Phytochemistry and lethality effect to brine shrimp of selected *Callicarpa* species

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ABSTRACT

Four *Callicarpa* species which includes *C. candicans*; CC, *C. arborea*; CA, *C. longifolia*; CL and *C. rubella*; CR were phytochemically investigated for a potential insecticidal component, callicarpone using TLC, HPLC-DAD and HPLC-ESI-MS techniques. Toxicity against *Artemia salina* (brine shrimp) was simultaneously tested for the water extract of the four plant species. HPLC fingerprint analysis was conducted using XBridge Shield RP18 column (150 mm ×4.6 mm i.d., 5 μm). The gradient mode of mobile phases with 0.005% trifluoroacetic acid in water and 0.005% trifluoroacetic acid in acetonitrile was used. The results showed that each plant species has typical terpenoid fingerprint. By comparing the UV absorbance spectral obtained from HPLC-DAD together with HPLC-ESI-MS with the literature data, the component at t_R of 21.76 min corresponding to callicarpone i.e. λ_{max} at 266 nm and m/z at 332 was only found in *C. candicans*. For toxicity assessment, percentage of brine shrimp lethality tested at the extract concentration of 1000 μg /ml from CC, CA, CL and CR were found to be 86.67, 16.67, 20 and 3.33, respectively.

Keywords: *Callicarpa* species, Callicarpone, HPLC-DAD, HPLC-EIMS, Brine shrimp

1. INTRODUCTION

The genus *Callicarpa* comprising about 140 plant species has been regarded as a significant member of Lamiaceae family [1,2]. Ethnomedical reports of several *Callicarpa* species indicated their uses for the treatment of hepatitis, rheumatism, fever, headache, indigestion, cancer and other ailments [3]. Moreover, some *Callicarpa* species have been traditionally used as fish poisoning agent due to the presence of a bioactive diterpenoid constituent named callicarpone. The pesticidal activity of callicarpone has been reported to be 10 times more potent than a natural insecticide rotenone [4,5]. Thus callicarpone containing *Callicarpa* species are believed to have potential for using as a natural insecticide. In this study we have investigated on chemical constituents of 4 *Callicarpa* species focusing on di- and triterpenoids using TLC, HPLC-DAD and HPLC-ESI-MS techniques. Preliminary evaluation on cytotoxic potential of extracts from these plant species also has been performed using brine shrimp lethality test.

2. MATERIALS AND METHODS

Plant materials

Leaf samples of 4 different *Callicarpa* species were collected from different locations in Thailand including *C. arborea* (CA, from Prachuapkhirikhan), *C. candicans* (CC, from Krabi), *C. longifolia* (CL, from Songkhla) and *C. rubella* (CR, from Chiang Mai) during December 2010 to February 2011. Plant samples were identified by Dr. Charan Leeratiwong, a botanist in Department of Biology, Faculty of Science, Prince of Songkla University, Songkhla, Thailand. Plant specimens were deposited at Pharmaceutical and Natural Products Department, Thailand Institute of Scientific and Technological Research (TISTR), Thailand. All plant samples were dried at 40^0 C for 6 h in hot air oven and pulverized.

Preparation of the extracts

Extract for chemical analysis

Plant samples (5 g) were separately extracted with 50 ml of methanol under sonication for 30 min and filtered. The process was repeated for 3 times. The combined filtrates were concentrated under reduced pressure and the final volume was adjusted to 5 ml with methanol, then filtered through a 0.45-µm PTFE syringe filter before applied to the TLC and HPLC system.

Extract for brine shrimp lethality test

Plant samples (15 g) were refluxed (80°C) with distilled water for 3 h (plant/solvent ratio 1:10 w/v), then filtered. The filtrate was taken to dryness by lyophilization.

Phytochemical analysis

Thin layer chromatography analysis [4, 5]

Thin layer chromatography of all extracts was performed on TLC silica gel 60 GF_{254} aluminium sheet, using chloroform-ether (4:1) as the solvent system. TLC plates were detected under UV at white R and 366 nm, vanillin sulfuric acid spray reagent.

High performance liquid chromatography analysis [6, 7]

HPLC-DAD: Shimadzu (Kyoto, Japan) LC-10ADvp series liquid chromatography with binary pump, a model 7725i manual injector valve with a 5 µL sample loop, thermostated column compartment and diode array detector (DAD), XBridge™ Shield RP18 column (150 mm x 4.6 mm i.d., particle size 5 µm) were used for the analysis. HPLC column was maintained at room temperature. HPLC analysis of the extracts was carried out using the conditions applied from the methods for terpenoid analysis previously reported. The gradient mode of mobile phases with acetonitrile containing 0.005% trifluoroacetic acid (solvent A) and Milli-Q water containing 0.005% trifluoroacetic acid (solvent B) was used. The flow rate was 0.6 mL/min, the injection volume was 5 µL and the chromatograms were monitored at 266 nm.

HPLC-ESI-MS: HPLC conditions were the same as described above. The MS analysis was performed using the Shimadzu (Kyoto, Japan) 2100 series mass selective detector (MSD) equipped with an electrospray ionization (ESI) source operated in the positive ion mode. Instrument control and data acquisition were performed using Shimadzu LC-MS solution software. The ion source temperature was 25°C, and the needle voltage was set at 1.5 kV. Nitrogen was used as the drying and nebulizer gases at a flow rate of 1.5 L/min and a backpressure of 27.6 MPa. The scanning range was starting *m/z* 100 and ending *m/z* 800.

Brine shrimp lethality test [8, 9]

Eggs of *Artemia salina* were hatched by incubating in saline water for 48 h at room temperature under light source. Stock solution of each plant extract was prepared by dissolving in saline water at the concentration of 10

mg/ml. An aliquot of 500 µL was added to well containing 4.5 mL of saline water and mixed thoroughly. Ten larvae were then added to the well and maintained at room temperature under light source for 24 h. A control treatment contained only saline water. The experiment was done in triplicate. Survivors were counted and calculated for percentage of lethality.

3. RESULTS

Phytochemical analysis

TLC analysis

TLC analysis of methanolic leaf extracts from *Callicarpa* species showed specific terpenoid pattern detected by vanillin sulfuric acid spray reagent. Based on the R_f value and color reaction, spots corresponding to triterpene oleanolic acid, *β*-sitosterol and diterpene abietic acid were observed in all samples, the most prominent one belonged to oleanolic acid (Figure 1).

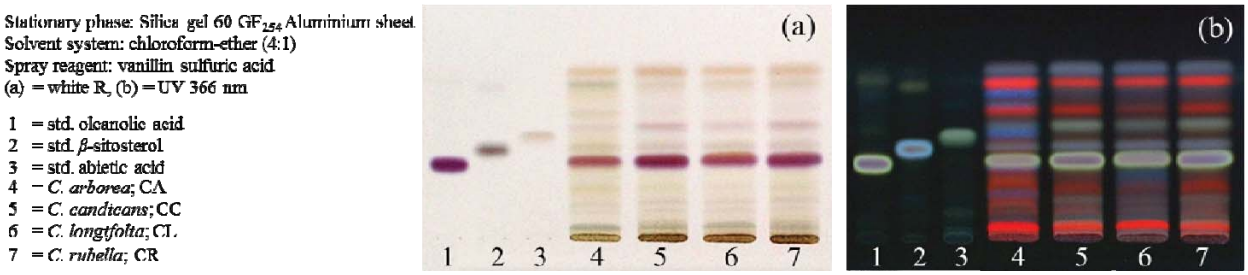

Figure 1. Thin-layer chromatography of leaf extracts from the 4 investigated *Callicarpa* species.

HPLC analysis

From HPLC-DAD analysis (Figure 2), typical terpenoid fingerprints of 4 test samples were observed at 266 nm and six common characteristic peaks (peaks 3, 4, 6, 8, 17 and 18) were found in all samples. By comparing their retention times (t_R) and UV spectra with the reference standards, three out of those six peaks were structurally confirmed as *β*-sitosterol (peak 3), abietic acid (peak 17), oleanolic acid and/or ursolic acid (peak 18) with the t_R of 12.68, 34.03 and 36.83 min, respectively. It is noteworthy that two main peaks at t_R 16.57 and 21.76 min (peaks 7and 9, respectively), were only presented in CC HPLC fingerprint. Positive mode ESI-MS spectrum of compound from peak 9 showed *m/z* at 332 (Fig. 3A) and DAD absorbance spectrum (Fig. 3B) showed λ_{max} at 266 nm corresponding to chemical character of callicarpone previously reported [4, 5]. ESI MS result has suggested a presence of the pesticidal active component, callicarpone in *C. candicans*.

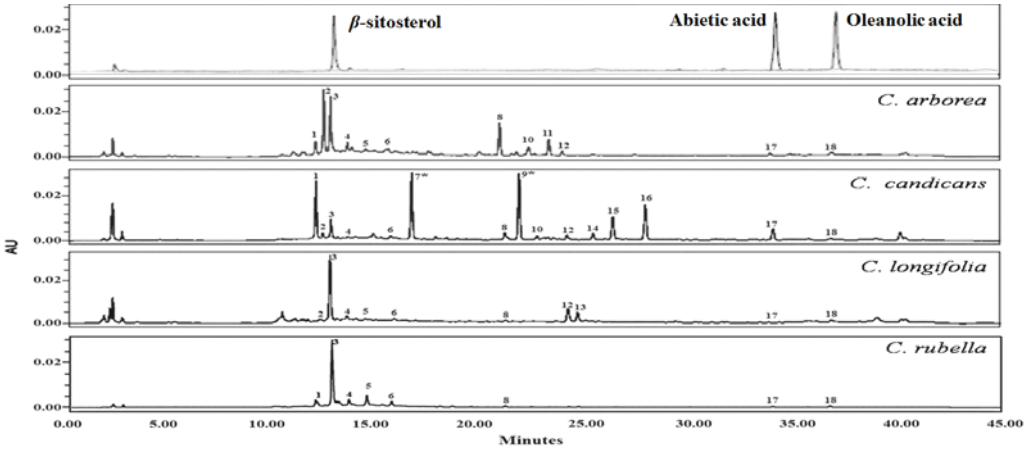

Figure 2. HPLC-DAD chromatograms of leaf extracts from the 4 investigated *Callicarpa* species at 266 nm.
* Only found in *C. candicans*

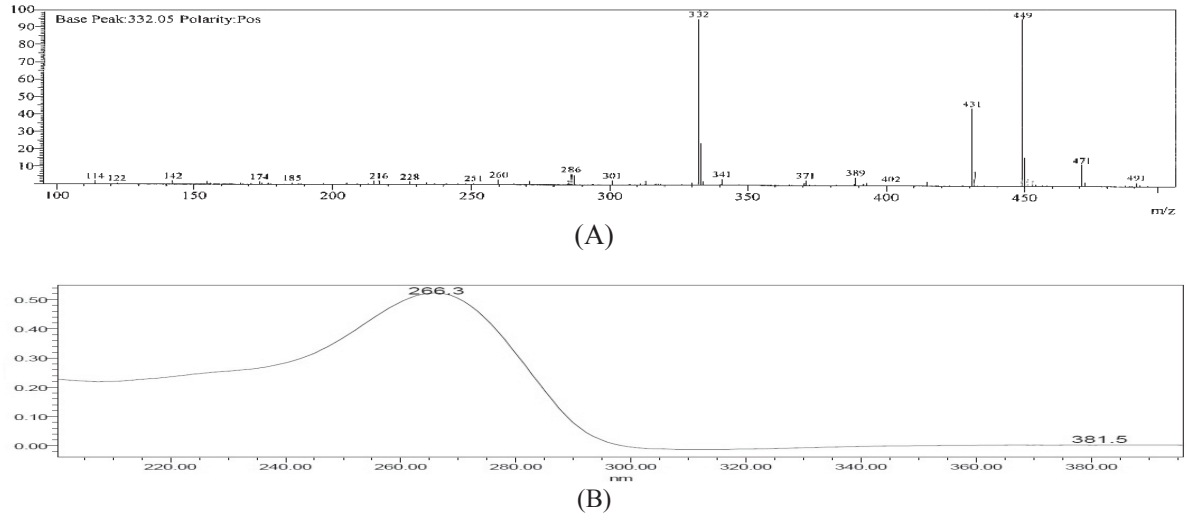

Figure 3. ESI-MS fragmentation pattern (A) and UV spectra (B) of compound from peaks 9.

Brine Shrimp Lethality Test

The results of toxicity to brine shrimp of aqueous leaf extract from *Callicarpa* species were shown in Table 1. Extract from CC showed the highest cytotoxic effect (86.67 % lethality) while that from CR was the lowest activity (3.33 % lethality).

Table 1. Toxicity to brine shrimp of leaf extracts from the 4 investigated *Callicarpa* species.

Test sample	% Lethality
CA	16. 67
CC	86.67
CL	20.00
CR	3.33
Control (saline water)	0.00

4. CONCLUSIONS

Our studies demonstrate HPLC-DAD fingerprints of di- and triterpenoid components of leaf extracts from the 4 investigated *Callicarpa* species. The fingerprints of all samples showed six similar chromatographic peaks. By comparing of retention time together with UV absorbance spectral data, three out of the six peaks were structurally confirmed as *β*-sitosterol, abietic acid, oleanolic acid and/or ursolic acid. Compound corresponding to callicarpone was observed only in *C. candicans* fingerprint. Preliminary toxicity evaluation of aqueous extract of 4 plant species showed a strongest effect in *C. candicans* which corresponded to its chemical profile.

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