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Original Article

Deep Eutectic Solvent as Green Alternative Solvents for Polygodial Extraction with Validated HPLC-UV Analysis and Evaluation of their Bioactivities

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ABSTRACT

Introduction: Akway (*Tasmannia piperita*), a native Papua plant is known for its benefits as stamina enhancer, antimicrobial, and antioxidant agents, which can be associated to polygodial, its marker compound. Green alternative solvents for polygodial extraction are required. **Objectives:** In this study, an HPLC-UV method was developed for polygodial analysis from akway barks extracted by deep eutectic solvent. **Materials and Methods:** Two types of DES were synthesized and used for extraction of akway barks, i.e. menthol:1-dodecanol (M-1D) (1:2) and Tetrabutylammonium Chloride: 1-Dodecanol (TBAC-1D) (1:2). Yields and antioxidant activities were compared with organic solvent (dichloromethane, ethanol, and hexane) extracts. **Results:** An HPLC-UV method was validated and applied for polygodial analysis under the following conditions: gradient mobile phase of acetonitrile-0.07% formic acid at a flow rate of 0.6 mL/min, detection at 230 nm, and separation using a C18 column (150 x 4.6 mm, 5 µm). Solvent polarity influenced polygodial yields, i.e. dichloromethane > hexane > M-1D > TBAC-1D > ethanol. With regards to DES, M-1D and TBAC-1D were able to extract polygodial with yields comparable to hexane. All DES extracts showed low phenolic content, and negligible antioxidant activities by DPPH, ABTS, and FRAP assays compared to organic solvent extracts. **Conclusion:** The HPLC-UV method can be applied for analysis of polygodial in akway barks extracted by both organic solvents and DES. This study can recommend M-1D and TBAC-1D for green extraction of polygodial from akway barks.

Keywords: 1-Dodecanol, Menthol, Nile Red Solvatochromic Probe, Tetrabutylammonium Chloride.

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INTRODUCTION

The genus Tasmannia or Drymis (family Winteraceae) is an endemic shrub plants native to Australia, New Zealand, and Papua. Among different *Tasmannia* spp., *Tasmannia piperita* Hook f. (locally known in Papua as akway) is valued as aromatic spices and traditional medicine. Local people in Arfak mountains of West Papua use akway barks for a range of health and medicinal purposes, including stamina booster, malaria, stomach and skin disorders (Pratiwi and Simaremare, 2020).

Pharmacological studies on akway revealed that the health benefits of this plant can be associated with drimane sesquiterpenes, which were identified in significant amount in Tasmannia species, including akway. Polygodial, a derivate of drimane sesquiterpenes, is the main bioactive compounds in akway (Figure 1). Studies revealed bioactivities of polygodial such as antiinflammation (Burgos *et al*., 2020; Ferreira *et al*., 2020), antibacterial (Montenegro *et al*., 2024), antifungal (Kipanga *et al*., 2021), antiparasitic (antihelmintic and antimalaria) (Umehara *et al*., 2025), antidiabetic (Belhadj *et al*., 2020), anticancer (Venkatesan *et al*., 2022), and mitochondrial-related antifatigue activities (Castelli *et al*., 2005). These findings highlight polygodial as a promising candidate for the development of natural therapeutic agents.

Polygodial has been extracted and isolated from *Tasmannia* spp. or *Drymis* spp., such as *Drimys winteri* and *Pseudowintera colorata* using hydroethanolic, chloroform, dichloromethane and hexane (Burgos *et al*., 2020; Ferreira *et al*., 2020; Kipanga *et al*., 2021), by conventional techniques, such as maceration, Soxhlation. While organic solvents are effective for extraction of polygodial, organic solvents combined with conventional techniques often require large amount of solvent and long duration of extraction.

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In addition, Soxhlation is operated at high temperature (60°C), thus not suitable for thermolabile compounds such as polygodial. Hence, there is a need for a more economical and environmentally friendly alternative extraction of drimane sesquiterpenes. The increasing demand for green approaches has stimulated the search for alternative solvents that are safer, biodegradable, and environmentally sustainable.

In recent years, Deep Eutectic Solvents (DESs) have emerged as promising alternative solvents for extraction (Bragagnolo *et al.*, 2024). DESs are formed by mixing a hydrogen bond acceptor and a hydrogen bond donor, resulting in a eutectic mixture with a melting point lower than that of its components. They are non-volatile, often biodegradable, and tunable in polarity and viscosity, making them versatile solvents for extracting a wide range of phytochemicals. Several studies have demonstrated the advantages of DES in recovering phenolics, flavonoids, alkaloids, terpenoids from plant matrices (Cao and Su, 2021; Simamora *et al.*, 2024). More recently, DES-based extraction of polygodial from *Pseudowintera colorata* was shown to be feasible, suggesting that such solvents can also be applied to drimane sesquiterpenes which are generally more hydrophobic in nature than polyphenolic compounds (Nadia *et al.*, 2018).

Along with the extraction, quantitative analysis is crucial to ensure accurate determination of plant bioactive compounds. High-Performance Liquid Chromatography (HPLC) is a well-established technique for separation and quantification of marker compounds from plant matrices. HPLC-UV methods for polygodial quantification are scarce and limited in scope to certain plant sources, often optimized for organic solvent extracts. Although an HPLC method for polygodial was previously reported, the method was validated and applied to leaf extract of *P. colorata* (Nadia *et al.*, 2018), which is different in terms of chemical composition from bark extract used in the present study. Bark tissues are rich in lignin and resin, whereas leaves are high in chlorophyll and polar plant metabolites. In addition, DES components in the studied extracts may introduce matrix effect and incompatibility to chromatographic conditions. The presence of plant matrices and DES components may affect selectivity of a method, highlighting the need for method validation and optimization.

This study aimed to validate an HPLC method for the quantification of polygodial in akway extracts obtained using organic solvents and DES, and to evaluate the antioxidant activities of these extracts.

MATERIALS AND METHODS

Chemicals and reagents

Polygodial certified-analytical standard (>98.744 % purity) was obtained from Med Chem Express (New Jersey, USA). HPLC-grade acetonitrile, methanol, formic acid were purchased from Smart Lab (Tangerang, Indonesia). Bi-distilled water pro injection was purchased from Ika Pharmindo Putramas (Jakarta, Indonesia). Whatman disposable nylon membrane filters (pore size 0.45 µm, diameter 25 mm) was used to filter test solution and was obtained from GE Healthcare UK Limited (Buckinghamshire, UK). 2,2-diphenyl-1-picrylhydrazyl (DPPH), Lactic acid (≥ 85%), DL-malic acid (≥ 99%), Citric acid (> 99.5), Nile red, and 2,4,6- Tris(2-Pyridyl)-S-Triazine (TPTZ) were obtained from Sigma Aldrich (St. Louis, MO, USA).

Bark samples were obtained from a commercial online source. The species was confirmed by plant DNA barcoding method on the akway barks. The DNA was extracted using the Cetyltrimethylammonium Bromide (CTAB) protocol, followed by amplification and electrophoresis. The Sanger sequencing results were analyzed and compared with the National Centre for Biotechnology Information (NCBI) barcode database, confirming the species identity as *Tasmannia piperita*.

The barks were pulverized using a mechanical grinder to obtain coarse powder, and stored at 4°C before use for extraction.

DES preparation

Two kinds of DES were synthesized with starting components as described in Table 1. DES components (Figure 1) were mixed in a pre-determined molar ratio. The mixture was continuously stirred at 70°C at 250 rpm agitation speed for 30 min to obtain a clear and homogenous liquid. DES was allowed to cool at room temperature and stored in capped bottle.

FTIR Analysis

The FTIR spectra of M-1D and TBAC-1D together with their starting components were obtained by an Agilent Cary 630 FTIR spectrophotometer. The instrument was operated at Attenuated Total Reflectance (ATR) mode, in the range of 4000-650 cm^{-1}, based on 4 cm^{-1} spectral resolution and the accumulation of 16 scans.

Determination of DES polarity

The polarity of prepared DES was determined using Nile red as a solvatochromic probe, as described previously (Simamora *et al.*, 2023). Polarity was determined by measuring the λ_{max} shift of Nile red (9-diethylamino-5-benzo[a]phenoxazinone) in DES. Nile red stock solution was prepared in ethanol (1 g L^{-1}) and stored at 4°C. The stock solution was diluted 100 times with ethanol and added to each DES. Maximum absorbance in the visible region (400-800 nm) was measured by a spectrophotometer using a 1 cm quartz cell. The molar transition energy was calculated using the following formula (Reichardt, 1994):

$$E_{NR} \text{ (kcal mol}^{-1}) = 28{,}591 / \lambda_{max^{-1}} \quad (1)$$

Preparation of DES and organic solvent extracts

Extraction of akway stem bark with DES was conducted based on a method by Duan *et al.* (2016) under the conditions as described in Table 1 (Duan *et al.*, 2016). In a 5 mL snap tube was placed 200 mg akway powder. DES (3 mL) was added (1:15, S/L ratio) into the powder and mixed. The mixture was sonicated at 50°C for 20 min in a Bandelin Sonorex Digitec ultrasound apparatus. Each tube was then placed in a tube rotator and rotated overnight. The extracts were centrifuged at 10000 rpm for 10 min, then the supernatant was separated from the biomass.

Extraction by conventional organic solvents were carried out for comparison. Organic solvents (100 mL) was added into akway powder (20 g). The mixture was sonicated for 20 min. Extraction mixture was shaken in a shaker overnight. The ensuing supernatant was separated from the biomass by filtration using a Whatman filter paper No 1. Solvent was removed from the extract by a Buchi R^2 rotavapor (Flawil, Switzerland) to obtain dry crude extract.

HPLC-UV Analysis of DES extracts

Chromatographic separation was performed using a Zodiac C18 column (150 x 4.6 mm, 5 µm) with a gradient elution method run at ambient temperature. An HPLC grade acetonitrile (eluent A) and formic acid 0.07% in pro injection water (eluent B) were used as mobile phase, on a Schambeck S9425 HPLC system, integrated with S8515 vacuum degasser. The conditions for gradient elution were as follows: initial to 10 min was 60-70% A; 10-20 min 80% A; then 20-30 min 100% A. Samples of 20 µL were loaded and eluted at a flow rate of 0.6 mL/min at room temperature. Peak detection was set on 230 nm on a 4245 UV detector. Data acquisition and processing were performed using a Clarity software by DataApex (Prague, Czech Republic).

Standard solutions and sample preparation

A primary stock solution of polygodial (5000 µg/mL) was prepared in DMSO by accurately weighing polygodial standard. The secondary stock solution (500 µg/mL) was prepared by diluting the primary stock solution with ethanol. Working standard solutions were made in ethanol by precise dilution of the secondary stock solution to make six concentration points of 6.25-200 µg/mL. The secondary stock solution was also used for the preparation of quality control samples of polygodial alone at three concentration levels (5, 50, and 75 µg/mL). These solutions were also used for spiking aliquots of plant extracts. All solutions were filtered through 0.45 µm nylon filter prior to injection. Areas under the curves for each standard were plotted against concentrations to generate polygodial calibration curves. Each standard was analyzed in duplicate measurements.

Method validation

The analysis method was validated in accordance with the ICH guidelines (ICH Guidelines, 1996). In order to show the acceptability of the analysis method, the following procedures were applied during the method evaluation.

System suitability

The standard solution of 50 µg/mL was used for system suitability test, which was conducted six repeats. Retention time, area under curve, tailing factor, and theoretical plate number of the analyte peaks were determined as a criterion of system suitability. Based on the ICH guidelines, the HPLC system is suitable for analyses if the Relative Standard Deviation percentage (%RSD) of the retention time is < 1%, the tailing factor ≤ 2, and the theoretical plate number > 2000.

Linearity

This procedure determined a linear relationship between analyte signals and analyte concentrations. Six polygodial calibration solutions (6.25, 12.5, 25, 50, 100, and 200 µg/mL) were freshly prepared. A calibration graph was created by plotting peak areas against the corresponding concentrations of polygodial. The regression equation and correlation coefficient (R^2) were calculated.

Sensitivity

The sensitivity of the HPLC-UV analysis method was determined by calculating Limit of Detection (LOD) and Limit of Quantification (LOQ). The LOD is 3.3 (/S), whereas the LOQ is 10(/S), where is the standard deviation of the response and S is slope of regression equation.

Precision and Accuracy

Three levels of standard concentrations (5, 50, and 75 µg/mL) in three replicates were analyzed for evaluation of precision and accuracy of the method. In each evaluation, intra- and inter-day analysis were determined over 1 and 2 different days, respectively. Un-spiked stem bark solution (500 µg/mL) was prepared in ethanol from the ethyl acetate crude extract. Spiked samples were prepared by spiking akway extract solution by each standard concentration. Polygodial standard solutions at their respective concentrations (5, 50, and 75 µg/mL) were added to the stem bark solution at a 1:1 ratio. Prior to injection, the mixture was filtered by a 0.45 µm nylon membrane.

The precision of the method was evaluated based on variances of analysis, expressed as %RSD of the peak area for each concentration level. In the accuracy study, the recovery percentages for each concentration point were calculated by subtracting the spiked sample area from un-spiked samples (only akway extract), and their respective %RSD were calculated.

Antioxidant assays

DPPH radical scavenging assay

An aliquot of 50 µL of akway extracts of different concentrations or trolox standard solution was mixed with 80 µL of 0.6 mM DPPH solution in methanol. The mixtures were incubated at 25°C for 30 min in a Thermostar BMG LabTech microplate shaker/incubator at 300 rpm. Absorbance was recorded at 515 nm by an iMark BioRad microplate reader (Hercules, California, USA). Radical scavenging activity (%) was calculated relative to the DPPH control. IC_{50} values were calculated from linear regression equation of concentration-response plot. The procedure was conducted in triplicates and results were presented as mean±SD.

Total Phenolic Content (TPC) assay

The TPC of akway extracts were determined using the Folin-Ciocalteu colorimetric method (Simamora *et al.*, 2020). In brief, sample (0.5 mL) was mixed with Folin-Ciocalteu reagent (10% v/v). After 10 min of incubation at room temperature, Na_2CO_3 (75 g/L, 2.5 mL) was then added, and the mixture was further incubated at room temperature for 2 hr. The absorbance was read using a UV-vis spectrophotometer at 769 nm (Libra S22, Cambridge, UK). Gallic acid (12.5-200 µg/mL) was used to generate a standard calibration curve (R^2 = 0.9977, y = 0.0084x+0.0478). Results were expressed as mgGAE (Gallic Acid Equivalence)/g dried extract.

ABTS radical cation scavenging assay

The 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was carried out by a microtiter assay (Hikmawanti *et al.*, 2024). The $ABTS^+$ solution were prepared mixing ABTS solution (7 mM in phosphate buffer solution) with potassium persulphate (2.45 mM) in a 1:1 ratio. The mixture was let stand in the dark at room temperature for 12-16 hr before use. The $ABTS^+$ solution was diluted to obtain an absorbance of 0.700±0.02 at 750 nm. For the assay, in a 96-well microplate, 20 µL of samples of different concentrations or Trolox standard solution was added to $ABTS^+$ solution (180 µL). The plate was shaken using a Thermostar BMG LabTech microplate shaker/incubator at 300 rpm for 5 min. The absorbance was read at 750 nm by an iMark BioRad microplate reader. Experiments were conducted in three replicates and results were expressed as IC_{50} values (mean±SD).

Ferric Reducing Antioxidant Power (FRAP) assay

The FRAP assay was carried out in a 96-well microplate according to a previous method (Simamora *et al.*, 2023). FRAP working solution was prepared by mixing 2,4,6-Tripyridyl-S-Triazine (TPTZ) solution (10 mM) in HCl (40 mM), $FeCl_3$ (20 mM), and acetate buffer (300 mM, pH 3.6) in a 1:1:10 ratio. For the assay, 20 µL of samples was transferred into the wells, followed by the working FRAP solution (280 µL), then shaken using a closed microplate shaker/incubator (Thermostar BMG LabTech) at 300 rpm for 30 min at 37°C. The absorbance was read at 569 nm by an iMark BioRad microplate reader. A standard curve was generated using Trolox standard solutions (12.5-200 ppm) and results were expressed as µgTE (Trolox Equivalence)/mg dried extract. The procedures were conducted in triplicate and data were presented as mean±SD.

Ethical Statement

Not applicable to this paper

Statistical Analysis

Experiments were conducted in three replicates. Results were presented as mean ± Standard Deviation (SD). Significant differences were analyzed by one-way ANOVA with Tukey's *post*

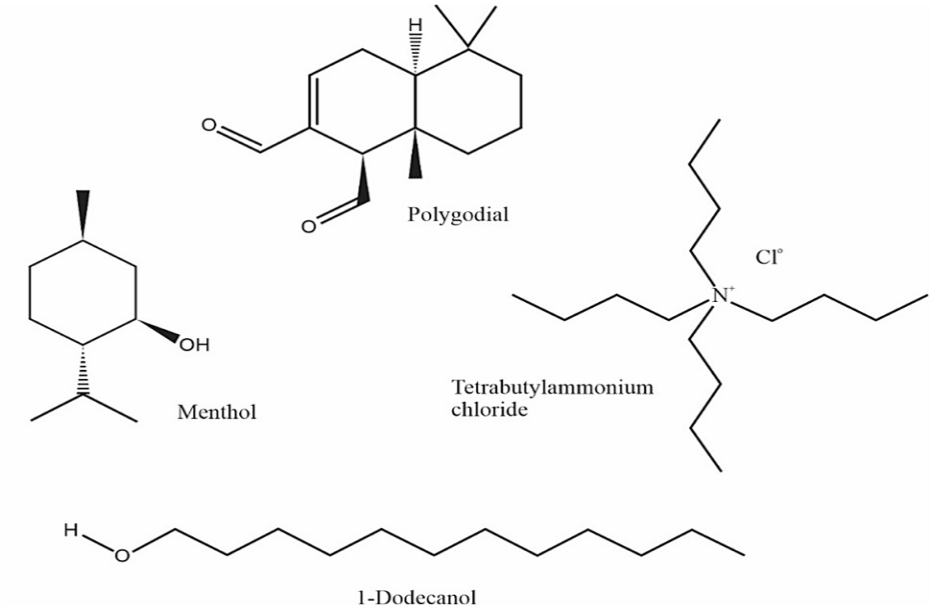

Figure 1: Polygodial and DES components used in this study.

hoc test using an IBM SPSS v25. A p value< 0.05 was considered statistically significant.

RESULTS

FTIR analysis of DES

FTIR analysis of the DES systems and their starting components were conducted to investigate DES formation. The FTIR spectrum of M-1D was dominated by the spectra of 1D, although the C-C skeletal vibrations of the menthol backbone were noticeable at 750-850 cm^{-1} in M-1D spectrum (Figure 2A). The O-H stretching at 3326.65 cm^{-1} of 1D was shifted to 3332.24 cm^{-1} in M-1D. The C-O vibration of 1D alcohol group at 1051.31 cm^{-1} shifted to lower frequency of 1047.33 cm^{-1} in M-1D.

Interaction between TBAC and 1D also resulted in shifts of the absorbance of several functional groups (Figure 2B). The 1D O-H stretching vibration appeared at 3345.29 cm^{-1}, shifted to 3317.33 cm^{-1} in TBAC-1D. Spectral shift was also observed of the $C\text{-}N^{+}$ group, from 881.51 cm^{-1} in TBAC to 894.56 cm^{-1} in TBAC-1D. The peaks at 2957.64 and 2871.91 cm^{-1} in TBAC spectrum were attributed to the stretching vibration of C-H. These peaks were shifted to shorter frequency i.e. 2927.82 and 2858 cm^{-1} in TBAC-1D.

Polarity of DES

The polarity of solvents used for polygodial extraction was determined using the Nile red solvatochromic probe. The UV-vis spectra of the probe in different solvents can be seen in Figure 3. The absorbance maxima and the calculated transition energy of the Nile red (E_{NR}) can be seen in the Supplementary Table 1. By the E_{NR} values, water was the most polar whereas hexane was the least polar. Among DES, M-LevA (1:2) and CC-EG (1:2) was more polar than M-1D (1:2) and TBAC-1D (1:2).

Validation of HPLC method

In HPLC-UV analysis, peak outputs (peak area, shape, and resolution) are sensitive to changes in chromatography system such as column conditions, UV detector, and solvent system. For this reason, chromatographic analysis was optimized for the determination of polygodial. A reverse phase C18 column was used in this study, which was run at ambient temperature. The mobile phase used consisted of acetonitrile (solvent A) and 0.07%

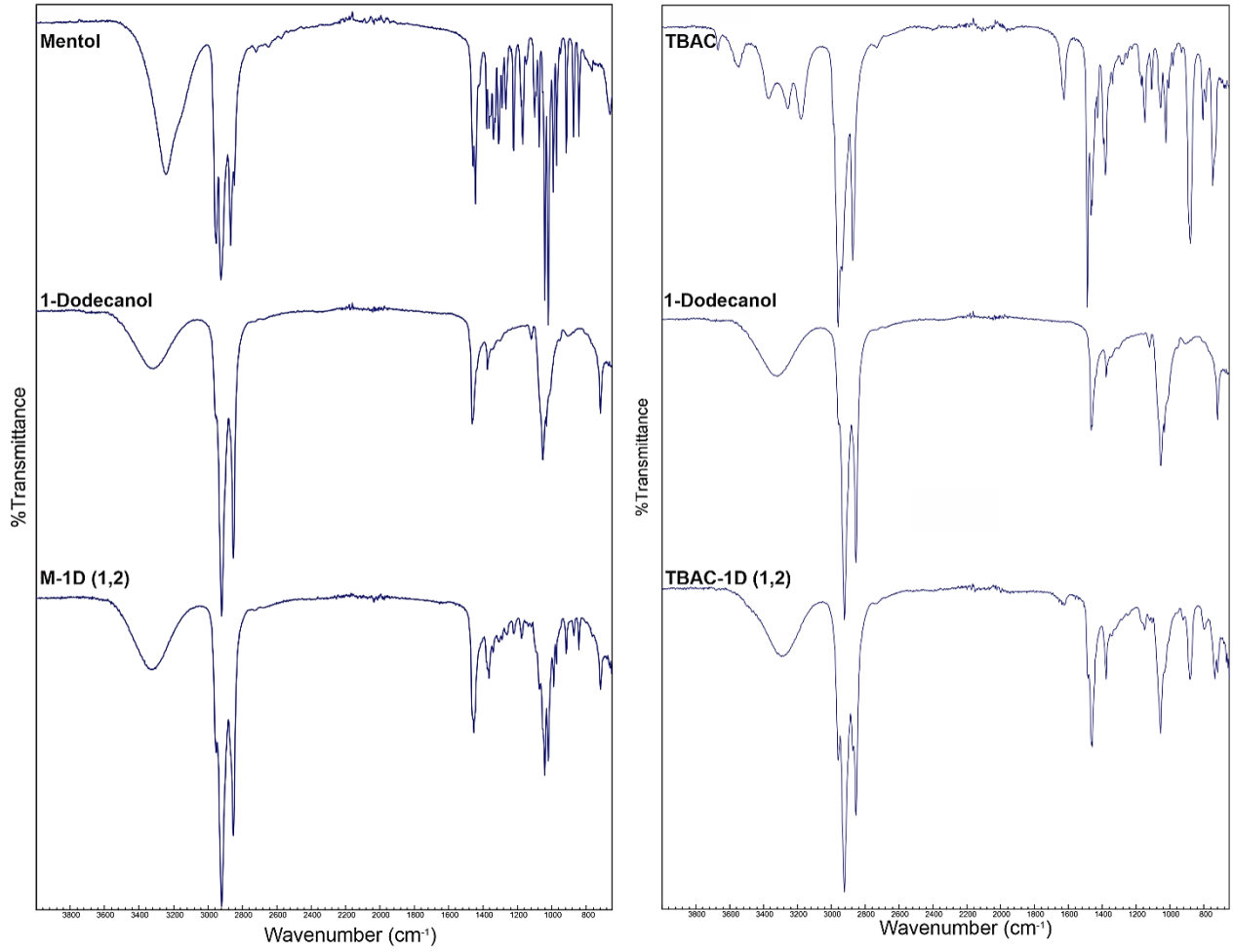

Figure 2: The FTIR spectra of (A) menthol:1-dodecanol (1:2), and (B) tetrabutylammonium chloride: 1-dodecanol (1:2). The first and second spectra refers to the spectra of DES components, whereas the third spectra are DES.

Table 1: DES used in this study.

DES name	DES Components		HBD: HBA	Liquid appearance
	HBA	HBD	ratio	
TBAC-1D	Tetrabutylammonium chloride	1-Dodecanol	1:2	Light yellow, slightly viscous
M-1D	Menthol	1-Dodecanol	1:2	Light yellow, slightly viscous

Table 2: Evaluation of precision and recovery of polygodial quantitative analysis.

Standard concentration	Precision (RSD, %)		Average recovery (%)	
(µg/mL)	Intra-day (*n*=3)	Inter-day (*n*=3)	Intra-day (*n*=3)	Inter-day (*n*=3)
5	1.459	7.525	77.347	73.086
50	1.001	2.715	99.185	96.591
75	4.387	2.506	97.822	99.222

formic acid (solvent B), run under gradient elution. Under these conditions, polygodial was detected at retention time 13.06 min at 230 nm (Supplementary Figure 1).

System suitability

System suitability was tested to ensure that the HPLC method met the minimum criteria for quantitative analysis. Repeat analysis (six times) of polygodial standard solution (50 µg/mL) resulted in low variations in the tested parameters, as follows (presented as mean±%RSD), retention time 13.06±0.474, area under curve 2832.77±1.375, tailing factor 0.799±0.272, and plate number 6598.33±0.878. The %RSD values were < 2%, which met the ICH Guidelines criteria (ICH Guidelines, 1996).

Linearity and sensitivity

Linearity test was performed to evaluate whether HPLC method gives linear responses to analyte concentrations. In the present study, calibration curve was generated, consisting of various levels of polygodial working solution (Supplementary Figure 2). The linearity of the method was validated by R^2 = 0.9999 in the range of 6.25-200 µg/mL, which obtained a regression equation y = 60.179x + 99.856. High R^2 value indicate that the method is able to measure responses proportionally according to analyte concentrations.

Sensitivity in terms of Limits of Detection (LOD) and Quantification (LOQ) is defined as the lowest concentration of analyte that can be reliably detected and quantified. The method obtained LOD and LOQ of 2.69 µg/mL and 8.16 µg/mL, respectively.

Precision and accuracy

Unlike pure standard solution used in the generation of calibration curves, extracts contain matrix components that may affect analytical responses to analyte of interest. Matrices may affect chromatographic separation which can alter retention time and peak shapes. Matrix compounds may bind to analytes or stationary phase, thus reducing effective analyte concentrations. For these reasons, precision and accuracy study were performed, assessing the differences in response between akway extract spiked with standard solutions and non-spiked akway. Three levels of polygodial standard solutions were used for quality control, i.e. 5, 50, and 75 µg/mL. Intra- and inter-day assay variations were assessed by analyzing spiked and non-spiked samples.

Intra- and inter-day precision of the method was assessed based on Relative Standard Deviation (%RSD) values. Results were presented in Table 2. Intra-day precision based on %RSD values at 5 and 50 µg/mL was within acceptable variability according to ICH guidelines (< 2.5%). High variability was seen for inter-day precision results, in particular at low concentration (5 µg/mL).

Accuracy study results were presented as recovery percentage. Intra-day results were within acceptable recovery by the ICH guidelines; however, low recovery was obtained at low concentration (5 µg/mL). Similar results were observed for inter-day recovery. At low concentrations which are close to LOD and LOQ values, the matrix becomes more dominant compared to the analyte amount that may cause interference to the analyte signal.

Polygodial analysis in akway extracts

Figure 4 shows representatives of HPLC chromatograms of hexane and DES extracts. Table 3 summarizes the yields of polygodial extracted from akway by different solvents.

Antioxidant activity

The antioxidant activities of akway barks extracted by different solvents were summarized in Table 4. DES extracts, TBAC-1D and M-1D both at 20 mg/mL, did not show detectable phenolic content. DES extracts did not show measurable radical scavenging and reducing activities either within the tested range of concentrations. For example, by DPPH assay, the scavenging activities of DES extracts were 19.67% at 35.40 mg/mL of M-1D,

whereas for TBAC-1D at 24.30 mg/mL, the activity only reached 14.08%. By ABTS radical scavenging assay, at 20 mg/mL, DES extracts showed 2.28±0.17% and 3.08±0.15%, for M-1D and TBAC-1D, respectively. In contrast, organic solvents extracts showed good antioxidant activity. Among the organic solvents, DCM extract showed the highest content of phenolic compounds. DCM extract showed the strongest DPPH radical scavenging activity and reducing capacity compared to other organic solvents. Hexane akway extract which is a nonpolar solvent, showed comparable activities to DCM extract. More polar extractant, i.e. ethanol obtained the least amount of total phenolic content, and in line with this result, very weak scavenging activities as observed in DPPH and ABTS assays (Table 4), although ethanol extract showed moderate FRAP activity. Interestingly, water extract obtained substantial TPC, coupled with strong DPPH scavenging activity, however obtained the lowest FRAP activity among organic solvents used. Polygodial, the marker compound of akway, did not exhibit measurable antioxidant activity in the tested assays, whereas Trolox (a positive control) showed the strongest activity in all assays.

DISCUSSION

The driving force of eutectic solvent formation is hydrogen bond interaction between HBA and HBD components, which may be indicated by structural changes observed in the FTIR spectra of the DES system, for example shifts of O-H stretching vibrations can be an indication of hydrogen bonding formation (Kyriakoudi *et al.*, 2024). In M-1D, hydrogen bond interactions are expected between the -OH groups of menthol and 1D. A blue shift (higher frequency) was observed of the -OH group in M1D compared to that of 1D, indicating stronger O-H bond. It is likely that the bulky structure of menthol inhibits intermolecular hydrogen bond interactions between menthol and 1-dodecanol, resulting in weak hydrogen bond between components, as also observed by others (Ivanova *et al.*, 2025). In addition, slight shift was observed in the C-O stretching of alcohol group which supports the alteration of the hydroxyl environment. In contrast,

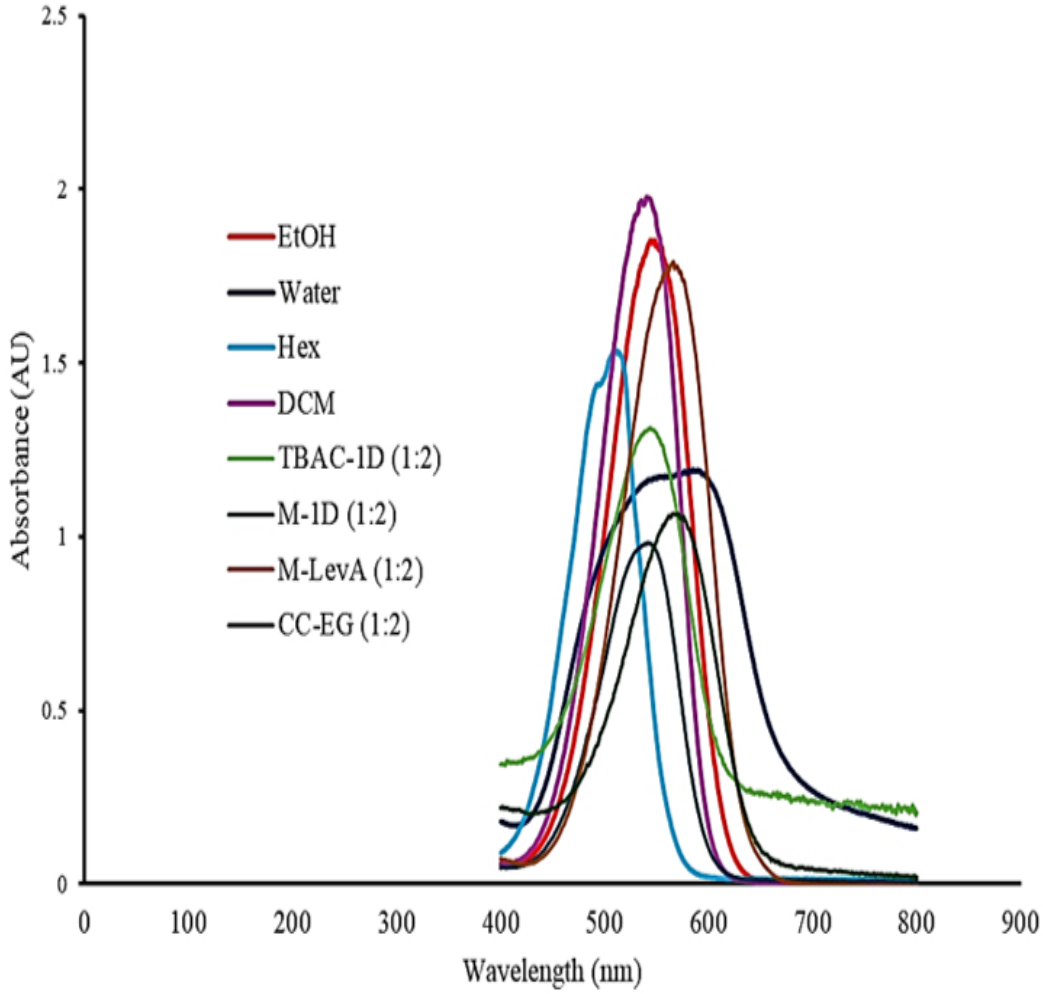

Figure 3: The UV-vis spectra of the Nile Red solvatochromic probe in different solvents. Water, EtOH (ethanol), Hex (hexane), DCM (dichloromethane), TBAC-1D (1:2), and M-1D (1:2), M-LevA (1:2), and CC-EG (1:2).

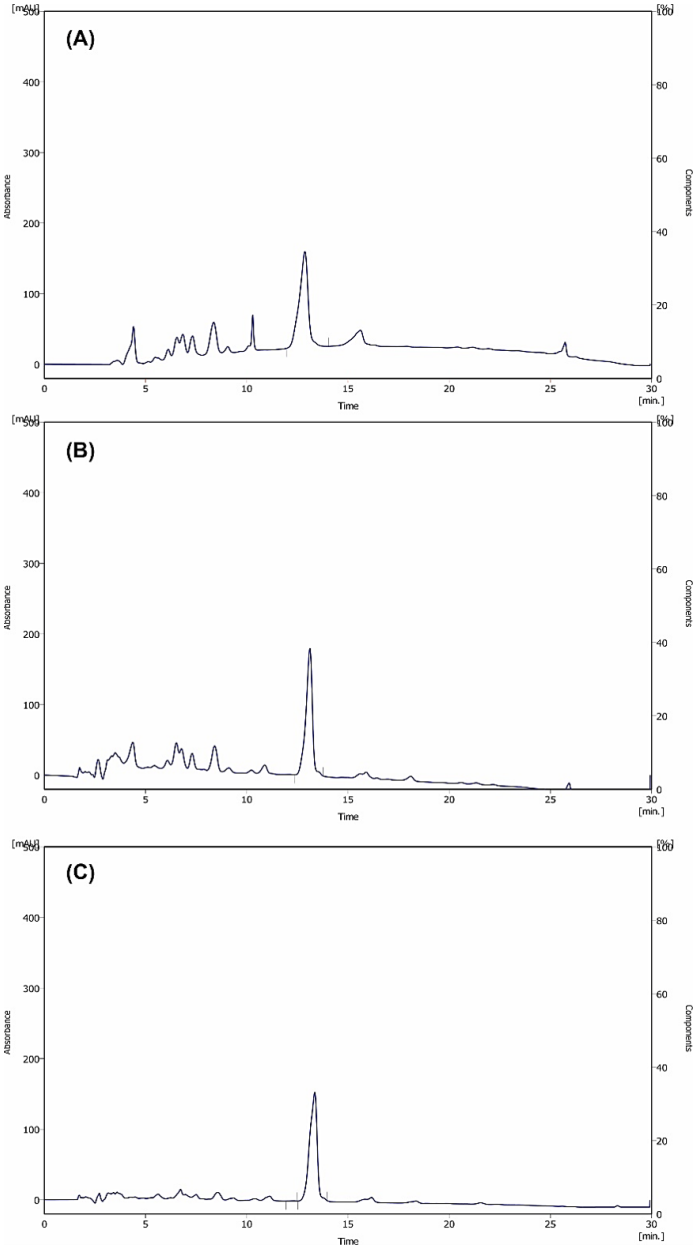

Figure 4: The chromatograms of polygodial extracted from akway barks by different solvents i.e. (A) methol:1-dodecanol (1:2), (B) tetrabutylammonium chloride:1-dodecanol (1:2), and (C) hexane. Detection was performed at 230 nm.

the O-H vibration in TBAC-1D occurred in lower frequency (red shift), indicating weaker O-H bond due to the formation of strong hydrogen bonding between TBAC and 1D. Stronger intermolecular bonding is likely due to interaction of the OH group with the positively charge C-N^+. Meanwhile, blue shift was observed for C-N^+ vibration consistent with the strengthening of C-N^+ bond due to hydrogen bond formation.

Solvent polarity is a crucial factor that determines the capacity of solvents to dissolve and extract bioactive compounds from plant matrices. To characterize solvent polarity, Nile red was used as a solvatochromic probe, due to its high sensitivity to the polarity of its environment. The absorbance maximum of Nile red shifts in accordance with the polarity of solvents. For example, in nonpolar solvents, the λ_{max} of Nile red shifts to shorter wavelength (blue shift) due to stabilization of its excited state. Results in Figure 3 show varying polarity among solvents which reflects their ability to bind to polygodial, through hydrogen binding or nonpolar interactions. Polygodial which is a sesquiterpene dialdehyde, is of low polarity; thus, it was expected to be more soluble in nonpolar or semipolar solvents.

Based on the HPLC-UV method validated in the present study, most of the solvents used in this study can extract polygodial with different extraction capability. Polygodial was detected at similar RT, both in conventional solvents and DES. It should be noted that the presence of DES in the extract did not alter the chromatogram obtained. Previously, other researchers observed similar results (Nadia *et al.*, 2018). However, when M-LevA (1:2) was used for extraction, polygodial in the extract could not be analyzed. The peak of polygodial could not be well separated indicating that M-LevA was not suitable for extracting polygodial.

From Table 3, it is clear that extraction solvents had a significant impact on the recovery of polygodial from akway bark. Among organic solvents used for extraction, dichloromethane yielded the highest polygodial content (10.56 mg/g dried bark), indicating the efficiency of extraction. Although hexane was less polar than dichloromethane, the polygodial yield was lower than that of dichloromethane. Polygodial is relatively nonpolar. Its large solubility in dichloromethane may be aided by interactions between functional groups. Two aldehyde functional groups of polygodial may contribute to good solubility of polygodial in dichloromethane, by interacting with polar sides of chlorinated solvents. On the other hand, the terpenoid backbone of polygodial may interact with the lipophilic nature (methane groups) of dichloromethane. These interactions therefore are expected to cause good solubility of polygodial in dichloromethane. Good solvation capability toward polygodial (4.74 mg/g dried bark) by hexane indicate nonpolar side of polygodial (terpenoid backbone) serve as effective binding side to hexane. However, weaker solvation towards the aldehyde groups of polygodial explains smaller yield of hexane compared to dichloromethane. In contrast, ethanol yielded negligible amounts of polygodial (0.06 mg/g), highlighting the incompatibility of polar protic solvents with this relatively nonpolar polygodial. It was noted that polygodial was not detected in water extract. This finding aligns with previous reports that alcohols preferentially extract polar phenolic compounds, while being less effective for sesquiterpenes and dialdehydes (González-Hernández *et al.*, 2024).

Interestingly, the two types of DES namely TBAC-1D (1:2) and M-1D (1:2) achieved comparable extraction efficiency (5.17 and 4.85 mg/g dried bark) to hexane. Although lower than dichloromethane, these results demonstrated that these DES are capable of solubilizing predominantly nonpolar bioactive

compound. It has been reported that menthol and TBAC are good HBA for hydrophobic DES (Cao and Su, 2021).

In addition to similar lipophilicity between DES and polygodial, hydrogen bonding formation between polygodial and DES is likely to contribute to good solubility of polygodial in DES. It should be noted that TBAC-1D outperformed hexane, suggesting that DES can serve as "green" alternatives to conventional organic solvents.

Taken together, these results demonstrate that solvent polarity and solute-solvent interactions govern polygodial extraction. Dichloromethane remains the most effective solvent, but DES show promises as sustainable alternatives, especially considering their lower toxicity, and tunability compared to conventional organic solvents. Further optimization, such as adjusting DES composition may enhance their extraction performance toward levels comparable with dichloromethane.

The antioxidant potential of akway is strongly influenced by the extraction solvent. Non-polar solvents such as dichloromethane and hexane extracted higher levels of phenolic compounds, which correlated with the observed strong radical scavenging and reducing activities across assays. These findings agree with previous literature that have established a strong positive relationship between TPC and radical scavenging activity (Muflihah *et al.*, 2021).

Interestingly, although water yielded a relatively modest TPC compared to dichloromethane and hexane, it exhibited notable DPPH activity, suggesting that certain water-soluble non-phenolic constituents may contribute to its antioxidant effect. In contrast, ethanol extraction resulted in low TPC and weaker radical scavenging capacity, despite a moderate FRAP value, indicating selective solubilization of reducing agents that are not necessarily phenolics, such as terpenoids, which have been shown to have a strong correlation with FRAP activity (Baptista *et al.*, 2024).

With regard to DES extracts, the absence of measurable activity in the DES extracts suggests that these solvent systems were not effective for extracting antioxidant constituents from akway bark under the conditions applied. The long-chain alcohol-based DES systems (1-dodecanol) tested here appear unsuitable for phenolic or polar antioxidant extraction. Furthermore, the lack of activity observed for polygodial confirms that the antioxidant capacity of akway is not attributable to its major marker compound, but rather to other secondary metabolites enriched in non-polar and moderately polar solvent extracts. Polygodial, a sesquiterpene dialdehyde lacks hydroxy aromatic structure found in phenolic compound, thus does not have hydrogen donating capacity. In contrast, the OH functional group of phenolic compounds allows for hydrogen or electron donating ability, in addition to resonance stabilization that contribute to the antioxidant activity of phenolic compounds.

Overall, these findings emphasize that akway's antioxidant activity is solvent-dependent and not directly associated with its marker compound polygodial. They also suggest that dichloromethane and hexane are more efficient for recovering antioxidant constituents, than DES.

Table 3: The yields of polygodial extracted from akway barks by different solvents.

Akway extracts	Polygodial content (mg/g dried bark)
TBAC-1D (1:2)	4.85±0.04
M-1D (1:2)	5.17±0.13
M-LevA (1:2)	ND*
CC-EG	3.32±0.09
Dichloromethane	10.56±0.02
Ethanol	0.06±0.01
Hexane	4.74±0.08

*ND: not determined.

Table 4: Antioxidant activity of different akway extracts.

Akway extract	TPC (mg GAE/G dried extract)	DPPH (IC_{50}, µg/mL)	FRAP (mgTE/G dried extract)	ABTS (IC_{50}, µg/mL)
TBAC-1D (1:2) (1.25-20 mg/mL)	ND[a]	ND[a]	ND[a]	ND[a]
M-1D (1:2) (1.25-20 mg/mL)	ND[a]	ND[a]	ND[a]	ND[a]
Water	187.58±2.96	28.35±0.41	6.26±0.00	381.87±13.53
Ethanol	8.57±0.19	1304±32.28	33.45±0.09	346.73±8.64
Hexane	1263.92±8.72	14.46±0.19	64.87±2.70	23.05±1.29
Dichloromethane	1474.44±1.73	10.80±0.11	94.52±4.04	34.35±3.12
Polygodial (78.13-1250 µg/mL)	NA[b]	ND[a]	ND[a]	ND[a]
Trolox	NA[b]	7.47±0.15	NA[b]	3.29±0.03

[a]IC_{50} value could not be determined because inhibition did not reach 50% in the tested range of concentration.

CONCLUSION

Akway extracts were analyzed for polygodial content by a validated HPLC-UV method. Findings show that while conventional organic solvents, particularly dichloromethane and hexane, effectively extracted polygodial, M-1D and TBAC-1D showed promising yields comparable to that of hexane. Dichloromethane and hexane extracted high amounts of polygodial, along with other antioxidant constituents, as shown by strong antioxidant activity. In contrast, M-1D and TBAC-1D did not yield measurable activity. The lack of antioxidant activity in pure polygodial indicates that the observed radical scavenging and reducing effects of akway extracts arise from compounds other than the marker constituent. Results herein suggest M-1D and TBAC-1D are attractive from a sustainability standpoint to extract polygodial from akway.

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ABBREVIATIONS

DES: Deep Eutectic Solvent; **HPLC:** High Performance Liquid Chromatography; **UV:** Ultraviolet; **M-1D:** Menthol:1-Dodecanol; **TBAC-1D:** Tetrabutylammonium chloride:1-Dodecanol; **DPPH:** 2,2-diphenyl-1-picrylhydrazyl; **TPC:** Total phenolic content; **GAE:** Gallic acid equivalence; **ABTS:** **2**,2′-azin o-bis(3-ethylbenzothiazoline-6-sulfonic acid); **FRAP:** Ferric reducing antioxidant power; **TE:** Trolox equivalence; **FTIR:** Fourier-transform infrared spectroscopy; **ATR:** Attenuated total reflectance; **CTAB:** Cetyltrimethylammonium bromide; **NCBI:** National Centre for Biotechnology Information; **HBD:** Hydrogen bond donor; **HBA:** Hydrogen bond acceptor; **LOD:** Limit of detection; **LOQ:** Limit of quantification; **RSD:** Relative standard deviation; **DMSO:** Dimethyl sulfoxide; **DCM:** Dichloromethane; **EtOH:** Ethanol; **Hex:** Hexane.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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AUTHORS CONTRIBUTION

AS: Concept, design, literature search, data acquisition, data analysis, statistical analysis, manuscript preparation, manuscript editing and manuscript review. **AWS:** Design, literature search, data acquisition, data analysis, manuscript editing and manuscript review. **MM:** Design, data acquisition, data analysis, manuscript editing and manuscript review. **KHT:** Concept, design, literature search, data analysis, manuscript preparation, manuscript editing and manuscript review.

SUMMARY

A deep eutectic solvent was used for polygodial extraction from the barks akway (*Tasmannia piperita)*. Analysis of polygodial content in *T. piperita* extracts by a validated HPLC-UV method indicates that solvent polarity influenced polygodial yields, i.e. dichloromethane > hexane > M-1D > TBAC-1D > ethanol. Menthol: 1-dodecanol (1:2) and tetrabutyl ammonium chloride: 1-dodecanol (1:2) can be used as green alternatives to extract polygodial.

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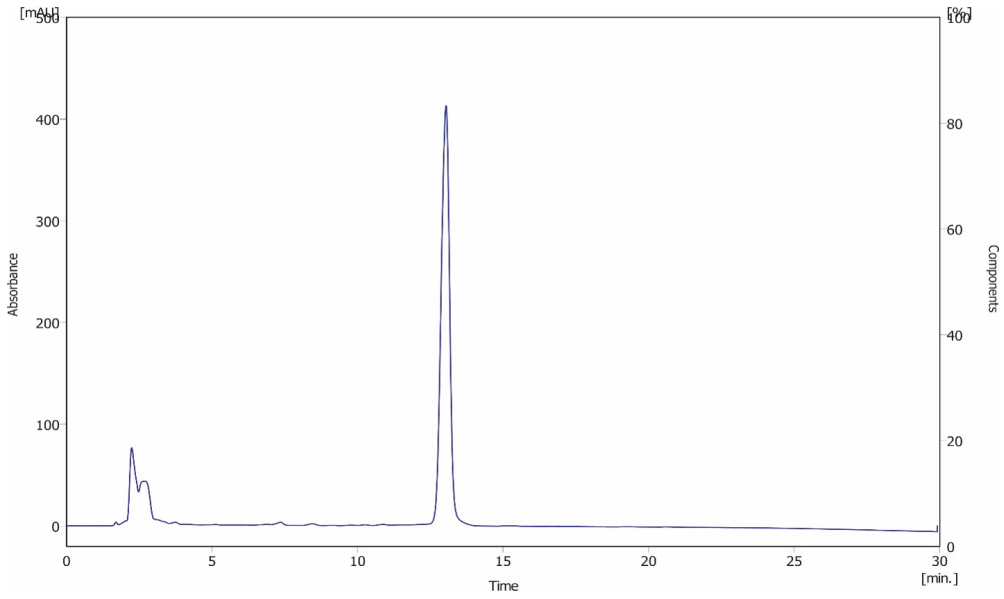
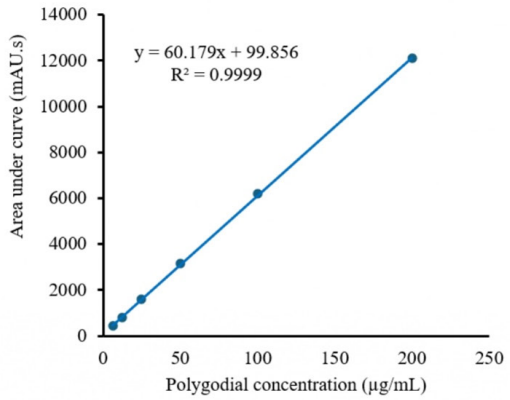


Supplementary Figure 1: Representative of polygodial chromatogram standard (200 µg/mL). Peak was detected at retention time 13.06 min at 230 nm.



Supplementary Figure 2: The Calibration curve of polygodial standards.

Supplementary Table 1: Maximum absorbance of the Nile red in different solvents.

Solvents	$_{max}$ (nm)	E_{NR} (Kcal/mol)
TBAC-1D (1:2)	543	52.65
M-1D (1:2)	545	52.46
M-LevA (1:2)	566	50.51
CC-EG	566	50.51
Water	592	48.30
Dichloromethane	540	52.95
Ethanol	544	52.56
Hexane	497	57.53