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Qualitative study of *Curcuma xanthorrhiza* using RP-HPLC-DAD

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Abstract

The chemical analysis of *Curcuma xanthorrhiza* was performed via reversed phased high performance liquid chromatography-diode array detection (RP-HPLC-DAD). Prior to the investigations, the extract was obtained via maceration. The chosen HPLC mobile phase was not similar with the one published in the national herbal monograph. Instead of utilising 0.1 % formic acid in water and methanol, this work involved a higher acidity of formic acid (0.3 %) and acetonitrile. The chromatograms were studied, and two major peaks were observed in the stepwise gradient, with the retention times recorded at t_R = 14.868 and 29.777 minutes. These signals represent two individuals, yet, unidentified constituents, since the chromatographic patterns were not satisfactory and non-comparable with the peaks in the fast, non-isocratic mode. In summary, more experiments should be conducted to understand the elution of the *Curcuma*'s targeted compounds, particularly xanthorrhizol and curcuminoids.

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1.0 Introduction

The chemical analysis of *Curcuma* was initiated via analytical and preparative silica Thin Layer Chromatography (TLC) (Abdul Zahar et al., 2020). The fractions of *C. longa* (local turmeric) were purified by using a solvent mixtures of chloroform:methanol (95:5). The compounds were subjected to Nuclear Magnetic Resonance (NMR) spectroscopy. Following the spectral elucidations, the natural biomolecules were identified as curcumin (C), demethoxycurcumin (DMC) and *bis*-demethoxycurcumin (BDMC) (Fig. 1). The values of the refractive index (R_f) were 0.75, 0.55, and 0.27 for C, DMC, and BDMC, respectively. Earlier, Ashraf et al. (2012) validated the high-performance thin layer chromatographic (HPTLC) method for the quantification of curcumin in *C. longa* from different geographical regions in India. A ternary solvent system of toluene: chloroform: methanol (5:4:1) was utilised to obtain lower R_f value for curcumin (0.3). Li et al. (2014) also isolated all three curcuminoids by reversed phased high performance liquid chromatography (RP HPLC), by consuming a ternary gradient solvent system, comprising of methanol, tetrahydrofuran and 0.1 g/100 ml phosphoric acid in water. The order of elution was recorded firstly by C, DMC, and followed by BDMC. An ultra-high performance liquid chromatography (UPLC) set up has involved acetonitrile and 10 mM ammonium formate (70: 30; v/v) in a gradient mode, to determine the curcuminoids (Ashraf et al., 2015). These compounds were also chemically modified via molecular engineering to obtain the analogues, hence, to improve the biological activities, enhance stability, reduce the rotational freedom, and minimise the metal-chelation (Ali et al., 2021).

In addition to the local tumeric, another sample of *Curcuma* species, *C. xanthorrhiza* (known as temulawak) receives increasing attention, due to their secondary metabolites. Automated liquid chromatography systems were also selected to study *C. xanthorrhiza* extracts (Rahmat et al., 2021). The rhizomes of *C. longa* and *C. xanthorrhiza* could be easily discriminated, physically. The rhizome's size of *C. xanthorrhiza* is much bigger than *C. longa*. However, it is becoming difficult, once they are pulverised, or powdered, or cut into slices (Rafi et al., 2015). The biological activities of *C. xanthorrhiza* are also due to the presence of its main components, likewise, the curcuminoids, and xanthorrhizol, a sesquiterpenoid (Fig. 1). Rafi et al. (2015) investigated the curcuminoid's content in the Indonesian specimens of *C. xanthorrhiza* by using Reversed Phased High Performance Liquid chromatography-diode array

detector (RP HPLC-DAD). The mobile phase consisted of acetonitrile and 0.5 % acetic acid in water. Later, Erpina et al. (2017) and Badrunanto et al. (2019) performed simultaneous quantification of curcuminoids and xanthorrhizol. Nevertheless, the RP column was conditioned in a very low acidity (0.001 % formic acid).

The combinations of methanol-water and acetonitrile-water are the most commonly used solvent mixtures in RP-HPLC analysis (Setyaningsih et al., 2021). However, curcuminoids are poorly resolved in methanol-water mixtures at a volume ratio of 50:50 and 60:40. Therefore, methanol was replaced by acetonitrile. Although with an acetonitrile-water volume ratio of 60:40, a sharp peak was obtained, all curcuminoids (C, DMC and BDMC) were co-eluted at a retention time of 12.54 minutes (Setyaningsih et al., 2021). Therefore, an isocratic mobile phase, comprising of an acetonitrile-methanol-water mixture with a volume ratio of 65:5:30, was set to assist the chromatographic separation of the three curcuminoids (Setyaningsih et al., 2021). In another trial, Hadi et al. (2018) run the RP column isocratically, with methanol only, to study the curcuminoid content in both *C. longa* and *C. xanthorrhiza* rhizome. For this study, the constituents of the aerial part of *C. xanthorrhiza* were screened by using RP-HPLC-DAD. It is aimed to study the elution pattern of the components in the extract of a local specimen. The mobile phase was not similar with one published by the national herbal monograph (Globinmed, 2022). Instead of utilising 0.1 % formic acid in water and methanol, this experiment involved a higher acidity of formic acid (0.3 %) and acetonitrile. It is hoped that the plant's constituent could be recognised by recording the extract's chromatograms.

2.0 Methodology

The rhizomes of *C. xanthorrhiza* were obtained from a local market at Puncak Alam, Selangor Darul Ehsan. The plant material was dried, chopped into small pieces and crushed by using a mortar and pastel, to increase the surface area. Prior to the liquid chromatography, the sample was macerated and sonicated in methanol (Rafi et al., 2015). The beaker containing *C. xanthorrhiza* sample was placed in a benchtop sonicator (Liarre Starsonic 90 Easy, 80 W, 30 kHz) for 20 minutes. Later, the extract was filtered through a 0.45 µm Millipore filter, into the vials. Then, the HPLC profiling was performed by using the Reverse-Phase High-Performance Liquid Chromatography (Agilent 1200 RP-HPLC) (Globinmed, 2022). About 10 µl of the sample was injected for the HPLC, which was duplicately ran through a column (Thermo C-18; 250 mm × 4.6 mm and 5 µm of pore size) as a stationary phase. Two approaches were employed to compare the extract profiles, between a rapid and a non-isocratic, stepwise gradient (Table 1) elutions in RP-HPLC methods. The mobile phase consisted of various composition of 0.3% formic acid in water and acetonitrile, with a flow rate of 1 ml/min. The data were recorded via dual wavelengths (254 and 360 nm) through diode array detector (DAD) and the column temperature was maintained at 36°C throughout the experiment. The running time was set at 30 and 50 minutes, for the rapid and stepwise gradient modes, respectively. The data acquisition was performed by using ChemStation software (Li et al., 2014).

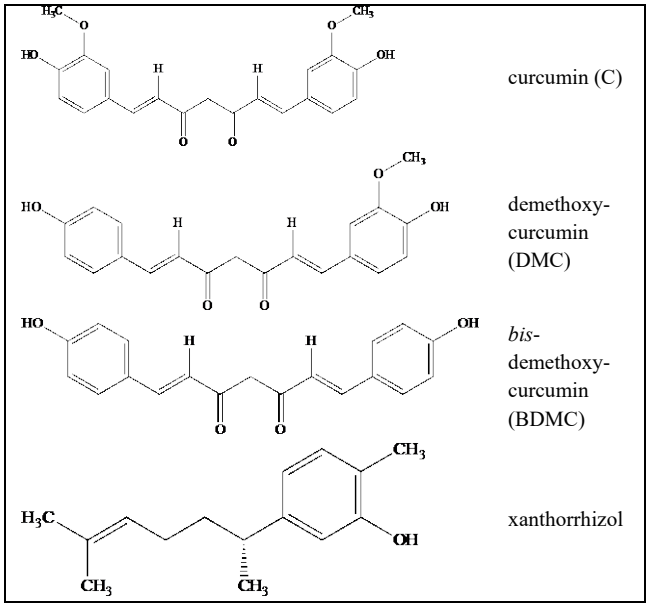

Fig. 1: The chemical structures of some constituents from *Curcuma* extract

Table 1: The solvents for the stepwise gradient elution

Time (minutes)	Acetonitrile (%)	0.3% formic acid in water (%)
0	10	90
5	10	90
20	40	60
22	40	60
30	70	30
32	70	30
40	90	10
45	90	10
47	10	90
50	10	90

3.0 Results and discussion

Fig. 2 displays the chromatogram of the methanol extract in a fast gradient. Meanwhile, Fig. 3 shows the chromatogram in a stepwise gradient mode. There was no much difference in the retention times (t_R) for the peaks that emerged below t_R = 2.5 minutes, in both methods. Nevertheless, two major, isolated signals were found at t_R = 14.868 and 29.777 minutes, when the stepwise gradient mode was applied (Fig. 3). RP column was found suitable, as recently reported (Salem et al. 2021), by engaging both C8 and C18 RP columns in analyzing *Curcuma*'s compound separation.

Fig. 2 displays a group of chromatographic signals at t_R from 8 to 11 minutes, at 20% of acetonitrile. Unfortunately, the baseline separation was not achieved for the major peak at t_R = 9.749 minutes. Comparatively, Badrunanto et al. (2019) reported that the curcuminoids' peaks could be individually separated, within t_R from 7 to 10 minutes, while the elution order was recorded firstly by BDMC, DMC and finally by C, in a RP HPLC system of 20 minutes gradient run, employing 42–58% acetonitrile. Likewise, the order for increasing peak intensities is similar as above, hence, the concentration of the individual curcuminoids, were as mentioned. The optimum chromatographic separation was similarly achieved (Rafi et al., 2015) by using linear gradient elution of 45–75% acetonitrile in 0.5% acetic acid. The total analysis time for quantitative analysis of curcuminoids was longer, i.e. 35 minutes. The three peaks were isolated at t_R = 9.07, 9.84, and 10.65 minutes, however, it is reversely for C, DMC and BDMC, respectively.

According to the online monograph (Globinmed, 2022), an RP-HPLC condition was set for an ethanol extract of *C. xanthorrhiza*. The mobile phase comprised of methanol and 0.1% formic acid in water. Fig. 4 displays the overlay chromatogram of the extract and the RP HPLC solvent profile (Globinmed, 2022), to graphically view the run. It shows that two unknown peaks were recorded, followed by the compound of interest, xanthorrizol, a non-polar molecule (Fig. 1), at t_R = 13.975 minutes. This event was after the fast gradient elution of 10–90% methanol for 12 minutes, or during the RP column conditioning (90–10% methanol for 5 minutes, at running time, t = 12 to 17 minutes). It is hypothesised that xanthorrizol would be eluted later from the C18 column, i.e. at a high percentage of the organic solvent (methanol) and low percentage of water.

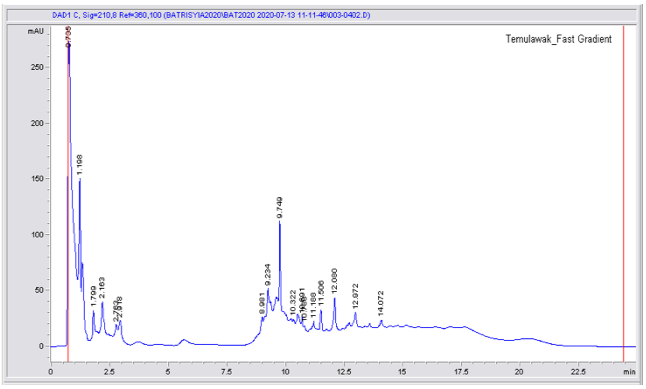

Fig. 2: The chromatogram of methanol extract of *C. xanthorrhiza* (fast gradient)

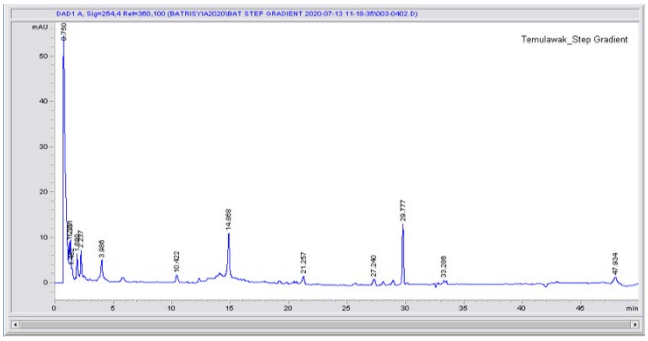

Fig. 3: The chromatogram of methanol extract of *C. xanthorrhiza* (stepwise gradient)

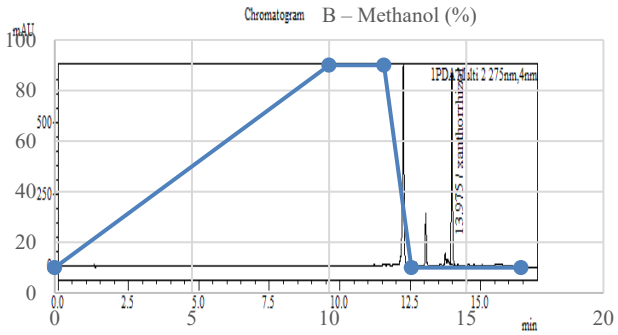

Fig. 4: The chromatogram of methanol extract of *C. xanthorrhiza* (Source: Globinmed, 2022)

There would be a concern on the chromatographic method for the standard itself (xanthorrizol). Similar RP HPLC procedure was applied to both standard and the solution test of *C. xanthorrhiza* extract. It was found that the standard was eluted during the column conditioning (Globinmed, 2022).

The method in the online monograph (Globinmed, 2022), could not be considered as a linear gradient, since the elution of xanthorrhizol happened at the isocratic period, i.e. during the column conditioning (10% MeOH) (Fig. 4). Instead, it is recommended that a stepwise gradient is to be planned from the beginning of the experiment, and it might be more suitable for *C. xanthorrhiza*. It is also proposed that the procedure

should be re-optimised, by setting a re-run of the experiment, for an example, by starting with 10 % of MeOH and maintaining the column situation for at least 5 minutes, at the beginning of the trial. It is anticipated that xanthorrhizol could be eluted earlier in this step. Moreover, the time and cost could be reduced, since lesser solvents would be used for the experiment, should xanthorrhizol be eluted at 10% MeOH.

4.0 Conclusions

An automated liquid chromatography system was selected for *C. xanthorrhiza* in this research. The identification of curcuminoids and xanthorrhizol was initially suggested by comparing the HPLC spectral data and the retention times of each compound or peak in this *Curcuma* sample, to those of the published spectra. Two major, separated signals were found, when the stepwise gradient was applied. Yet, these peaks are representing two unidentified compounds, since the chromatographic pattern were not satisfactory and non-comparable with the peaks in the fast, non-isocratic mode.

It is unfortunate that the components from this natural product resource could be not deduced. The curcuminoids and sesquiterpenoid could be introduced, due to the absence of the standards, in this chemical investigation. The method in the monograph could not be fully agreed upon, as a linear gradient mode, since the compound of interest, xanthorrizol, and the other two unidentified compounds were eluted during the column conditioning. Hopefully, there could be a room for improvement for this herbal documentation, specifically for *C. xanthorrhiza*. Finally, more experiments should be made to understand the compounds' elution of this *Curcuma* sample. Should future adaptation be made based on the data from the monograph, the extension of HPLC run could be programmed in order to reduce the probability of the compounds' elution during column flushing and conditioning. HPLC solvent mixture with an inclusion of the double distilled water might also offer some changes on the retetntion time (Yantih et al., 2022).

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