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°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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