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Polyphenol content and antioxidant capacities of *Graptophyllum pictum* (L.) extracts using *in vitro* methods combined with the untargeted metabolomic study

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ABSTRACT

Graptophyllum pictum (L.) Griff contains polyphenols and pharmacological activity for health. In this research, various solvents were employed to examine the total polyphenols, antioxidant capacity, and metabolite profile of *G. pictum* leaf extract. The chemometric analysis evaluated secondary metabolites from liquid chromatography with tandem mass spectrometry from *G. pictum* with various solvents on total polyphenol and antioxidant capacity. Analysis of total polyphenols revealed that the highest total phenolic content was 32.17 mg gallic acid equivalent g^{-1} DW in ethanol solvent, while the highest total flavonoid content was 9.14 quercetin equivalent g^{-1} DW in ethyl acetate solvent. Meanwhile, the highest radical scavenging capacity of 2,2-diphenyl-1-picrylhydrazyl was 29.71 µmol TE g^{-1} DW in ethanol solvent, and the highest radical scavenging capacity of 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) was highest at 33.27 µmol TE g^{-1} DW in ethanol solvent. Furthermore, the reduction capacity value used a ferric-reducing agent. The highest ferric reducing antioxidant power was 52.54 TE g^{-1} DW in ethanol solvent, and the highest reduction of cupric reducing antioxidant capacity was 65.94 µmol TE g^{-1} DW in ethanol solvent. The results of chemometric analysis using hierarchical cluster and principal component analysis showed that secondary metabolites such as phenolic and flavonoid group in ethanol extract had the best potential for various antioxidant activities.

INTRODUCTION

Graptophyllum pictum (L.) Griff, often referred to as purple leaf, is a herbaceous plant from the Acanthus family, often used in traditional medicine to relieve menstrual pain, stomach pain, and ulcers [1]. This plant is endemic to Papua New Guinea and is spread across the equator, such as Asia (especially Indonesia), India, Africa, and Latin America [2]. Many studies state that *G. pictum* has various pharmacological activities, such as free radical scavenging capacity, anti-inflammatory, anti-hemorrhoidal, and anti-inflammatory [2,3]. On the other hand, *G. pictum* contains phenolic and flavonoid phytochemical compounds that have anti-inflammatory activity by reducing free radicals and inhibiting enzymes that play a role in inflammatory processes, such as cyclooxygenase-2 and inducible nitric oxide synthase [4]. In addition, other studies also mention that *G. pictum* contains steroid class metabolites, terpenoids, alkaloids, tannins, and volatile compounds that play a role in the treatment of infections [5,6]. The compounds contained can be extracted through an extraction process. Extraction separates certain compounds or active substances from a solid or liquid mixture using a particular solvent [7].

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Solvents with varying polarities can be used in extracting various classes of compounds with different polarities. Huliselan [8] reported that the type of solvent is one of the factors that can affect the content of extracted compounds. Besides that, the bioactive components in a sample will move to the solvent with intensive contact. Polyphenolic compounds (phenolics and flavonoids) are polar compounds that tend to dissolve in polar solvents such as water, ethanol, methanol, and ethyl acetate. On the other hand, steroid and terpenoid compounds tend to dissolve in nonpolar solvents such as n-hexane.

Many studies on the bioactivity of purple plants have been carried out. The study in Masyita *et al.* [9] reported that wungu leaves have the potential for sun protection, and that in Kusumaningsih *et al.* [7] also reported that purple leaves extracted using 70% ethanol had better antibacterial activity than 96% ethanol. In addition, the optimization of purple leaf extract for flavonoid content and antioxidant activity using the simplex centroid design has also been reported [2]. However, metabolomics studies using liquid chromatography with tandem mass spectrometry (LC-MS/MS) in the comprehensive analysis of secondary metabolites of *G. pictum* in various solvents on antioxidant activity [2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), ferric reducing antioxidant power (FRAP), and cupric reducing antioxidant capacity (CUPRAC)] in *G. pictum* extracts have been little reported. Therefore, this study used chemometric analysis in the form of Pearson correlation, hierarchical clustering (HCA), and principle component analysis (PCA) to evaluate metabolic data from LC-MS/MS results on the antioxidant capacity of purple plants. In addition, this study also aims to determine the most optimal type of solvent in purple plant extracts with total phenolic (TPC) and flavonoid content (TFC) and antioxidant activity (DPPH, ABTS, FRAP, and CUPRAC) with the highest values.

MATERIALS AND METHODS

Chemicals and reagents

All extraction solvents in the form of ethanol, ethyl acetate, and n-hexane using analytical grade were purchased from MERCK (Germany); Folin-Ciocalteu phenol reagent, Na_2CO_3, $AlCl_3$, methanol, ABTS, $K_2S_2O_8$, hydrogen chloride (HCl), $CuCl_2$, and buffer ammonium acetate (pH 7.0) were purchased from MERCK (Germany); glacial acetic acid and DPPH powder were purchased from Sigma-Aldrich (St. Louis, MO); $FeCl_3$, 2,4,6-tripyridin-s-triazine (TPTZ), and 2,9-dimethyl-1,10-phenanthroline (neocuproine) were purchased from Sisco Research Laboratories Pvt. Ltd. (India); buffer acetate (pH 3.6) was purchased from Mallinckrodt plc (Cruiserath, Blanchardstown, Dublin 15, Ireland); gallic acid (98.0%) and quercetin standard (99.0%) were purchased from Sisco Research Laboratories Pvt. Ltd. (India); and 6-hydroxy-2,5,7,8-tetramethyl-3,4-dihydrochromene-2-carboxylic acid (Trolox) standard and Aquabidest were purchased from MERCK (Germany).

Plant *Simplicia* preparation and extraction

Samples came from the collection of the Tropical Biopharmaca Research Center, Bogor Agricultural University, West Java, Indonesia (6°32′25.47″ N, 106°42′53.22″ E, 142.60 m above sea level), and were authenticated at the same place through the brochure number BMK0164092016. *Graptophyllum pictum* is a shrub with a height of 1–2 m, has characteristic green elliptical leaves with reddish-purple spots, herbaceous-type stems with purplish-red coloring and four-cornered branches. Sample preparation based on Nurcholis *et al.* [10] was performed as follows: first, samples were dried using an oven at 45°C for 2 days and pulverized to obtain *Simplicia* powder (80 mesh). Extraction *G. pictum* using the maceration method with ethanol, ethyl acetate, and n-hexane solvents, using a randomized complete block design with three replications for each solvent treatment. Maceration was carried out by dissolving 25 g of powdered *Simplicia* in 250 ml of solvent [1: 10 (m/v)], followed by a sonication process using a sonicator (Decon, Hove, Sussex, UK) for 30 minutes, and a maceration process was carried out for 24 hours at a speed of 150 rpm using a water bath shaker (Wisebath SSB, South Korea). The extract was filtered using Whatman No. 4 filter paper and vacuum. The filtrate was concentrated using a rotary evaporator (LabTech, Germany) with a temperature of 45°C and a rotational speed of 35 rpm. Stock solutions were prepared at a concentration of 2,000 ppm (20 mg paste extract in 10 ml ethanol).

Determination of total polyphenol content

TPC measurements

The TPC was determined [11] using the Folin-Ciocalteu reaction reagent and standard gallic acid. Preparation of phenolic test using Foline reagent 10% (v/v) was prepared by dissolving 5 ml of 100% folin solution in 50 ml of distilled water; 10% (m/v) Na_2CO_3 was prepared by dissolving 5 g of Na_2CO_3 in 50 ml of distilled water. The gallic acid standard was prepared by dissolving 0.02 g of gallic acid in 20 ml of pro-analytical ethanol, so a concentration of 1,000 ppm was obtained. The curve is made by varying the concentration of gallic acid in the range of 20–300 ppm. The TPC test was carried out by reacting 20 µl of the sample or standard gallic acid with 120 µl of 10% Folin-Ciocateu reagent and then incubating for 5 minutes; after incubation, 80 µl of Na was added with Na_2CO_3 10% and then incubated for 30 minutes. Absorbance was read at a wavelength of 750 nm by nano spectrophotometry (SPECTROstar Nano BMG LABTECH). TPC is calculated based on gallic acid equivalent (GAE) concentration parameters is shown using the following equation:

$$\text{TPC (mg GE g}^{-1}\text{ DW)} = \frac{\text{GAE (mg l}^{-1}\text{)} \times \text{Sample volume (l)} \times \text{Dilution factor}}{\text{Sample weight (g)}}.$$

TFC measurements

The method for determining the TFC based on Khumaida *et al.* [11] used aluminum chloride reagent and quercetin standards. Preparation of 10% (m/v) $AlCl_3$ reagent was prepared by dissolving 5 g of $AlCl_3$ into 50 ml of distilled water. Quercetin standard solution was prepared by dissolving 0.02 g of quercetin in 20 ml of ethanol to obtain a concentration of 1,000 ppm. The curve was made by varying the concentration of quercetin in the range of 25–500 ppm. The total content of

flavonoids (TFC) was determined by adding 120 µl of distilled water with 10 µl of sample or quercetin standard and 10 µl of $AlCl_3$ 10%, 10 µl glacial acetic acid, and 50 µl ethanol pro-analyzed, then incubated for 30 minutes. The absorbance was read at a wavelength of 415 nm using nanospectrophotometry (SPECTROstar Nano BMG LABTECH). TFC is calculated based on quercetin equivalent (QE) concentration parameters using the following equation:

$$\text{TFC (mg QE g}^{-1}\text{ DW)} = \frac{\text{QE (mg l}^{-1}) \times \text{Sample volume (l)} \times \text{Dilution factor}}{\text{Sample weight (g)}}.$$

Determination of antioxidant capacity

The antioxidant capacity of the samples was measured by nanospectrophotometer-based calorimetry (SPECTROstar Nano BMG LABTECH) using DPPH, ABTS, FRAP, and CUPRAC methods with trolox equivalent antioxidant capacity (TEAC) based on modification [9,11]. Antioxidant capacity is calculated based on TEAC concentration parameters using the following equation:

$$\text{TEAC (µM TE g}^{-1}\text{ DW)} = \frac{\text{TE (µM l}^{-1}) \times \text{Sample volume (l)} \times \text{Dilution factor}}{\text{Sample weight (g)}}.$$

DPPH scavenging capacity method

The solution of DPPH radicals was prepared by mixing 2.5 mg of DPPH powder in 50 ml of methanol to obtain a final volume of 1.25 µM. Trolox standards were made in various concentrations of 20–90 µM. The test was carried out by adding 100 µl of sample or trolox standard and 100 µl of DPPH reagent (1.25 µM), then incubated for 30 minutes, and the absorbance was measured at a wavelength of 515 nm.

ABTS scavenging capacity method

The solution of ABTS radicals was prepared by mixing ABTS stock solution (7.7 mM) and potassium persulfate (2.4 mM) in a ratio of 2:1 (v/v). Dilution was carried out by adding Aqua Bidest to obtain an absorbance of 0.7 ± 0.2 at a wavelength of 734 nm. Trolox standards were made with various concentrations of 100–500 µM. The test was carried out by adding 20 µl of the sample or trolox standard and 180 µl of ABTS reagent and then incubating it for 6 minutes. Absorbance was measured at a wavelength of 734 nm.

FRAP method

The ferric-reducing antioxidant assay reagent was carried out by mixing acetate buffer pH 3.6, TPTZ, and ferric chloride, with a ratio of 10:1:1 (v/v). The mixture was then incubated for 30 minutes. Trolox standards were made with various concentrations of 100–600 µM. The test was carried out by adding 180 µl of FRAP reagent with ten µl of sample or trolox standard and then incubating it for 30 minutes. Absorbance was measured at a wavelength of 593 nm.

CUPRAC method

The cupric reducing antioxidant assay reagent was carried out by preparing a solution of neocuproine (0.0075 M) in distilled water, copper sulfate ($CuCl_2$) (0.01 M) in distilled water, and ammonium acetate buffer (pH 7). Trolox standards were made with various concentrations of 100–500 µM. The test was carried out by adding 50 µl of the sample or trolox standard, 50 µl of $CuCl_2$ solution (0.01M), 50 µl 2,9-dimethyl-1,10-phenanthroline (neocuproine) reagent (0.0075 M), and 50 µl ammonium acetate buffer solution (pH 7.0). Absorbance was measured at a wavelength of 450 nm.

Liquid chromatography with tandem mass spectrometry

Analysis of secondary metabolites is based on Li *et al.* [12]. A total of 10 mg of the sample was dissolved in 5 ml of methanol and homogenized using an ultrasonicator for 30 minutes. The sample solution was then filtered using a 0.2 µm PTFE membrane. A total of 2.0 µl of the sample was then injected into the LC-MS/MS using a syringe. The eluent in the mobile phase [H_2O + 0.1% formic acid (A) and 0.1% formic acid (B)] with a flow rate of 0.2 ml/minute, using the gradient method 0–1 minute (5% B), 1–25 minutes (5%–95% B), 25–28 minutes (95% B), and 28–30 minutes (5% B). The stationary phase consisted of accucore C18, 100 × 2.1 mm, 1.5 µm (ThermoScientific) at 30°C. The tool is set to detect a mass size of 100–1,500 m/z with the positive (+) ionization method. The LC-MS/MS analysis data were then processed using Compound Discoverer 3.2 software and matched to the output spectra of MS1 and MS2 using various online databases (mzCloud, ChemSpider, and PubChem) to obtain predictions of the chemical structure of the metabolites.

Statistical analysis

Data on polyphenol content and antioxidant capacity were expressed as three replicates' mean ± standard error (SE). Analysis of variance and Tukey's follow-up test were conducted using SPSS version 25. Pearson correlation analysis between polyphenol and pharmacological parameters was generated using the performance analytics package in R-Studio version 4.2.2 [13]. Characterization of secondary metabolites *G. pictum* based on differences in solvents was carried out using various chemometric approaches such as HCA and PCA [13–15]. PCA analysis was performed using MetaboAnalyst 5.0. Data preparation begins by creating a data frame consisting of the secondary metabolite area as a result of the chromatogram and the antioxidant capacity of each sample, and then by normalizing the data using logarithmic transformation and auto-scaling of the data. Then, hierarchical clustering features were selected in the clustering analysis option, and PCA features were selected in the chemometric analysis option.

RESULTS AND DISCUSSION

Extraction yield

The results showed that the ethanol solvent produced the highest total yield of 19.62% compared to the hexane solvent at 15.54% and ethyl acetate at 16.29% (Table 1); however, the obtained percent yield did not show a significant difference from each other at p <0.05. This research was in line with Jiangseubchatveera *et al.* [16], which states that the extraction is influenced by the type of solvent used.

Total polyphenol content

The polyphenol content of *G. pictum* is shown in Table 2. Extracts with polar solvents, such as ethanol, have the highest TPC compared to other solvents. Furthermore, TPC has a higher value when compared to TFC in all solvent treatments. Phenolics are compounds containing hydroxyl groups and double rings in the benzene structure that undergo resonance stabilization, stabilizing the radical state in reactions with free radicals [17]. It has various pharmacological activities, such as antioxidant, anti-inflammatory, and preventing degenerative diseases [18,19]. Phenolics are one of the polyphenol group compounds that are very abundant in nature and have a variety of structures. Quantifying TPC using the Folin-Ciocalteu reagent and GAE as a standard can determine the TPC of herbal sample 2. In this study, the highest yield was 32.17 mg GAE g^{-1} in ethanol solvent, and the lowest yield was 1.87 mg GAE g^{-1} in n-hexane solvent. The difference in TPC in each sample is due to the difference in polarity of the extraction solvent, so metabolites with the same degree of polarity as the solvent can be easily extracted. This research lines with Mohammed *et al.* [20], where TPC tends to have a high value in organic polar solvents such as ethanol and methanol. Various studies have reported that using polar solvents to extract *G. pictum* produces a higher yield of polyphenolic compounds [16]. The highest TPC of *G. pictum* was 102.5 mg GAE g^{-1} in the ethyl acetate fraction, and the lowest TPC was 11.7 mg GAE g^{-1} in the n-hexane fraction.

The highest TFC was found in extracts with semipolar solvents such as ethyl acetate. Extracts in nonpolar solvents such as n-hexane showed the lowest results for phenolic and flavonoid content. Flavonoids are a group of polyphenols that are very abundant in nature. In addition, the same phenolic group of flavonoids has various pharmacological activities, such as antioxidants, and in preventing degenerative diseases [21,22]. Flavonoid activity in scavenging free radicals involves hydroxyl groups at numbers 3, 5, 3', and 4', covering various electron and proton transfer reactions [23]. Quantification of TFC of leaves *G. pictum was* the highest at 9.14 mg QE g^{-1} DW in ethyl acetate solvent, and the lowest was 0.51 mg QE g^{-1} DW in n-hexane solvent. Flavonoids tend to have a broad solubility in various solvents, and this is due to the varied structure of the flavonoids. The study in Makkiyah *et al.* [2] reported that extraction using aqueous solvents increased TFC by 10.46 mg QE g^{-1}. On the other hand, the research in Jiangseubchatveera *et al.* [16] reported the highest TFC of 28.21 mg QE g^{-1} in the n-hexane fraction and the lowest TFC of 2.02 mg QE g^{-1} in the water fraction.

Table 1. Percentage yield of *G. pictum* extract.

Solvent	Yield (%)
Ethanol	19.62 ± 2.92[a]
Ethly acetate	15.54 ± 0.10[a]
n-Hexane	16.29 ± 0.64[a]

The same letter in the same column shows the results are not significantly different from the results at $p < 0.05$.
Percentage yield of *G. pictum* based on differences in solvents. Results are expressed in mean ± SE ($n = 3$) for each solvent-treated sample.

Table 2. Total phytochemical content of *G. pictum* extracts based on various solvents.

Solvent	TPC (mg GAE/g DW)	TFC (mg QE/g DW)
Ethanol	32.17 ± 4.03[a]	5.49 ± 0.13[a]
Ethyl acetate	16.52 ± 3.33[b]	9.14 ± 1.23[b]
n-Hexane	1.87 ± 0.09[c]	0.51 ± 0.18[c]

Total phytochemical extracts of *G. pictum* based on differences in solvents. Results are expressed in mean ± SE ($n = 3$) for each solvent-treated sample. Different letters in the same column show statistically different results at $p < 0.05$.

Antioxidant capacity

The antioxidant capacity of the TEAC *G. pictum* method is shown in Table 3. Extracts with polar solvents, such as ethanol, showed the highest antioxidant capacity results in all TEAC tests, followed by ethyl acetate and n-hexane extracts, and this is in line with Makkiyah *et al.* [2], which stated that polar solvents such as water in *G. pictum* extract showed optimum antioxidant capacity results. This study used *in vitro* antioxidant screening approaches such as DPPH, ABTS, FRAP, and CUPRAC in Trolox equivalents. This approach was intended because each antioxidant assay has advantages and limitations [24–26]. Analysis of the free radical scavenging capacity of samples using two radical-based methods such as 2,2-di(4-tert-octylphenyl)-1-picrylhydrazyl radical ($DPPH^{\cdot}$) and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid radical cation ($ABTS^{+\cdot}$). In addition, the reduction capacity of ferric metal (Fe^{3+}) to ferrous (Fe^{2+}) is also used in the FRAP assay. The reduction of cupric ion metal (Cu^{2+}) to cuprous (Cu^{+}) in the CUPRAC assay [16] indicated that *G. pictum* had the highest DPPH absorption capacity of 0.78 (IC_{50}, mg ml^{-1}) and ABTS absorption capacity of 69.19 mg TE g^{-1} DW; in Makkiyah *et al.* [2], the best DPPH capacity was found in water solvent of

Table 3. TEAC of *G. pictum* extracts based on various solvents.

Solvent	DPPH (μM TE g^{-1} DW)	ABTS (μM TE g^{-1} DW)	FRAP (μM TE g^{-1} DW)	CUPRAC (μM TE g^{-1} DW)
Ethanol	29.71 ± 0.23[a]	33.27 ± 1.40[a]	52.54 ± 4.93[a]	65.94 ± 0.37[a]
Ethyl acetate	19.71 ± 0.53[b]	12.30 ± 3.27[b]	24.11 ± 2.49[b]	64.17 ± 3.83[b]
n-Hexane	3.94 ± 0.15[c]	1.64 ± 4.79[c]	8.66 ± 0.90[c]	18.76 ± 2.34[b]

Extract antioxidant capacity *G. pictum* based on differences in solvents. Results are expressed in mean ± SE ($n = 3$) for each solvent-treated sample. Different letters in the same column show statistically different results at $p < 0.05$.

2.21 µmol TE g^{-1} DW and the best FRAP capacity was found in acetone solvent of 27.60 µmol TE g^{-1} DW.

Correlation of polyphenol content and antioxidant capacity

The Pearson correlation between total phytochemical content and antioxidant capacity is shown in Figure 1. TPC correlates very strongly with DPPH capacity and strongly correlates with FRAP, CUPRAC, and ABTS, in line with the research of Indradi *et al*. [27]. Meanwhile, TFC has a strong correlation with CUPRAC, a moderate correlation with DPPH, and a weak correlation with FRAP and ABTS, in line with that reported by Insanu *et al*. [28]. However, TPC and TFC are moderately correlated but not significantly, in line with Batubara *et al*. [13]. In addition, the correlation of all antioxidant capacity tests shows a good correlation. Phenolics have a reasonable correlation with all antioxidant assays compared to flavonoids because phenolic compounds tend to have a simple structure that minimizes steric hindrance [29]. Furthermore, differences in metabolite polarity and redox potential between phenolic compounds and flavonoids also affect the results of each antioxidant assay [24,26].

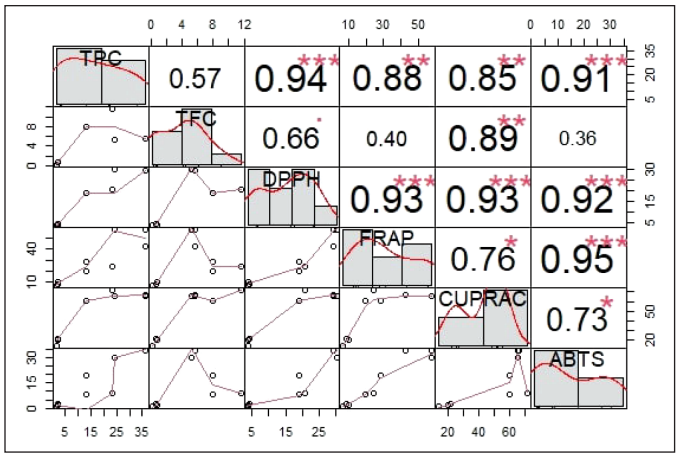

Figure 1. Diagonal matrix of correlation of total polyphenol (TPC and TFC) content and antioxidant capacity (DPPH, ABTS, FRAP, and CUPRAC) of various solvent treatments *G. pictum*. The upper diagonal shows the Pearson correlation coefficient, while the lower diagonal shows the scorpion. *, **, *** indicated significance at the level of $p < 0.05$, <0.01 and 0.001.

Metabolite composition

Secondary metabolite fingerprinting analysis using LC-MS/MS based on the elucidation of chemical components on liquid chromatography and combined with mass spectrophotometry. This research is based on an untargeted metabolomics line with Lee *et al*. [30], which compares all identified metabolites at different extraction solvents *G. pictum*. All sample LC-MS/MS analysis data were chromatograms in the positive (+) ionization treatment based on the treatment of the three solvents (ethyl acetate, ethanol, n-hexane) (Fig. 2). Differences in spectral patterns on each solvent's chromatogram indicated differences in each extract's secondary metabolites.

Secondary metabolite analysis *G. pictum* using LC-MS/MS based on differences in the polarity of solvents (ethanol, ethyl acetate, n-hexane). Metabolic compounds were identified based on parameters such as molecular weight, the chemical formula of the compound, retention time, and area. In total, 138 active secondary metabolites were found in the ethanol extract, 88 compounds in the ethyl acetate extract, and 67 compounds in the n-hexane extract. The profiling process was carried out to separate the secondary metabolites from the impurities, in addition to determining the chemical structure of the metabolites using the PubChem, ChemSpider, and mzCloud

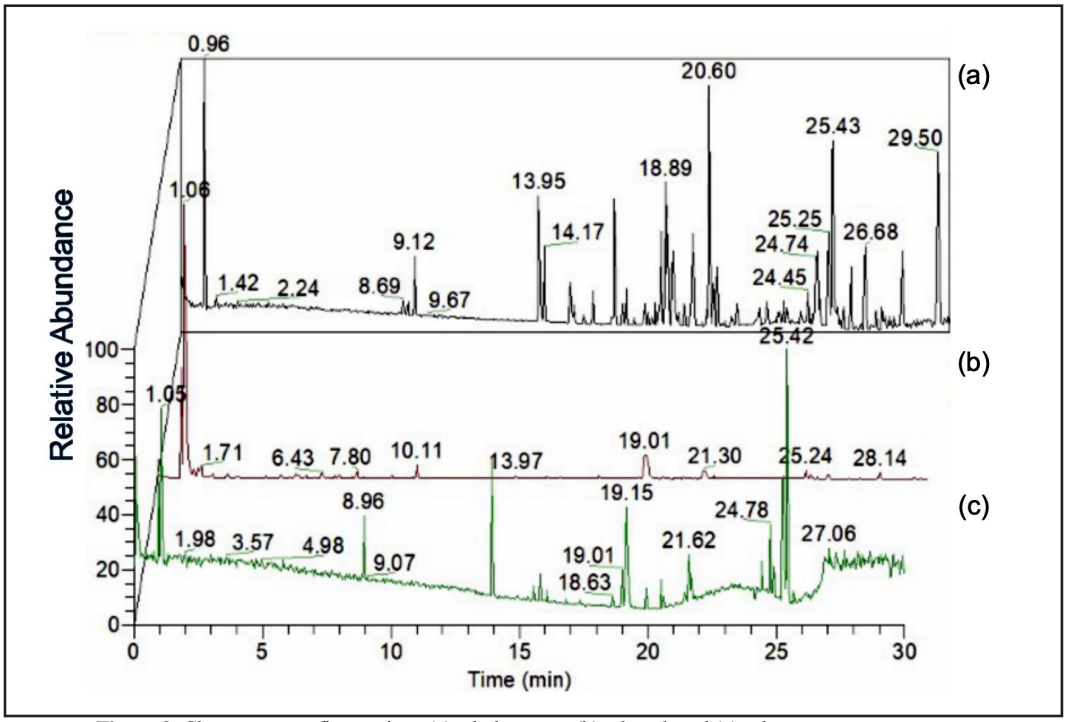

Figure 2. Chromatogram fingerprints: (a) ethyl acetate, (b) ethanol, and (c) n-hexane.

databases to obtain 27 active secondary metabolites in all 3 types of solvents. Twenty-seven selected compounds were divided into several groups (Table 4), namely, phenolics (5), flavonoids (3), terpenoids (3), carotenoids (1), alkaloids (1), fatty acids and their derivatives (8), amino (5), and indole (1).

Characteristics of metabolites, total polyphenol content, and antioxidant capacity of herbal plant samples are influenced by factors such as the extraction method and the type of solvent used [31]. This research uses *G. pictum* as one of the traditional medicinal plants that are empirically and experimentally helpful in treating various diseases ranging from infectious to degenerative [2,32–34]. In addition, this research was conducted to investigate the type of solvent based on polarity on the characteristics of secondary metabolites, total polyphenol, and antioxidant capacity so that it can become a reference in the development of standardized and validated herbal products.

Multivariate analysis

This study used multivariate analysis (HCA and PCA) to process quantitative data for various analyses (total polyphenol, antioxidant capacity, secondary metabolites) of the samples *G. pictum*. The chromatograms of which the compounds had been identified were subjected to PCA and HCA chemometric analysis. HCA was carried out to see the intensity level of the compound based on different solvents. The HCA analysis is shown on the heatmap (Fig. 3a); there are

Table 4. Secondary metabolite of *G. pictum* in all three solvents.

No	Compounds	Class	MF	MW (g mol^{-1})	RT (minute)	Group Areas								
						EtAc 1	EtAc 2	EtAc 3	EtOH 1	EtOH 2	EtOH 3	Hex 1	Hex 2	Hex 3
1	O-ureido-D-serine	Amino acid derivatives	$C_4H_9N_3O_4$	163.06	1.15	√	√			√	√			
2	Betaine	Amino acid derivatives	$C_5H_{11}NO_2$	117.08	1.27				√	√	√	√	√	√
3	DL-β-Leucine	Amino acid derivatives	$C_6H_{13}NO_2$	131.09	1.34				√	√	√			
4	p-coumaric acid	Phenolics	$C_9H_8O_3$	164.05	2.15					√	√			
5	L-Phenylalanine	Amino acid derivatives	$C_9H_{11}NO_2$	165.08	2.74				√	√	√			
6	Proacaciberin	Amino acid derivatives	$C_{16}H_{25}N_{10}$	391.15	3.56						√			
7	(E)-Ferulic acid	Phenolics	$C_{10}H_{10}O_4$	194.06	3.86					√				
8	Cinnamic acid	Phenolics	$C_9H_8O_2$	148.05	3.86					√				
9	4-Methoxycinnamic acid	Phenolics	$C_{10}H_{10}O_3$	178.06	5.12					√	√			
10	4-(4-Hydroxy-2,6,6-trimethyl-3-{[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl) oxan-2-yl]oxy}cyclohex-1-en-1-yl)butan-2-one	Terpenoids	$C_{19}H_{32}O_8$	388.21	7.04					√				
11	Methyl Jasmonate	Fatty acid derivates	$C_{13}H_{20}O_3$	224.14	7.32					√	√			
12	shaftosides	Flavonoid	$C_{26}H_{28}O_{14}$	564.15	8.01				√	√	√			
13	P-cymene	Terpenoids	$C_{10}H_{14}$	134.11	8.22					√				
14	Corymboside	Flavonoid	$C_{26}H_{28}O_{14}$	564.15	8.23					√				
15	4-Indolecarbaldehyde	Indole	C_9H_7NO	145.05	9.69					√				
16	12-oxo Phytodienoic Acid	Fatty acid derivates	$C_{18}H_{28}O_3$	292.20	12.62					√	√			
17	9S,13R-12-Oxophytodienoic acid	Fatty acid derivates	$C_{18}H_{28}O_3$	292.20	13.67					√				
18	Xanthohumol	Flavonoid	$C_{21}H_{22}O_5$	354.15	18.34	√								
19	Stearidonic acid	Fatty acid	$C_{18}H_{28}O_2$	276.21	18.97	√		√		√	√			
20	Anacardic acid	Phenolics	$C_{22}H_{36}O_3$	348.27	19.29					√				
21	α-Eleostearic acid	Fatty acid	$C_{18}H_{30}O_2$	278.22	20.21	√	√	√		√	√			
22	Nootkatone	Terpenoids	$C_{15}H_{22}O$	218.17	21.84							√		
23	Linoleum	Fatty acid	$C_{18}H_{33}NO$	279.26	23.75	√		√						
24	Violaxanthin	Carotenoid	$C_{40}H_{56}O_4$	600.42	25.06	√	√							
25	Hexadecanamide	Fatty acid derivates	$C_{16}H_{33}NO$	255.26	25.14		√	√		√				
26	Oleamide	Fatty acid derivates	$C_{18}H_{35}NO$	281.27	25.67	√	√	√	√	√	√	√	√	
27	Haplophytine	Alkaloid	$C_{37}H_{40}N_4O_7$	652.29	30.01					√	√			

MF: molecule form; MW: molecular weight; RT: retention time; EtAc: ethyl acetate; EtOH: ethanol; Hex: n-hexane.

25 compounds that are the most dominant in the treatment of three types of solvents (ethanol, ethyl acetate, and n-hexane). HCA shows that the three types of solvents were form different clusters. Besides that, based on secondary metabolites, secondary metabolites are divided into three clusters. The first cluster consists of 11 compounds, including cinnamic acid, p-coumaric acid, (E)-ferulic acid, betaine, corymboside, proacaciberin, schaftoside, haplophytine, 4-methoxy cinnamic, DL-beta-leucine, and L-phenylalanine. The second cluster consists of three compounds: nootkatone, oleamide, and linoleamide. The third cluster consists of 11 compounds, including 4-(4-hydroxy-2,6,6-trimethyl-3-{[(2R,3R,4S,5S,6R)-3,4,5-trihydroxy-6 (hydroxymethyl) oxan-2-yl]oxy}cyclohex-1-en-1-yl) butane-2-one, P-cymene, 12-oxo phytodienoic acid, 4-indolecarbaldehyde, O-ureido-D-serine, methyl jasmonate, 9S,13R-12-oxophytodienoic acid, violaxanthin, alpha-allosteric, anacardic acid, and stearidonic acid. HCA analysis regarding the relationship between TEAC and solvent type is shown in Figure 4a. Heatmap analysis shows the extraction method with various solvents shows a different correlation with the order of ethanol > ethyl acetate > n-hexane.

PCA was carried out to reduce the data obtained so that the distribution of chromatogram data could be explained. The PCA results can correlate the possibility of predicting active compounds through loading (Fig. 4a) and score plots (Fig. 4b), with PC1 and PC2 values of 63.2% and 27.9%,

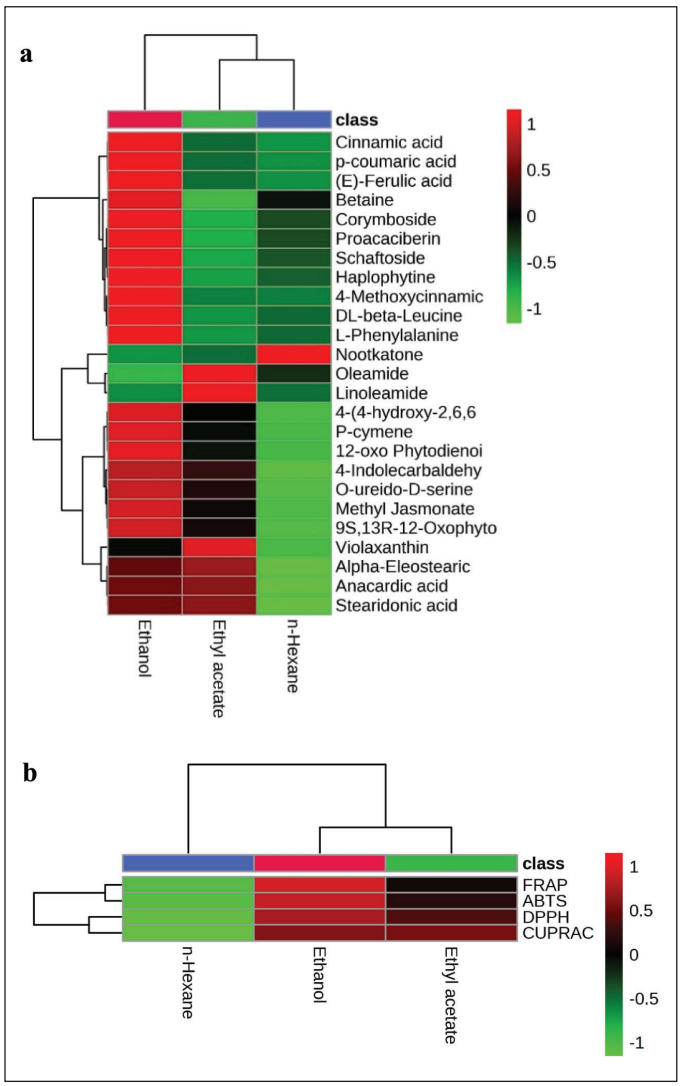

Figure 3. HCA of *Orthosiphon aristatus*. (a) HCA-heatmap dendrogram based on 25 dominant metabolites and (b) TEAC antioxidant capacity against solvent treatment. Red color indicates a positive correlation (+1), green color indicates a negative correlation (−1), and black color indicates no correlation (0).

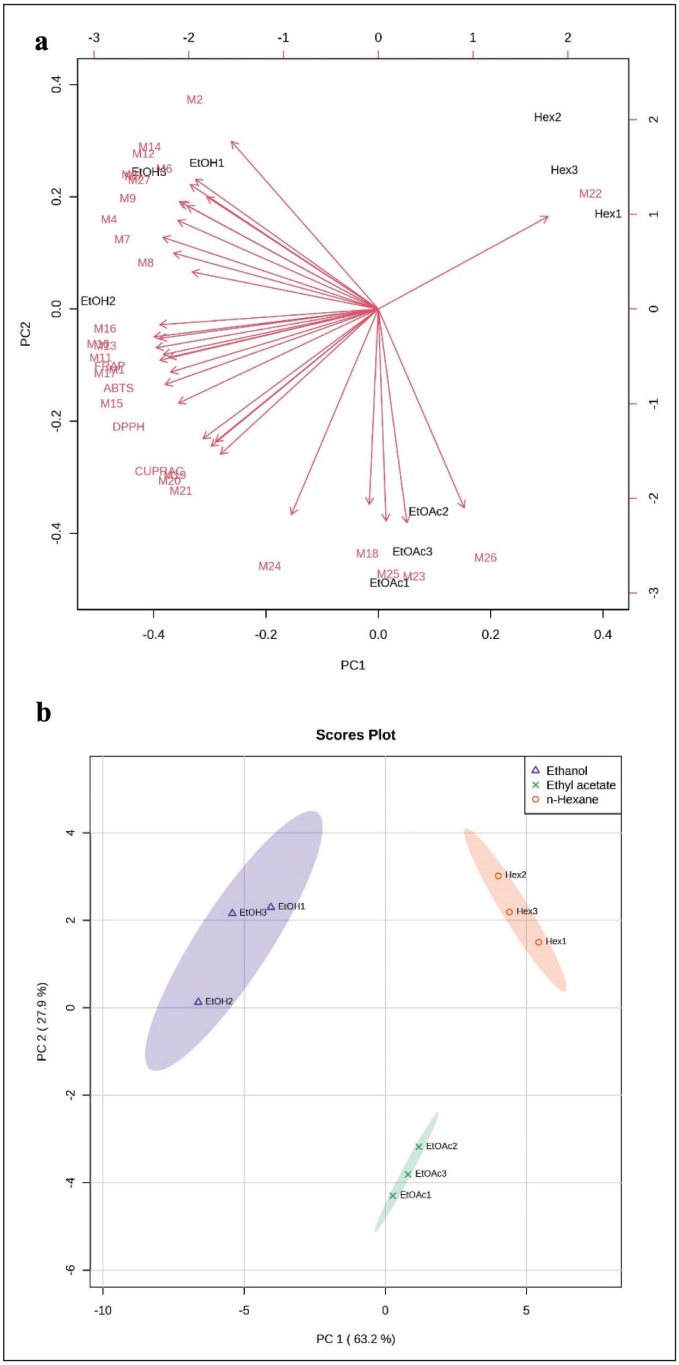

Figure 4. PCA of secondary metabolites of *G. pictum*: (a) loading plot and (b) score plot of PCA based on differences in secondary metabolites (1–27, Table 4) in solvent treatment samples *G. pictum*.

respectively, so that the combined value of PC1 and PC2 (91.1%) is greater than 70%, indicating a high data diversity (peak chromatogram area), which can be explained well by PCA [35]. Furthermore, the loading plot is the contribution of various secondary metabolites to the PCA. Based on their close correlation, the data are visualized as a vector between all secondary metabolites [36]. Based on the data on the loading plot, the secondary metabolites of the ethanol extract have the highest contribution to the overall observed TEAC test analysis (DPPH, ABTS, FRAP, and CUPRAC), consistent with the HCA analysis (Fig. 3). The score plot shows differences between the solvent treatment data, forming three data clusters. Figure 4b shows that the identified compounds tend toward ethanol solvents for all antioxidant methods; this correlates with the high antioxidant capacity of ethanol solvents. The identified compounds show the dominance of phenolic group compounds, which play a role in antioxidants. This is appropriate with Sadeer *et al.* [25], who evaluated several antioxidant methods and showed that the phenolic group had a significant role with a correlation value greater than 0.8. According to reports [13], using a chemometric approach showed that phenolics and flavonoids play a role in various antioxidant activities. This research shows that ethanol extract can be fractionated to isolate active compounds with antioxidant activity.

CONCLUSION

This study shows that the type of solvent determines the optimal conditions for TPC and TFC and antioxidant activity in *G. pictum* extracts. The ethanol solvent showed optimal conditions for the TPC of *G. pictum* extract of 32.17 mg GAE/g DW. In comparison, the optimal condition for TFC was indicated by ethyl acetate solvent with a value of 9.14 mg QE/g DW. On the other hand, overall, based on its pharmacological activity as an antioxidant, the four methods used, namely, DPPH, ABTS, FRAP, and CUPRAC, showed that the *G. pictum* extract with ethanol solvent showed the highest antioxidant activity, respectively 29.79, 33.27, 52.54, and 65.94 µM TE/g DW. This shows that the most optimal antioxidant ability of *G. pictum* extract is obtained from the ethanol extract of the *G. pictum*. This study helps develop industrial and medicinal products containing *G. pictum* leaves.

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AUTHOR CONTRIBUTIONS

Waras Nurcholis, Feda Anisah Makkiyah, and Eldiza Puji Rahmi carried out the concept, design, and drafting of the article. Acquisition of data, interpretation of data, statistical analysis, critical revision, supervision, and final approval were carried out by Waras Nurcholis, Fachrur Rizal Mahendra, Rini Anggi Arista, and Faizal Maulana.

CONFLICTS OF INTEREST

The authors report no financial or any other conflicts of interest in this work.

ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included in this research article.

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