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Bioactive triterpenoids, antimicrobial, antioxidant and cytotoxic activities of *Eclipta prostrata* Linn.

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ABSTRACT

Bioactive triterpenoids; 3-acetylaleuritolic acid, stigmasterol, a mixture of triterpenoids, fatty esters and aromatic components were isolated from the aerial parts of *Eclipta prostrata* by column chromatography. The plant extracts were investigated for their antimicrobial activity (agar dilution method) against twenty-eight strains of gram-positive and gram-negative bacteria, including diploid fungus. In addition, antioxidant and cytotoxic activities were also evaluated**.** The extracts and isolated fractions exhibited antimicrobial activity against *Morexella catarrhalis*, *Corynebacterium diphtheriae* NCTC 10356 and *Streptococcus pyogenes* with the MIC of 64 μg/mL including *Saccharomyces cerevisiae* ATCC 2601 (MIC 256 μg/mL). The ethyl acetate extract and isolated fractions displayed antioxidant effect. In addition, the plant extracts showed cytotoxic activity (ED_{50} > 100 μg/mL) toward HuCCA-1 and KB cells**.** The results demonstrate beneficial effects of *E. prostrata* as the antimicrobials and bioactive compounds for medicinal usages.

INTRODUCTION

Eclipta prostrata Linn. (Compositae), a medicinal plant, has been used for treatment of hyperlipidemia, atherosclerosis, hepatic disorders (Kim *et al.,* 2008, Chokotia *et al.,* 2013), inflammatory conditions, ophthalmic and digestive disorders (Arunachalam *et al.,* 2009) as well as skin diseases. The plant species is well recognized as the best remedy for hair dying and hair growth (Tewtrakul *et al.,* 2007). The extract of *E. prostrata* was reported to inhibit toxic or lethal action of snake venom (Mors *et al.,* 1989; Melo *et al.,* 1994; Pithayanukul *et al.,* 2004; Pithayanukul *et al.,* 2007; Samy *et al.,* 2008). Many studies showed that triterpenoids isolated from such plant displayed antiproliferative (Lee *et al.,* 2008) and antimicrobial (Gopiesh-Khanna *et al.,* 2008) potentials. Previously, biological and chemical investigations of *E. prostrata* (hexane extract) was reported by our research group (Prachayasittikul *et al.,* 2010). As our continuing study on medicinal plants, herein, isolated bioactive compounds, antimicrobial, antioxidant and cytotoxic effects of *E. prostrata* extracts obtained from polar solvents have been reported.

MATERIALS AND METHODS

General

Melting points were determined on a Griffin melting point apparatus, UK and were uncorrected. ^{1}H NMR spectra were

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recorded on a Bruker AVANCE 300 NMR spectrometer (operating at 300 MHz). Infrared spectra (IR) were obtained on a Perkin Elmer System 2000 FTIR. Column chromatography was carried out using silica gel 60 (0.063–0.200 mm). Analytical thin layer chromatography (TLC) was performed on silica gel 60 PF_{254} aluminum sheets (cat. No. 7747 E., Merck).

Solvents were distilled prior to use. Reagents for cell culture and assays were of analytical grade as the following: Ham's/F12 (nutrient mixture F-12) and FBS (fetal bovine serum) from Hyclone laboratories, USA. L-glutamine, penicillin-streptomycin, sodium pyruvate, glucose, crystal violet, α-tocopherol and DPPH (2,2-diphenyl-1-picrylhydrazyl) from Sigma, USA; and DMSO (dimethyl sulfoxide) from Merck.

Plant material

E. prostrata (aerial parts) were collected from Nakornratsrima province, Thailand. The plant has been identified (BKF 075505) by The Forest Herbarium, Royal Forestry Department, Bangkok.

Cancer cells

Two human cancer cells, cholangiocarcinoma (HuCCA-1) and epidermoid carcinoma (KB), were grown in Ham's/F12 medium containing 2 mM L-glutamine supplemented with 100 U/mL penicillin-streptomycin and 10% FBS.

Extraction

The dried powder of *E. prostrata* (1.2 kg) was extracted with hexane (4L × 5days × 3) followed by filtration. The filtrates were combined and evaporated *in vacuo* to give a hexane extract (25 g). Similar extraction was performed using chloroform and ethyl acetate to give the corresponding chloroform (33 g) and ethyl acetate (29 g) extracts, respectively. In this study, chloroform and ethyl acetate extracts were investigated.

Isolation

Isolation of the extracts was performed by a silica gel column chromatography using gradient elution with increasing polarity of the solvents. Fractions were collected and combined based on TLC chromatograms and evaporated to dryness.

Chloroform extract (27 g) was separated using the silica gel (650 g) column, then eluted with hexane: ethyl acetate, chloroform : ethyl acetate, ethyl acetate, and ethyl acetate : methanol to provide seven fractions of yellow oil, dark brown and black semi-solids of C1 (0.29 g), C2 (0.79 g), C3 (1.30 g), C4 (5.58 g), C5 (2.36 g),C6 (0.90 g) and C7 (10.16 g). Fraction C6 was further isolated by the silica gel (30 g) column to give eight fractions (C6.1-C6.8).

Yellow solid fraction C6.4 (37.2 mg) was recrystallized in methanol to give 3-acetylaleuritolic acid (**1**, 3.1 mg, mp 298-300°C) (Prachayasittikul *et al.*, 2009; Prachayasittikul *et al.*, 2009). Fraction C6.6 (58.5 mg of yellow solid) was washed with hexane-methanol and then recrystallized in dichloromethane to afford stigmasterol (**2**, 11.2 mg, mp 150-152°C) (Prachayasittikul *et al.*, 2010; Prachayasittikul *et al.*, 2009; Prachayasittikul *et al.*, 2009; Srisung *et al.*, 2013). Ethyl acetate extract (23 g) was chromatographed on the silica gel (600 g) column using gradient elution of solvent as above to give seven fractions of dark yellow solid and dark green solid of E1 (0.37 g), E2 (1.13 g), E3 (0.52 g), E4 (0.93 g), E5 (11.43 g), E6 (1.35 g) and E7 (1.79 g). The fraction E2 was re-chromatographed on the silica gel (40 g) column to afford fractions E2.1 (250 mg), E2.2 (42 mg) and E2.3 (54 mg) as yellow solid. The fraction E2.2 was recrystallized in methanol to give white solid of 3-acetylaleuritolic acid (**1**, 4.3 mg).

Bioactivities

Antimicrobial Assay

Antimicrobial activity of the tested compounds was performed using the agar dilution method as previously described (Srisung *et al.*, 2013). The tested compound (10.24 mg) was dissolved in DMSO (0.2 mL) and then added to the Müller Hinton (MH) broth (1.8 mL). A two-fold dilution was prepared, and 1 mL of each diluted compound was transferred to the MH agar (19 mL) to give the initial concentration of 256μg/mL. The DMSO (0.5%) as a control, was added to the MH agar. Microorganisms cultured in the MH broth at 37°C for 24 h, were diluted with 0.9% normal saline solution to adjust the cell density of 1.5×10^8 CFU/mL. The microorganisms were inoculated onto each plate and further incubated at 37°C for 18-48 h. Compounds which exerted high efficacy to inhibit cell growth of the microorganisms were determined. Twenty-eight strains of tested microorganisms were gram negative bacteria: *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Salmonella typhimurium* ATCC 13311, *Salmonella choleraesuis* ATCC 10708, *Pseudomonas aeruginosa* ATCC 15442, *Edwardsiella tarda*, *Shigella dysenteriae*, *Citrobacter freundii*, *Morganella morganii*,*Vibrio cholerae*, *Vibrio mimicus*, *Aeromonas hydrophila*, *Plesiomonas shigelloides*, *Stenotrophomonas maltophilia*, *Neisseria mucosa*, *Moraxella catarrhalis*; gram positive bacteria: *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Enterococcus faecalis* ATCC 29212, *Micrococcus luteus* ATCC 10240, *Corynebacterium diphtheriae* NCTC 10356, *Bacillus subtilis* ATCC 6633, *Streptococcus pyogenes*, *Listeria monocytogenes*, *Bacillus cereus*, *Micrococcus flavas* and diploid fungus (yeast): *Candida albicans* ATCC 90028 and *Saccharomyces cerevisiae* ATCC 2601.

Antioxidant assay

The compounds were tested for antioxidant property using 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay. The DPPH (a stable purple color radical) reacts with an antioxidant to form a light-yellow colored of diphenylpicrylhydrazine, the reduced product that can be spectrophotometrically detected. The assay (Prachayasittikul *et al.*, 2012) was initiated by adding 1 mL solution of DPPH in methanol (0.1 mM) to a sample solution (0.45 mL, 1 mg/mL dissolved in DMSO). The reaction mixture was incubated for 30 min in a dark

room. The absorbance at 517 nm was measured using UV-visible spectrophotometer (UV-1610, Shimadzu),and the percentage of radical scavenging activity (RSA) was calculated from the following equation:

$$\text{RSA (\%)} = \left[1 - \frac{Abs._{sample}}{Abs._{control}}\right] \times 100$$

where $Abs._{control}$ is the absorbance of the control reaction and $Abs._{sample}$ is the absorbance of the tested compound.

Cytotoxic assay

Cytotoxic assay was performed using the modified method as previously described (Prachayasittikul *et al.*, 2008). Briefly, the confluent cell monolayers were trypsinized and diluted with appropiate culture medium to a final concentration of 3×10^5 cells/mL. Portions (100 µL) containing approximately 3×10^4 cells were added into 96-well flat-bottomed tissue plates and incubated overnight at 37°C in a humidified 5% CO_2 incubator. Solution (100 µL) containing various concentrations of tested compounds, positive control (etoposide) or negative control (DMSO) were added to each well and the plates were incubaed for an additional 48 h. After the incubation, each well was washed three times with phosphate-buffered saline (pH7.2) and then stained with crystal violet. The excess dye was removed, the stained cells were lysed with 100 mM HCl (100 µL) in absolute methanol and the optical density was determined at 540 nm by a microtitre plate reader (Titertek, Multiskan MCC/340). All assays were carried out in quadruplicate and the mean values were calculated. The cytotoxic activity was expressed as an ED_{50} (the effective dose that inhibits 50% of cell growth).

RESULTS

Isolation

The extracts (chloroform and ethyl acetate) of *E. prostrata* were isolated by the column chromatography. Isolated fractions (C1-C6) of the chloroform extract were mainly found to be a mixture of triterpenoids and long chain hydrocarbons together with trace amount of aromatic components as seen by ^{1}H NMR and IR spectra (data not shown).

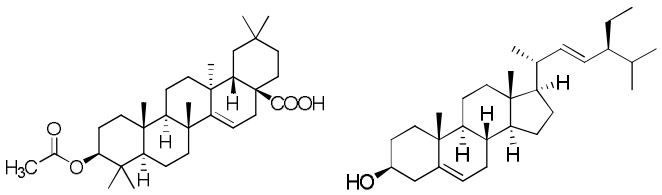

Fig. 1: Chemical structures of compounds **1** and **2**

Repeated chromatographic separation or purification was performed on some selected fractions in which the fraction C6 was further isolated to provide eight fractions (C6.1-C6.8) of yellow solids. Fraction C6.4 was purified by recrystallization to give 3-acetylaleuritolic acid (**1**). Purification of fraction C6.6 gave stigmasteriol (**2**), chemical structures are shown in Figure 1. Additionally, compound **1** was also found in fraction E2.2 of the ethyl acetate extract. Their structures were confirmed by comparison of ^{1}H NMR and IR spectra with the authentic samples.

Biological activities

Antimicrobial activity

The plant extracts (chloroform and ethyl acetate) and isolated fractions (C2-C7) were evaluated for their antimicrobial potency against twenty-eight strains of microorganisms using the agar dilution method. Results (Table 1) showed that the bioactive fractions (C3, C4, C6, and C7) exhibited inhibiroty activity against both gram positive and gram negative bacteria with their minimum inhibitory concentrations (MIC) of 64 μg/mL. The chloroform and ethyl acetate extracts displayed activity against *S. cerevisiae* ATCC 2601 with the MIC value of 256 μg/mL. Frations C2 and C5 were found to be inactive antimicrobials. However, the DMSO (0.5%) was tested in parallel with the compounds and showed no effect on the tested microorganisms.

Table 1: Antimicrobial activity (MIC) of *E. prostrata*

Compound	Microorganism	MIC [a, b] (μg/mL)
C2	inactive	-
C3[c], C4[d]	*M. catarrhalis*, *C. diphtheriae* NCTC 10356	64
C5	inactive	-
C6	*M. catarrhalis*, *S. pyogenes*	64
C7	*S. pyogenes*	64
Chloroform extract	*S. cerevisiae* ATCC 2601	256
Ethyl acetate extract	*S. cerevisiae* ATCC 2601	256

[a] Ampicillin at 10 μg/mL was used as a control of the antimicrobial testing system; it showed 100% inhibition against*S. aureus* ATCC 25923, *B. subtilis* ATCC 6633, *S. epidermidis* ATCC 12228, *S. pyogenes*, *E. tarda*, *N. mucosa* and *M. catarrhalis*. [b] MIC: Minimum inhibitory concentration was the lowest concentration that inhibited the growth of microorganisms. [c] At 64 μg/mL, C3 showed 75% inhibition against *N. mucosa*. [d] At 128 μg/mL, C4 showed 75% inhibition against *S. pyogenes*.

Antioxidant activity

Radical scavenging activity of the plant extracts and fractions (C2-C7) was carried out using the DPPH assay. It was found that (Table 2) ethyl acetate extract displayed the highest RSA (IC_{50} 151.7 μg/mL) as compared to fractions C4, C5 and C6 with IC_{50} range of 223.9 - 467.7 mg/mL. However, chloroform extract and fractions C2,C3 and C7 were shown to be inactive antioxidants.

Table 2: Radical scavenging activity (IC_{50}) of *E. prostrata*

Compound	IC_{50}[a] (μg/mL)
C2, C3, C7	-[b]
C4	223.9 [c]
C5	358.1 [c]
C6	476.7 [c]
Chloroform extract	-[b]
Ethyl acetate extract	151.7

[a] α-tocopherol was used as the control (IC_{50} = 5.57 μg/mL)
[b] Compounds exhibited < 50% inhibition at 333.33 μg/mL were denoted as inactive antioxidants
[c] IC_{50} value was determined as mg /mL

Cytotoxic activity

Cytotoxic assay was performed toward HuCCA-1 and KB cells. The chloroform and ethyl acetate extracts of *E. prostrata* showed inhibitory effect on the two tested cells with ED_{50}> 100 μg/mL.

DISCUSSION

The extracts (aerial parts) of *E. prostrata* were chromatographic separated to provide many combined fractions containing a mixture of triterpenoids and fatty esters together with aromatic compounds as observed by ^{1}H NMR and IR spectra. Extensive repeated isolation and purification afforded bioactive triterpenoids namely 3-acetylaleuritolic acid (**1**) and stigmasterol (**2**) as well as a mixture of β-sitosterol and stigmasterol. However, compound **1** has never been reported to be isolated from the *E. prostrata*. Previously, compound **1** was isolated from other medicinal plants such as *Spilanthes acmella* Murr. (Compositae) (Prachayasittikul *et al.,* 2009, Prachayasittikul *et al.,* 2013), *Polyalthia debilis* (Annonaceae) (Prachayasittikul *et al.,* 2009). Compound **2** is a known phytosterol found in soy beans and other plant species i.e., *Hydnophytum formicarum*Jack. (Rubiaceae) (Prachayasittikul *et al.,* 2008), *S. acmella* Murr. (Prachayasittikul *et al.,* 2009), *P. debilis* (Prachayasittikul *et al.,* 2009), *Saraca thaipingensis* Cantley ex Prain (Leguminosae) (Prachayasittikul *et al.,* 2012) and *Pterocarpus indicus* Willd (Leguminosae) (Prachayasittikul *et al.,* 2013). Biological activity results of *E. prostrata* showed that the plant extracts (chloroform and ethyl acetate) displayed antifungal activity against *S. cerevisiae* ATCC 2601 (MIC = 256 μg/mL), but not showed antibacterial activity. However, most fractions (C3, C4 and C6) showed inhibitory effect against *M. catarrhalis* while fraction C7 selectively inhibited the growth of *S. pyogenes*.

In addition, C3 and C4 also displayed antimicrobial activity against *C. diphtheriae* NCTC 10356; and fraction C6 exhibited activity against *S. pyogenes* with the same MIC (64 μg/mL) value. Previously, ethyl acetate extract of *E. alba* (*E. prostrata*) was reported to exhibit antimicrobial activity against many strains of microorganisms with MICs of 1.56-25.00 mg/mL (Borkataky *et al.,* 2013). These investigated microorganisms i.e., *M. catarrhalis*, *S. pyogenes* and *C. diphtheriae* are pathogenic bacteria causing various kinds of diseases. *M. catarrhalis* has been found to be a true pathogen in respiratory tract of human, and is the most common bacteria that causes ototis media in children after *Haemophilus influenzae* and *Streptococcus pneumoniae*. However, it can cause chronic obstructive pulmonary disease in adults (De Vries *et al.,* 2009; Buskirk *et al.,* 2014). *C. diphtheriae* is a causative agent of Diphtheria which can be spread from human to human. Although this disease can cause high fatality rate, however, DPT vaccine has been generated for a successful protection. Nevertheless, this disease is not disappear because of the unvaccinated people in some developing countries (Adler *et al.,* 2013; Meera *et al.,* 2014). *S. pyogenes* is the most common causative agent of bacterial pharyngitis. However, this bacteria can cause a wide range of disease such as scarlet fever, impetigo, necrotizing fasciitis, streptococcal toxic shock syndrome and also cause the autoimmune diseases, acute poststreptococcal glomerulonephritis as well as acute rheumatic heart disease (Morefield *et al.,* 2014; Walker *et al.,* 2014). The results could suggest the potential use of *E. prostrata* as traditional medicine for these infectious diseases.

Antioxidant activity (DPPH assay) of *E. prostrata* was noted for the ethyl acetate extract while the other tested samples were found to be slightly active to inactive antioxidants. In our previous study, the hexane extract showed relatively weak antioxidant effect (Prachayasittikul *et al.,* 2010). It was reported that the whole plant extract (ethyl acetate) of *E. prostrata* diplayed antioxidant activity (ferric reducing ability) (Chauhan *et al.,* 2012)

Both of the aerial plant extracts (chloroform and ethyl acetate) exerted cytotoxic activity against the HuCCA-1 and KB cells with ED_{50} values > 100 μg/mL. Previously, the hexane extract (aerial part) of the plant species was reported to show cytotoxic activity (ED_{50} = 100 μg/mL) against the two human cancer cell lines (Prachayasittikul *et al.,* 2010). The whole plant extract (ethyl acetate) of *E. alba* was documented to exert moderate cytotoxic activity against human lung epithelial adenocarcinoma cell line (HCC-827) (Chauhan *et al.,* 2012). Regarding biological activities, compound **1** isolated from other plant species was reported to exhibit antibacterial activity against *S. aureus* and *S. typhimurium* (Peres *et al.,* 1997) , and to show significant inhibition on vitality of adult male worm of *Onchocerca gutturosa* (Nyasse *et al.,* 2006). Furthermore, 3-acetylaleuritolic acid (**1**) displayed strong inhibition of DNA topoisomerase II, and strong cytotoxic activity against human lung carcinoma A549 cells (Wada *et al.,* 2006). Compound **2** was previously reported to exert antioxidant activity as determined by the thiocyanate method (Hung *et al.,* 2001) and by the lipid antioxidant property (Ramadan *et al.,* 2007). In addition, stigmasterol (**2**) significantly suppressed HMG-CoA reductase activity (~11% reduction) in plasma cholesterol levels in Wistar and WKY rats feeding 0.5% of compound **2** (Batta *et al.,* 2006). A mixture of β-sitosterol and stigmasterol exhibited antimicrobial activity against *S. cerevisiae* ATCC 2601 with MIC of 64 μg/mL (Prachayasittikul *et al.,* 2009).

In conclusion, the *E. prostrata* exerts antimicrobial and antioxidant activities, and constitutes bioactive triterpenoids (**1** and **2**) with diverse biological effects. The results demonstrate benificial effects of the plant species as antimicrobials and bioactive compounds for medicinal uses.

CONFLICT OF INTEREST STATEMENT

We declare that we have no conflict of interest.

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