including hydrolysis and isomerization (Yang & Eilerman, 1999). These reactions are crucial as they influence the profile of bioactive compounds in galangal, with ACA being of particular interest. The drying process conducted at temperatures between 40-50°C might cause some reduction in ACA content.

2. Extraction of GRP

ACA, being an ester, is susceptible to hydrolysis due to the instability of ester bonds. Consequently, the use of water in the extraction process should be avoided, especially in the water distillation extraction of essential oil containing ACA from fresh galangal rhizomes. Therefore, this study utilized absolute ethanol as the extraction solvent. Additionally, the degradation of ACA from galangal can be influenced by the extraction technique. Strong acidic or basic solvents should be avoided during extraction, as they can hydrolyze ACA or cause other undesirable reactions. High extraction temperatures might accelerate the breakdown of ACA, particularly through isomerization and hydrolysis processes. Extended extraction times can gradually degrade ACA, even at mild temperatures. Identifying effective extraction techniques that reduce processing time can help minimize this issue (Kojima-Yuasa & Matsui-Yuasa, 2020). As a result, ACA is absent in galangal essential oil obtained by steam distillation (Yang & Eilerman, 1999). To achieve high ACA content from GRP, the correct extraction technique is essential.

Each sample of 100 g GRP was extracted with ethanol 1,000 mL for 6 hr by four techniques (ME, UAE, RE and SE). The ethanol extract solution was dried with a vacuum rotary evaporator. All extraction experiments with dried ethanol CEs resulted in a yield of brownish semi-solid mass with a galangal odor. The yields of CEs per GRP from the experiment were expressed as mean ± SD (w/w) and shown in the second column of Table 1. The data demonstrated that the technique of extraction had a significant effect on the %(w/w) yield of CE per GRP. The best technique of extraction was SE. This technique demonstrated the highest %(w/w) yield of CE per GRP (19.15 ± 0.66%) with a significantly difference (P-value < 0.05) from all other techniques, RE (9.05 ± 0.44%), ME (7.44 ± 0.60%) and UAE (4.91 ± 0.77%), respectively. The SE technique is a continuous extraction of all compounds by soaking plant materials in a thimble with a solvent for a short period and repeating extraction many times with a fresh solvent recycles (Chibuye et al, 2023; Azwanida, 2015; Patra et al, 2022; Patel et al, 2019). In this study, the thimble packed with GRP was placed in the compartment of the Soxhlet apparatus. Ethanol was filled into the round bottom flask, which was then heated on a heating mantle, causing the ethanol to boil. Its vapors flowed through a vapor duct to a condenser, where they condensed and fell as droplets onto the GRP thimble in the extraction chamber (percolation). The extraction chamber gradually filled with ethanol, allowing the GRP to remain in contact with the ethanol (maceration). When the ethanol level reached above the siphon tube, it flowed back to the round bottom flask through the siphon tube and the cycle continued. Each time the extraction chamber received cooled, recycling ethanol, preventing saturation of ethanol with compounds. Therefore, the Soxhlet extraction technique provided a continuous and exhaustive extraction of GRP. Extracting compounds in the round bottom flask resulted in a higher concentration compared to other techniques. In contrast, the RE technique employed a single-step ethanol extraction process aided by heat to enhance the solubility of compounds, resulting in a higher yield of extracted ACA compounds. However, this yield was typically lower compared to the SE technique, which employed continuous extraction with multiple cycles of solvent recycling. ME relied on static soaking of the GRP in single-step ethanol, which might result in incomplete extraction, particularly for compounds that require prolonged contact for solubilization. UAE technique applied high-frequency ultrasound waves to enhance the extraction process in single-step ethanol, but it might not achieve the same level of thoroughness as the SE technique in extracting all constituents from GRP. Therefore, the SE technique yielded a higher percentage of all compounds due to its continuous and efficient extraction process. However, it's worth noting that the extraction chamber did not directly receive heat, potentially resulting in a restricted dissolution of certain compounds with low solubility in ethanol. Furthermore, compounds extracted and dissolved in the round bottom

flask, which received direct heat from the heating mantle, might undergo degradation.

Table 1 Yield of CEs per GRP (%w/w), ACA per CEs (%w/w) and ACA per GRP (%w/w) in four extraction techniques

Extraction technique	Yield of CEs per GRP (%w/w)	ACA per CEs (%w/w)	ACA per GRP (%w/w)
ME	7.44±0.60	33.78±3.80	2.50±0.10
UAE	4.91±0.77	33.99±5.94	1.64±0.01
RE	9.05±0.44	41.77±4.58*	3.79±0.57*
SE	19.15±0.66*	12.31±0.22	2.38±0.06

Remark: *Significant difference (P-value < 0.05) when compared in the same column

3. Quantitative measurement of ACA in CE

The high degree of automation inherent in HPTLC enables its proficient utilization in analyzing complex mixtures of natural products. Acknowledgment of its significance by authorities such as the United States Pharmacopeia (United States Pharmacopeial Convention, 2008) and the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission, 2009) point out its pivotal role as the preferred method for tackling complex analytical challenges associated with herbal drugs and botanicals. This recognition highlights HPTLC's efficacy in providing accurate and reliable results, thus solidifying its position as a cornerstone technique in pharmaceutical and botanical research and analysis.

The quantitative analysis of ACA in CEs was conducted utilizing the HPTLC densitometric technique, exploring various solvent systems including hexane-ethyl acetate (8:2 v/v), hexane-ethyl acetate-ethanol (8:2:1 v/v), chloroform-ethyl acetate (8:2 v/v) and dichloromethane-ethyl acetate (8:2 v/v). Among these, the dichloromethane-ethyl acetate (8:2 v/v) system demonstrated optimized results, exhibiting sharp, symmetrical and well-resolved peaks of ACA at retention factor (R_f) 0.72, effectively separating it from other sample components. Confirmation of ACA bands' specificity in GRP was achieved by overlaying absorption spectra of samples with ACA reference standards (Fig. 1). The analyzed peaks' specificity was further validated at three different levels within the peak-start, apex and end positions-corresponding to the ACA reference standard (Fig. 2). The maximum absorbance of ACA occurred at 219 nm, thus guiding the choice of wavelength for analysis. HPTLC profiles of ACA in GRP and reference standards at 219 nm were illustrated, providing a comprehensive visual representation of the analytical findings (Fig. 3).

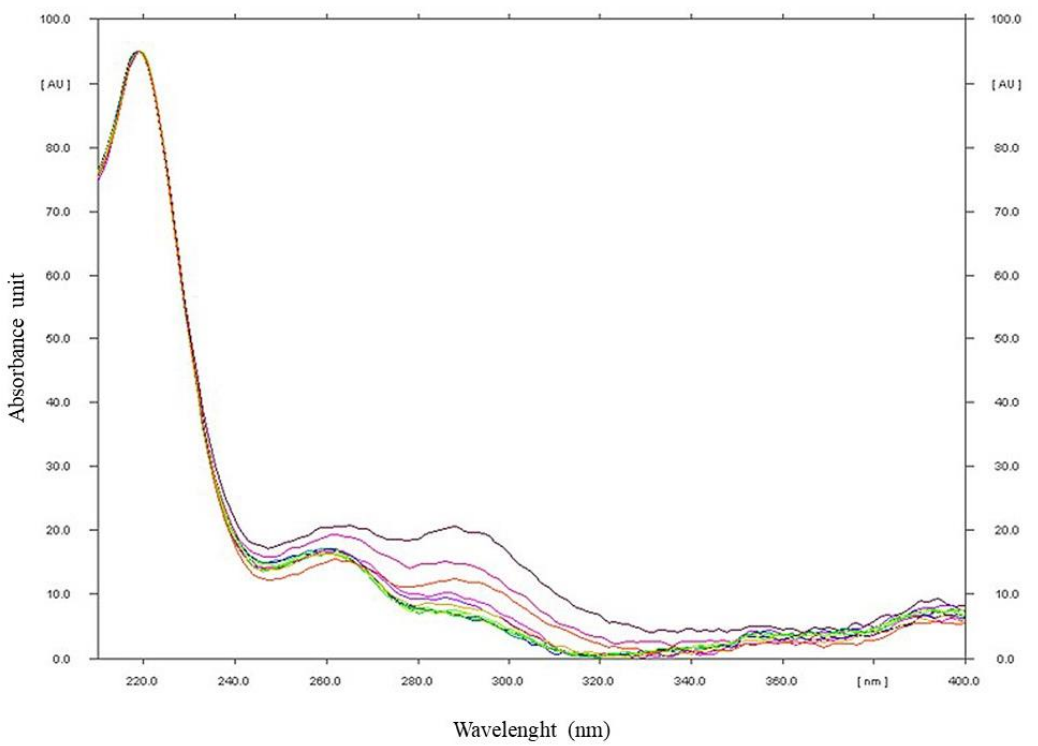

Fig. 1 The overlay of UV spectra, scanned from 205 to 400 nm using the HPTLC method, for the ACA reference standard (shown in green and dark green lines) and the ACA extracted using ME (pink and yellow lines), UAE (dark blue and orange lines), RE (purple and red lines) and SE (blue and brown lines)

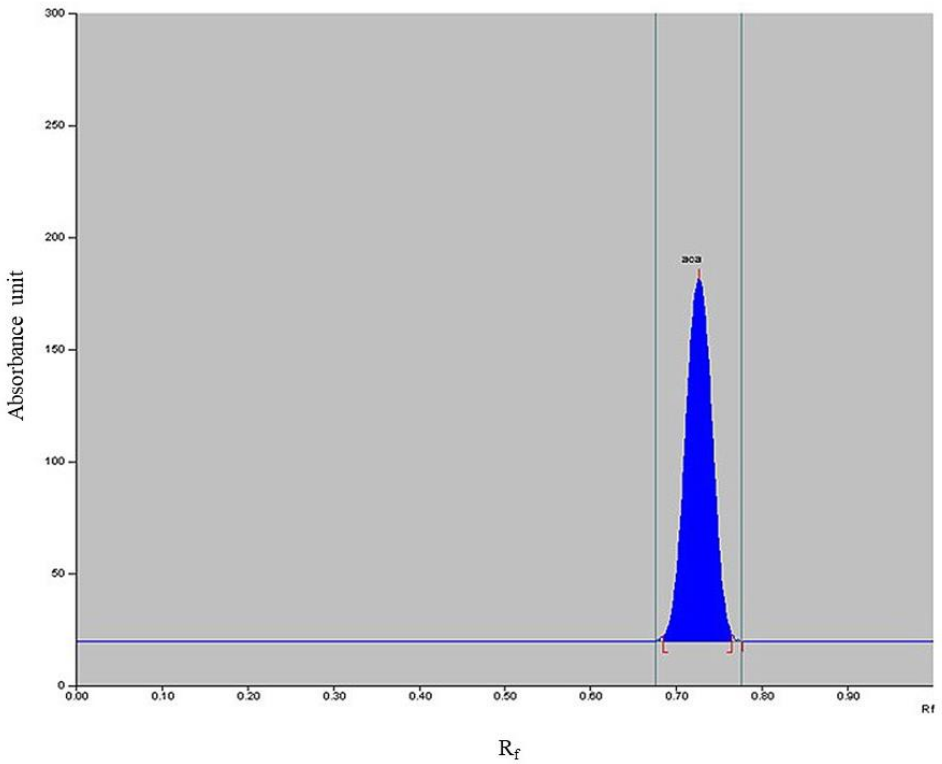

Fig. 2 HPTLC chromatograms of ACA reference standard

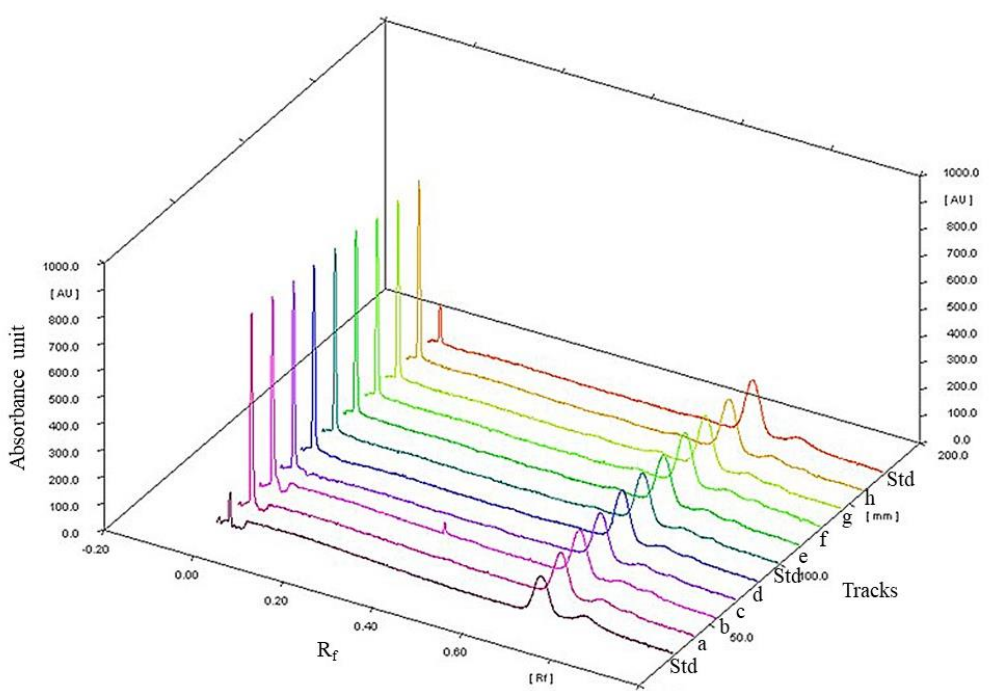

Fig. 3 The HPTLC chromatogram, displaying the ACA reference standard (Std tracks) alongside ACA extracted using UAE (tracks a and b), ME (tracks c and d), SE (tracks e and f) and RE (tracks g and h)

The study demonstrated a good linear relationship between the quantity of ACA reference standard and the absorbance of peak area, exhibiting a high coefficient of determination (R^2 = 0.9980), as depicted in Fig. 4. Utilizing the HPTLC densitometric technique and employing a linear regression equation f(x) = 1.1041x + 2693.6, the ACA content in each extract was accurately determined relative to the ACA reference standard. The ACA contents were then expressed as mean ± SD (%w/w) per the CE and GRP, detailed in the third and fourth columns, respectively, of Table 1. These findings underscore the reliability and precision of the analytical methodology employed, providing valuable insights into the ACA content of the extracts analyzed.

To prepare proper sample solutions for the quantification of ACA content, the sample solutions must be clear and free from precipitation. Since the crude extracts (CEs) obtained from each extraction technique have different solubility values, we prepared sample solutions from the crude extracts in each extraction technique with varying concentrations as follows: 2 mg/mL for ME, 2 mg/mL for UAE, 10 mg/mL for RE and 6 mg/mL for SE. Subsequently, the sample solutions obtained from the four extraction techniques were applied onto HPTLC plates in appropriate quantities to ensure that the amount of ACA fell within the linearity range. Preliminary experiments indicated that the sample solutions should be applied in the following quantities: ME 10 µL, UAE 10 µL, RE 2 µL, and SE 8 µL.

The CEs from each extraction technique were investigated for ACA content with an HPTLC densitometer. The %(w/w) ACA content per CE was calculated and expressed as ACA (ng) using the equation from the calibration curve shown in Fig. 4. The data of %(w/w) ACA content per CEs were shown in the third column of Table 1. We found that the best extraction technique for the highest %(w/w) yield of CEs (SE technique) did not give the highest ACA content (12.31 ± 0.22%) per CE. However, the best extraction technique for the highest %(w/w) ACA content (41.77 ± 4.58 %) per CEs was the RE technique. This %(w/w) ACA content was significantly different (*P*-value < 0.05) from other extraction techniques, UAE (33.99 ± 5.94%), ME (33.78 ± 3.80%), SE (12.31 ± 0.22%). It indicated that the best technique for the highest %(w/w) yield, SE, did not give the highest ACA content because the increased masses were from impurities other than ACA. The situation might be attributed to the fact that the GRP in RE technique was directly extracted in boiling ethanol which was not found in other techniques in this study. The boiling ethanol facilitates better penetration and solubilization of ACA in the RE technique than ethanol at room temperature in other techniques (Chibuye et al, 2023; Azwanida, 2015; Patra et al, 2022; Patel et al, 2019). Interestingly, the SE technique, despite producing the highest %(w/w) yield, had the lowest percentage of ACA per CEs when compared to other methods. This could be due to the fact that the Soxhlet chamber extraction involves percolation and maceration, processes that do not utilize heat to dissolve ACA. Consequently, not only was the quantity of ACA extracted reduced, but also the ACA present in the round bottom flask was

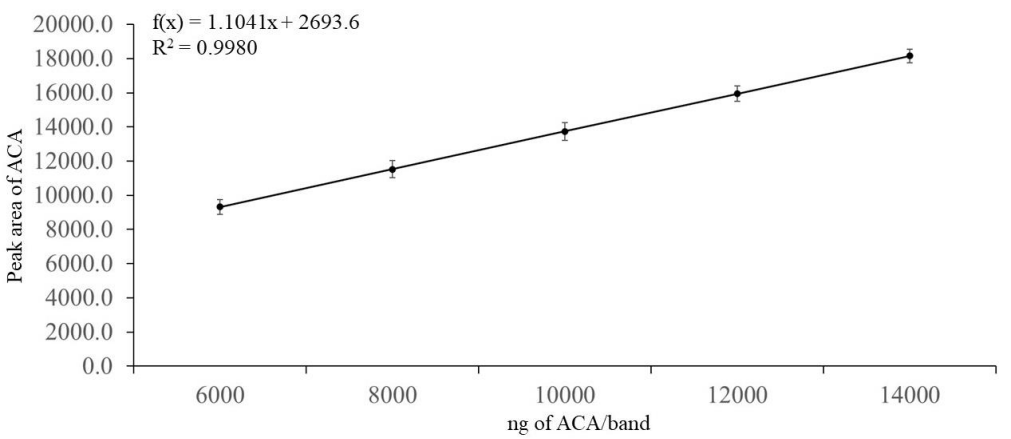

Fig. 4 Linear range of calibration plots for ACA reference standard

continuously exposed to heat during the extraction process, further diminishing the ACA quantity. Thus, the SE technique exhibited the lowest percentage of ACA per CEs when compared to other techniques.

The ACA content was calculated as %(w/w) ACA content per GRP as shown in the fourth column of Table 1. The result found that the best extraction technique for the highest %(w/w) ACA content (3.79±0.57%) per GRP, was significantly different (P-value < 0.05) from other extraction techniques, ME (2.50±0.10%), SE (2.38± 0.06%), and UAE (1.64 ± 0.01%), was RE technique. Although the RE technique produced significantly less %(w/w) yield of CEs than the SE technique (second column in Table 1), it produced the quantity of ACA with a significantly different (P-value < 0.05) higher %(w/w)ACA content per GPR than the SE technique (fourth column in Table 1). The RE technique, involving the extraction of ACA from 100 g of GRP using 1,000 mL of ethanol and aided by heat to enhance ACA dissolution, yielded significantly lower impurities in comparison to the SE technique. In a single-step extraction process lasting 6 hr, the RE technique produced minimal impurities, unlike the SE technique which required repetitive ethanol recycling for multiple extractions, resulting in higher impurity levels and ACA yield. However, the ACA yield from the RE technique, facilitated by direct heat application, was superior to that of the SE technique, indicating the efficacy of direct heat-assisted extraction.

The degradation of ACA derived from galangal is sensitive to the extraction technique used. It is advised to avoid the use of strong acidic or basic solvents during extraction to prevent hydrolysis or other unwanted reactions that could degrade ACA (Yang & Eilerman, 1999). Additionally, high extraction temperatures may accelerate ACA breakdown, particularly via isomerization and hydrolysis reactions (Yang & Eilerman, 1999). Even at moderate temperatures, extended extraction times can lead to a gradual degradation of ACA. Therefore, developing efficient extraction methods that minimize processing time is essential to mitigate this issue.

Conclusion

The conclusion of the study on the extraction of ACA from GRP using four different extraction techniques indicates significant variations in the obtained ACA content. The HPTLC analysis revealed distinct differences in the ACA content among the extraction techniques. Specifically, the SE technique yielded the highest %w/w of CE (19.15±0.66%). Conversely, the RE technique demonstrated superior performance in terms of ACA content per CE (41.77±4.58%) and GRP (3.79±0.57%). Despite this, the RE technique did not yield the highest %w/w of CEs. These findings underscore the influence of extraction methodology on both the quantity and quality of CEs from GRP, providing valuable insights for optimizing extraction processes in future endeavors.

The primary limitation of this study is the potential degradation of ACA due to its sensitivity to extraction conditions, such as high temperatures and extended times, which can lead to hydrolysis or isomerization. The exclusive use of ethanol as the solvent may overlook other solvents that could enhance ACA extraction efficiency. Additionally, the extraction techniques may introduce varying levels of impurities, affecting the CEs' purity and quality. These issues highlight the need for optimizing extraction parameters and exploring alternative solvents for better ACA yield and stability.

Acknowledgments

The authors extend their heartfelt gratitude to the Pharmacognosy and Pharmaceutical Chemistry Laboratory at Huachiew Chalermprakiet University, Thailand, for providing the essential research facilities that made this study possible. Special thanks to Dr. Rumrada Meeboonya, a botanist from the College of Allied Health Sciences, Suan Sunandha Rajabhat University, for her expertise in identifying and authenticating the plants used in this research.

References

Aljobair M.O. (2022). Chemical composition, antimicrobial properties, and antioxidant activity of galangal rhizome. *Food Science and Technology, 42,* e45622.

Al-Snafi, A.E. (2014). The pharmacological activities of *Alpinia galanga*l - A review. *International Journal for Pharmaceutical Research Scholars (IJPRS), 3*(1), 607-617.

Azwanida, N.N. (2015). A review on the extraction methods use in medicinal plants, principle, strength and limitation. *Medicinal & Aromatic Plants, 4*(3), 1-6.

Chibuye, B., Singh Sen, I., Luke, C., & Kakoma, M. (2023). A review of modern and conventional extraction techniques and their applications for extracting Phytochemicals from plants, *Scientific African, 19,* e01585.

Chinese Pharmacopoeia Commission. (2009). *TLC atlas of Chinese crude drugs in pharmacopoeia of the People's Republic of China*. Beijing, Chaina: Chemical Industry Press.

Chouni, A., & Paul, S. (2018). A review on phytochemical and pharmacological potential of *Alpinia galanga*, *Pharmacognosy Journal, 10*(1), 9-15.

Eram, S., Mujahid, M., Bagga, P., Arif, M., Ahmad, M.A., Kumar, A., … Akhter, M.S. (2019). A review on phytopharmacological activity of *Alpinia galanga*. *International Journal of Pharmacy and Pharmaceutical Sciences, 11*(3), 6-11.

GlobinMed. (2018). *Herbal medicine use in primary health care (PHC)*. Retrieved June 1, 2021, from https://globinmed.com/atm/herbal-medicine-or-medicinal-plants-recommended-for-the-primary-health-care-in-thailand/

Jiratchariyakul, W., & Mahady, G.B. (2005). Alpinia galanga: Overview of its chemistry and biological activity. *Journal of Ethnopharmacology, 45*(2), 201-208.

Khairullah, A.R., Solikhah, T.I., Ansori, A.N.M., Fadholly, A., Ramandinianto, S.C., Ansharieta, R., … Anshori, A. (2020). A Review of an important medicinal plant: *Alpinia galanga* (L.) willd, *Systematic Reviews in Pharmacy, 11*(10), 387-395.

Kojima-Yuasa, A., & Matsui-Yuasa, I. (2020). Pharmacological effects of 1′-Acetoxychavicol acetate, a major constituent in the Rhizomes of *Alpinia galanga* and *Alpinia conchigera*. *Journal of Medicinal Food, 23*(5), 465–475.

Nampoothiri, S.V., Menon, A.N., Esakkidurai, T., & Pitchumani, K. (2016). Essential oil composition of Alpinia calcarata and *Alpinia galanga* Rhizomes-A comparative study. *Journal of Essential Oil Bearing Plants, 19*(1), 82-87.

Patel, K., Panchal, N., & Ingle, P. (2019). Review of extraction techniques extraction methods: microwave, ultrasonic, pressurized fluid, soxhlet extraction, Etc. *International Journal of Advanced Research in Chemical Science, 6*(3), 6-21.

Patra, A., Abdullah, S., & Pradhan, R.C. (2022). Review on the extraction of bioactive compounds and characterization of fruit industry by-products. *Bioresources and Bioprocessing, 9*(1), 14.

Patwekar, M., Quazi Aamir, S., Faheem, I.P., Maheen, S., Mukim, M., & Siddiuque, A. (2022). *Alpinia Galanga*: A review of its ethnomedicinal, phytochemical and pharmacological activity. *Current Trends in Pharmacology and Clinical Trials, 5*(1), 180042.

Ramu, B., & Chittela, K.B. (2018). High performance thin layer chromatography and its role pharmaceutical industry: Review, *Journal of Bioscience and Bioengineering. 5*(3), 29-34.

Reich, E., & Schibli, A. (2007). *High-performance thin-layer chromatography for the analysis of medicinal plants,* New York: Thieme.

Sing, S., & Negi, A. (2022). A review on phytopharmacological activity of *Alpinia galanga*. *Indo American Journal of Pharmaceutical Research, 12*(8), 499-508.

Thongchai, S., Wongpoomchai, R., & Saenphet, K. (2013). The wound healing properties of *Alpinia galanga*. *International Journal of Medicinal Mushrooms, 15*(2), 203-211.

United States Pharmacopeial Convention. (2008). *USP 31–NF 26: United States Pharmacopeia and National Formulary* (15th ed.). Rockville, MD: United States Pharmacopeial Convention.

Van, H. T., Thang, T.D., Luu, T.N., & Doan, V.D. (2021). An overview of the chemical composition and biological activities of essential oils from *Alpinia genus* (Zingiberaceae). *RSC Advances, 11*(60), 37767–37783.

Verma, R.K., Mishra, G., Singh, P., Jha, K.K., & Khosa, R.L. (2011). *Alpinia galangal* - An important medicinal plant: A review. *Der Pharmacia Sinica, 2*(1), 142-154.

Verma, R., & Sharma, N. (2022). Phytochemical and Pharmacological activities of *Alpinia galanga*l: A review, *Asian Journal of Pharmacy and Pharmacology, 8*(3), 74-85.

Yan, W., Ying, W., Zhi Hua, L., Cheng Fang, W., Jian Yu, W., Xiao Lan, L., . . . Zhi Wei, D. (2014). Composition of the essential oil from *Alpinia galanga* rhizomes and its bioactivity on *Lasioderma serricorne*. *Bulletin of Insectology, 67*(2), 247-254.

Yang, X., & Eilerman, R.G. (1999). Pungent principal of *Alpinia galanga*l (L.) swartz and its applications. *Journal of agricultural and food chemistry, 47*(4), 1657–1662.

Zhang, W.J., Luo, J.G., & Kong, L.Y. (2016). The genus Alpinia: A review of its phytochemistry and pharmacology, *World Journal of Traditional Chinese Medicine, 2*(1), 26–41.