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Research Article

STANDARDIZATION AND PHYTOCHEMICAL STUDIES OF *CURCUMA XANTHORRHIZA* ROXB.

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ABSTRACT

The present study reports on the comprehensive phytochemical analysis from a potential medicinal plant, *Curcuma xanthorrhiza* Roxb. Standardization, qualitative and quantitative determination of phytochemicals, and thin layer chromatography (TLC) profiling of secondary metabolites from *C. xanthorrhiza* rhizome were carried out. Furthermore, GC-MS characterization of the compounds present in *C. xanthorrhiza* was also studied. The generated data provides the basis for its wide usage as the therapeutant and preliminary information towards the development of potential products based on this plant.

Keywords: *Curcuma xanthorrhiza*, Secondary metabolites, Standardization, Phytochemical analysis, Xanthorrhizol.

INTRODUCTION

Plants have an almost unlimited ability to synthesize diverse secondary metabolites that have a wide-range biological functions that are useful to human[1]. *Curcuma xanthorrhiza* (Roxb.) is an important and potential medicinal plant belonging to the family Zingiberacea and shares the same genus as *Curcuma longa* Linn., commonly known as turmeric. In Malaysia and Indonesia this plant is locally known as 'temu lawak' or 'koneng gede'.

C. xanthorrhiza is commonly used in the local food industry and possesses a variety of therapeutic values[2]. One of the constituent, xanthorrhizol is a unique marker for *C. xanthorrhiza* and thus its presence differentiates this plant from other Curcuma species. Xanthorrhizol has been reported to exhibit a wide range of biological activities such as anticandidal, antibacterial, and antimetastatic[3-5].

It is important to establish the chemical profiles and determined the phytochemicals content of this plant as it is popularly used in traditional medicines. Although previous studies of phytochemicals of this plant had been conducted[6,7], a comparison of phytochemicals obtained from an organic extraction solvent to those obtained from an aqueous extraction solvent had not been done. Thus, the present study is aimed to systematically examine the phytochemicals of *C. xanthorrhiza* rhizome prepared with an organic solvent (ethanol) and aqueous extracting solvent (water) as a preliminary approach for standardization, characterization, qualitative and quantitative determination of phytochemical, and TLC profiling of secondary metabolites from the rhizomes of *C. xanthorrhiza.*

MATERIAL AND METHODS

Plant Material

Fresh rhizomes of *C. xanthorrhiza* were purchased from Temerloh, Pahang, Malaysia in February 2010. The plant was authenticated by an expert and deposited at the Herbarium Unit of the School of Biological Sciences, Universiti Sains Malaysia. The rhizomes were air dried, ground into fine powder and stored prior usage.

Solvents and Chemicals

All solvents used were of analytical grade. Hexane, chloroform, deuterated chloroform, 95% ethanol, dichloromethane, glacial acetic acid, n-butanol, diethyl ether, ammonium hydroxide and sulphuric acid were from QreC Chemicals (Malaysia). Basic bismuth nitrate, polyethylene glycol-400, diphenylboryionylamine, vanilin, catechin standard, potassium iodide and feric (III) chloride were purchased from Sigma Chemical Company (St Louis, MO, USA) whereas Folin & Ciocateu reagent, sodium hydroxide, aluminium chloride, sodium nitrite, sodium carbonate, gallic acid, and sodium chloride were purchased from R & M Chemicals (Canada). Water was purified by Purelab Option-S System (ELGA, UK). Analytical chromatographic TLC aluminium sheets, silica gel 60 F254, silica gel 7734 (0.063-0.200 mm) and silica gel 9385 (0.040-0.063 mm) were from Merck (Darmstadt, Germany).

Preparation of extracts

Ethanol extract

Extraction was performed by continuous soaking of powdered plant material (50 g) in a 1 L beaker covered with aluminium foil with ethanol as the extracting solvent. The solvent was drained out after three days and this procedure was repeated twice (Total = 3 x 72 h, 2.1 L). The extract obtained was filtered with Whatman filter paper (no 1) and concentrated using rotary evaporator (Buchi Rotavapor R215, Switzerland) until ethanol crude extract formed. The extract was stored at 4 °C until further analysis.

Aqueous extract

Powdered plant material (50 g) was extracted with 800 ml of warm purified water (60 °C) for 30 min. The extract was filtered with Whatman filter paper (no 1) and the residue was re-extracted under the same conditions twice. The filtrates were combined and freeze-dried (LABCONCO 7753037, Free Zone 6 Liter, USA) into resultant powder which was then stored at 4 °C prior analysis.

Reference compounds

Ar-curcumene and xanthorrhizol were isolated from the hexane extracts of *C. xanthorrhiza* in Centre for Drug Research, Universiti Sains Malaysia. Briefly, the powdered rhizomes (100 g) were ground and extracted with n-hexane (2 L) in the Soxhlet extraction apparatus for 24 h. Removal of solvent by rotary evaporator under reduced pressure gave crude hexane extract

(9.8 g). It was then separated chromatographically by applying the extract (4.0 g) to gravity column chromatography (GCC) using silica gel 7734 for separation and silica gel 9385 for purification. Elution with petroleum ether-chloroform solution (9:1, v/v) produced colorless oil known as ar-curcumene (0.32 g). Moreover, xanthorrhizol (0.35 g) were collected as yellowish oil from 6:4, v/v elution with petroleum ether-chloroform solution. The compounds were identified by direct comparison of the electron ionization (EI) mass spectral result, ^{1}H and ^{13}C nuclear magnetic resonance (NMR) (Bruker Avance 400 MHz) and concur with previously published data[8-12].

Gas chromatography-mass spectrometry (GC-MS) analysis

Analysis was carried out on an Agilent GC-MS consisted of a HP 6890 N gas chromatograph coupled with a HP 5973I mass selective detector. Separations were accomplished with a HP-5MS column (30 m long, 0.25 mm i.d, film thickness 0.25 µm, 60 – 350 °C temp. limit).

The inlet temperature was set at 280 °C and the detector at 250 °C. GC was performed in the splitless mode. The oven temperature was initiated at 70 °C for 2 min, then increased to 280 °C at 20 °C/min and kept at 280 °C for 2 min. The carrier gas was helium at a flow rate 1.0 ml/min with 1 µl of sample injected. Mass spectrometer was operated in the positive ion electron impact (EI) mode with 70eV ionization energy. Identification of compounds was performed in full scan mode within the scan range of 35-550 m/z. Quantification was done by single-ion monitoring (SIM) mode using 119 m/z for ar-curcumene and 132 m/z for xanthorrhizol in three replicates. The compounds were identified using National Institute of Standard Technology (NIST) library together with comparison of their respective retention times and mass spectra.

Preliminary Phytochemical Screening

The standardized extracts were used for all preliminary phytochemical screening. Each extract was diluted with ethanol and separated on the thin layer chromatography (TLC) plates using a solvent mixture of chloroform and dichloromethane (4.5:0.5). The developed TLC plates were then tested using various spray reagents. The colour of the spots was recorded both under ultraviolet (UV $_{254nm}$ and UV $_{365nm}$) and visible light[13].

Determination of total phenol content (TPC) and total flavonoid content (TFC).

Folin-Ciocalteu method with slight modification[14] was used in the determination of TPC and the values were expressed as mean (±SD) miligram per gram of gallic acid equivalent

(mg GAE/g samples). Meanwhile, TFC in the samples were determined based on colorimetric method describe previously[15] and TFC value were expressed as mean (±SD) miligram per gram of catechin equivalent (mg CE/g samples). The absorbance was measured using a UV-visible spectrophotometer (Shimadzu UV-1800, Kyoto, Japan) for determination of both TPC and TFC. All determinations were performed in triplicates.

Determination of total saponin content (TSC) and total alkaloid content (TAC).

TSC in the samples were acquired by method designate previously[16] from rhizomes of *C.xanthorrhiza*. The sample obtained was dried in an oven to a constant weight. Similarly, TAC was conducted based on the following method[17] by extraction from the rhizomes. Sample was kept in the oven to a constant weight. The weight for TSC and TAC were expressed as mg/g plant sample.

RESULT AND DISCUSSIONS

Typical GC-MS separation of ar-curcumene and xanthorrhizol in both extracts are as shown in Figure 1. Table 1 shows the major constituents found in the extracts, their retention times and mass fragmentation pattern (at nominal concentrations). Four major constituent peaks were observed representing ar-curcumene (m/z 202), α-cedrene (m/z 204), β-elemenone (m/z 218), and xanthorrhizol (m/z 218) along with other minor peaks. The chemical markers (ar-curcumene and xanthorrhizol) were identified by comparing their mass spectra and retention times to that of the isolated standard, respectively. Our separation methods established the retention time of ar-curcumene and xanthorrhizol as 8.46 min and 10.04 min respectively. The mass spectral patterns and chemical structures of these compounds were as illustrated in Figure 2.

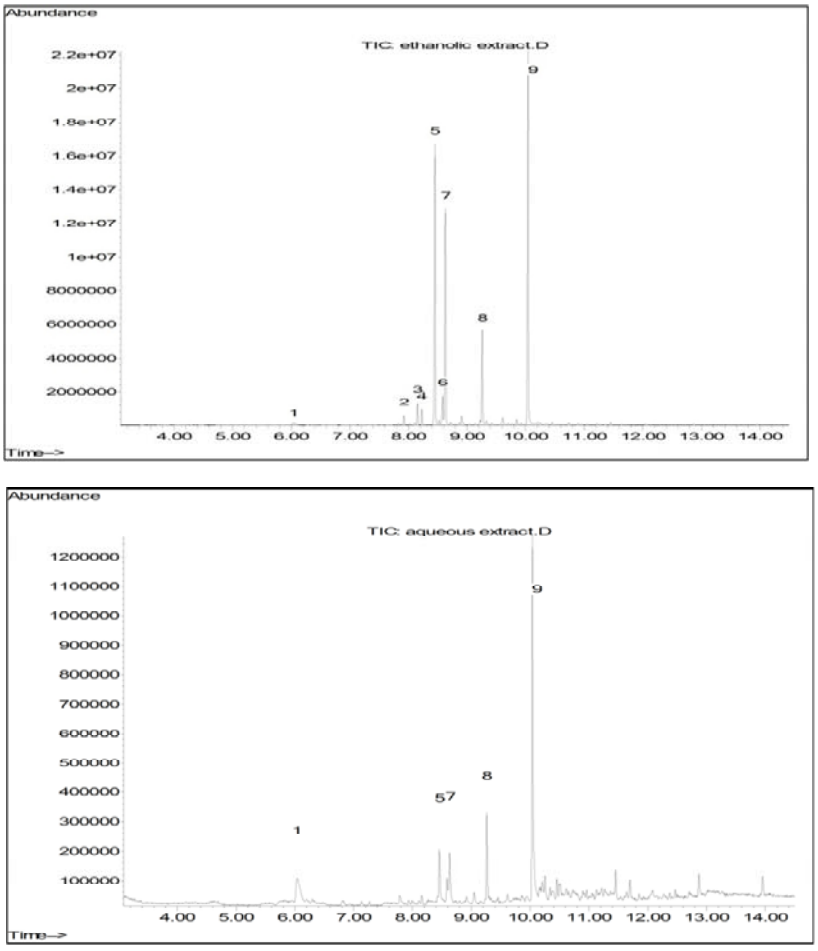

Fig. 1: Typical chromatograms from GC-MS analysis of *C. xanthorrhiza* (a) ethanol extract, (b) aqueous extract.

Table 1: Major chemical constituents of *C. xanthorrhiza* by GC-MS

Peak No.	Retention time (min)	Mass m/z	Constituents
1	6.03	152	Camphor
2	7.93	204	Zingiberene
3	8.16	204	γ-elemene
4	8.23	204	Trans β-farnesene
5	8.46	202	Ar-curcumene
6	8.58	216	Benzofuran
7	8.63	204	α-cedrene
8	9.23	218	β-elemenone
9	10.04	218	Xanthorrhizol

Under the experimental conditions described earlier, linear calibration curves of peak areas versus concentration of ar-curcumene ($y = 1e^{+06}x - 2e^{+07}$) and xanthorrhizol ($y = 856491x - 2e^{+07}$) were obtained respectively. Eight point of concentrations were determined in the range of 1-500 µg/ml. Regression analysis of the calibration curve demonstrated acceptable linearity for ar-curcumene and xanthorrhizol with r^2 of 0.996 and 0.995, respectively. The amount of ar-curcumene and xanthorrhizol from the ethanol extract is higher compared to the amount in the aqueous extract (Table 2). A higher amount of xanthorrhizol (84.9%) was determined in this study compared to our previously published data[7]. This may be due to the different sources of plant materials. These identified and quantified compounds can be used as markers in standardizing formulations containing *C. xanthorrhiza* as the base ingredients.

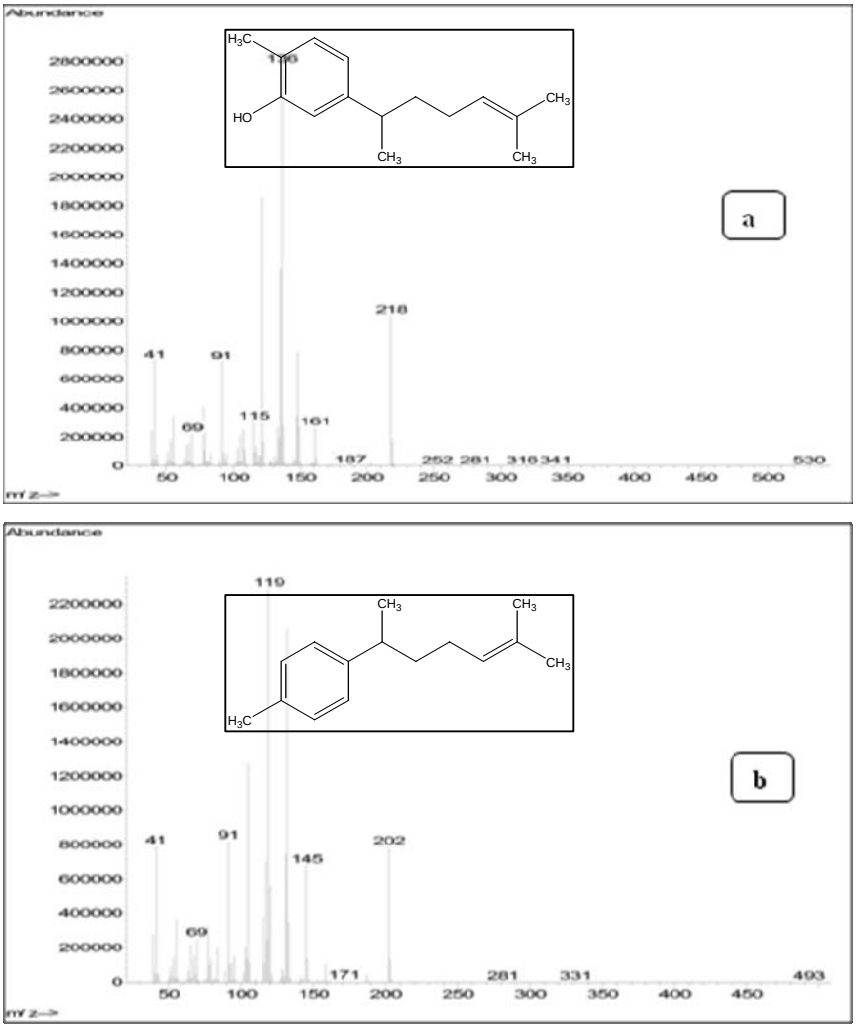

Fig. 2: Mass spectrum and chemical structure of (a) xanthorrhizol and (b) ar-curcumene.

For TLC, various solvent systems were tested starting from non-polar solvents to polar solvents. The best solvent system chosen consisted of chloroform-dichloromethane solution with the ratio of 4.5:0.5. TLC profiling for marker compounds were successfully obtained based on their retardation factor (R_f). Ar-curcumene and xanthorrhizol shows R_f at 0.97 and 0.72 (data not shown),

respectively. Qualitative phytochemical screening using a set of standards was performed and the result revealed that extracts of *C. xanthorrhiza* mainly consisted of terpenoids, phenols, flavonoids, saponins, cardiac glycosides, alkaloids, and coumarins. Anthraquinones, anthrones and tannins were absent in the extract of *C. xanthorrhiza*. The organic extract of ethanol had shown the presence of more groups of compounds whereas the aqueous extract showed less positive observations and weak presence of groups of compounds from each test conducted. The results were summarized in Table 3.

Table 2: Extraction yield, ar-curcumene content, xanthorrhizol content, TPC and TFC values of *C.xanthorrhiza* extract.

Samples	Extraction yield (g)	Cc (µg/ml)	Xc (µg/ml)	TP content mg GAE/g	TF content Mg CE/g
CXEE	5.9	136.02 ± 5.11	228.86 ± 16.10	199.00 ± 1.31	101.66 ± 0.83
CXAE	4.5	21.08 ± 0.10	34.09 ± 0.93	19.99 ± 0.16	10.58 ± 0.83

CXEE; CXAE, *C. xanthorrhiza* ethanol extract; *C.xanthorrhiza* aqueous extract; Cc, ar-curcumene content; Xc, xanthorrhizol content

The secondary metabolites in the *C. xanthorrhiza* were further studied for the determination of phenol, flavonoid, saponin and alkaloid content. Table 2 shows the TPC and TFC for both extracts. From the calibration curves of gallic acid ($y = 6.3983x - 0.0259$, $r^2 = 0.9997$), the ethanol extract showed a high amount of TPC followed by aqueous extract. Our findings revealed that the ethanol extract yielded a higher amount of TPC compared to a previous study which had used methanol as the extracting solvent[18] and comparable with the finding that demonstrated high polarity of solvents yielded higher amount of polyphenolics[19]. Previous study reported that phenols and phenolic compounds are highly used in skin infections, wound treatments and healing purposes compared to other bactericides[20]. Thus, the enormous amount of polyphenolics in *C. xanthorrhiza* may have contributed to the antibacterial, antifungal[21] and wound healing treatment[22] properties.

Table 3: Preliminary screening of secondary metabolites from *C. xanthorrhiza*

Test	Positive Result	Ethanol Extract	Aqueous Extract
Terpenoid	Strong blue, green, red and brown color	+++	+
Phenols	Blue color	+++	+
Flavonoid	orange, orange-yellow, yellow-green and blue violet color	++	+
Saponin	Blue, violet and green (UV-365 nm)	++	±
Cardiac glycoside	Blue, brown, green and fluorescent zones (UV-365 nm)	++	-
Alkaloid	Brown, orange brown	±	-
Coumarin	Yellow-green	±	-
Anthraquinone	Red, fluorenscent red (UV-365 nm)	-	-
Anthrone	bright yellow (UV-365 nm)	-	-
Tannin	Dark colored zone	-	-

Key: +++ = abundance; ++ = moderately present; + = present; ± = weakly present; - = absent

For TFC, using the calibration curve generated from catechin ($y = 2.7668x - 0.0086$, $r^2 = 0.9950$), a similar order as TPC was observed; ethanolic extract shows a higher amount of TFC compared to the aqueous extract. Flavonoids have been associated with the inhibition of glutathione S-Transferase (GST) activity in human blood platelets, rat liver and rat kidney[23]. Our previous study reported that aqueous extract of *C. xanthorrhiza* exhibited no significant inhibition towards GST activity[24]. This may be due to the low amount of TFC present therein.

Furthermore, our results showed that total saponin and total alkaloid contents present in the rhizomes of *C. xanthorrhiza* were 80.90 mg/g and 14.06 mg/g, respectively. Saponins are naturally occurring glycosides which are typically characterized by their foam-forming ability in water, hemolytic activity, and cholesterol binding properties[25]. The interesting feature of saponins was the ability of this compound to inhibit or kill cancer cells without destroying normal cells in the process. Cancer cells possess more of cholesterol type-compounds on their membrane compared to that of normal cells. Saponins therefore bind to the cholesterol and thus interfere with cell division and cells growth[26]. Our finding lends support the important fact that this plant possesses a serum cholesterol-lowering effects[27]. Furthermore, its tendency to ward off microbes endorses them as good candidate in treating fungal and yeast infections, in concurrence also with the plants traditional use in treatment of bacterial infections[28].

Our data has shown a trace amount of alkaloids in rhizomes of *C. xanthorrhiza*. Previous study proved that alkaloids exist in the genus of curcuma such as in *Curcuma longa*[30]. Alkaloids are important due to its toxicity, in a way, to combat against microbe[29]. To date, there are still no findings on alkaloids in *C. xanthorrhiza* and their therapeutic value. Further experiments could lead to new findings of potential alkaloid compounds along with their activities.

CONCLUSION

In summary, the standardization, qualitative and quantitative determination of phytochemicals, and TLC profiling were successfully accomplished. Furthermore, the result may be useful in developing potential phytopharmaceutical products based on *C. xanthorrhiza*. However, there is a need to further identify specifically the flavonoid, saponin and alkaloid compounds that are present in the extracts.

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