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RESEARCH ARTICLE

Isolation, characterisation, and determination of capsaicin compound levels in red chilli fruit (Capsicum annum fructus) in IPB CH3 and carvi agrihorti varieties

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Abstract

Background: Capsaicin, the active compound responsible for the pungency of chilli (Capsicum annuum), has significant pharmacological applications. However, its content varies depending on the chilli variety and extraction method. **Objective:** This study aims to isolate, characterise, and quantify capsaicin in two Indonesian chilli varieties, IPB CH3 and Carvi Agrihorti, using different extraction techniques. **Method:** Capsaicin was extracted using reflux and shaking water bath methods with ethanol and acetone as solvents. The extracts were analysed using phytochemical screening, fractionation, purification, and FTIR spectroscopy. **Result:** The shaking water bath method with acetone yielded the highest capsaicin concentration (45.172%), surpassing the reflux method (33.012%). Phytochemical screening confirmed the presence of alkaloids, quinones, and steroids. FTIR analysis confirmed the structural identity of capsaicin, with the IPB CH3 variety yielding a significantly higher isolate (29 mg) than Carvi Agrihorti (<1 mg). **Conclusion:** The IPB CH3 variety contains a higher capsaicin concentration than Carvi Agrihorti. The shaking water bath method using acetone is the most effective extraction technique for obtaining capsaicin.

Introduction

Red chilli peppers have been utilised for many years as a spice to enhance the flavour of various dishes. They can be cultivated in diverse regions, with Asia being the largest producer, followed by Mexico and the United States (NASS, 2009; Chinn *et al.,* 2011). In 2021, Indonesia ranked as the fourth-largest producer of chilli in Asia, with a production volume of 1.36 million tons. East Java contributed the highest percentage, accounting for 36.17% of the national total, followed by Central Java and West Java (Kementan, 2021). Corresponding to the production levels, chilli consumption in Indonesia increased by 9.94%, reaching approximately 490.83 thousand tons in 2021. Chilli has become one of the most widely used spices in Indonesian cuisine (Kusnandar, 2022). Despite its frequent use in cooking, extracts from this plant, such as oleoresin, have also been employed as colourants in food, textiles, and cosmetics (Santamaria *et al.,* 2000; Huntrods, 2008).

Chilli peppers are a rich source of phytochemical compounds, particularly capsaicin. Capsaicin (trans-8-methyl-N-vanillyl-6-nonenamide), an alkaloid or capsaicinoid constituent of vanilloids, is found in the highest concentrations among all types of red chilli peppers and is characterised by an amide group and a hydrophobic side chain (Xing *et al.,* 2006). Capsaicin is the compound responsible for the spiciness of chilli, which is commonly measured using the Scoville heat value (SHV) index. The spiciness of a chilli correlates directly with its capsaicin content, which also varies with the ripeness of the fruit (Gnayfeed *et al.,* 2001;

Estrada *et al.,* 2002). The quality and quantity of capsaicin obtained depend on various isolation process parameters, including the preparation of the material and the specific parts used, contact time, sample particle size, storage duration, temperature, and time of extraction, as well as the solvent employed (Kruawan *et al.,* 2022). Solid-liquid extraction using solvents such as hexane, acetone, chloroform, ethanol, methanol, and acetonitrile, or their combinations, is the most commonly used method (Tapia *et al.,* 1993; Catchpole *et al.,* 2003).

Research conducted by Attuquayefio and Buckle (1987) and Chinn et al. (2011) demonstrated that extraction with acetone yields the highest capsaicin content, reaching 20 mg/g of dry material, followed by ethanol and acetonitrile. However, fresh chilli samples produced the highest capsaicin levels when using ethanol and acetonitrile as solvents. Efforts to determine capsaicin levels in chilli have led to various studies employing different extraction techniques to enhance efficiency, such as liquid-liquid extraction (LLE) (Tapia *et al.,* 1993), enzymatic extraction (Santamaria *et al.,* 2000), solid-phase microextraction (SPME) (Spicer and Almirall, 2005), and ultrasonic-assisted extraction (Karnka *et al.,* 2002).

Each chilli variety possesses genetic differences that can influence growth and adaptation processes, as well as capsaicin content. However, the production and quality of chilli in Indonesia remain relatively low. Several factors contribute to this, including poor environmental management, which affects growth, low resistance to pest and disease attacks, and inadequate adaptation to varying environmental conditions. Consequently, these issues lead to a decline in chilli production. The adoption of superior new chilli varieties (VUB) has become a standard solution among chilli farmers facing these challenges (Minanti *et al.,* 2020).

The advancement of biotechnology has facilitated the development of various chilli seed varieties with distinct advantages. The IPB CH3 and Carvi Agrihorti varieties are superior red chilli types that are widely cultivated in the Bogor Regency, Indonesia (Utami *et al.,* 2019; Safitri *et al.,* 2023). Despite this, research on determining capsaicin levels in these two varieties remains limited. Given this gap, the authors are interested in comparing the capsaicin content in these two superior red chilli varieties. To address this issue, both qualitative and quantitative experimental research will be conducted with the aim of isolating, characterising, and comparing the capsaicin levels between the two varieties.

Methods

Plant determination

The determination of Big Red Chilli (Capsicum annuum) for the IPB CH3 and Prima Agrihorti varieties was conducted at the Phytochemistry Laboratory of the Military Pharmacy Faculty, Republic of Indonesia Defence University, Bogor. The plants' morphological characteristics were aligned with the International Code of Nomenclature for Cultivated Plants (ICNCP), and references were made to the Ministry of Agriculture's Centre for Plant Variety Protection and Agricultural Licensing (PPVTPP) to ensure accuracy.

Capsaicin isolation

The isolation method was based on the studies by Rahmawati et al. (2020) and Thaib et al. (2015) with various modifications.

Sample collection

Fresh red chilli fruits from the IPB CH3 and Carvi Agrihorti varieties, which are superior varieties typical of the Bogor region, were collected. The IPB CH3 variety was sourced from the Dramaga District, while the Carvi Agrihorti variety was obtained from the Cibungbulang District in Bogor Regency, which serves as a primary cultivation and research development centre.

Sample preparation

Fresh red chilli fruits from both varieties were thoroughly washed and then separated into seeds and the outer fruit parts. The outer parts (shell) were dried in an oven at 100°C for three hours and 30 minutes, followed by grinding and sieving using a mesh size of 20-30. The seeds were not subjected to the drying process.

Extraction

The obtained red chilli powder was dissolved in acetone solvent at a ratio of 1:10 (grams:ml) and stirred on a shaking water bath at 120°C, 150W, for 15 minutes with periodic stirring. The resulting extract was then evaporated using a rotary evaporator at 60°C with a speed of 30 rpm to obtain a concentrated extract. The final extract was weighed and recorded.

Phytochemical screening

Reagent preparation

Mayer's reagent: 1.36 grams of $HgCl_2$ was dissolved in 60 mL of distilled water (Solution I). Five grams of KI was dissolved in 10 mL of distilled water (Solution II). Both

solutions (I and II) were mixed and then diluted to 100 mL (Moelyono, 1996).

Dragendorff's reagent: 2.72 grams of KI was dissolved in 100 mL of distilled water, followed by the addition of one gram of Bi(NO3)3 and 20 mL of HNO3 (Harborne, 1987).

Testing

Alkaloid testing: A 2-gram sample was mixed with 10 mL of 2N HCl, ground, and then filtered. The filtrate was then added to 5 mL of 25% ammonia and extracted using 20 mL of chloroform. The chloroform layer was collected and dropped onto filter paper, followed by the addition of Dragendorff's reagent. The formation of an orange colour indicated a positive alkaloid result. The remaining chloroform was then treated with 2N HCl, and the aqueous layer was separated from the chloroform layer. Five mL of the aqueous layer was taken and reacted with Mayer's and Dragendorff's reagents in separate test tubes. The formation of a white colour indicated a positive result with Mayer's reagent, while a brick-red colour that developed within 15 minutes showed a positive outcome with Dragendorff's reagent (Farnsworth, 1996).

Flavonoid testing: Five grams of the sample were boiled in 100 mL of hot water for 15 minutes before being filtered while hot. The resulting filtrate was then used to test for the presence of flavonoids, tannins, quinones, and saponins. For the flavonoid test, 5 mL of the filtrate was mixed with 0.1 grams of magnesium powder, 1 mL of concentrated hydrochloric acid, and 5 mL of amyl alcohol in a test tube. After shaking, the solution was allowed to separate. The formation of yellow, orange, or red colours indicated the presence of flavonoid compounds (Farnsworth, 1966).

Tannin testing: The filtrate from the sample was divided into three test tubes, each containing 5 mL. In the first tube, 1 mL of 5% ferric chloride solution was added; in the second tube, 1 mL of 10% gelatin solution was added; and in the third tube, 1 mL of Stiasny's reagent was added. A positive test for phenolic compounds was indicated by a colour change to blue-black after the addition of ferric chloride. A positive test for tannins was marked by the formation of a white precipitate after gelatin was added. The presence of catechin tannins in the third tube could be confirmed by the formation of a pink precipitate after Stiasny's reagent was added and heated. Subsequently, after filtration, the filtrate was treated with 1 mL of 1M sodium acetate and 1 mL of 5% FeCl3. The formation of a blue-black colour indicated a positive result for gallic tannins (Farnsworth, 1966).

Quinone testing: To test for the presence of quinones, 5 mL of the filtrate was prepared in a test tube, and then one drop of 1N sodium hydroxide solution was added. The formation of a red colour indicated the presence of quinones in the sample (Farnsworth, 1966).

Saponin testing: To test for the presence of saponins, 10 mL of the filtrate was placed in a test tube and shaken vigorously for ten seconds until a stable foam with a height of one to ten cm was formed. The sample was considered to contain saponins if the foam remained stable and did not dissipate after the addition of a few drops of 2N hydrochloric acid solution (Ministry of Health of the Republic of Indonesia, 1995).

Steroid/Triterpenoid testing: To test for the presence of steroids/triterpenoids, one gram of the sample was ground in 20 mL of ether in a mortar, then filtered. The filtrate was placed in an evaporating dish and allowed to evaporate. Subsequently, a few drops of Liebermann-Burchard reagent were added. The formation of a green-blue or red-purple colour indicated the presence of steroid/triterpenoid compounds (Farnsworth, 1966).

Monitoring of extracts

The monitoring of the extracts was performed using Thin Layer Chromatography (TLC). The chamber was first saturated with a mobile phase consisting of a 4:1 mixture of hexane and ethyl acetate. A sample of 1 mg was dissolved in 5 mL of acetone until it was homogeneous, and then it was spotted onto a 5x5 cm TLC plate coated with silica gel 60 F254. The plate was placed in the chamber, and the mobile phase was allowed to rise until it reached the marked upper limit. The plate was then removed, and the spots were visualised under UV light at wavelengths of 254 and 366 nm, along with specific spot visualisation using Dragendorff and Mayer reagents, compared against a standard capsaicin sample based on the Rf values (Deepa and Subhashini, 2020).

Fractionation, sub-fractionation, and screening of fraction results

Fractionation was conducted using a liquid-liquid extraction method. The concentrated extract obtained from the evaporation process was first weighed and recorded, then dissolved in acetone. After achieving homogeneity, the solution was placed in a separating funnel with two liquid phases: chloroform and distilled water. The mixture was shaken gently for ten minutes, and the stopcock was periodically opened to release gas. The separating funnel was then placed on a ring stand until the two solvents were separated entirely,

and the extract was collected. This process was repeated five times with chloroform: water ratios of 5:1, 4:2, 3:3, 2:4, and 1:5. The resulting fractions were concentrated, weighed, and analysed using TLC with the same system as before. A pure sample was indicated by the presence of a single spot on all three chromatograms when viewed under UV light and with specific spot visualisation.

Sub-fractionation was performed in a similar manner, but the liquid-liquid phase ratios were changed to a mixture of n-hexane and petroleum ether in ratios of 1:1, 4:1.5, and 8:2 (Al-Samydai *et al.,* 2019). Screening of the fraction results was conducted using the same method, with a mobile phase of ether:chloroform: methanol in a ratio of 75:19:6.

Purification

Purification was carried out using the recrystallisation method. The concentrated extract was weighed and recorded, then dissolved in diethyl ether and cooled in an ice bath until crystals formed. The obtained crystals were dried using a desiccator. The process of removing impurities was performed through recrystallisation using 96% ethanol, and the resulting crystals were weighed and recorded. The samples were stored at -20°C.

Purity testing

The obtained crystals were subjected to physical property tests using single development TLC, melting point determination, and refractive index measurement.

Single development TLC

A chamber lined with filter paper was first saturated with a mobile phase consisting of a 4:1 mixture of hexane and ethyl acetate. A sample of 1 mg was dissolved in 5 mL of acetone until it was homogeneous, and then it was spotted onto the TLC plate, which was then placed in the chamber. The mobile phase was allowed to rise until it reached the marked upper limit. The plate was then removed, and the spots were visualised under UV light at wavelengths of 254 and 366 nm, along with general visualisation using 10% sulfuric acid in methanol. The same procedure was repeated with two different mobile phase ratios of 3:2 and 2:3.

Melting point determination

The crystals were placed in a capillary tube. The capillary tube was then heated using a melting point apparatus (Innotech). The temperature was gradually controlled until the melting point of capsaicin was reached, which is between 62-65°C (Sukma *et al.,* 2020).

Refractive index

The crystals were dissolved in ethanol and placed on an Abbe refractometer. The sample and the instrument were allowed to equilibrate to the same temperature. The scale was read with an accuracy of 0.02, and a correction factor of 0.000385 was applied. The refractive index was calculated based on the equation (1), with R' being the refractive index reading at temperature T' °C, T being the standard temperature, and T' being the reading temperature. The results were referenced from the study by Tandon et al. (1964), which reported a refractive index of capsaicin at 1.4690.

$$R = R' - K\,(T - T')\ (2)$$

Characterisation and identification

Structural identification using FTIR

Fourier Transform Infrared Spectroscopy (FTIR) was calibrated using potassium bromide (KBr) standards. The sample was placed on a sample plate and analysed using infrared spectroscopy. Peaks observed in the infrared spectrum were selected for further analysis in accordance with the functional groups associated with capsaicin (Thaib *et al.,* 2015).

Quantitative analysis using UV-vis spectrophotometry

A standard solution of 1 mg of capsaicin was prepared in ethanol to create a series of standard solutions with concentrations ranging from 10 to 100 micrograms, which were measured at a wavelength of 280 nm. Linear regression calculations were performed using the Online Statistics and Forecasting Software (www.wessa.net). The samples were subsequently analysed for capsaicin content after a 300-fold dilution in the same solvent (Amruthraj *et al.,* 2014).

This comprehensive approach to the isolation, characterisation, and quantification of capsaicin in the IPB CH3 and Carvi Agrihorti varieties of red chilli fruit provides valuable insights into the chemical properties and potential applications of this critical phytochemical.

Results

Extraction

As shown in Figure 1, there is a significant colour difference between the whole dried chilli sample and the fresh samples from both varieties. This colour

concentration is attributed to the presence of capsicum oleoresin and dihydrocapsaicin, which are abundant in the samples (Chinn *et al.,* 2011). Capsicum oleoresin is a colouring agent contained within the chilli samples.

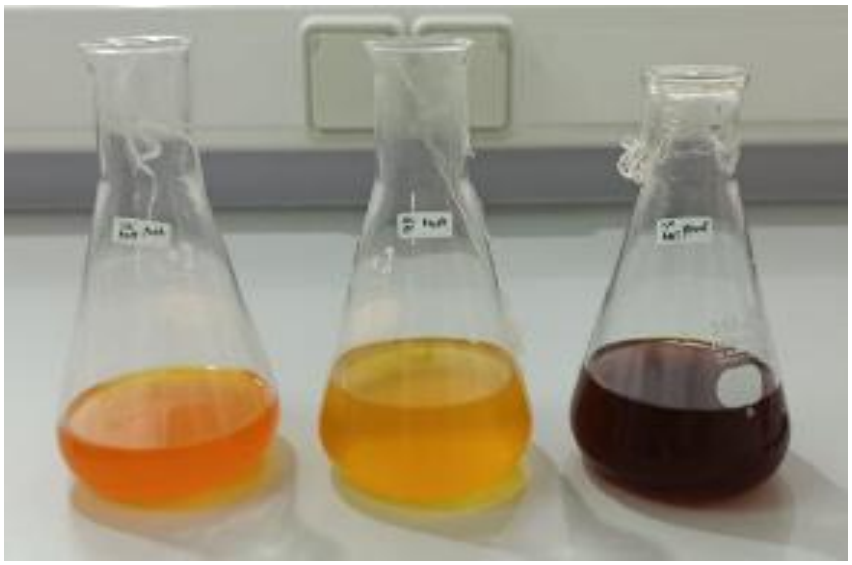


Figure 1: Process and results of extraction using the shaking water bath method

Table I shows that the reflux method using 96% ethanol produced a yield of 33.012% of the total dry powder, while the stirring method in a water bath with acetone yielded a higher yield of 45.172%. Extraction from fresh placenta IPB and fresh placenta carvi also showed variations in results, with yields of 25.541% and 21.076%, respectively. These findings confirm that selecting the correct extraction method and solvent is crucial in the development of natural ingredient-based pharmaceutical products. The higher yield of the stirring method in a water bath indicates the potential to improve extraction efficiency, which may contribute to more effective drug formulations.

Table I: Extraction results

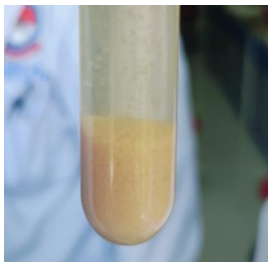
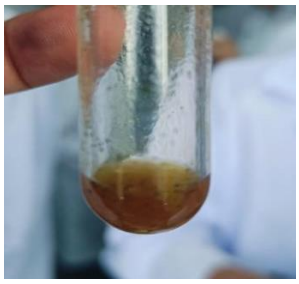
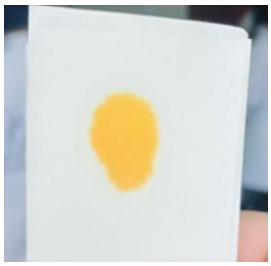
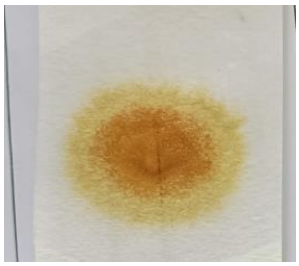
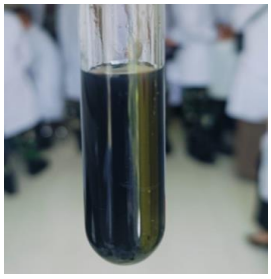
Extraction	Simplicia	Sample weight (g)	Solvent	Solvent volume (ml)	Extract weight (g)	Yield (%)
Reflux†	Whole dry powder	25	Ethaol 96%	250	8.253	33.012
Shaking waterbath††	Whole dry powder	25	Aceton	250	11.293	45.172
	Fresh placenta IPB CH3	170	Aceton	250	4.342	25.541
	Flesh placenta carvi agrihorti	170	Aceton	250	2.583	21.076

†Four cycle, 80ºC; ††20 rpm, 100ºC, 18 mintes

Phytochemical screening

Table II presents the qualitative phytochemical analysis, which revealed a diverse composition of bioactive compounds with varying concentrations. The presence of quinones (+++), tannins (+++), alkaloids (+), and steroids (+++) indicated a rich phytochemical profile with potential pharmacological applications.

Table II: Phytochemical screening results

	Control		Sample	
Quinon	+++		+	
Tannin	+++		-	
Alkaloid	+		+++	
Steroid	+++		++	

(-): The compound was not found; (+): The number of compounds found was small; (++): The number of compounds was large; (+++): The number of compounds found was very large

Quinone assay showed very high concentrations (+++) of these compounds, indicating their significant presence, which is generally associated with antimicrobial and antioxidant properties. However, lower intensity (+) was observed in the secondary confirmation assay, implying possible variations in solubility or reactivity under different test conditions.

Tannins were also detected in large amounts (+++), indicating their abundance. However, the absence (-) of positive results in the second assay suggests potential interference or selective solubility of specific tannin subtypes in the extraction medium. Given the well-documented astringent, antimicrobial, and anti-inflammatory properties of tannins, these findings are of great interest for pharmaceutical development.

Alkaloids, known for their diverse pharmacological activities, were detected in small amounts (+) in the primary test but showed a very high presence (+++) in the confirmatory test. This difference may indicate variations in extraction efficiency or the presence of specific alkaloid subtypes with differing reactivity under different conditions. Alkaloids are widely studied for their central nervous system activity, antimicrobial properties, and potential anticancer effects.

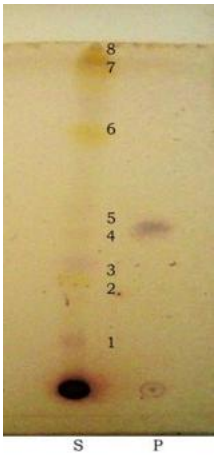
Steroid compounds were present in very high concentrations (+++) in the primary test, with slightly lower but still significant detection (++) in the confirmatory test. The presence of steroids is essential due to their role in anti-inflammatory, immunomodulatory, and hormone-regulating

activities, making them valuable for pharmaceutical and nutraceutical applications.

Fraction monitoring

The fraction monitoring results presented in Table III provide a comprehensive chromatographic profile of the analysed sample, highlighting distinct separation patterns across different mobile phase systems. The application of Toluene:Ethyl Acetate (1:1) and Chloroform:Diethyl Ether (9:2) as mobile phases demonstrates their efficiency in resolving bioactive compounds based on their polarity and differential affinity to the stationary phase.

Table III: Fraction monitoring results

No.	Standard Rf†	Standard plate image	Fraction Rf††	Refraction plate image	Refractionation Rf††	Refractionation plate image
1	0.15		$\frac{2.3}{6} = 0.38$		$\frac{0.9}{7} = 0.12$	
2	0.28		$\frac{3.1}{6} = 0.51$		$\frac{2.8}{7} = 0.4$	
3	0.31		$\frac{4.3}{6} = 0.71$		$\frac{5.9}{7} = 0.84$	
4			$\frac{5}{6} = 0.83$		$\frac{6.4}{7} = 0.9$	
5	0.53		$\frac{5.5}{6} = 0.91$		$\frac{6.7}{7} = 0.95$	
6	0.74					
7	0.91					
8	0.96					

† Toluene mobile phrase: Ethyl acetate = 1:1 (Dielectric constant = 4.2); ††Chloroform mobile phase: Diethyl ether = 9:2 (Dielectric constant = 4.05)

The standard plate establishes the initial reference Rf values for the primary components, ranging from 0.15 to 0.67, which indicates the presence of multiple analytes with varying polarities. Subsequent fractionation reveals an improved separation, with observed Rf values ranging from 0.38 to 0.91, indicating selective enrichment of compounds. The fraction migration is particularly evident in the refraction and refractionation stages, where the retention factor R values shift slightly, suggesting increased purification and resolution of distinct chemical constituents.

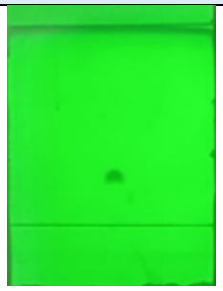
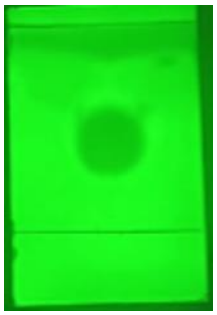
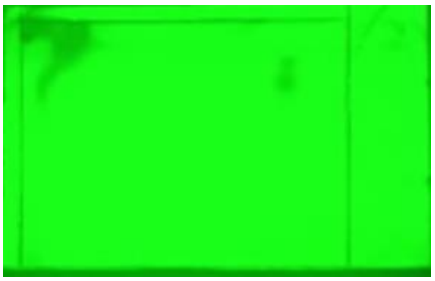
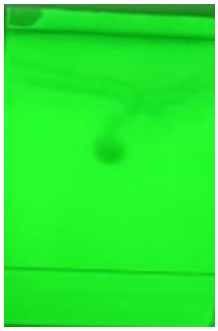
Of particular interest is the fraction with Rf = 0.67, which demonstrates a consistent migration pattern across all stages, suggesting it represents a stable and potentially dominant bioactive compound. Additionally, the observation of Rf variations in the range of 0.71 to 0.91 during the refractionation phase indicates the presence of less polar constituents, which aligns with the dielectric properties of the selected solvent systems.

The visualisation of bands under UV light further confirms the presence of well-resolved fractions, which may correspond to structurally distinct molecules of pharmaceutical relevance. The consistency in separation efficiency across different stages supports the robustness of the chromatographic method used.

Purification test

The purification test results presented in Table IV provide a comprehensive evaluation of the compound's purity through thin-layer chromatography (TLC) and Two-dimensional TLC (2D-TLC) using different mobile phase systems. The data reveal distinct purification patterns across the three phases, highlighting variations in compound homogeneity and separation efficiency.

Table IV: Purification test results

Phase no.	TLC development single	Description	Two-dimensional TLC	Description
Phase 1		+		+
Phase 2		+		++
Phase 3		+		

+ = There is one spot (pure); ++ = There are two spots (impure); †Mobile phase = chloroform: Ethyl acetate = 1:1; ††Mobile phase = Chloroform: Methanol = 3:2; †††Mobile phase = Ethyl acetate: Methanol = 3:7

In Phase 1, the TLC analysis exhibits a single well-defined spot, indicating a high degree of purity (+) when using Chloroform: Ethyl Acetate (1:1) as the mobile phase. The corresponding 2D-TLC confirms this result, further supporting the compound's homogeneity. This suggests that the selected mobile phase effectively isolates a single component with minimal contamination or co-elution.

Phase 2, developed using Chloroform: Methanol (3:2), also shows a single spot (+) in the TLC analysis, indicating an apparently pure compound. However, the 2D-TLC results reveal the presence of two distinct spots (++), signifying the existence of an impurity or a structurally similar compound that was not resolved in the initial TLC. This finding suggests that while the first TLC method indicates purity, a more refined chromatographic technique is necessary to confirm the true homogeneity of the sample.

Phase 3, utilising Ethyl Acetate: Methanol (3:7), again displays a single spot (+) in both TLC and 2D-TLC, indicating that this mobile phase system efficiently isolates a pure compound. The absence of additional spots in both tests confirms the effectiveness of this purification method in removing impurities, making it a suitable candidate for further structural elucidation and pharmaceutical applications.

Results of peak analysis of isolate samples using FTIR

The Fourier Transform Infrared (FTIR) spectroscopic analysis presented in Table V provides a detailed characterisation of the functional groups present in the isolated compound, offering critical insights into its structural composition. The spectral data reveals key absorption bands corresponding to various chemical bonds, confirming the molecular identity and purity of the isolate.

Table V: Results of peak analysis of isolate samples using FTIR

Frequency (cm^{-1})	Intensity	Functional group	$C_{18}H_{27}NO_3$
2927.23	Medium (should be strong)	C-H stretch	$C_17H_26NO_3$
2851.17	Medium (should be strong)	C-H stretch	$C_16H_25NO_3$
2119.37	Weak (safe)	C=C	$C_14H_23NO_3$
1615.71	Medium (safe)	N-H (Amida) + (C=O) (bend) (harusnya stretch)	C_13H_22O2
1066	Strong	C-O stretch	$C_12H_{27}O$
806.42	Medium (should be strong)	Pengarah Para	-
800.68	Medium (safe)	Aromatis C-C (C6H3)	$C6H_24O$

The presence of C-H stretching vibrations at 2927.23 cm^{-1} and 2851.17 cm^{-1} with medium intensity, which ideally should be stronger, suggests the existence of aliphatic hydrocarbon chains ($C_17H_26NO_3$ and $C_16H_25NO_3$), which are characteristic of lipid-derived or long-chain organic molecules. The weak C=C stretching absorption at 2119.37 cm^{-1} is indicative of an alkene functional group ($C_14H_23NO_3$), suggesting partial unsaturation in the structure.

A notable peak at 1615.71 cm^{-1}, corresponding to N-H (amide) bending and C=O bending, confirms the presence of amide functional groups (C_13H_22O2), which are fundamental to peptide bonds or nitrogen-containing heterocyclic compounds. However, this band ideally should appear as a stretching vibration rather than a bending mode, which might indicate structural constraints or molecular interactions affecting vibrational modes.

A strong absorption at 1066 cm^{-1}, assigned to C-O stretching, is consistent with ether or ester functionalities (C2H7O), reinforcing the possibility of oxygenated heterocycles or carbohydrate moieties. Additionally, the bands observed at 806.42 cm^{-1} (medium intensity) suggest a para-substituted aromatic ring system, which is further supported by the 800.68 cm^{-1} peak, indicating aromatic C-C stretching ($C6H_24O$).

Discussion

Extraction

As previously shown in Figure 1, there is a significant colour difference between the whole dried chilli sample and the fresh samples from both varieties. This colour concentration is attributed to the presence of capsicum oleoresin and dihydrocapsaicin, which are abundant in the samples (Chinn *et al.,* 2011). Capsicum oleoresin is a colouring agent contained within the chilli samples. Table I indicates a significant difference between the samples extracted using the reflux method and those extracted using the shaking water bath method, which is due to the different solvents used. In the reflux method, 96% ethanol was employed as the solvent, while acetone was used in the shaking water bath. Additionally, there were differences in the results among the three samples in the shaking water bath method. The whole dried chilli powder yielded a percentage yield of 45.172%, while the fresh chilli from the IPB CH3 variety yielded 25.541%, and the fresh chilli from the Carvi Agrihorti variety yielded 21.076%. This discrepancy is due to the difference in sample weights used for the dried and fresh samples. The dried sample weighed 25 mg, while the fresh sample weighed 170 g. The difference in weights is based on the conclusion that the dried sample underwent a drying process using equipment, whereas the fresh sample did not undergo drying, resulting in the moisture content remaining in the placenta and seeds of the fruit. Consequently, a significant difference exists between the sample weights used and the volume of solvent required, resulting in a notable variation in the percentage yield obtained.

Another difference observed is between the fresh samples of the IPB CH3 variety and the Carvi Agrihorti variety using the shaking water bath method, with % yields of 25.541 and 21.076, respectively. This indicates that the capsaicin content in the fresh chilli from the IPB CH3 variety is higher than that in the fresh chilli from the Carvi Agrihorti variety.

According to data from the Tropical Horticulture Study Centre of the Research and Community Service Institute – Bogor Agricultural Institute, the capsaicin content in the IPB CH3 variety is reported to be 377.66 ppm, which translates to 377.66 mg in 1000 mL of water. In contrast, data from the Ministry of Agriculture of the Republic of Indonesia indicate that the capsaicin content in the Carvi Agrihorti variety is 2.6 ppm, meaning there are 2.6 mg of capsaicin in 1000 mL of water. This clearly demonstrates that the capsaicin content in the IPB CH3 variety is greater than that in the Carvi Agrihorti variety. Furthermore, it can be observed that the reflux method utilised four cycles at a temperature of 80°C for two hours, while the shaking water bath method operated at 20 rpm with a temperature of 100°C for 18 minutes. This suggests that the shaking water bath method is more efficient than the reflux method when using the same samples,

considering the effectiveness of the time used and the percentage yield obtained.

Research conducted by Amruthraj et al. (2014) indicates that the capsaicin content using the shaking water bath method with acetone as a solvent yields higher values compared to using ethanol as a solvent, specifically 84.22 ± 0.02 for acetone and 77.91 ± 0.07 for ethanol. This aligns with these findings that acetone is a more effective solvent than 96% ethanol.

Additionally, according to Chinn et al. (2011), the highest capsaicin content is obtained from the seeds of the chilli fruit. This finding does not align with the results of this study, as the authors used whole, dried chilli samples and fresh samples consisting of both the placenta and seeds. The difference in results may also be attributed to the fact that in the study by Chinn et al. (2011), the chili samples were dried using an oven, resulting in only the outer surface of the chili being thoroughly dried, while the placenta and seeds that were not directly exposed to the drying process still retained moisture. Another factor contributing to the difference in results in this study is that the authors did not dry the fresh chilli samples used.

Phytochemical screening

The phytochemical screening revealed that the samples contained saponins, quinones, alkaloids, and steroids, with the highest concentrations being steroids and alkaloids.

Saponin screening was conducted using a foam test, which involved shaking the sample in a test tube. The presence of saponins is indicated if the foam produced remains stable and does not dissipate after the addition of a few drops of 2N hydrochloric acid (Ministry of Health of the Republic of Indonesia, 1995). The reaction that occurs when concentrated hydrochloric acid (HCl) is added to the saponin solution does not break down the saponin molecules; rather, it enhances the electrical conductivity of the solution and strengthens foam formation. Saponin molecules dissolve in water and form micelles, where the hydrophilic (polar) head dissolves in water while the hydrophobic (non-polar) tail extends outward, stabilising the air bubbles formed during shaking, resulting in stable foam. The H+ ions from HCl increase the solution's conductivity, thereby enhancing the interactions between saponin molecules and facilitating the formation of more stable micelles. More stable micelles produce stronger and longer-lasting foam. The foam test results for saponins in this extract indicated that the saponin content in the sample was lower (+) compared to the standard (+++).

The next step in phytochemical screening was the quinone screening. In this process, the sample was treated with 1 N sodium hydroxide. A positive result was indicated by the appearance of a red colour in the sample (Farnsworth, 1966). The standard used for quinone screening was quinine, which contains a conjugated carbonyl group that is easily oxidised. Quinones in the quinine solution react with OH- ions from NaOH, producing phenolate ions and water. These phenolate ions can absorb specific wavelengths of light, resulting in a red colour (Harborne, 1987).

In this instance, the sample contained quinone compounds; however, the expected red colour was obscured by the brown hue of the capsaicin extract. The standard used, quinine, contained a higher concentration of quinones (+++) compared to the extract sample (+). This was evident from the more intense red colour produced by the standard compared to the sample, which was muted by the dominant brown colour of the extract.

Phytochemical screening for tannins was conducted using Stiasny's reagent to indicate the presence of tannin compounds in the sample. Stiasny's reagent typically contains zirconyl chloride ($ZrOCl_2$) and sodium pyroantimonate ($NaSbO_3$). In the tannin screening, zirconyl chloride provides zirconium ions (Zr^{4+}) that react with tannins to form a tannin-zirconium complex. This complex is then coloured by antimonate ions (SbO_3^-) from sodium pyroantimonate, resulting in a pink precipitate.

The screening results indicated that the standard contained a higher concentration of tannins (+++) compared to the extract sample, which did not show any pink precipitate (-). This suggests that the extract sample did not contain tannin compounds.

Following the tannin screening, steroid examination of the extract was performed using Liebermann-Burchard reagent. This reaction produces a green-blue or red-purple colour in the presence of steroids or triterpenoids. The Liebermann-Burchard reagent contains concentrated sulfuric acid as a catalyst and vanillin as a chromophore that imparts colour.

In this screening, a tea solution was used as the standard for steroid examination. The steroid compounds in tea react with vanillin in concentrated sulfuric acid, producing a green-purple complex known as carboxyphenyl quinone. The results indicated that the standard (tea) had a higher steroid content (+++) compared to the extract sample (++).

The final phytochemical screening involved the examination of alkaloids in the extract using Dragendorff's reagent. Alkaloids in the standard (kratom) react with bismuth ions (Bi^{3+}) from Dragendorff's reagent, forming an insoluble alkaloid-bismuth complex. This complex then binds with iodide

ions (I-) from potassium bismuth iodide, resulting in an orange precipitate.

In this phytochemical screening, the results obtained from the standard (kratom) showed a brighter orange colour compared to the extract sample. This indicates that the standard contains a higher concentration of alkaloids (+++) compared to the extract sample (+). The presence of a greater amount of alkaloids in the extract sample also confirms that the extract contains capsaicin as the desired analyte.

Fractionation and sub-fractionation

Following the phytochemical screening, efforts were made to purify capsaicin through fractionation and sub-fractionation. Fractionation was performed using the liquid-liquid extraction (LLE) method. According to Mirwan (2013), the principle of the LLE method is based on the separation of liquid phases, which exploits the differences in solubility of the solute between the original solution and the extracting solvent. Referring to the research by Al-Samydai et al. (2019), fractionation was conducted using a separating funnel with a combination of chloroform and water, repeated five times with chloroform:water ratios of 5:1, 4:2, 3:3, 2:4, and 1:5.

The desired extract is the non-polar alkaloid capsaicin, which is more soluble in chloroform than in water. Due to the higher density of chloroform compared to water, the chloroform phase settles at the bottom of the separating funnel, facilitating the collection of fractions since the dissolved capsaicin is located at the bottom. The resulting extracts were then combined into a single fraction.

The subsequent process involved sub-fractionation using classical column chromatography. This method is based on the principle of separating mixtures according to the distribution differences of their components between the mobile phase and the stationary phase. A glass column was filled with a stationary phase, such as silica gel or alumina, and an eluent was used to separate the components of the mixture. The fundamental principle of classical column chromatography involves the diffusion and adsorption of elements between the mobile phase and the stationary phase, the use of an eluent to separate components, and the detection and collection of fractions. In this study, three types of solvents were used: chloroform, ether, and methanol, as referenced in the research by Al-Samydai et al. (2019). The fraction separation process was based on volume, colour, and separation time.

Before and after sub-fractionation, fraction monitoring was conducted using Thin Layer Chromatography (TLC) in accordance with the Herbal Pharmacopoeia, Edition 2. In this monitoring, a 10% sulfuric acid visualisation was used under UV light at 366 nm. The basic principle of TLC involves separating components based on their differing affinities for the stationary and mobile phases. The stationary phase typically consists of a thin layer of adsorbent, such as silica gel or alumina, coated on glass, plastic, or metal plates. The mobile phase is a solvent or a mixture of solvents that moves upward through the adsorbent layer by capillary action.

According to the Herbal Pharmacopoeia, the mobile phase should ideally be a mixture of toluene and ethyl acetate. However, due to constraints with toluene, the mobile phase was replaced with a mixture of chloroform and diethyl ether. The monitoring results indicated that the chloroform-diethyl ether mixture had a lower dielectric constant compared to toluene-ethyl acetate, making it slightly more non-polar. Chloroform-diethyl ether was used for two rounds of fraction monitoring, both before and after sub-fractionation, using the same mobile phase system.

Based on the Herbal Pharmacopoeia Edition II, the standard Rf value for capsaicin is 0.46. Since the mobile phase used is slightly more non-polar, the resulting Rf values were somewhat lower. The Rf values of 0.38 for monitoring before sub-fractionation and 0.4 after sub-fractionation indicate the presence of capsaicin compounds. As seen in the results presented in Table V earlier, there were no significant differences in the number of spots before and after sub-fractionation. However, the sub-fractionation process using classical column chromatography resulted in much clearer spots for the capsaicin isolate.

Purification and purity testing

The next stage after fractionation is the purification of the obtained samples. The purification method is a process of separating substances to eliminate impurities and get a purer substance from a sample. In this study, purification was performed using recrystallisation and sublimation methods. The fundamental principle of recrystallisation is based on the difference in solubility between the substance to be purified and the solvent or contaminating substance. The resulting solution is separated, and the desired substance is crystallised by saturation, which is achieved by evaporating the solvent (Rositawati, 2013). The type of solvent used in the recrystallisation process is crucial, as it significantly affects the outcome of the recrystallisation. The solubility of a component in a solvent can be determined by the polarity of each solvent. Polar solvents will dissolve polar compounds, while non-polar solvents will dissolve non-polar compounds.

Given that capsaicin is suspected to be in crystalline form, the recrystallisation method is suitable for purifying capsaicin. Recrystallisation is a method used to purify solids (Mohrig, 1979), based on dissolving the solid in an appropriate solvent and then re-crystallising the substance from the solvent. The difference in solubility between the substance to be purified and its impurities is a critical factor in achieving a truly pure result. Capsaicin has a melting point ranging from 62°C to 68°C (Purseglove *et al.,* 1981). However, in this experiment, capsaicin was dissolved at 100°C to facilitate the dissolution of the solvent and to expedite the evaporation process. When capsaicin was dissolved at 100°C, the solution was filtered immediately before the solvent thickened and was evaporated entirely.

Once the isolated powder was obtained, the authors conducted purity testing using single development TLC and two-dimensional TLC. The results from the three TLC plates for the single development method showed only one spot, indicating that a pure isolate was obtained. However, this differed from the results obtained in the subsequent two-dimensional TLC test. In the two-dimensional TLC results, two spots were observed in the second phase, indicating that the compound is still not pure.

Identification and characterisation

To confirm that the obtained isolate is indeed capsaicin, identification was performed using instruments based on the presence of functional groups. In this identification, the authors utilised FTIR spectroscopy without the use of KBr powder. This identification stage is necessary because the previous purity tests did not provide distinctive characteristics of capsaicin, particularly due to the Rf values not being analysed based on various factors that could differentiate the obtained Rf values from the reference Rf values.

The FTIR chromatogram results showed characteristic peaks in the frequency range of 4000-400 cm^{-1}. Based on Table V, the analysis revealed six critical functional groups, including C-H stretch (2927.23 and 2815.17 cm^{-1}), C=C (2119.37 cm^{-1}), amide (1615.71 cm^{-1}), C-O (1066 cm^{-1}), aromatic (800.68 cm^{-1}), with one peak indicating a para orientation (806.68 cm^{-1}). The six functional groups identified have been verified to exist in capsaicin, with the para orientation indicating a bond between the long-chain group and the OH group on the aromatic ring. The impurity of the isolate can also be verified here by the presence of a peak at a frequency of 1456.44 cm^{-1}, indicating a nitro group, and at 1994.53 cm^{-1}, indicating an isocyanate group. The impurity of the isolate results was also confirmed by the differences in response of the functional groups to the types of vibrations and the intensity of the peaks produced, such as the C-H group peak and the para orientation having medium intensity when it should have vigorous intensity, and the amide peak experiencing a shift from stretch to bend vibrations. The presence of hydrogen bonding or interactions between impurity molecules in different environments can shift the frequency of the N-H peak (Ekhlas *et al.,* 2016).

Conclusion

The shaking water bath method, utilising acetone as a solvent, proved to be more effective than the reflux method with 96% ethanol, yielding a higher extract concentration and facilitating a faster extraction process. Analysis of the extraction results revealed that the whole dried chilli powder is richer in oleoresin and dihydrocapsaicin, while the fresh placenta and seeds contain higher levels of capsaicin. Notably, the capsaicin isolate from the PB CH3 variety reached 29 mg, significantly surpassing the yields of other samples, which were below 1 mg. However, it is essential to note that the obtained isolate remains impure, indicating the need for further purification efforts.

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