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# Optimised Method for Purification of Allylpyrocatechol from *Piper Betle* L. Ethanolic Extract using HPLC and $H^1$-NMR

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**Abstract**
We previously reported isolation of allylpyrocatechol (APC) from *P.betle* ethanolic extract using HPLC-UV in gradient mode with adequate yield. The method was optimised to purify APC at much higher yield and purity, in isocratic mode, using HPLC-NMR. Isolation of APC from betel extract was initially optimized on analytical Agilent 1100 RP-HPLC using Zorbax Eclipse C18 column (250 X 4.6 mm, 4µm), which showed allylpyrocatechol as the major constituent in the extract. The optimized method was applied to preparative Agilent 1200 RP-HPLC using Zorbax Eclipse Prep Ht C18 column (250 x 21.2 mm, 7µm) with 100% acetonitrile-0.1% aqueous phosphoric acid (45:55, v/v) as mobile phase, at flow rate of 22 ml/min for larger scale purification. APC was monitored by UV-Vis (222 nm) and fractions with retention times matching standard APC (Sigma) were collected and pooled. Pooled fractions were characterized using analytical HPLC which revealed APC yield of 78% with 97% purity. Structural verification of the recovered compound using NMR spectral analysis showed identical pattern compared to standard APC. We put forth an optimised RP- HPLC technique combined with NMR spectral identification as an alternative choice for APC isolation, which is faster and gives high yield of APC.

**Keywords** *P.betle* Linn, ethanolic extract, allylpyrocatechol, RP HPLC, $H^1$ NMR

## INTRODUCTION

Allylpyrocatechol (APC) is one of the major phenolic constituents of *Piper betle* Linn. (*P.betle* L.) which possesses a wide range of pharmacological properties including antioxidant, antimicrobial and anti-inflammatory activities [1-3]. It consists of two hydroxyl groups as a component of its chemical structure which confers overall polarity to the compound and interact with the hydroxyl groups in ethanol which facilitates its isolation when used as the extraction solvent [4] .

In view of its activities, large quantities of the purified compound are needed for in-depth pharmacological investigations and for use as "marker compounds", for chemical evaluation and standardization of *P. betle* L. In view of its potential for development as an alternative therapeutic agent, an effective and sustainable method for isolation and purification of APC is warranted. Preparative high-performance liquid chromatography (HPLC) and related technology is an effective method for the isolation and purification of active components from herbal plants. However, few researchers have optimized isolation and purification method of allylpyrocatechol from betle leaf extract using the preparative HPLC method compared to column chromatography [5].

HPLC is a solid-liquid partition chromatographic technique and its advantage is that it allows shorter separation time and use of various solvent systems for purification of compounds with diverse characteristics [6]. When coupled with Nuclear Magnetic Resonance (NMR) for characterization, the HPLC technique allows optimization leading to increased yield and high purity of isolated compounds.

This study effectively performed isolation of APC from *P. betle* L. ethanolic extract by the following steps; oven drying, ethanolic extraction, optimization of APC isolation using analytical HPLC, adaptation of optimized method to a larger scale purification using preparative HPLC for higher yield and purity, and structural verification of the recovered compound as APC using spectral $^1$H NMR analysis. The overall effects of different drying temperatures during the initial extraction procedure on APC yield were also investigated. We report an optimized method for isolation and purification of APC from *P.betle* ethanolic extract by HPLC and NMR which shows encouraging yield and high purity. With further optimisation, we envision to develop a fully optimized method for isolation of APC with better yield and purity.

## MATERIALS AND METHODS

### *Apparatus*

Analytical reverse-phase (RP) -HPLC was carried out using Agilent 1100 HPLC (Agilent Technologies, USA) equipped with a quaternary pump (G1311A), micro degasser (G1379A) and variable wavelength UV-vis detector (G1314A). The preparative RP-HPLC system consisted of binary preparative (gradient) pump (G1361A/G1391A), autosampler preparative (G2206A), column organizer (G1383A), multiple wavelength detector preparative (G1364B), preparative fraction collector (G1364B) with HPLC Chemstation B 03 01 SR1 and Purification/Hi throughput SW module (G2262AA). Nuclear magnetic resonance (NMR) spectrometer used was Ultra Shield Bruker FT NMR 500 MHz (Bruker, Switzerland).

***Reagents***

Ethanol used was analytical grade (Sigma, USA)). Acetonitrile, phosphoric acid (HPLC grade) and acetone D6 for NMR analysis were purchased from Merck (Germany). Reverse osmosis Milli-Q water (Millipore, USA) was used for all solutions and dilutions. Analytical allylpyrocatechol standard was obtained from Sigma (USA).

***Preparation of Crude Extract***

Crude ethanolic extract was prepared according to Mokhtar et al. [7] with modifications. *Piper betle* L. leaves were collected from Selangor and authenticated at the Forest Research Institute Malaysia (PID 541213-31). Leaves were cleaned with water and oven dried (Labtech LCO-3050H, Korea) at 40$^0$C (Sample A) and 60$^0$C (Sample B) for 24 hours. Dried leaves were ground via mechanical blender (Khind BL-1012, Malaysia) and stored at – 20$^0$C until use. Powdered leaves were extracted with 95% ethanol (1 g: 20 ml) and placed on a flat rotator, (3017 GFL, 90 rpm) at room temperature for 24 hours. The mixtures were filtered (Whatman No. 1) and evaporated to dryness using rotary evaporation (Eyela OSB-2100, Japan) at 60$^0$C under reduced pressure. Dried crude extracts were collected in separate clean vials and stored at 4$^0$C protected from light prior to analysis.

***Optimization from Gradient to Isocratic Mode in Analytical Mode***

Initial optimization of APC isolation from crude extract was performed on the analytical ion-pair RP-HPLC (ZORBAX Eclipse plus C18 column (250 mm x 4.6 mm I.D., 5 µm)). Column conditioning was performed with three empty column volumes flushing with solvent (95% purified water and 5% acetonitrile). In order to reduce the solvent consumption and retention time of eluted APC, with good resolution on chromatogram, the gradient solvent system (Table 1) used in our previous study was changed to isocratic mode. Four different ratios in isocratic mode, 35:65 (v/v), 40:60 (v/v), 45:55 (v/v) and 50:50 (v/v), consisting of 100% acetonitrile to 0.1% aqueous phosphoric acid were tested at flow rate 1ml/min. The chromatogram was monitored using UV-Vis at 222 nm. Elution results obtained suggested optimized recovery at solvent ratio of 45:55 (v/v) and this was applied to the preparative HPLC for larger scale purification. It was also used in APC quantification on analytical HPLC with increased flow rate at 1.5 ml/min.

**Table 1 Change in mobile phase constituent ratios with time**

| Time (min) | 100% acetonitrile(%) | 0.1% phosphoric acid(%) |
|---|---|---|
| 0.0 | 5 | 95 |
| 1.0 | 5 | 95 |
| 11.0 | 50 | 50 |
| 15.0 | 50 | 50 |

***Calibration Curve and APC Quantification by Analytical HPLC***

APC standards were prepared by diluting stock APC in acetonitrile to concentrations ranging from 250 to 1800 ng/µl. Five microlitres of each concentration was injected into analytical column at column temperature of 25$^0$C. APC peaks were eluted in mobile phase with solvent ratio of 45:55 (v/v) at a flow rate of 1.5 ml/min. Crude extract was dissolved in acetonitrile (1 mg/ml) and corresponding volume was injected into the same column and eluted under similar conditions. APC in crude extracts were quantified in reference to the APC standard calibration curve and calculated as follows [8, 9]:

Calculation of APC yield, given by:

Concentration of the compound: $\frac{A - I}{M}$

where $A$ : the peak area of the compound in test solution,

$I$ : the y-intercept of the calibration curve

$M$ : the slope of the calibration curve

Percentage of APC in *P. betle* ethanolic extract was calculated using the following equation:

Percentage of APC: $\frac{C \times V \times D}{10000\ W}$

Where, $C$ = the concentration, in mg/L, of the analyte in the test solution

$V$ = dilution factor, if any

$D$ = the final make up volume, in mL, of the test solution

$W$= weight, in g, of the sample used for the preparation of the test solution

***Purification of APC by Preparative HPLC***

Crude extract was dissolved in acetonitrile (20 mg/ml) and filtered (17 mm, porosity: 0.45 mm, PVDF membrane) before HPLC analysis. The optimized elution parameters that were developed in analytical HPLC were applied to preparative HPLC on Zorbax Eclipse Prep Ht C18 column (250 x 21.2 mm, 7µm). Higher sample injection volume (400 µl per injection) and flow rate (22.0 ml/min) was applied and the separation was achieved using 45:55 (v/v) ratio of 100% acetonitrile: 0.1% aqueous phosphoric acid at column temperature of 25$^0$C. 20 injections of sample were repeated for the APC purification. The effluent was monitored at 222 nm using UV-vis and peak fractions were collected according to APC standard elution profile. Fractions collected were evaporated to dryness under vacuum and stored protected from light at 4$^0$C.

***Spectral Identification of Compound***

Dried eluted fractions (approximately 3 mg) collected from preparative HPLC column was dissolved in 0.6 ml acetone D6. Sample mixture was transferred into Norell standard 5 mm NMR tube and sonicated prior to $^1$H NMR analysis. The data was verified against with mass spectrum of APC from literature [10].

## RESULTS

***Optimization from Gradient to Isocratic Mode in Analytical HPLC Mode***

The mobile phase system used in our previous study consisted of 100% acetonitrile and 0.1% aqueous phosphoric acid, with a gradient change in 15 minutes running time. The retention time of APC was at 13.00 min with a flow rate of 1.0 min/min (Fig. 1). Therefore, 50% acetonitrile and 50% aqueous phosphoric acid were used to elute the target compound. In the present study, the mobile phase ratio was optimized to reduce the running time on HPLC, hence reducing the retention time of APC [11].

Several ratios of the mobile phase were tested on analytical HPLC. The isocratic mode was used with the ratio of 35:65 (v/v), followed by 40:60 (v/v), 45:55 (v/v) and 50:50 (v/v) of acetonitrile-aqueous phosphoric acid. Sample injection volume was 5µl and flow rate was fixed at 1ml/min. The chromatograms in Fig. 2 showed continuous reduction on APC retention time and different resolution on each chromatogram.

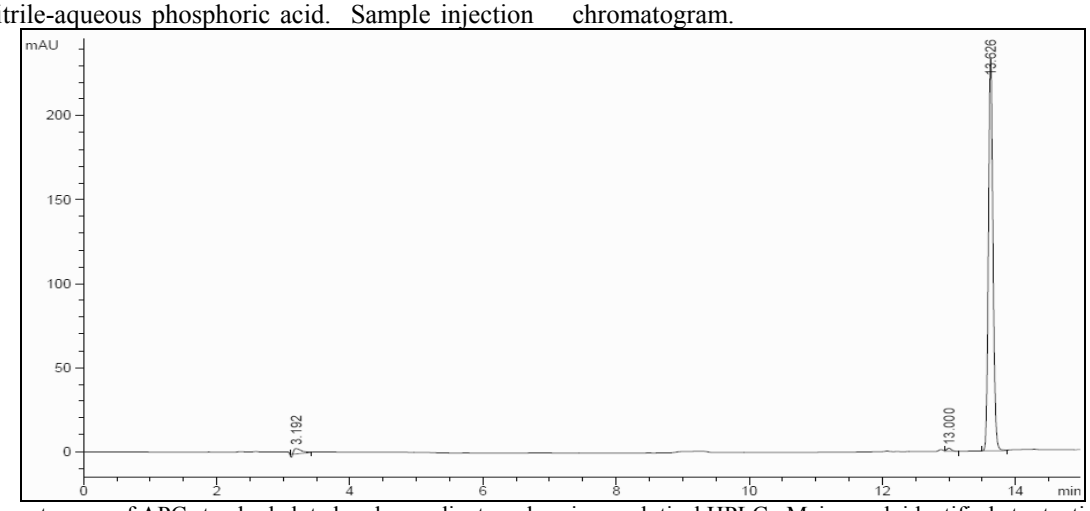


Fig. 1 Chromatogram of APC standard eluted under gradient mode using analytical HPLC. Major peak identified at retention time 13.6 mins. 5 µl of sample was injected into the HPLC at flow rate of 1.0 ml/min and eluted peaks were monitored at 222 nm..

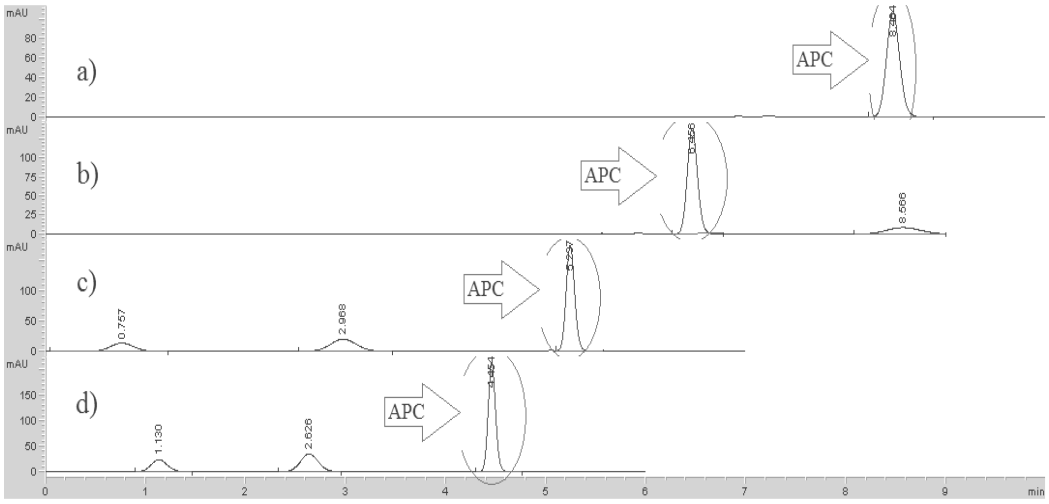


Fig. 2 Chromatograms of APC standard tested with different ratios of acetonitrile-aqueous phosphoric acid under isocratic mode using analytical HPLC. Sample injection volume was 5µl and flow rate was fixed at 1ml/min a) Isocratic mode with mobile phase 35:65 (v/v) of 100% acetonitrile – 0.1% aqueous phosphoric acid, APC peak showed retention time at 8.4 min. b) Isocratic mode with mobile phase 40:60 (v/v) of 100% acetonitrile – 0.1% aqueous phosphoric acid, APC peak showed retention time at 6.4 min. c) Isocratic mode with mobile phase 45:55 (v/v) of 100% acetonitrile – 0.1% aqueous phosphoric acid, APC peak showed retention time at 5.2 min. d) Isocratic mode with mobile phase of equal volume of 100% acetonitrile – 0.1% aqueous phosphoric acid, APC peak showed retention time at 4.5 min.

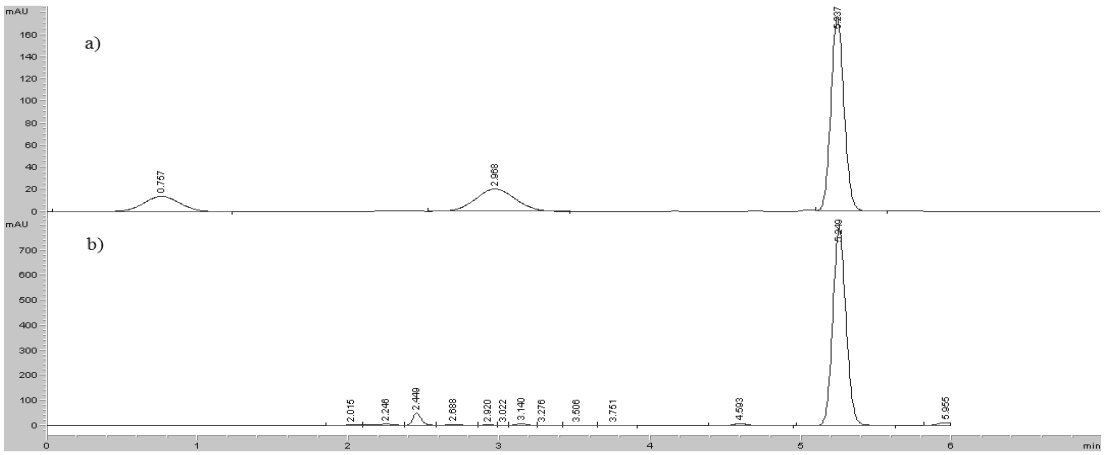

Fig. 3 Chromatograms from isocratic mode using 45% acetonitrile: 55% aqueous phosphoric acid as mobile phase on analytical HPLC at flow rate of 1ml/min and sample injection volume of 5µl by analytical HPLC. a) APC standard chromatogram showing APC peak at Rt 5.237 min and b) Chromatogram of crude *P. betle* L. ethanolic extract with the major APC peak at Rt 5.249 min.

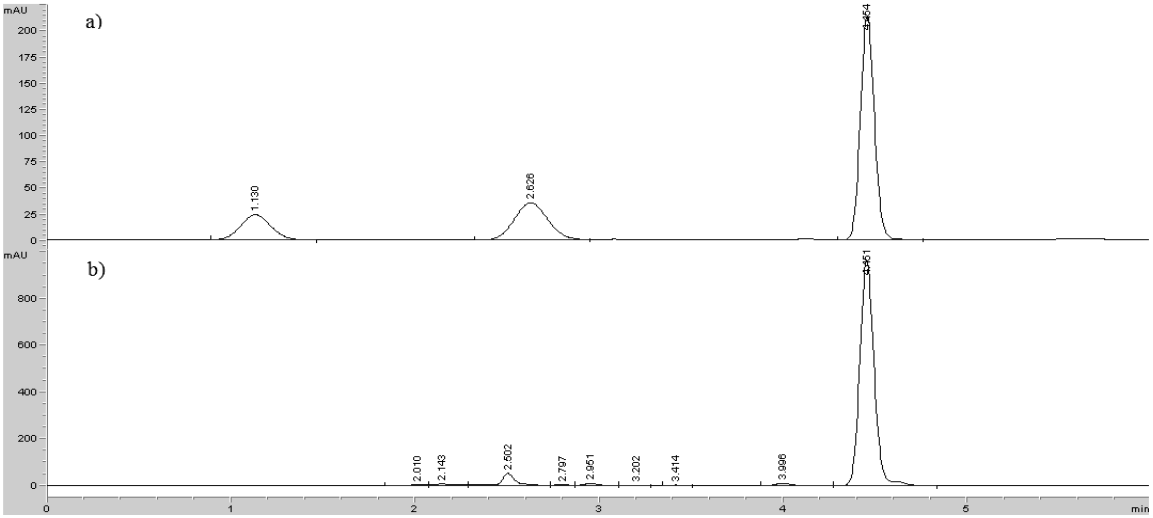


Fig. 4 Chromatograms from isocratic method of equal volumes acetonitrile:aqueous phosphoric acid (50:50, v/v) as mobile phase on analytical HPLC with flow rate of 1ml/min and sample injection volume of 5µl a) APC standard chromatogram showing APC peak at Rt 4.454 min and b) Chromatogram of crude *P. betle* L. ethanolic extract with APC peak unresolved (with tailing).

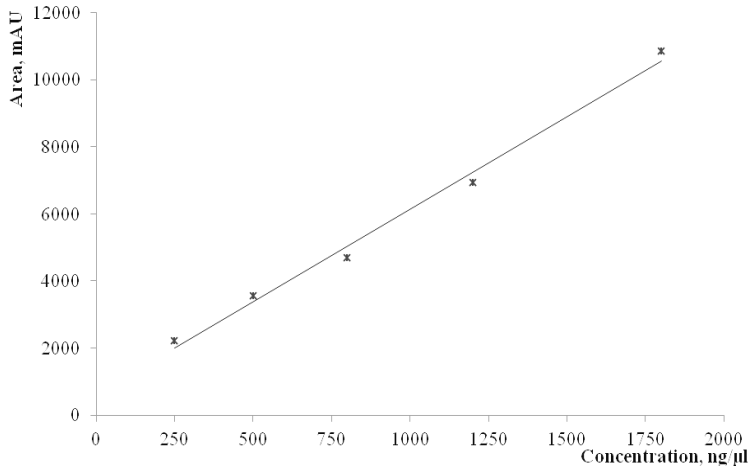


Fig. 5 Calibration curve of allylpyrocatechol standards ranging from 250, 500, 800, 1200 and 1800 ng/µL.

Isocratic mode with mobile phase ratio; 45:55 (v/v) and 50:50 (v/v) of acetonitrile–aqueous phosphoric acid eluted APC peak at retention time of 5.2 min and 4.5 min respectively. Both method resulted in shorter separation time for eluted APC and indirectly reduced the sample running time. Fig. 3 and Fig. 4 show the separation of APC standard and crude sample using 45:55 (v/v) and 50:50 (v/v) respectively. The 45:55 (v/v) of acetonitrile –aqueous phosphoric acid ratio showed better separation of eluted APC compared to 50:50 (v/v) ratio which showed poor resolution of the APC peak, due to peak tailing from a mixture of compounds present in the crude extract. Two peaks that were eluted in the chromatogram of the APC standard are "ghost peaks" that may have resulted from elution of analytes retained from previous injection, sample preparation or contamination of HPLC column or system [12].

Based on the elution characteristic of APC shown in the chromatogram above, the 45:65 (v/v) ratio of acetonitrile to aqueous phosphoric acid was selected to be used for APC isolation from Sample A, which had a better separation and resolution of APC peak at retention time of 5.2 min.

***Calibration Curve using Analytical HPLC***

***Response linearity***

A calibration curve was generated to establish the linear relationship between UV absorption intensity and the amount of APC. APC standard was prepared in five different concentrations; 250, 500, 800, 1200 and 1800 ng/µl. The peaks of APC standards were integrated and identified, and the peak areas were plotted against concentration for the calibration curve (Fig. 5). Within the relevant range, the correlation coefficient was very close to value 1. The correlation coefficient from the regression analysis was determined to be 0.9914 which was within the acceptance limits ($r^2 > 0.98$).

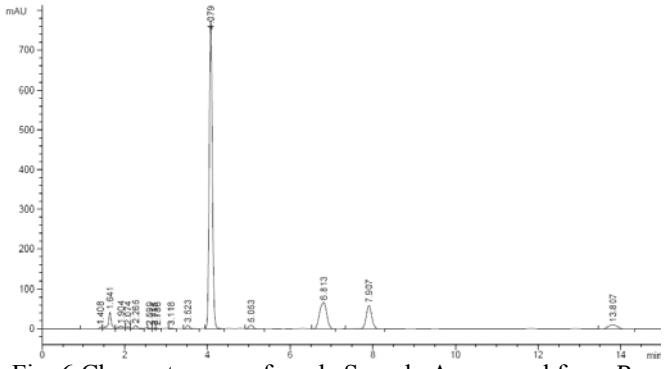

Fig. 6 Chromatogram of crude Sample A prepared from *P. betle* L. ethanolic extract leaf dried at 40ºC on analytical RP-HPLC in isocratic mode using 45% acetonitrile: 55% aqueous phosphoric acid at flow rate of 1.5ml/min.

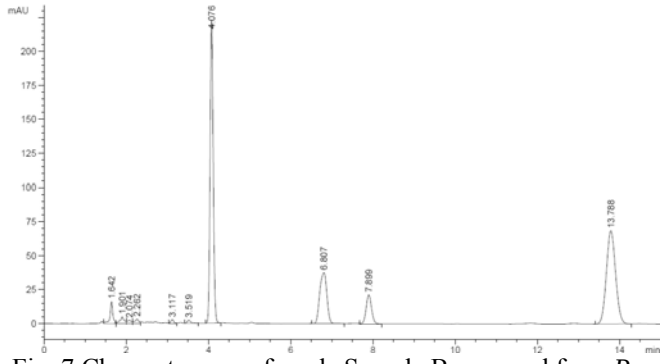

Fig. 7 Chromatogram of crude Sample B prepared from *P. betle* L. ethanolic extract leaf dried at 60ºC on analytical RP-HPLC in isocratic mode using 45% acetonitrile: 55% aqueous phosphoric acid at flow rate of 1.5ml/min.

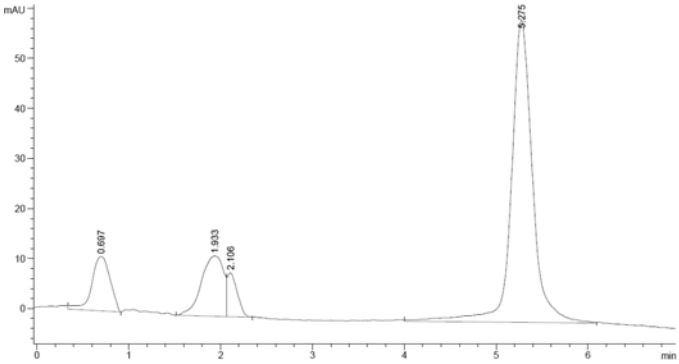

Fig. 8 Chromatogram of APC standard used as a reference for elution time for APC purification by prep RP-HPLC using isocratic mode ( 45% acetonitrile and 55% aqueous phosphoric acid) at flow rates of 22 ml/min and sample injection volume of 400µl. Major peak on chromatogram at Rt 5.275 min is APC.

**Table 2 APC concentrations and percentages in Sample A and B.**

| Sample | APC concentration (mg/L) | Percentage of APC (%) |
|---|---|---|
| A | 632.1014 | 63.21 |
| B | 97.0920 | 9.71 |

***Calculation of APC Yield***

Calculation of APC yields and percentages from Sample A and Sample B on analytical column using optimized method.

APC in crude extracts of Sample A and Sample B were quantified in reference to the APC standard calibration curve and calculated as shown in Table 2. Chromatograms of Crude A (dried leaves at $40^0$C) and Crude B (dried leaves at $60^0$C) are shown in Fig. 6 and Fig. 7 respectively. Both chromatograms from separation on the analytical column showed similar retention time of APC peaks at 4.07 min. From the calculations, APC yields in Crude A and Crude B were determined to be 632.1014 mg/L (63.21%) and 97.0920 mg/L (9.71%) respectively. The chemical profiles of Sample A and Sample B were similar but the peak for APC was higher in Sample A extract indicating higher APC content in the extract obtained from leaves that were dried at $40^0$C, despite being stored at -$20^O$C for three months prior to extraction.

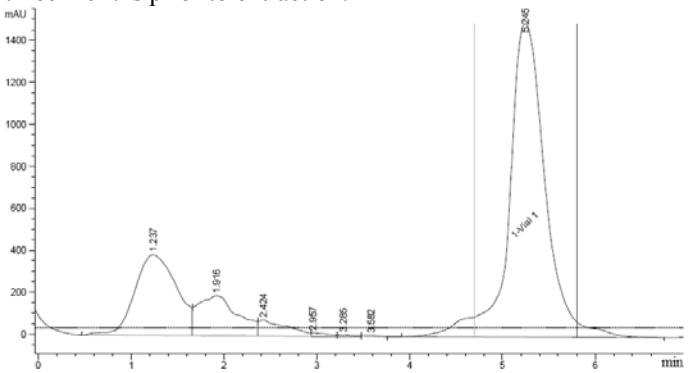

Fig. 9 Chromatogram of crude Sample A (0.01mg/ml) of APC purification by prep RP-HPLC using isocratic mode (45% acetonitrile: 55% aqueous phosphoric acid) at flow rate of 22 ml/min and 400µl sample injection volume. APC fraction was collected from 4.7 min to 5.8 min .

***Purification of APC from P. betle using Preparative HPLC***

The optimized parameters that were obtained from the analytical HPLC were then applied to preparative HPLC for purification of APC from *P.betle* ethanolic extract. Chromatogram of APC standard showed a major peak corresponding to retention time of 5.275 min (Fig. 8). A similar APC peak with retention time of 5.245 min was observed in the *P. betle* L. ethanolic extract (Sample A) as shown in Fig. 9. The fraction collector was set up using a time-based trigger mode between 4.7 to 5.8 mins with 1.1 min duration. Volume of single fraction collected from each injection was 24.2 ml at a flow rate of 22.0 ml/min. Twenty running analysis was performed on preparative HPLC with a total combined yield of 50 mg of brown compound. Fractions were pooled and verified using analytical HPLC followed by APC structural confirmation using NMR.

In preparative HPLC, APC concentration was found to be 7805.04 mg/L or 78.05% of APC yield. 97% of purity was recovered from concentrated extracts in acetonitrile.

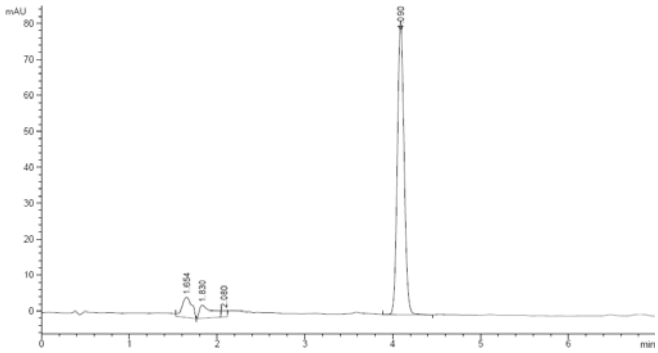


Fig. 10 Chromatogram of APC standard on analytical RP-HPLC isocratic mode 45% acetonitrile: 55% aqueous phosphoric acid at flow rate of 1.5ml/min. Major APC peak on chromatogram is eluted at Rt 4.090 min.

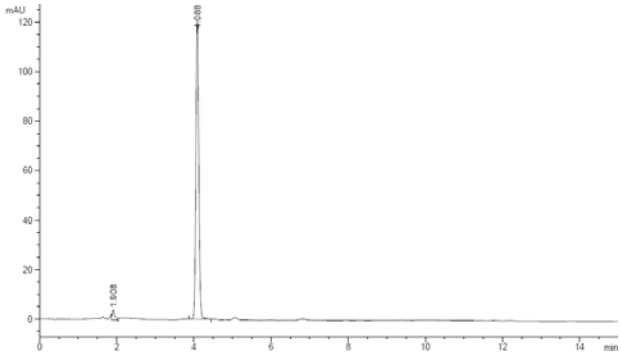


Fig. 11 Chromatogram of isolated dried compound from prep RP-HPLC on analytical RP-HPLC isocratic mode 45% acetonitrile: 55% aqueous phosphoric acid at flow rate of 1.5 ml/min. Major peak has 97.3% of peak area percentage with Rt 4.088 min.

***Confirmation of APC Peak Fractions***

The mobile phase was evaporated from each of the fractions that were collected from the preparative HPLC column before the purification step. Fractions were pooled and characterised using analytical HPLC (Fig. 11) which showed a similar chromatogram to the APC standard (Fig. 10). Based on retention times, the brown compound was confirmed as APC with a retention time of 4.088 which was almost identical to the retention time of the APC standard, 4.090. S11 shows chromatogram of the isolated compound with only two peaks (one major and a minor) visible even when analysis time was held for 15 minutes. Each compound present in the sample is represented by a peak area which was used to determine the purity of isolated APC. Based on the peak area, the purity of APC recovered from the extract was determined to be 97.3% .

***Spectral Identification of Compounds***

Structural identification of peak fraction was performed with $^{1}$H-NMR. Data of each compound are as follows: δ 3.23 (2H, d, J= 6.5 Hz), 5.063-4.976 (2H, m), 5.956-5.902 (1H, m), 6.513 (1H, dd, J=8, 2 Hz), 6.682 (1H, d, J=2 Hz) and 6.737 (1H, d, J=8 Hz), the results were similar to those in literature [7]. As the APC structure has eight protons or Hydrogen atoms attached to the main structure, NMR spectra analysis predicted all eight hydrogens, labelled $H_1$ to $H_8$ (Fig. 12) and was found comparable to the NMR spectra of APC recovered from the extract (Fig. 13). Each proton is identified based on its unique constant coupling which is determined by its bond to the carbon atom in APC and neighbouring proton (s).

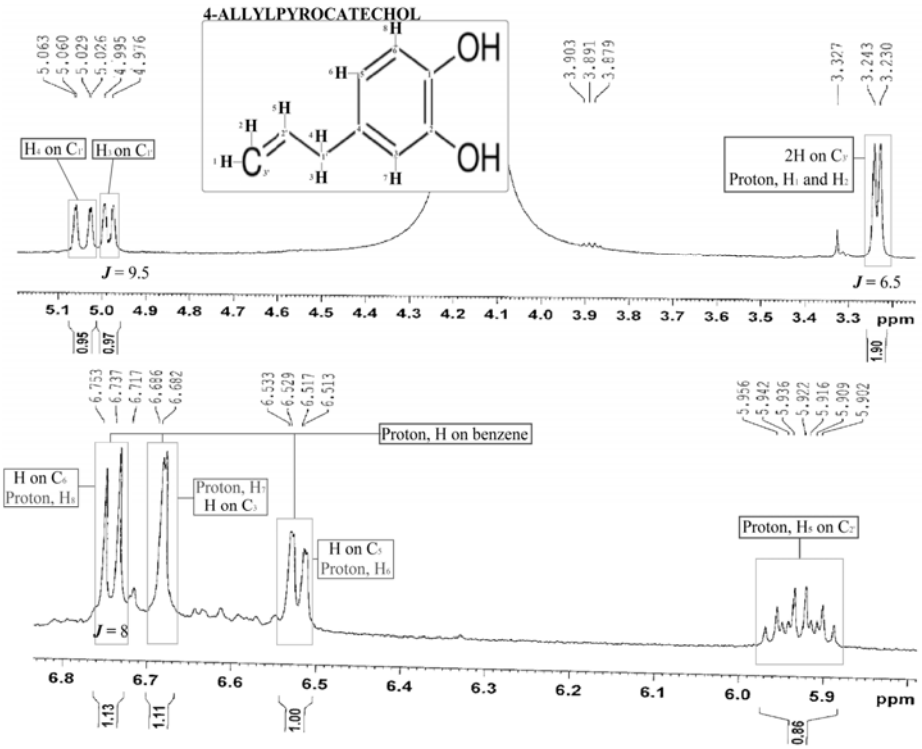


Fig.12 NMR analyses of proton in APC standard, 8 protons are detected. Proton are labelled as H1 to H8.

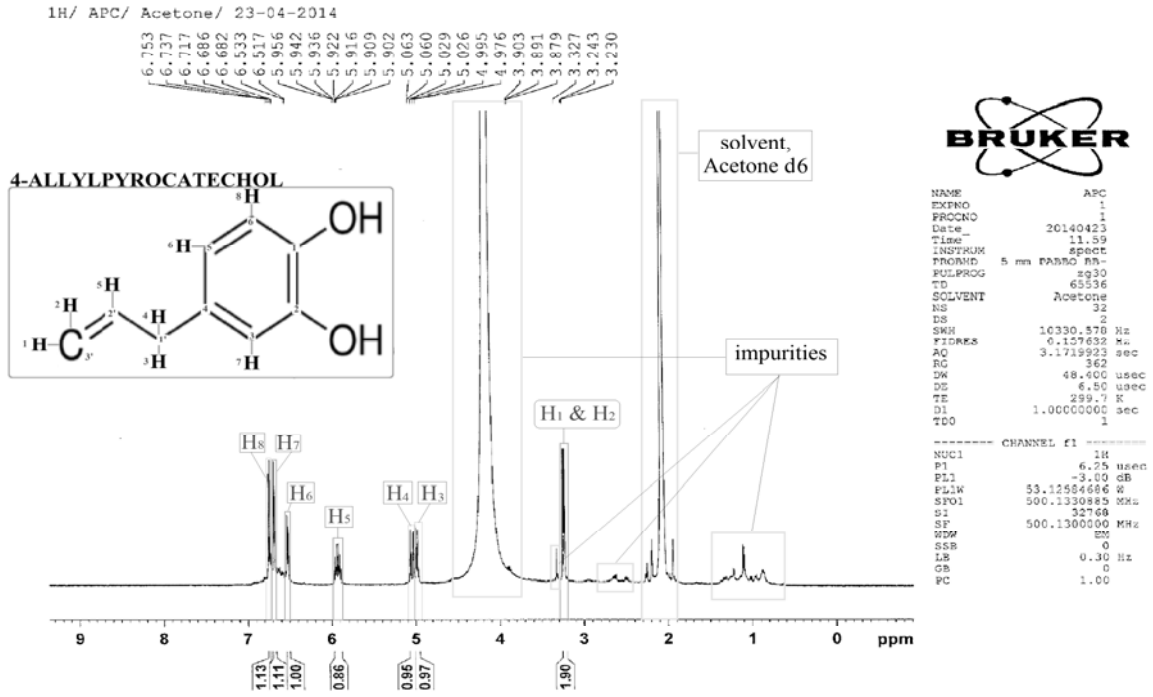


Fig. 13 Full NMR signal for dried compound (*P.betle* ethanolic extract) from APC purification using prep RP-HPLC with strong signal at 4 ppm may due to water or hydroxyl group on APC, signals at 1 ppm, 2.5 to 2.6 ppm and 3.4 ppm are impurities from HPLC peak tails. Eight protons on APC present with predicted constant coupling of each proton location on APC structures.

## DISCUSSION

Extensive studies on the constituents of *Piper betel* L. leaves have identified five chemical groups which can be classified into polyphenols, alkaloids, terpenes, steroids and prenylated hydroxybenzoic acid group [13]. Allylpyrocatechol (APC) was identified as the major active constituent belonging to polyphenols group [14]. An important consideration in drying leaves is the preservation of the phytochemicals, which are mostly heat sensitive and contribute to the biological activity of the plant materials. The effects of drying temperature on the quality of *P. betle* L. was investigated previously based on the content of two compounds; allylpyrocatechol (APC) and eugenol [15]. Apart from removing moisture that causes leaf damage from decomposition, oven drying was chosen because it yields a chemical profile that is similar to fresh leaves [16]. The concentration of APC increased with the extraction temperature, but the percentage of increase dropped when the temperature over $60^0$C was applied. Drying temperature of $60^0$C produced lower APC yield (9.71%), possibly due to the effect of temperature on the nature and amount of the bioactive compound [17]. In this study, the increase in APC yield (63.21%) indicated that temperature of $40^0$C was ideal for drying betle leaves and thus in our extraction procedure, *P. betle* L. leaves were oven dried at 40 ºC to preserve the active compounds and for optimised yield.

Most secondary metabolites reside in the cells, thus grinding of raw material before extraction increases extraction yield. Extraction of active constituent from the insoluble matrix in which they are soaked needs to take into account the polarity, toxicity and purity of the extraction solvent [18]. Methanol and ethanol are commonly used in the isolation of active constituents specifically to separate the polar and semipolar compounds due to the polarity of these solvents [19]. The capability of ethanol, ethyl acetate and hexane in extracting more types of phytochemicals from betel leaves extract in comparison with water has been proven. Partitioning of methanol extract into the ether, ethyl acetate and aqueous methanol fractions have been done to identify active constituents in *P. betle* leaves. APC was identified as the major constituent (80% w/w) in the ether fraction which was subjected to bioassay-guided fractionation using column chromatographic purification. 58% (w/w) of APC yield were obtained by using ethyl acetate refluxed extraction of liquid-liquid extraction (LLE), while the supercritical fluid extraction (SFE) method showed moderate APC yield (48.6%). The difference in APC yields may be due to other factors such as extraction temperature and ratio of solid to solvent in extraction procedure [20].

Identification and quantification of active compounds from *P. betle* leaf extracts has been routinely performed by HPLC, HPTLC, GC-MS [21]. Analytical methods based on DART-MS [22] have been reported for the profiling of phytoconstituents in *P. betle* leaf extracts and recently, UPLC-ESI-MS/MS was developed for characterization and quantitative determination of eugenol, eugenyl acetate and allylpyrocatechol-3,4-diacetate [23]. These methods are limited only for qualitative and quantitative analysis, but are not feasible for isolation and purification of the active

compounds. Extraction methods using different solvents and chromatographic conditions have also been conducted to isolate allylpyrocatechol (APC) from *Piper betle* leaf extracts. However, isolation and purification of active compounds using conventional methods such as low pressure column chromatography and thin layer chromatography (TLC) requires several steps resulting in low recoveries of the products [24]. These methods have difficulties due to low column efficiency, poor separation repeatability and inefficient isolation using manual sample collections. In addition, the selective separation is difficult to realize [25].

In this work, a strategic approach of isolation procedure through direct use of techniques in the laboratory such as HPLC and NMR shortened its duration and enabled a simplified process of fractionation, isolation and purification. To effectively purify the compounds, the separation condition was optimized using analytical RP-HPLC. The separation condition, including sample volume, flow rate of mobile phase and gradient condition, were optimized in this work. Successful separation by HPLC largely depends upon the selection of suitable two-phase solvent system. As a rule of thumb, the column was flushed with at least 10 column volumes of initial eluent before reliable separation can be obtained in the following injection [26]. The use of acetonitrile resulted in better resolution in a shorter analysis time than methanol, resulting in a sharper peak shape [27].

Isocratic elution is a faster technique, in terms of separation speed, compared to gradient elution, Different solvent ratios at 35:65 (v/v), 40:60 (v/v), 45:55 (v/v) and 50:50 (v/v) (acetonitrile: aqueous phosphoric acid) were tested initially in isocratic mode with adequate flow rate applied to the column to reduce retention time of target compound [28]. Flow rate of 1.0 ml/min was applied throughout analysis due to its efficiency in resolving APC peak. Based on retention time and good resolution of APC peak on chromatogram obtained from analytical HPLC, isocratic condition of mobile phase consisting of solvent ratio 45% acetonitrile: 55% aqueous phosphoric acid, at flow rate of 1 ml/min and sample injection of 5 µl by was chosen as the optimized method.

Larger scale preparation required transfer of extraction from analytical column to a preparative column which possesses similar characteristics to the analytical column, except with larger particle size, column length and increased diameter [29]. According to the formula of changing flow rate from analytical column to prep column by Schulte and Epping (2005) [30], without regard to which method is chosen flow rate at prep RP-HPLC must be at least 21 mlmin-$^{1}$ to achieve the same elution time as that which appears on chromatograms from the analytical RP-HPLC column. Lower flow rate causes the retention time to increase because prep column has larger diameter and larger particles than the analytical column [31]. In order to reach greater column efficiency in separation with preparative column, higher volume loads and concentration were used, compared to analytical column that is mainly focused for better separation and low volume loads. Flow rate of 22 ml/min with high sample injection volume (400 µl) was adapted to preparative HPLC. Because the flow rate for the prep column used in this study was limited to maximize at 22 ml/min for the RP-HPLC system used, no further research was performed using higher flow rate even though the prep column could withstand flow rate up to 60 ml/min. A shorter prep column with larger particle size and diameter such as column from Waters (Atlantis HILIC T3 OBD Prep Column, 100Å, 10µm particle size, 30mm X 75mm diameter) would have permitted adaptation to a higher flow rate.

To achieve separation of high purity of APC, purging effectively washes the column so that the next purification is not overloaded by previous samples. Purge functions to wash off the column before new sample injection is performed for each analysis without any extension of analysis time that leads to higher solvent consumption. For this purpose three different time settings were tried for buffer purging while solvent purging was fixed at one minute. It was found that the purging time equivalent to one empty prep column volume effectively got rid of carryover from previous sample into chromatogram. Six minutes of buffer or purified water purging time at flow rate 14 ml/min or equal to one prep column empty volume showed better chromatogram resolution (data not shown) without cross over from previous purification. Purging of acetonitrile was kept at minimum, from half to one minute of purging time for column reconditioning before next analysis.

Concentration overload trials were performed to investigate the highest concentration possible to be separated and maximise APC yield from crudes on a single purification step. Five crude concentrations were tried; 400µl each at 0.3 mg/µl, 0.2 mg/µl, 0.1 mg/µl, 0.02 mg/µl and 0.01 mg/µl were injected into the system. The three highest concentrations failed to be separated and were terminated at initial time of separation due to poor peak resolution and appearance of peaks as soon as minute in one. Under these circumstances, concentrations 0.02 mg/µl and 0.01 mg/µl were used for purification and in optimization of volume overload. Several trials on volume overloads were conducted by varying sample injection volumes up to 700µl. However, chromatograms of crudes and APC standard were observed to have lost peak symmetry and chromatogram from the crude showed merging of peaks with the APC major peak, indicating a similarity in Rt to the APC standard, indicating that the APC peak was not separated either in the APC standard nor crude extract.

Compound recovery from eluted fractions is a more challenging task because one complete run through the column may insufficiently obtain a pure compound and may require additional steps using recycling or simulated moving bed processor chromatography [31]. Immediate recovery of eluted fractions was performed by drying under pressure to preserve the quality of active compound as degradation of the compound may occur if it is stored under prolonged period of time in the mobile phase. Final characterization of pooled fractions was analyzed by analytical HPLC, which resulted 78% of APC yield with 97% purity. The overall results indicated that this method has ability to purify APC in short duration of time. This

work has shown that HPLC had no influence on the quality of APC, but obtaining better yields must be studied further.
The purified compound was confirmed as APC based on elution time identity compared to APC standard and its chemical structural identity to 4-Allylpyrocatechol as determined by spectroscopic analyses. Purity of compounds was calculated from the percentage of area represented by the peak on chromatogram. Since each peak represent a component therefore each of component has a percentage of peak area [32]. Purity assessment in chromatography using multi wavelength UV-visible absorbance detection is related to spectral absorbance of every compounds present. Compound purity is directly proportional to visible and detectable peaks on chromatogram. As the APC standard has 98% purity ("4-Allylpyrocatechol analytical standard | Sigma-Aldrich," 2014) , based on the eluted peak are on the chromatogram the purity of APC in the extracted compound was determined to be 97.3%.
Analytical HPLC with UV detector does not accurately measure percentage purity as many substances are invisible [33]. Therefore, UV light detection should include reconfirmation of compound purities by qNMR [34]. According to Rizzo and Pinciroli [33], qNMR analysis of compound purity is equal to interested compound in powder minus the impurities from extraction, excess of HPLC solvent, water and impurities from solvent. However, researchers query that qNMR is not a sensitive method in checking purity due to high concentration limit of detection which is above 0.1mM. In this study, one dimension proton NMR and isocratic mode analytical RP-HPLC coupled to UV-vis were performed to confirm and obtain compound purity. From HPLC analysis, all the three compounds showed peaks that match Rt of the major peak on APC standard chromatogram so all compounds were confirmed as APC.
Structure elucidations of APC was carried out by comparison of NMR spectral data of purified compound with literature and NMR database [35]. NMR elucidation succeeded to give all eight proton H signals that are present in the APC chemical structure. Matching proton locations on Carbon of APC and constant coupling proton to proton was also observed. For further NMR confirmation Carbon ($^{13}C$) profile is necessary to quantify the exact amount of total carbon present and carbon-carbon constant coupling in the structure because proton test is insufficient due to its inability to distinguish H/H gauche rotamers [36,37].

## CONCLUSION

We determined that oven drying of *P.betle* leaves at 40$^{o}$C was most conducive in preparation of the crude ethanolic extract to enhance APC yield. In this study, an optimised method was developed for isolation from *Piper betle L*. leaf ethanolic extract based on elution from analytical RP-HPLC in isocratic mode using ratio 45% acetonitrile: 55% aqueous phosphoric acid at flow rate of 1.5 ml/min, where we proved allylpyrocatechol (APC) to be the major constituent. Adapting these optimised parameters to preparative HPLC, we purified APC from the extract with 78% yield and 97% purity and verified the recovered compound as APC which showed nearly identical NMR spectral pattern compared to standard APC (Sigma). APC is commonly purified mostly using column chromatography and although this method is cheaper to set up. However, it is time consuming as it is involves complicated separation work at each extraction step that requires use of solvents with different polarities. HPLC-NMR should ideally enable the complete structural characterization of any molecule directly in an extract. The RP- HPLC technique combined with NMR spectral identification is an alternative choice for APC separation, which provides a speedier and cleaner method providing high yield of APC as end product.

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