Chromatographic Fingerprint and Free Radical Scavenging Activity of *Boesenbergia xiphostachya* (Gagnep.) Loes Rhizome Extract

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ABSTRACT

Boesenbergia xiphostachya (Zingiberaceae) is commonly known as Ngon nark. The rhizome is used in Thailand as laxative and flatulence remedy. In this study, the one- and two-dimensional thin-layer chromatographic (TLC) fingerprint profiles of hexane, dichloromethane and ethanolic extracts of *B. xiphostachya* rhizome were studied for the first time. Distinct spots were visualized under visible light, UV 254 nm, UV366 nm and after spraying with specific reagents for preliminary phytochemical screening propose. The slightly different chemical profiles of all extracts were observed by TLC. The results indicated the presence of diverse classes of secondary metabolites i.e. essential oils, terpenoids, steroids, flavonoids, alkaloids and coumarins. Three extracts were also *in vitro* tested on anti-oxidant activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay. Among extracts tested, the ethanolic and dichloromethane extracts were found to exhibit the DPPH radical formation with IC_{50} values of 565.18 ± 37.85 and 695.14 ± 84.70 µg/mL, respectively. The further isolation and structural elucidation of the anti-oxidant compounds in this extract is interesting. This finding might be a useful supporting for further studied and expanded the utilization of this plant in the pharmaceutical and cosmetics fields.

Keywords: Boesenbergia xiphostachya, fingerprint, phytochemical, free radical scavenging

INTRODUCTION

Boesenbergia xiphostachya (Gagnep.) Loes, locally known in Thai as "Ngon nark" or "Ngon praya nark", belongs to the Zingiberaceae family and is widely distributed in Indo-chica (Chayamarit, Pooma, and Pattharahirantricin 2014). This plant is a perennial ground herb that grows to 70 cm height and has brown rhizome. The leaf is simple with lanceolate shape. The inflorescence is terminal which usually peduncle enclosed by the uppermost leaf-sheath and extended to 25 cm long. Calyx lobes are truncated whereas the corolla tubes are extended and dilated to the top. The anther is connective glandular-hairy, crest bilobed and not produced beyond thecae. The ovary is glabrous and placenta free central (Sirirugsa 1992). From the local literature survey reported, *B. xiphostachya* rhizome has been medicinally used in Thailand as laxative and flatulence remedy (Chuakul and Boonpleng 2003). There were reports on the evaluation of genetic variation, evolutionary relationships and phylogenetic relationships of *Boesenbergia* in Thailand using multilocus DNA fingerprints generated by AFLP analysis. The molecular markers for identifying species origin of these taxa were also studied (Techaprasan et al. 2006, Ngamriabsakul and Techaprasan 2006, Techaprasan, Klinbunga, and Jenjittikul 2008). Literature survey revealed that the *Boesenbergia* genera under the Zingiberaceae family is commonly used as a food ingredient and in ethnomedicinal preparations. The diversity of secondary metabolite constituents like essential oils, terpenes, flavonoids, etc. were found and demonstrated many biological activities (Eng-Chong et al. 2012). Interestingly, no work has been done on phytochemicals screening of *B. xiphostachya*. There is a need for the chemical profiling of secondary metabolites in aid of rapid identification. In this study, *B. xiphostachya* rhizome was collected to study the thin-layer chromatography (TLC) fingerprint profile and to establish preliminary phytochemical screening for the first time. The *in vitro* anti-oxidant activity of extract was also investigated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay which is a convenient and fast method of measuring free radical scavenging activity. Information on the qualitative compositions and anti-oxidant property of *B. xiphostachya* extract might provide the scientific basis for further therapeutic uses.

METHODOLOGY

Plant materials and Extraction

Fresh rhizomes of *B. xiphostachya* (50 kg) were collected from Amphur Khaokhor, Phetchabun province, Thailand (March, 2014). The plant material was identified by Assit.Prof.Dr. Pranee Nangngam. The voucher specimen (collection number: 004071) is kept at Department of Biology, Faculty of Science, Naresuan University, Phitsanulok, Thailand.

The dried powder rhizomes (4.17 kg) were macerated for 3 days with hexane (20 L x 3 cycles) at room temperature. The filtrates were evaporated under reduced pressure to afford crude hexane extract (82.72 g). The marc was further extracted by increasing the polarity of solvents as followed dichloromethane (CH_2Cl_2) and 95% ethanol (EtOH), respectively using the same extraction procedure. The obtained filtrates were evaporated to produce 84.04 g of CH_2Cl_2 extract and 115.64 g of EtOH extract.

TLC analysis

Chromatographic fingerprints and preliminary phytochemical screening of *B. xiphostachya* rhizome extracts were performed on TLC. The fingerprints were studied on both one- and two-dimensional TLC. For one-dimensional study, about 200 µg of each extracts was loaded on the 3 x 5 cm silica plate (TLC silica gel 60 F_{254} aluminium sheet, Merck, Damstadt, Germany). The plate was developed to a distance 35 mm at ambient temperature. The mixture solution of hexane and ethyl acetate (7:3 v/v) was used as mobile phase. After the development, the plate was observed under visible light, short wave UV at 254 nm and long-wave at 366 nm. The plate was then sprayed with various specific reagents for detecting the classes of phytochemical constituents including aluminium chloride (detection of flavonoids), anisaldehyde-sulfuric acid (detection of essential oils, steroids and saponins), dragendroff reagent (detection of alkaloids, heterocyclic nitrogen compounds and quaternary amines), ferric chloride (detection of tannins), potassium hydroxide (detection of anthraquinones, anthones and coumarins) and vanillin-sulfuric acid (components of essential oils; terpenoids, phenylpropane derivatives, etc.) (Nandhasri and Parawach 2006).

For two-dimensional study, about 200 µg of each extracts was also loaded on the 5 x 5 cm silica plate. Two eluent systems consisting of hexane:ethyl acetate (7:3 v/v) was used in the first direction of developing, and CH_2Cl_2:acetone (9:1 v/v) was used in the second direction. After the development, the plate was observed using the same procedure as mentioned.

Free radical scavenging assay

To investigate the *in vitro* anti-oxidant capacity of three *B. xiphostachya* extracts, DPPH free radical scavenging assays based on TLC-DPPH detection and high-throughput analysis with a 96-well microplate reader were performed according to the method described in previous study (Piangpraichom et al. 2014). For TLC-DPPH method, the developed TLC plate (as above mentioned) was carried out with DPPH solution (500 µM in absolute ethanol) for 3 s in immersion chamber and then incubated for 10 min at room temperature. The TLC plate was then observed with visible light. The active anti-oxidant constituents were detected as yellowish white spots produced by bleaching of DPPH. All detected active spots were noted according to their retardation factor (R_f) values.

For 96-well plate assay, the series of concentrations of extracts (9.76–20,000 µg/mL) and two positive controls; L-ascorbic acid and 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) (0.20-200 µg/mL) were prepared in absolute ethanol. The sample solutions at 100 µL were then transferred to 96-well microplate. The reaction was started by adding of 100 µL of DPPH solution (500 µM in absolute ethanol) to wells. The plate was kept in the dark (at room temperature) for 30 min and absorbance (Abs) at 515 nm was read post-incubation using microplate reader (Hybrid Multi-Mode detection Synergy H1, model H1MF, Bio-TeK Instruments, USA). The percentage inhibition of radical formation was calculated using the following formula:

$$\text{Inhibition (\%)} = [(Abs_{control} - Abs_{sample}) / Abs_{control}] \times 100$$

Determinations were done in triplicate and the inhibition values were then plotted against the final concentrations of tested samples in reaction. The half maximal inhibitory concentration (IC_{50}) were determined using OriginPro 8.6 program.

Statistical analysis

The experiments of free radical scavenging test were performed in triplicate and the data were expressed as mean ± Standard Deviation (SD). Statistical significance was determined by Student's t-test. A significance level of $p \leq 0.05$ denoted significance in all cases.

RESULTS AND DISCUSSION

The thin-layer chromatography (TLC) is the conventional technique for analysis of the chemical components in the extract. The TLC fingerprint was used for the quality control of plant material. In this study, TLC fingerprint profiles of hexane, CH_2Cl_2 and EtOH rhizome extracts of *B. xiphostachya* were studied for the first time. Many TLC mobile phase systems were tested and the mobile phase hexane: ethyl acetate (7:3 v/v) seemed to give the most suitable fingerprints (Fig. 1).

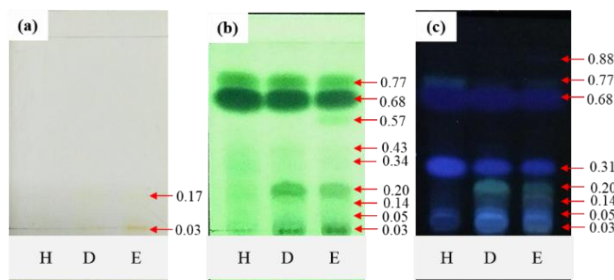

Fig. 1 TLC fingerprints of *B. xiphostachya* rhizome extracts using hexane:ethyl acetate (7:3 v/v) as mobile phase: visualized without coloration under (a) visible light, (b) 254 nm UV light and (c) 366 nm UV light. R_f values are indicated. (H = hexane extract, D = CH_2Cl_2 extract and E = EtOH extract)

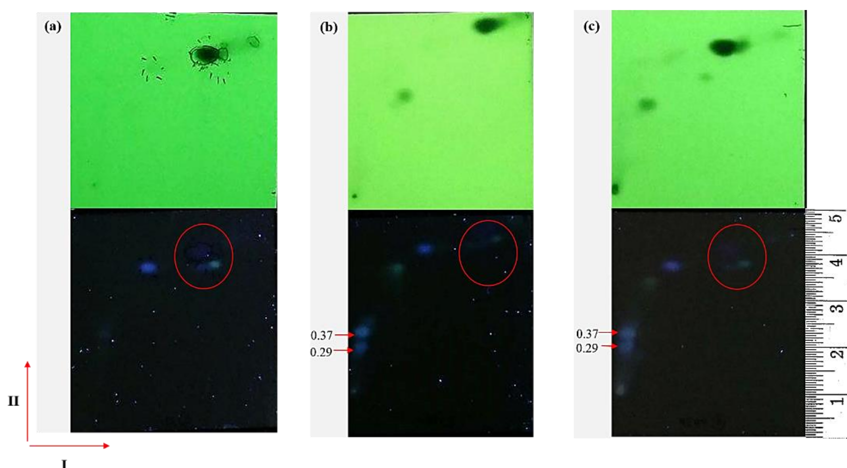

Fig. 2 TLC fingerprints of *B. xiphostachya* rhizome extracts, the silica plate were double developed with hexane:ethyl acetate (7:3 v/v) and CH_2Cl_2:acetone (9:1 v/v), respectively. The plates of (a) hexane extract, (b) CH_2Cl_2 extract and (c) EtOH extract

were visualized without coloration under 254 nm UV light (upper) and 366 nm UV light (lower).

Under illumination at visible light, few spots for three extracts were observed. Quenching spots under short UV wavelength (254 nm), and the fluorescence spots under long UV wavelength (366 nm), were detected as shown in Fig. 1b and 1c. Visualization at 254 nm yielded the greatest number of observable spots. The CH_2Cl_2 showed similar TLC profile to that of EtOH extract and they also yielded more spots than that of hexane extract. As can be seen in Fig. 1, the visualized spots at R_f values about 0.03 to 0.20 and 0.68 to 0.77 might contain the structural analogues/ identical polarity compounds, single development may not be sufficient. Application of multiple development (two-dimensional TLC) was performed. When the plate were visualized under 366 nm (R_f values about 0.29 to 0.37 and about 0.82 to 0.88), it was clear that the second dimension yielded the partly separated spots as shown in Fig. 2.

The preliminary study on phytochemical constituents of extracts were also tested according to the earlier procedure for one-dimensional TLC method. The qualitative phytochemical analysis gives information on the presence or absence of different classes of secondary metabolites. Most of detected spots gave color after sprayed with anisaldehyde-sulfuric acid and vanillin-sulfuric acid reagents (Fig. 3a and 3e) which mainly could be essential oils, terpenoids, phenylpropane derivatives and steroids. The results also suggested that the alkaloids (orange spots on yellow background, Fig. 3c) and coumarins (light blue spot, Fig. 3e) are present in all samples. Flavonoids were found on both CH_2Cl_2 and EtOH extracts. There are observable overlaps in the methods of visualization method. However, tannins was not detected in any samples as shown in Fig. 3d.

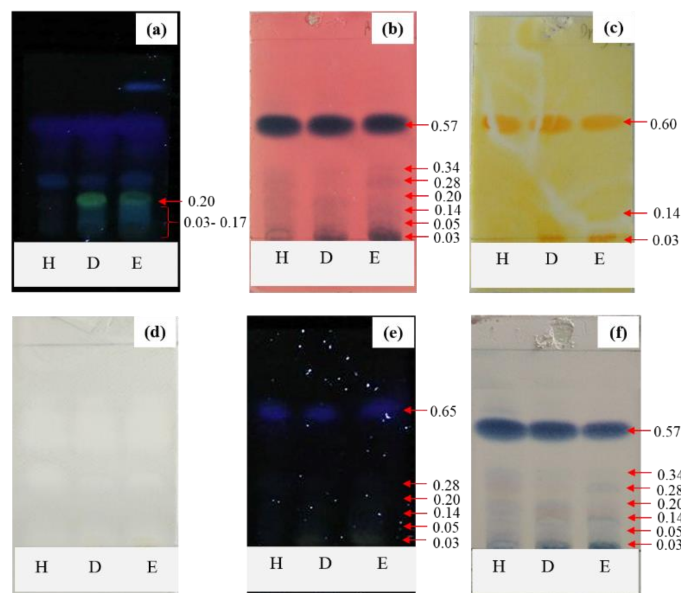

Fig. 3 TLC fingerprints of *B. xiphostachya* rhizome extracts using hexane:ethyl acetate (7:3 v/v) as mobile phase: visualized after coloration with (a) aluminium chloride, (b) anisaldehyde-sulfuric acid, (c) dragendroff reagent, (d) ferric chloride reagent, (e)

potassium hydroxide and (f) vanillin-sulfuric acid. R_f values are indicated. (H = hexane extract, D = CH_2Cl_2 extract and E = EtOH extract)

Radical scavenging activities of *B. xiphostachya* rhizome extracts were screened against DPPH radical which is widely used to investigate the scavenging activities of several natural compounds. To screen the antioxidant capacity of three extracts, a TLC-DPPH method was performed. After developed TLC plate was sprayed with DPPH solution and incubated, the samples producing yellowish bands on the purple background were considered as anti-oxidants. All detected active antioxidant constituents were noted according to their R_f values (Fig. 4). The results demonstrated that only few active spots were observed on TLC of hexane extract while that in CH_2Cl_2 and EtOH extracts exhibited many potent active zones (R_f = 0.03-0.23, 0.34 and 0.63).

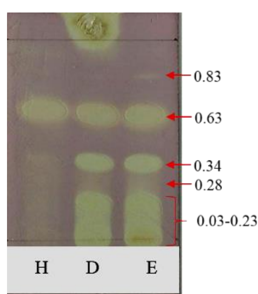

Fig. 4 TLC fingerprints of *B. xiphostachya* rhizome extracts using hexane:ethyl acetate (7:3 v/v) as mobile phase and visualized after coloration with 500 µM DPPH. (H = hexane extract, D = CH_2Cl_2 extract and E = EtOH extract)

To investigate the free radical scavenging activity of three extracts (hexane, CH_2Cl_2 and EtOH extracts), the series of final concentrations of extracts (4.88–10,000 µg/mL) and two positive controls; L-ascorbic acid and trolox (0.10-100 µg/mL) were performed using 96-well plate assay. All the extracts and standards showed a dose dependent inhibition of the DPPH radicals. As shown in Table 1, the radical scavenging activities of all tested samples were calculated in terms of IC_{50} value.

Table 1 DPPH free radical scavenging activity of *B. xiphostachya* extracts and two positive controls. The data are expressed as means ± SD. Determinations were done in triplicate.

Samples	IC_{50} (µg/mL)
Hexane extract	6,998.72 ± 80.57 [a]
CH_2Cl_2 extract	695.14 ± 84.70 [a,b]
EtOH extract	565.18 ± 37.85 [a,b]
L-ascorbic acid	10.17 ± 0.32
trolox	10.99 ± 0.83

Remark: [a] $p < 0.05$ represents a significant difference when compared with two positive controls (L-ascorbic acid and trolox)
[b] $p < 0.05$ in comparison with hexane extract

Among three extracts tested, EtOH extract expressed the most potent inhibitory activity (IC_{50} = 565.18 ± 37.85 µg/mL) while that of CH_2Cl_2 was 695.14 ± 84.70 µg/mL. However, its activity showed no significant difference from EtOH extract. For the hexane extract, the highest concentration tested (10,000 µg/mL) was limited by it solubility in absolute ethanol. It gave % inhibition on free radical formation only 63.86 ± 0.50. Therefore, its IC_{50} value could approximately calculated as 6,998.72 ± 80.57 µg/mL. The IC_{50} values of L-ascorbic acid and trolox in our assay system were found to be 10.17 ± 0.32 and 10.99 ± 0.83 µg/mL, respectively which were in agreement with results reported in previous (Li, Wu, and Huang 2009, Luanchoy et al. 2014, Piangpraichom et al. 2014). Although the radical scavenging activity against DPPH radical of all extracts were lower than that of positive controls, they exhibited higher inhibitory activity than that of ethanolic extract of *Boesenbergia rotunda* (L.) Mansf (IC_{50} = 76.3 mg/mL or 76,300 µg/mL) (Jitvaropas et al. 2012). Interestingly, our finding revealed that *B. xiphostachya* (CH_2Cl_2 and EtOH extracts) also showed the same level of anti-oxidant activity to that of crude methanol and its fractionated extracts (hexane, ethyl acetate and water) of *Alpinia mutica* rhizome (IC_{50} were ranging from 0.22 to 0.68 mg/mL or 220 to 680 µg/mL)(Phang et al. 2011).

Since three extracts of *B. xiphostachya* showed different anti-oxidant activity, their class of constituents were observed. As shown in Fig.4, it was clearly observed that hexane extract slightly contained the band in the range of R_f = 0.03 to 0.34 which related to the presence of essential oils, terpenoids, steroids and flavonoids. These constituents are likely accountable for various bio-activities. They also has been previously found and reported in herb from the *Boesenbergia* genera under the Zingiberaceae family such as *B. rotunda* which is widely found in Asian countries where it is commonly used as a food ingredient and in ethnomedicinal preparations (Eng-Chong et al. 2012). Therefore, the DPPH assay guided fractionation might lead to the further isolation and identification of anti-oxidant compounds from *B. xiphostachya*. In addition, the other biological activities are still needed and expanded the utilization of this plant in the related fields.

CONCLUSION

In conclusion, the one-and two-dimensional TLC systems were developed for qualitative determination of chemical constituents in *B. xiphostachya* extract. The phytochemical screening demonstrated the presence of diverse classes of constituents. The CH_2Cl_2 and EtOH extracts were found to exhibit free radical scavenging activity. The further isolation and structural elucidation of the anti-oxidant compounds in this extract is interesting.

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