Pharmacognostic Investigation, Optimization of Extraction Condition, and Determination of Piperine in Black Pepper Fruit

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Abstract: This study investigated the standardization parameters of black pepper fruit (*Piper nigrum* Linn.) through qualitative analyses. Microscopic characteristics of the crude drug were examined. Various parameters were determined, including loss on drying (7.46 ± 0.16 %w/w), total ash (4.29 ± 0.12 %w/w), acid-insoluble ash (0.15 ± 0.03 %w/w), water-soluble extractives (8.35 ± 0.45 %w/w), ethanol-soluble extractives (4.30 ± 0.18 %w/w), and volatile oil content (1.60 ± 0.00 %w/w). Piperine content in the ethanol extract was evaluated using Thin Layer Chromatography (TLC) fingerprint analysis and validated High-Performance Liquid Chromatography (HPLC) analysis. The calibration curve for piperine showed good linearity (R^2 = 0.9991) in the range of 10-100 µg/ml. The limits of detection and quantification were 0.01 µg/ml and 0.04 µg/ml, respectively. Intra-day and inter-day precision were 0.01-0.12% RSD and 0.52-0.90% RSD, respectively. Recovery ranged from 95.85% to 101.85%. Optimal extraction conditions determined through experimental design were 70°C for 60 minutes, yielding a piperine content of 38.98 ± 0.05%. The study provides quantitative pharmacognostic specifications for the fundamental standardization of black pepper fruit.

Keywords: Pharmacognostic investigation; optimization; black pepper fruit

1. Introduction

Piper nigrum Linn., commonly known as black pepper (Piperaceae), is utilized as a spice in many countries worldwide, primarily due to the presence of piperine (Figure 1), which enhances its value as a food additive. The spicy heat of black pepper predominantly arises from piperine obtained from both the outer fruit and the seed. Traditionally, black pepper has been employed as an herbal remedy for alleviating various ailments such as pain, muscular aches, chills, rheumatism, influenza, and fever. Additionally, black pepper tea has been administered to relieve headaches, migraines, strep throat, and digestive issues [1]. It has also been used to address conditions like asthma, chronic indigestion, colon toxins, obesity, sinus congestion, fever, intermittent fever, cold extremities, colic, gastric ailments, and diarrhea. Moreover, black pepper exhibits potent antioxidant activity, aiding in relieving oxidative stress induced by a high-fat diet and acetaminophen-triggered liver damage in mice [2].

ASEAN J. Sci. Tech. Report. **2024**, 27(4), e253186.

2 of 8

The major constituent of black pepper, piperine, possesses various pharmacological properties, including central nervous system depression, antipyretic, analgesic, antioxidant, and hepatoprotective effects. Piperine has also demonstrated anti-inflammatory properties by reducing inflammation in animal models [3]. In humans, piperine enhances the bioavailability of antitubercular drugs when administered together [4].
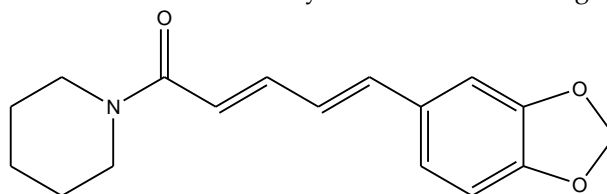

Figure 1. Structure of piperine

The concentration of piperine varies among different types of pepper. Its content in *P. nigrum* L. fruit and root ranges from 3,000 to 6,650 mg/100 g and 790 mg/100 g, respectively [5-9]. Black pepper typically contains between 4.6 and 9.7% piperine by mass [10]. Lee *et al.* (2021) determined the concentration of piperine in black pepper using high-performance liquid chromatography–ultraviolet detection, validating their findings through performance parameter measurements [11]. They observed that the concentration of piperine in black pepper was 4,418 mg/100 g. Bhardwaj *et al.* (2002) investigated the functional properties of piperine through human liver microsomal studies, revealing its inhibition of human P-glycoprotein [12].

Considering the beneficial properties of piperine, particularly its utility in drugs and preservatives, there is a need for its standardization and quality evaluation using a short, fast, reliable, and economical tool. Therefore, this study aimed to investigate the standardization parameters through qualitative analyses of black pepper fruit.

2. Materials and Methods

The pharmacognostic parameters were examined using standard methods outlined in the World Health Organization (WHO) guidelines, "Quality Control Methods for Medicinal Plant Materials."

2.1 Microscopic Characterization

The microscopic characteristics of the black pepper fruit were examined in cross-section. The tissue section was mounted onto a glass slide in water for microscopic observation.

2.2 Loss on Drying

Determination of Loss on Drying: The ground sample was weighed in a pre-weighed small beaker and dried with heat at 105 °C until a constant weight was achieved.

2.3 Determination of Total Ash

The ground sample of black pepper fruit powder was weighed in a pre-weighed crucible and incinerated for 5 hours at 500 °C until it turned white. The crucible was cooled at room temperature in a desiccator, and the total ash content was calculated as a percentage.

2.4 Determination of Acid Insoluble Ash

The crucible containing the total ash was treated with 25 mL of 10% hydrochloric acid, covered with a watch glass, and boiled gently for 5 minutes. The insoluble matter was filtered through ashless filter paper No. 40, dried on a hot plate, and then incinerated for 5 hours at 500 °C. The crucible was cooled at room temperature in a desiccator, and the acid-insoluble ash content was calculated as a percentage.

2.5 Determination of Volatile Oil Content

The ground sample was subjected to volatile oil distillation in a Clevenger apparatus for approximately 4-6 hours.

2.6 Determination of Water and Ethanol Soluble Extractive Value

The ground sample of dried black pepper fruit was macerated with either 100 mL of water or 95% ethanol in a closed conical flask for 6 hours, followed by standing for 18 hours. The extract was filtered rapidly,

ASEAN J. Sci. Tech. Report. **2024**, 27(4), e253186.

3 of 8

and 20 mL of the filtrate was evaporated to dryness in a water bath, then dried at 105 °C until a constant weight was achieved. The extractive value was calculated as a percentage.

2.7 Thin Layer Chromatographic Fingerprint

One gram of black pepper fruit dried powder was macerated in 20 mL of 95% ethanol, shaken for 6 hours, and left to stand for 18 hours at room temperature. The extract was then evaporated to dryness and dissolved in 1 mL of 95% ethanol. The resulting solution was applied to a TLC silica gel 60 GF254 plate, developed in a saturated TLC chamber with hexane: ethyl acetate (1:1), and observed under short wavelength (254 nm) and long wavelength (366 nm) ultraviolet light. The plate was then sprayed with anisaldehyde, developing a reagent.

2.8 Experimental Design and Plant Extraction

Extraction conditions, including temperature (X_1) and time (X_2), were designed based on a spherical composite design. X1 and X2 were varied from 45-95 °C and 10-60 minutes, respectively. The ground sample (10 g) of black pepper fruit powder was mixed with 50 mL of 95% ethanol in a closed Erlenmeyer flask. The flask was then placed in a water bath shaker and extracted under specific conditions. The mixture was filtered using Whatman No. 1 filter paper, and the residue was extracted twice before evaporating to dryness.

Table 1. Two-factor spherical composite experimental design.

Condition No.	Temperature (X_1)		Time (X_2)	
	Coded	Actual (°C)	Coded	Actual (min)
P1	-1	52.3	-1	17.3
P2	1	87.7	-1	17.3
P3	-1	52.3	1	52.7
P4	1	87.7	1	52.7
P5	$-\sqrt{2}$	45.0	0	35.0
P6	$\sqrt{2}$	95.0	0	35.0
P7	0	70.0	$-\sqrt{2}$	10.0
P8	0	70.0	$\sqrt{2}$	60.0
P9	0	70.0	0	35.0
P10	0	70.0	0	35.0

2.9 Chromatographic Conditions

The study utilized an HPLC instrument (Agilent 1260 Infinity) with a photodiode array detector coupled to a UV detector set at 342 nm. A ZORBAX Eclipse Plus C18 (4.6 x 5.5 mm, 3.5 μm) column was eluted using a mixture of methanol and water (70:30) as the mobile phase. The injection volume, flow rate, and column temperature were set at 10 μL, 1 mL/min, and 25 °C, respectively.

2.10 Preparation of Standard Piperine

A stock solution of piperine was dissolved in ultrapure water at a concentration of 1 mg/mL. From this solution, a series ranging from 10-100 μg/mL was prepared to construct a calibration curve.

2.11 Method Validation

Method validation was performed following the ICH Harmonised Tripartite Guideline 6, covering five topics: linearity and range, specificity, limit of detection (LOD) and limit of quantitation (LOQ), precision, and accuracy.

2.11.1 Linearity and Range

The standard solution was prepared at five concentrations (10, 25, 50, 75, and 100 μg/mL) and injected for analysis. The linearity was assessed by the coefficient of determination (R^2), and the relationship between peak areas and standard piperine concentrations was determined.

2.11.2 Specificity

Specificity was evaluated by scanning UV absorption spectra in the range of 190-400 nm. Similar UV spectra obtained across the interest peak's up-slope, apex, and down-slope indicated specificity.

2.11.3 Limit of Detection (LOD) and Limit of Quantitation (LOQ)

ASEAN J. Sci. Tech. Report. **2024**, 27(4), e253186.

4 of 8

LOD represents the lowest concentration detectable but not necessarily quantifiable, while LOQ represents the lowest concentration detectable and measurable. Formulas were utilized based on the standard deviation of y-intercepts and the slope of the calibration curve.

LOD = (3.3 × σ)/S, LOQ = (10 × σ)/S

2.11.4 Precision

Precision was assessed at three standard solution levels (25, 50, and 75 µg/mL) by determining intra-day and inter-day precision. Percent relative standard deviation (% RSD) was calculated to quantify precision.

% RSD = SD × 100/mean

2.11.5 Accuracy

Accuracy was evaluated using the spike method, where different standard levels (low, medium, and high) were spiked into the sample. Recovery percentages were calculated based on adding standard piperine to the known amount of black pepper fruit extract.

3. Results and Discussion

The anatomical structure in the transverse section of the *Piper nigrum* Linn fruit reveals several key features. Anatomical characteristics include: a) Epidermis: Comprising an outer layer of polygonal cells with a distinct cuticle containing dark brown to blackish contents. b) Parenchyma: Consisting of two layers of thin-walled cells interspersed with considerably thickened isodiametric to radically elongated stone cells. c) Stone cells are considerably thickened and vary in shape from isodiametric to radially elongated. d) Oil cells: Larger oil cells with suberized walls are in a layer beneath the parenchyma cells. e) Starch grains: Certain starch grains are scattered among the parenchyma cells. f) Pericarp: The mesocarp, a comparatively broad zone, comprises most of the pericarp. The outer layers of cells are parenchymatous, and larger secretion sacs with suberized walls and oil contents may be observed. g) Perisperm: The inner zone of perisperm cells is radial and contains largely oleoresin starch and protein substances. The morphological characteristics observed in this study are similar to those reported in a previous study conducted in India. The epicarp has polygonal cells with a cuticle and dark contents, followed by 2-3 layers of parenchyma and thickened stone cells. The mesocarp is a broad zone with parenchymatous cells containing starch grains, secretion sacs, oil/resin, and fibrovascular bundles. The endocarp consists of stone cells with pronounced inner walls. The testa has compressed elongated cells and a pigment layer with tannin [13].
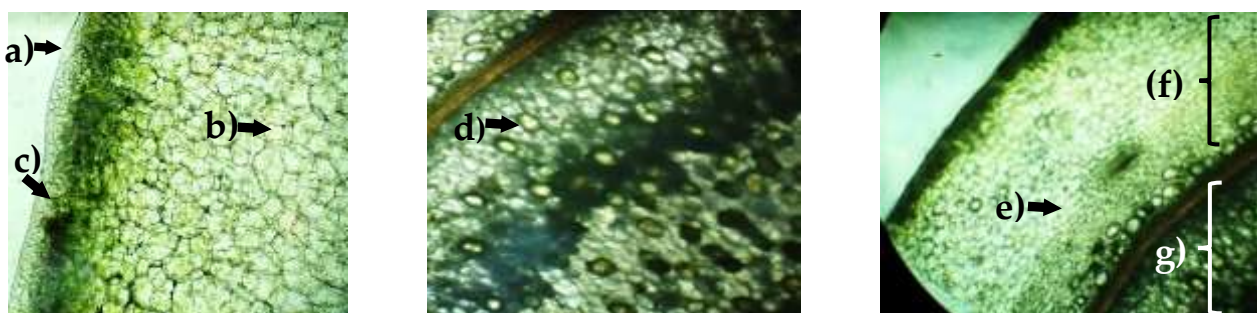

Figure 2. Transverse section of the fruit of *Piper nigrum* Linn.

Table 2. The pharmacognostic parameters of black pepper fruit

Parameters	Content (%w/w)
Loss on drying	7.46 ± 0.16
Total ash	4.29 ± 0.12
Acid-insoluble ash	0.15 ± 0.03
Water extractive value	8.35 ± 0.45
Ethanol extractive value	4.30 ± 0.18
Volatile oil	1.60 ± 0.00

ASEAN J. Sci. Tech. Report. **2024**, 27(4), e253186.

5 of 8

The pharmacognostic parameters of black pepper fruit were determined, including loss on drying, total ash, acid-insoluble ash, water-soluble extractives, ethanol-soluble extractives, and volatile oil content. The results are as follows: 7.46 ± 0.16, 4.29 ± 0.12, 0.15 ± 0.03, 8.35 ± 0.45, 4.30 ± 0.18, and 1.60 ± 0.00 %w/w, respectively, as shown in Table 2.

A TLC fingerprint of the Piper nigrum Linn fruit was conducted using a TLC silica gel 60 GF254 plate as the stationary phase. The development was performed in a saturated TLC chamber with a hexane and ethyl acetate ratio of 1:1. The spots were observed using ultraviolet light under both short (254 nm) and long (366 nm). Subsequently, the plate was sprayed with anisaldehyde developing reagent and observed (Figure 3). Thin-layer chromatography is a simple, rapid, and inexpensive method with promising potential for analyzing and quantifying the constituents in herbal drugs [14].
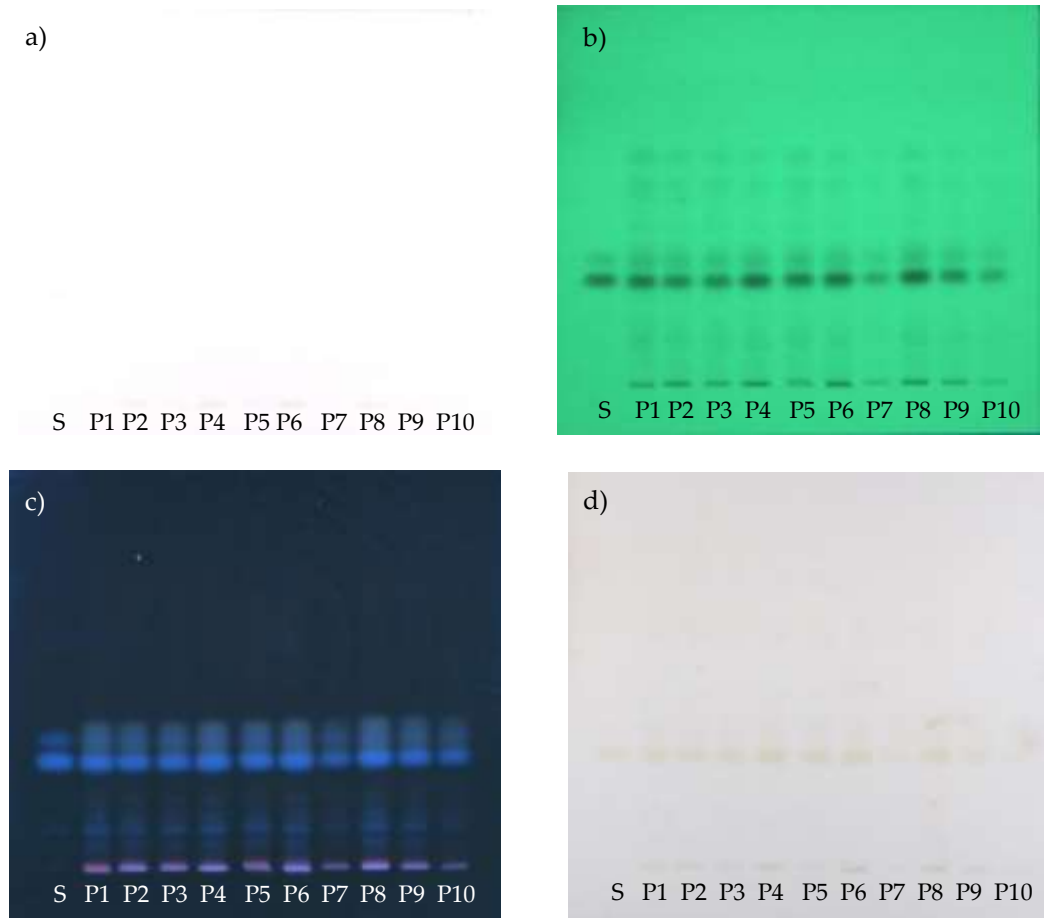

Figure 3. TLC fingerprint of *Piper nigrum* Linn. Fruit a) detection under daylight, b) detection under UV 254 nm, c) detection under UV 366 nm, d) detection with anisaldehyde reagent, (S) piperine standard and (P1-P10) extract of *Piper nigrum* Linn. Fruit

Table 3. HPLC validation of piperine content in black pepper fruit

Parameter	Piperine in black pepper fruit
Calibration curve	$y = 137356x - 447800$ ($R^2 = 0.9991$)
Range	10-100 µg/ml
Accuracy	95.85-101.85% recovery
Precision	
Intra-day	0.01-0.12 % RSD
Inter-day	0.52-0.90 %RSD
LOD	0.01 µg/ml
LOQ	0.04 µg/ml

The HPLC analysis utilized in this study for the quantitative determination of piperine was validated in terms of linearity, LOD (limit of detection), LOQ (limit of quantification), accuracy, precision, and specificity.

The regression line of the method demonstrated linearity in the range of 10-100 µg/ml, with a coefficient of determination (R^2) of 0.9991. The recovery of spiked known concentrations of standard piperine was assessed accurately. The percent recovery ranged from 95.85% to 101.85%, indicating the method's accuracy with a percent recovery between 80% and 120%.

Repeatability (intra-day precision) and intermediate (inter-day precision) were less than 2%. The precision of piperine quantitative analysis was determined by analyzing three concentrations on three different days.

The LOD and LOQ of the method were calculated using the residual standard deviation of the regression lines. The LOD was found to be 0.01 µg/ml, and the LOQ was 0.04 µg/ml (refer to Table 3).

The specificity of the method was validated through absorbance spectra within the range of 190-400 nm, comparing standard piperine with the extracts. The results revealed identical spectra obtained at the peak's up-slope, apex, and down-slope, indicating the piperine's chromatographic peak purity. The maximum absorption of piperine was observed at 342 nm, consistent with a previous study reporting an absorption peak at 343 nm [15].
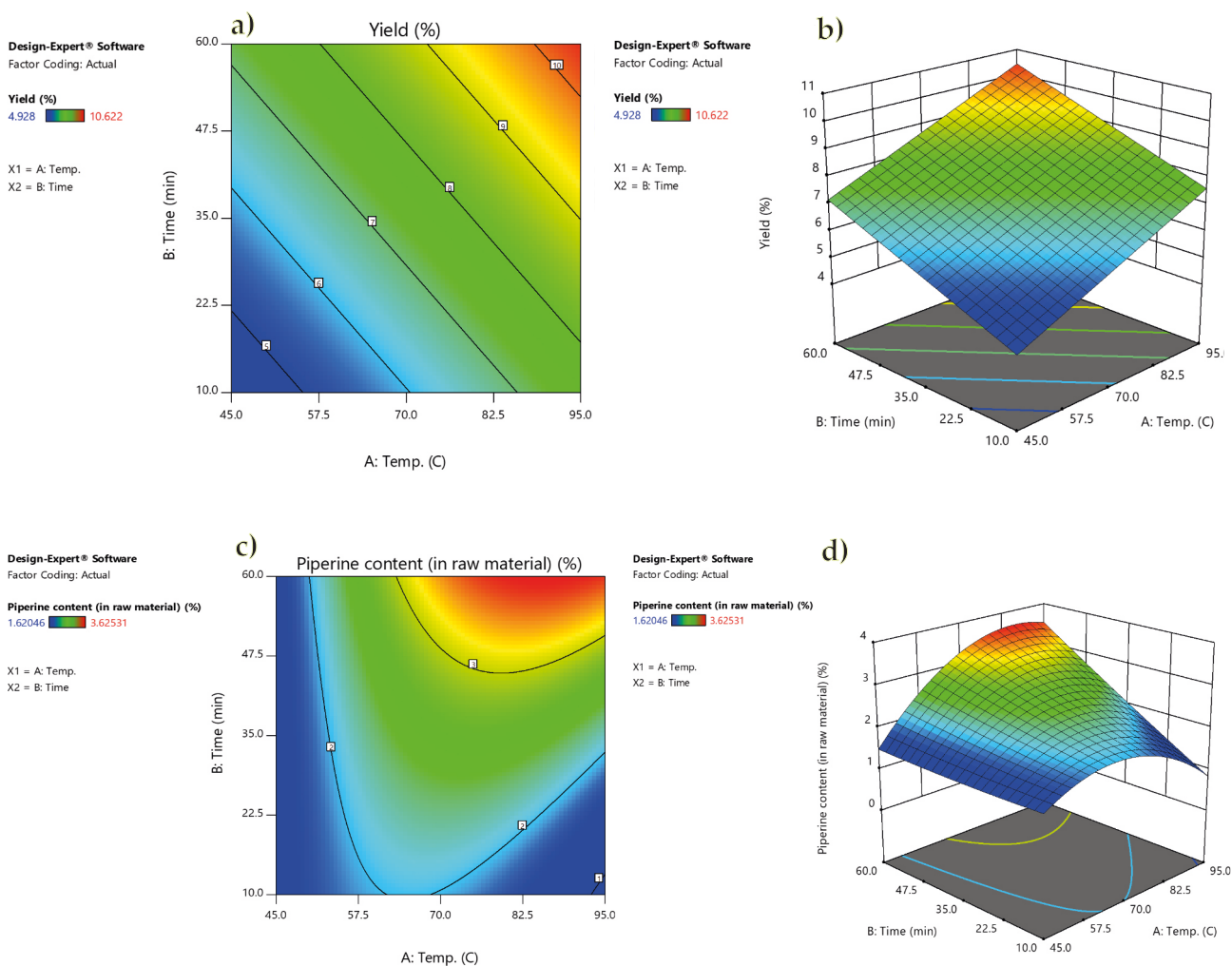

Figure 4. Contour plots of the model conditions of the content of (a-b) % yield and (c-d) piperine content

ASEAN J. Sci. Tech. Report. **2024**, 27(4), e253186.

7 of 8

A two-factor spherical composite experimental design was conducted to estimate piperine content. The response surface analysis revealed the complex relationship between the causal factors (Xn) and their responses (Yn). Higher piperine content was observed at higher temperatures and longer extraction times. The optimal conditions for achieving the highest piperine content were a temperature of 70°C and an extraction time of 60 minutes. The result indicated that the extract's piperine content was 38.98±0.05% (as shown in Figure 4). Previous studies have suggested that piperine content's extraction yield was lower than ultrasound-assisted extraction (37.0 mg/g) at 50°C for 30 minutes. Extraction yields of piperine obtained from Soxhlet extraction and batch extraction methods were much lower than those obtained from ultrasound-assisted extraction. Utilizing ultrasound-assisted extraction for natural phytochemicals can mitigate the issues of lower extractability and longer extraction times associated with conventional methods [16].

4. Conclusion

The pharmacognostic properties identified in this study can contribute to the quality control of black pepper fruit. Physicochemical parameters are crucial for herbal drug identification and quality control. The findings of this study, including pharmacognostic specifications, quality control parameters, and prediction of piperine content, can facilitate drug development.

5. Acknowledgements

References

[1] Parthasarathy, V.A., Chempakam, B.; Zachariah, T.J. (eds.), *Chemistry of spices*. Cabi. **2008**.

[2] Vijayakumar, R.; Surya, D.; Nalini, N. Antioxidant efficacy of black pepper (*Piper nigrum* L.) and piperine in rats with high fat diet induced oxidative stress. *Redox Report* **2004**, *9*(2), 105-110.

[3] Bang, J.S.; Oh, D.H.; Choi, H.M.; Sur, B.J.; Lim, S.J.; Kim, J.Y.; Yang, H.I.; Yoo, M.C.; Hahm, D.H.; Kim, K.S. Anti-inflammatory and antiarthritic effects of piperine in human interleukin 1β-stimulated fibroblast-like synoviocytes and in rat arthritis models. *Arthritis research & therapy* **2009**, *11*, 1-9.

[4] Srinivasan, K. Black pepper and its pungent principle-piperine: a review of diverse physiological effects. *Critical reviews in food science and nutrition* **2007**, *47*(8), 735-748.

[5] Ko, J. M. A study on quantitative method of piperine in pure ground black pepper. *Journal of Food Hygiene and Safety* **1995**, *10*, 169-174.

[6] Hu ShiLin, H. S.; Ao Ping, A. P.; Liu Dai, L. D. Pharmacognostical studies on the roots of *Piper nigrum* L. *III: Determination of essential oil and piperine* **1996**, 179-182.

[7] Pradeep, C. R.; Kuttan, G. Effect of piperine on the inhibition of lung metastasis induced B16F-10 melanoma cells in mice. *Clinical & experimental metastasis* **2002**, *19*, 703-708.

[8] Sunila, E. S.; Kuttan, G. Immunomodulatory and antitumor activity of *Piper longum* Linn. and piperine. *Journal of ethnopharmacology* **2004**, *90*(2-3), 339-346.

[9] Lee, J. G.; Kim, A. Y.; Kim, D. W.; Kim, Y. J. Determination and risk characterization of bio-active piperine in black pepper and selected food containing black pepper consumed in Korea. *Food Science and Biotechnology* **2021**, *30*, 209-215.

ASEAN J. Sci. Tech. Report. **2024**, *27*(4), e253186.

8 of 8

[10] Lawless, H. T.; Heymann, H. Sensory evaluation of food: principles and practices. Springer Science & Business Media. **2010**.

[11] Lee, J. G.; Kim, Y. J. Comparison study of validation parameters and measurement uncertainty of rapid analytical methods for piperine in black pepper by ultraviolet spectroscopy and high-performance liquid chromatography. *Food Science and Biotechnology* **2022**, *31*(9), 1133-1143.

[12] Bhardwaj, R. K.; Glaeser, H.; Becquemont, L.; Klotz, U.; Gupta, S. K.; Fromm, M. F. Piperine, a major constituent of black pepper, inhibits human P-glycoprotein and CYP3A4. *Journal of Pharmacology and Experimental Therapeutics* **2002**, *302*(2), 645-650.

[13] Trivedi, M. N.; Khemani, A.; Vachhani, U. D.; Shah, C. P.; Santani, D. D. Pharmacognostic, phytochemical analysis and antimicrobial activity of two Piper species. *Pharmacie Globale* **2011**, *7*(05), 1-4.

[14] Sueree, L.; Ruangrungsi, N. Pharmacognostic Specification and Pinocembrin Content of *Boesenbergia rotunda* Root. RSU International Research Conference 2016, Rangsit University, Thailand, 29 April 2016.

[15] Saingam, W.; Monton, C.; Suksaeree, J.; Chankana, N.; Kittiwisut, S.; Charoenchai, L. Development and validation of RP-HPLC method for determination of piperine in maha-solos tablet formulation. *The Thai Journal of Pharmaceutical Sciences* **2013**, *38*, 133-136.

[16] Gorgani, L.; Mohammadi, M.; Najafpour, G. D.; Nikzad, M. Sequential microwave-ultrasound-assisted extraction for isolation of piperine from black pepper (*Piper nigrum* L.). *Food and Bioprocess Technology* **2017**, *10*, 2199-2207.