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Effects of drying and extraction methods on phenolic compounds and in vitro assays of *Eclipta prostrata* Linn leaf extracts

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ABSTRACT: *Eclipta prostrata* Linn leaves have a potential to be an alternative source of commercial herbal extracts. The effects of different drying methods (oven-drying at 60 °C, freeze drying, and microwave drying) on polyphenol and antioxidant activities of *E. prostrata* were reported. The extractions with absolute ethanol and freeze-drying preserved higher levels of total phenolic content and total flavonoid content than those of the oven and microwave-drying methods. Correlation analysis indicated that phenolic and flavonoid compounds were the major antioxidants in *E. prostrata* extracts. Drying methods were a critical factor following the order of freeze-dried, microwave dried, oven-dried leaf extracts, for which the extraction methods seemed to have no correlation with the properties of the extracts. The *E. prostrata* ethanolic extracts contained flavonoids (luteolin, luteolin glucoside, luteolin sulphate, wedelolactone, dimethylwedelolactone, dimethylwedelolactone sulphate, 3′-hydroxybiochanin A), phenolic acids (gallic acid, protocatechuic acid, caffeoylquinic acid, dicaffeoylquinic acid), phenolic aldehyde (protocatechualdehyde), and triterpenoids (eclalbasaponin). High performance thin layer chromatography of the crude extracts from the freeze-dried leaves and soxhlet extraction gave the main wedelolactone, chlorophyll, and some non-phenolic compounds. The IC_{50} values of the crude extracts for anti-proliferation of HaCaT cells indicated less efficiency for psoriasis treatment. However, an appropriate extraction of *E. prostrata* leaves to obtain high luteolin and wedelolactone contents has been suggested for a further development of local and systemic treatments for other inflammatory skin diseases.

KEYWORDS: *Eclipta prostrata*, phenolic, flavonoid, drying method, anti-proliferation

INTRODUCTION

Eclipta prostrata L. belongs to the therophyte herb of the family *Asteraceae*, and it is widely distributed throughout Thailand. This plant is widely applied in traditional Thai and Chinese medication, and it is also a functional food[1,2]. It has been reported to possess antioxidant[3,4], anti-inflammatory[5], and antitumor[6] activities. Wedelolactone and luteolin are major polyphenols of methanol/ethanol extracts of *E. prostrata*[6–8]. Besides known as a natural compound with potent anti-inflammatory properties, luteolin has been firstly reported to reduce keratinocyte proliferation, which is related to an autoimmune disease psoriasis, without affecting the viability and intracellular aspects. More importantly, luteolin decreases nuclear factor-kappa B (NF-κB) activation, typically associated in inflammatory mediators, at both the transcriptional and translational levels[9]. Wedelolactone has been demonstrated a significant retrieval of anti-oxidative enzymes, its effects on alleviating inflammatory markers related to UVB exposure, and early tumour stimulating incidences in murine skin describing feasible role of the NF-κB pathway[10]. These studies provide a medical exploitation of this plant as a substantial treatment for skin diseases, especially psoriasis and inflammation.

The polyphenol contents in plants are affected by numerous factors, such as genetics, age, environmental factors (soil type, sun exposure, and rainfall), processing, and storage[11]. The postharvest drying method, extraction, and distillation affect the qualitative and quantitative aspects of the bioactive compounds from plant materials. Different postharvest methods have been used for drying different medicinal plants. Oven drying is the most cost

and time effective for drying most plant materials. Microwave drying can shorten the drying time and moderately lower the energy consumption, but sometimes it causes degradation of phytochemicals. Freeze-drying is a sublimation process that a plant sample is frozen prior to lyophilization, then reduced surrounding pressure allows frozen moisture to sublime directly out of the sample. Despite high cost, freeze-drying causes less damages of substances than other high temperature drying, resulting in general applications to preserve food and to increase shelf life of agricultural and pharmaceutical products. Nevertheless, this drying led to loss some volatile compounds capable of sublimation [12, 13]. Although modern extraction techniques, e.g., pressurized liquid extraction and supercritical fluid extraction, have been developed, existing practical techniques (soxhlet extraction, maceration and percolation) have been consistently applied for crude extraction [14, 15]. The drying and extraction methods are major factors contributing to radical scavenging activities of herbal plants and their extracts, i.e., *Cinnamomum zeylanicum* [16], *Betula pendula* [17], *Alpinia zerumbet*, *Etlingera elatior*, *Curcuma longa*, *Kaempferia galangal* [18], and *Thunbergia laurifolia* [19]. Since there are notable differences in secondary metabolites in plants, a particular plant needs suitable drying and extracting methods to achieve high levels of beneficially targeted phytochemicals.

This study investigated the effects of the postharvest drying processes (freeze drying, oven drying, and microwave drying) and conventional extraction processes (soxhlet extraction, maceration, and percolation) on the total phenolic content (TPC), total flavonoid content (TFC), and free radical scavenging activity (FRSA) of *E. prostrata* extracts. The levels of marker compounds (wedelolactone and luteolin) were analysed by high-performance liquid chromatography (HPLC). Liquid chromatography-electrospray ionization-quadrupole-time of flight-tandem mass spectrometry (LC-ESIQTOF-MS/MS) was used to characterize the phenolic compounds and their glycosides. Cell cytotoxicity and anti-proliferation assays of the *E. prostrata* extracts were assessed using HaCaT cells. Our experimental data could introduce a proper choice for producing *E. prostrata* crude extracts, which would be further developed and applied for skin health and treatment products, cooperating by small and medium-sized enterprises (SMEs).

MATERIALS AND METHODS

Plant materials and chemicals

Like other weeds, *E. prostrata* was simply collected from a paddy field in Koeng Subdistrict, Muang District, Maha Sarakham, Thailand (16°12′30″ N, 103°17′42″ E), in September 2014. The soil properties were pH 6.72 ± 0.06, electrical conductivity 16.67 ± 5.77 mS/cm, organic matter 3.28%, nitrogen 0.17 ± 0.02%, phosphorus 27.71 ± 2.89 mg/kg, potassium 114.52 ± 6.40 mg/kg and cation exchange capacity 17.46 ± 0.55 cmol/kg. This plant was authenticated and a voucher specimen was deposited at Department of Biology, Faculty of Science, Mahasarakham University, Maha Sarakham, Thailand. Collected whole plants were immediately transferred into polyethylene bags and stored at 4 °C for 20 min of transportation. Healthy leaves (not including seriously damaged or diseased leaves) were washed with an excess of tap water. The water was wiped off with clean tissue paper before drying processes.

The solvents and chemicals used in this study were analytical grade. Standard chemicals and mobile phases for HPLC and LC-MS/MS analyses were HPLC grade from Sigma-Aldrich and Fluka. Chemicals and media for in vitro assays were from GibcoTM Thermo Fisher Scientific.

Drying and extraction

The clean leaves were dried in a hot air oven (RI 53 Binder, Germany) at 60 °C for 20–24 h. Microwave drying was performed by a digital microwave (Samsung J7EV, Malaysia) at 600 watts for 4 min, in which the leaves were flattened on a few pieces of tissue paper laid between the leaves and a ceramic plate to absorb water vapour. For freeze-drying, the leaf samples were pre-frozen by liquid nitrogen before lyophilizing overnight at −40 °C, 0.5 psi in a freeze dryer (Heto Power Dry PL3000, Thermo Fisher Scientific, Japan). The dried leaves were ground to powder with a grinder, followed by storage in a dark closed container with silica absorber.

The extraction methods of soxhlet, maceration and percolation were carried out with the dried leaf powder and 99.9% (v/v) ethanol in a ratio of 1:100 (w/v). Dried samples (2.5 g) were packed in a cellulose thimble (33 × 80 mm) (Whatman, GE Healthcare, UK) with soxhlet apparatus and Mantle (MS-EAM M-TOP, Indonesia), and extracted with 250 ml of the ethanol for 10 h (one cycle per hour) at 80 °C. Maceration was performed by filling 0.2 g of dried leaf powder in a tightly closed 50 ml glass tube

containing 20 ml ethanol, then shaken at 150 rpm for 24 h at room temperature (30 ± 5 °C). A percolation column was formed in a disposable syringe (0.5 cm in diameter and 10 cm height) (NIPRO, Japan). A set was 0.1 g of leaf powder packed into the syringe to obtain a 0.2 ml bed volume. The effluent of 10 ml loading was collected as a fraction with the flow rate controlled at 0.1–0.2 ml/min by a vacuum manifold (12-Port Teknokrama, Spain). All extracts were filtrated through Whatman no. 4 paper, and the volume was made up to compensate for evaporation. All samples were kept in separated amber glass bottles with tight stoppers at 4 °C until analysis. Then 100 ml of each sample from soxhlet extraction were evaporated and dried at 60 °C to obtain a dried crude extract.

TPC, TFC and FRSA

The TPC was determined using a modified Folin-Ciocalteu method20. Briefly, a 100 µl extract was pipetted into 1.5 ml Eppendorf tube and 500 µl of 10% (v/v) Folin-Ciocalteu reagent was added. The mixture was left to stand in the dark for 3 min and then 100 µl of 7.5% (w/v) Na_2CO_3 and 300 µl of deionized water were filled in. After 2 h, the absorbance was measured at 731 nm using a UV/visible spectrometer (Beckman Coulter DU 730 Life Science, USA). A standard curve was constructed from 5, 20, 40, 80, and 100 mg/l of caffeic acid. The TPC was expressed in terms of a caffeic acid equivalent (µmol CAE/g dry wt).

The TFC was analysed using a modified colorimetric method[21]. Aliquots of 500 µl of deionized water and 100 µl of leaf extract were added to 1.5 ml Eppendorf tube. Then 30 µl of 5% (w/v) $NaNO_2$ was added. The mixtures were kept in the dark for 5 min before adding 60 µl of 10% (w/v) $AlCl_3$. After standing for 6 min, 200 µl of 1 M NaOH and 110 µl of deionized water were added. After 5 min in the dark, the absorbance was measured at 510 nm. A standard curve was prepared from 10, 20, 40, 80, and 100 mg/l of epicatechin. The TFC was expressed in terms of an epicatechin equivalent (µmol EPE/g dry wt).

The FRSA was evaluated based on the 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH·) method[22]. An amount of 100 µl of the leaf extract or blank was pipetted into separated 1.5 ml Eppendorf tube and 900 µl of 80 µM DPPH solution was added. The mixture was kept in the dark for 30 min, the absorbance was measured at 515 nm. The ability of the extract to scavenge DPPH free radicals was calculated by FRSA (%) $= (A_0 - A_1)/A_0 \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the test sample.

LC-MS/MS and HPLC

An extract was analysed by reverse phase HPLC (LC-20AC Shimadzu, Japan), modified from the previous method[23]. Each extract was filtered through a 0.22 µm nylon filter (VertiClean Vertical, Thailand) and injected onto the Inertsil ODS-3C18 column (4.6 × 250 mm, 5 µm, Hichrom Limited, UK) with an injection volume of 20 µl. The mobile phases were 3% (v/v) acetic acid (mobile phase A) and 99.9% (v/v) methanol (mobile phase B), with a flow rate of 1 ml/min. The components of the extract were separated using gradient elution at 40 °C and detected at 280 and 360 nm with a UV-diode array detector (SPD-M20A, Shimadzu, Japan). Peak identification was performed by comparing the retention times (RT) with the standard compounds. Wedelolactone and luteolin, which were confirmed the peaks by the RT of LC-MS, were quantitatively determined by external standard methods. The same HPLC conditions were operated with various concentrations for calibration curves, which were generated by linear regression based on the peak area.

The LC-MS/MS was conducted following a modified method[24]. The main components were assayed by LC-MS/MS, with quadrupole-time of flight mass analysers. The LC-QTOF-MS/MS analysis was performed on an Agilent HPLC 1260 series coupled with a QTOF 6540 UHD accurate mass (Agilent Technologies, Waldbronn, Germany). The separation of the sample solution was carried on a Luna C18(2) 150 × 4.6 mm, 5 µm (Phenomenex, USA). The solvent flow rate was 500 µl/min, and 5 µl of the sample solution was injected into the LC system. The binary gradient elution system was composed of water as solvent A and acetonitrile as solvent B, and both contained 0.1% formic acid (v/v). The linear gradient elution was 5–95% for solvent B at 35 min and a post run for 5 min. The column temperature was set at 35 °C. The conditions for the negative ESI source were as follows: drying gas (N_2) flow rate 10 l/min, drying gas temperature 350 °C, nebulizer 30 psig, fragmentor 100 V, capillary voltage 3500 V, and scan spectra from m/z 100–1500 amu. The auto MS/MS for the fragmentation was set with collision energies of 10, 20, and 40 V. All data analyses were controlled using Agilent Mass Hunter Qualitative Analysis Software B06.0 (Agilent Technologies, CA, USA).

High performance thin layer chromatography (HPTLC) analysis

The phenolic and flavonoid compounds were investigated by an HPTLC system (CAMAG, Muttenz, Switzerland) with TLC visualizer linked to vision CATS software. A plant crude extract and each chemical standard were dissolved in DMSO (dimethyl sulphoxide) 30 and 20 mg/ml, respectively. The filtered solutions were applied to silica 60F 254 on aluminium sheet, 10 × 20 cm (Merck, Darmstadt, Germany), and the conditions were syringe delivery speed of 10 s/µl; injection volumes of 2 µl for plant extract and 1 µl for standards; band width 8 mm; and distance from bottom 8 mm. The HPTLC plate was developed in a horizontal chamber after saturation in a mobile phase of toluene: ethyl acetate: formic acid (11:6:1 v/v/v) for 5 min at room temperature[25]. The length of the chromatograms was 75 mm from the applied spots. The developed plate was allowed to dry for 1 min before derivatizing with natural product reagent I (1% (w/v) ethanolamine diphenyl borate in methanol) and natural product reagent II (5% (v/v) polyethylene glycol-100 in ethanol)[26]. The plate before and after derivatization was observed under a UV lamp at 254 and 365 nm. The standards of chlorogenic acid, caffeic acid, p-coumaric acid, rutin, wedelolactone and luteolin were used for identification.

Cell cytotoxicity, anti-proliferation and MTT assays

Dried crude extracts were dissolved in DMSO as stock solutions at 50 mg/ml. The concentrations of DMSO were controlled to be less than 1%. The crude extracts were filtered through a 0.2 µm filter (Corning Inc., Corning, NY, USA). HaCaT cells (Cell Line Service, Heidelberg, Germany), the immortalized human epidermal keratinocyte cell line, were cultured in Dulbecco's Modified Eagle Medium/High glucose (DMEM/HG) (GibcoTM Thermo Fisher Scientific, USA) with 10% (v/v) fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were cultured at 37 °C in a humidified atmosphere, and 5% CO_2 for 24 h that gave 80% cell confluence. For the cell cytotoxicity test, the cells were trypsinized with 3 ml of 5% (w/v) trypsin for 15 min and seeded in a 96 well plate at a cell density of 5 × 104 cells/ml for 24 h that gave a cell confluence of about 50%. For the anti-proliferation test, the cells were seeded for 12 h, then replaced with fresh medium that contained 50 ng/ml of TNF-α to stimulate cell inflammation. After that, the cells were washed with phosphate buffer saline (PBS) before treatments with different concentrations of the plant extracts (62.5, 125, 250, 500, and 1000 µg/ml) for 24 h. Two marker compounds, wedelolactone and luteolin, were employed as standards at the concentrations of 6.3, 12.5, 25, 50, and 100 µg/ml. Paclitaxel, a chemotherapy medication for cancer treatment, at the concentrations of 0.3, 0.6, 1.3, 2.5, and 5.0 µg/ml was also used as a positive control.

After incubation for 24 h, the medium was removed from each treatment. Then the cells were washed twice with PBS and replaced in each well with 110 µl of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution at a final concentration of 0.5 mg/ml, before incubating in the dark for 2 h. The MTT solution was removed, washed with PBS, and replaced with 100 µl of DMSO to dissolve the intracellularly formed crystals of dark-blue formazan. The absorbance at 540 nm was measured for the cell viability[27]. Cell survival rate (%) was calculated from the fraction of alive cells relative to that of the control for each point, as cell survival rate (%) $= (A_S - A_B)/(A_C - A_B) \times 100$, where A_S, A_B, and A_C are the absorbances of the sample, blank, and control, respectively.

Table 1 Percentages of wet weight and dry weight of each plant part per whole plant.[†]

Plant part	Wet weight (%)	Dry weight (%)
Root	7.1 ± 2.2	11.8 ± 3.9
Leaf	24.2 ± 1.5	35.1 ± 0.4
Flower	6.8 ± 0.1	8.7 ± 1.1
Stem	61.9 ± 3.6	44.3 ± 3.2

[†] Data are given as mean ± SD ($n = 3$).

Statistical analysis

The data were reported as the mean ± standard deviations (SD) and were analysed using ANOVA. Significant differences between the means were determined by Duncan's new multiple range test (DMRT). Statistical analyses were performed using SPSS statistical software (SPSS 14, SPSS Inc., IL, USA).

RESULTS AND DISCUSSION

Plant parts

Whole plants of *E. prostrata* were separated to roots, leaves, flowers and stems, and they were dried to determine the dry mass production per plant (Table 1).

Table 2 Effect of drying methods on moisture removal, dried mass, and amount of luteolin and wedelolactone in the *E. prostrata* extracts from soxhlet extraction[†].

Drying method	Moisture (%)	Dried mass (%)	Lute.[*] (mg/g)	Wede.[*] (mg/g)	Ratio
Freeze dry	81.3 ± 0.3	18.7 ± 0.3	3.01	15.81	1:5.26
Microwave	81.5 ± 0.4	18.5 ± 0.4	1.52	5.69	1:3.75
Oven	81.4 ± 0.9	18.6 ± 0.9	0.21	0.28	1:1.29

[†] Data are given as mean ± SD ($n = 3$).
[*] Quantities (mg/g dry wt.) of luteolin (Lute.) and wedelolactone (Wede.) obtained from HPLC analysis.

The average percentages indicated that stems and leaves showed the major weights obtained from the whole plant after drying as 44.3% and 35.1%, respectively. Undoubtedly, the plant stems have been generally used for stem cutting propagation. Since phytochemical compounds have been reported in the leaves of *E. prostrata* to be higher than that in the stem[2], our preliminary testing of maceration extracts obtained from samples of oven-dried stems and leaves was done. It was also indicated that TPC and TFC in the stem extracts were 1–2 times lower than their contents in the leaf extracts (data not shown). Commercially, the separated leaf samples (not whole plant) were investigated to control the quality and quantity of the crude extract.

Effect of drying and extraction methods

Table 2 shows that the three-drying methods (oven, microwave and freeze dry) eliminated 81% of the moisture content, and the samples had no significant differences in dry mass obtained. In other words, 1 kg of fresh weight sample offers approximately 200 g of dried leaves. Ethanol and methanol have been proven to be better extraction solvents for phytochemicals, especially phenolic compounds. Since methanol is more polar than ethanol, and possesses cytotoxic nature, it is unsuitable for extraction for human product[14,16,28]. Thus ethanol was applied to extract *E. prostrata* leaves throughout this experiment.

The effects of drying and extraction methods on TPC, TFC and FRSA in the extracts are shown in Table 3. The highest values of TPC and FRSA were obtained from freeze dry/Soxhlet and freeze dry/maceration, respectively. The freeze-dried leaf extracts from soxhlet, maceration and percolation were not different in the high TFCs. Losses in the TPC, TFC and FRSA were the highest for oven-drying.

The correlation coefficients for relationships among extraction methods, drying methods and the values of TPC, TFC, and FRSA are presented in Table 4. There were positive values among TPC, TFC and FRSA ($r \approx 0.8$, $p < 0.01$), which indicated that phenolic and flavonoid compounds would be the major antioxidants in *E. prostrata* extracts. The extraction methods seem to have no correlation with TPC, TFC, and FRSA, whereas, the drying methods were considered as a critical factor for TPC, TFC, and FRSA as there was a negative correlation ($r \approx -0.9$, $p < 0.01$). The quantitative analysis of the leaf extracts from soxhlet extraction (Table 1) indicated that thermal heating during the microwave-drying method reduced the amounts of luteolin and wedelolactone three times, when compared to the freeze-drying method, while luteotin and wedelolactone in the oven-dried leaf extracts were destroyed, resulting in only very small amounts. In addition, the ratio of luteolin to wedelolactone in the extracts implied that high amounts of wedelolactone were thermally degraded when compared to luteolin.

Table 3 Effect of drying conditions and extractions on total phenolic content, total flavonoid content and free radical scavenging activity of the *E. prostrata* extracts[†].

Antioxidant properties	Drying methods	Extraction methods[‡] Soxhlet	Maceration	Percolation
TPC (mg GA/g)	Freeze dry	30.7 ± 1.1^{a}	20.3 ± 2.4^{b}	20.3 ± 0.9^{b}
	Microwave	15.0 ± 0.2^{c}	4.7 ± 0.3^{d}	16.2 ± 2.7^{c}
	Oven	1.3 ± 0.1^{f}	0.5 ± 0.0^{f}	1.8 ± 0.1^{df}
TFC (mg EP/g)	Freeze dry	32.9 ± 0.2^{a}	31.9 ± 4.8^{a}	28.8 ± 0.0^{a}
	Microwave	19.0 ± 1.0^{bc}	10.8 ± 0.4^{d}	22.8 ± 4.8^{b}
	Oven	1.1 ± 0.4^{f}	0.5 ± 0.6^{f}	14.1 ± 0.8^{cd}
FRSA (%)	Freeze dry	35.8 ± 3.1^{c}	55.1 ± 2.1^{a}	45.7 ± 6.7^{b}
	Microwave	44.4 ± 0.5^{b}	32.5 ± 1.9^{c}	46.2 ± 6.8^{b}
	Oven	8.9 ± 0.9^{d}	4.9 ± 0.1^{d}	8.7 ± 0.3^{d}

[†] Data are given as mean ± SD ($n = 3$).
[‡] Different letter(s) in each group of antioxidant properties are significant differences according to Duncan's new multiple range test ($p < 0.01$).

Table 4 Correlation coefficients (r) for relationships among extraction methods, drying method, total phenolic content, total flavonoid content, and free radical scavenging activity.

	Extraction	Drying	TPC	TFC	% FRSA
Extraction	1	.00	−.12	.15	.09
Drying		1	$-.91^{**}$	$-.90^{**}$	$-.85^{**}$
TPC			1	$.93^{**}$	$.77^{**}$
TFC				1	$.82^{**}$
% FRSA					1

[**] Correlation is significant when $p < 0.01$ (2-tailed).

The drying of herbs inhibits microbial growth and forestalls biochemical changes, and it can give rise to other changes that affect the herb quality. The loss of macromolecules, like phytochemicals, during the heat treatment might be due to harsh drying conditions from the temperature and duration. The thermal drying methods (oven- and microwave-drying) possibly induced oxidative condensation or decomposition of thermolabile phenolic compounds. Some easily volatile phenolics might be lost by volatilizing at higher temperatures[29]. Furthermore, it is possible that at high temperatures, certain phenolics (e.g., polyphenolic condensed tannin) may simply decompose or combine with other plant components that cause difficulty in getting free again during the extraction. Increasing the temperature above 50 °C lowered the phenolic amounts considerably in Salix cv. aquatica leaves[29]. In the case of microwave drying, fast drying at high temperatures can induce polymerization or degradation of thermolabile phenolics[30], and the critical aspect is water vapour, which sometimes causes degradation of thermolabile phytochemicals[13]. Freeze-drying may lead to a higher extraction efficiency of phenolic compounds because freeze-drying can cause the development of ice crystals within the plant matrix. Ice crystals can result in a greater rupturing of the plant cell structure, which may allow for better solvent access and extraction. On the other hand, with oven-drying there is little or no cell rupture and there is the added effect of heat, which can cause losses in phenolic compounds[17,31].

Effect of drying methods on LC-MS/MS of the *E. prostrata* extracts

Freeze-drying with prefreezing of the leaves resulted in no significantly lower concentration of flavonoid glycosides[17]. Hence the extracts from freeze-, microwave-, and oven-dried leaves with soxhlet extraction were investigated and compared. Base peak LC-MS chromatograms of the oven-, microwave-, and freeze-dried leaf extracts revealed interesting results (Fig. 1S).

Acquisition times (ATs) of major peak compounds of the three extracts remained relatively unchanged. The main peak assignments of the LC-ESIQ TOF-MS/MS (Table 5) allowed for the characterization of the constituents of *E. prostrata*, which were mainly flavonoids (luteolin, luteolin glucoside, luteolin sulphate, wedelolactone, dimethylwedelolactone, dimethywedelolactone sulphate, 3′-hydroxybiochanin A), phenolic acids (gallic acid, protocatechuic acid, caffeoylquinic acid, dicaffeoylquinic acid), phenolic aldehyde (protocatechualdehyde), and triterpenoids (eclalbasaponin). Previous LC-MS/MS studies of methanol/water and ethanol/water *E. prostrata* extracts have reported the compounds of luteolin, luteolin-glucoside, 3-hydroxybiochanin A, caffeoylquinic acid, luteoloside, dicaffeoylquinic acid, wedelolactone[32–34], luetolin sulphate, dimethylwedelolactone sulphate, dimethylwedelolactone[34] and eclalbasaponin[6,34]. Phenolics, flavonoids and their glycosides, which are compounds that yield one or more sugars upon hydrolysis, are presented in drugs as monoglycosides or diglycosides[26].

The short thermal microwave-drying might break and rebound the side groups. In comparison with the chromatogram of the freeze-dried leaf extract, the chromatogram of the microwave-dried leaf extract showed increased peaks at RT 15.4 and 16.7 min, and a decrease in peak 11 (16.5 min) implied the splitting and/or increase of peak 10 (16.3 min), which was the same compound as dimethylwedelolactone sulphate. In addition, the long thermal oven-drying at 60 °C for 20–24 h intensely decreased the phenolic and flavonoid compounds of *E. prostrata* leaves, as many peaks were lost without clear new peaks occurring (Fig. 1S). The LC-MS corresponded to the results of the TPC, TFC, and FRSA (Table 2). Declines in the properties were attributed to the thermal degradation of phytochemicals. There was a dramatic decrease in phenolic glycoside concentrations in Populus and Salix leaf extracts due to the thermal oven-drying of the leaf materials, when compared to the freeze-dried and fresh leaves[35]. Short thermal treatment by microwave drying could cause the release of bound phenolic compounds, and is brought about the breakdown of cellular constituents, and the formation of new compounds in leaf extracts of *A. zerumbet*, *Etlingera elatior*, *Curcuma longa*, and *K. galanga*[18].

HPTLC, cytotoxicity and anti-proliferation of dried crude extract

The dried crude extract obtained from the soxhlet extraction of the freeze-dried leaves was dissolved in DMSO. HPTLC was performed on the crude extract, which is not the case with other analytical methods[26]. The TLC profile of the crude extract and the chemical standards caused fluorescence quenching when irradiated with UV light at 254 nm and 366 nm depending on their structural type, as shown in Fig. 1a and Fig. 1b, respectively. The TLC profile following the retardation factor (Rf) indi-

Table 5 LC-ESI-QTOF-MS/MS analysis of phenolic compounds from the *E. prostrata* leaf (freeze dried) extracts obtained from 99.9% (v/v) ethanol fraction.

Peak	RT	ESI-MS m/z						Tentative identification	Formula	CID[†]	Error
No.	(min)	[M-H]	MS/MS fragment							(V)	(ppm)
1	8.8	169.0	125.0					Gallic acid	$C_7H_6O_5$	10	6.16
2	9.7	153.0	109.0					Protocatechuic acid	$C_7H_6O_4$	10	5.40
3	10.4	353.1	191.1	135.0	93.0	85.0		Caffeoyl quinic acid (Chlorogenic acida[†])	$C_{16}H_{18}O_9$	40	2.28
4	11.4	137.0	119.0	108.0	92.0	81.0	53.0	Protocatechualdehyde	$C_7H_6O_3$	20	5.92
5	11.9	447.1	285.0					Luteolin glucoside	$C_{21}H_{20}O_{11}$	20	2.42
6	12.8	447.1	285.0					Luteolin glucoside	$C_{21}H_{20}O_{11}$	20	2.42
7	13.5	515.1	353.1	335.1	191.1	173.0	135.0	Dicaffeoyl quinic acid	$C_{25}H_{24}O_{12}$	20	2.32
8	14.2	515.1	353.1	191.1	173.0	135.0		Dicaffeoyl quinic acid	$C_{25}H_{24}O_{12}$	20	2.32
9	16.1	1003.5	957.5	837.5	795.5	633.4		Eclalbasaponin	$C_{48}H_{78}O_{19}$	20,30	
10	16.3	379.0	299.0					Dimethylwedelolactone sulphate		10	
11	16.5	379.0	299.0					Dimethylwedelolactone sulphate		10	
12	17.3	285.0	285.0	151.0	133.0			Luteolin	$C_{15}H_{10}O_6$	20	3.36
13	17.6	313.0	298.0	270.0				Wedelolactone	$C_{16}H_{10}O_7$	20	2.79
	17.8	313.0	298.0					Wedelolactone	$C_{16}H_{10}O_7$	20	2.79
	18.0	313.0	298.0	270.0				Wedelolactone	$C_{16}H_{10}O_7$	20	2.79
14	18.1	299.1	284.0	255.0	239.0	227.0	211.0	3-Hydroxybiochanin A	$C_{16}H_{12}O_6$	40	3.04
15	19.8	365.0	285.0					Luteolin sulphate	$C_{15}H_{10}O_9S$	10,20,40	3.49

[†] Peaks are compared with standard compounds; CID = collision-induced dissociation.

cated the wedelolactone and luteolin in the crude extract. The detection via spraying with natural reagents (Fig. 1(c,d)) indicated that wedelolactone was highly present in the extract. In addition, the auto fluorescence bands under 366 nm (Fig. 1b) revealed that our plant extract also contained chlorophyll (red) and some compounds (yellow and blue), which were not phenolics and flavonoids.

The HaCaT keratinocyte cell line was used to study the cytotoxicity and anti-proliferation with the MTT assay. The cell survival rates after treatment with each concentration of the *E. prostrata* extract and chemical standards are shown in Fig. 2. The DMSO solvent had neither stimulatory nor inhibitory effects on the growth of HaCaT cells. Fig. 2(a, c, and e) gives evidence that the crude extracts had milder cytotoxic effects than pure marker compounds of wedelolactone and luteolin. Paclitaxel, which is a chemotherapy medication used to treat many types of cancer, showed cytotoxicity to the HaCaT cells, as a positive control.

Table 6 presents the cell cytotoxicity as IC_{50} values that mean the concentration causing half inhibition of the cell growth. The lowest IC_{50} value of paclitaxel was 2.4 ± 0.2 μg/ml followed by luteolin, wedelolactone, and the *E. prostrata* extract as 13.2 ± 2.0, 25.6 ± 1.5, and 271.4 ± 3.9 μg/ml, respectively. In the case of the anti-proliferation property, HaCaT cells were treated with TNF-α to stimulate cell inflammation before being treated with different concentrations of the crude extract and the chemicals. Fig. 2(b, d, f, and g) presents similar trends in comparison with the cell cytotoxicity test. The IC_{50} values of wedelolactone, luteolin and paclitaxel still present the same values on both non-stimulated and TNF-α stimulated cells after anti-proliferation. Interestingly, the *E. prostrata* extract showed a significantly lower IC_{50} value on

Table 6 Cell cytotoxicity and anti-proliferation after TNF-α treatment of the *E. prostrata* extract and standard compounds on HaCaT cell line[†].

Sample	IC_{50} (μg/ml)	
	Cell cytotoxicity	Anti-proliferation
E. prostrata extract	$271.4 \pm 3.9^{a,*}$	$241.5 \pm 5.8^{a,**}$
Luteolin	$13.2 \pm 2.0^{c,*}$	$14.7 \pm 0.9^{c,*}$
Wedelolactone	$25.6 \pm 1.5^{b,*}$	$24.9 \pm 0.6^{b,*}$
Curcumin	$5.7 \pm 0.5^{d,*}$	$6.2 \pm 1.1^{d,*}$
Paclitaxel	$2.4 \pm 0.2^{d,*}$	$2.5 \pm 0.1^{d,*}$

[†] Data are given as mean ± SD ($n = 3$). Different letter(s) in the same column are significant differences according to Duncan's new multiple range test ($p < 0.01$). *,** Indicate significant differences ($p < 0.01$) between cytotoxicity and anti-proliferation for each sample.

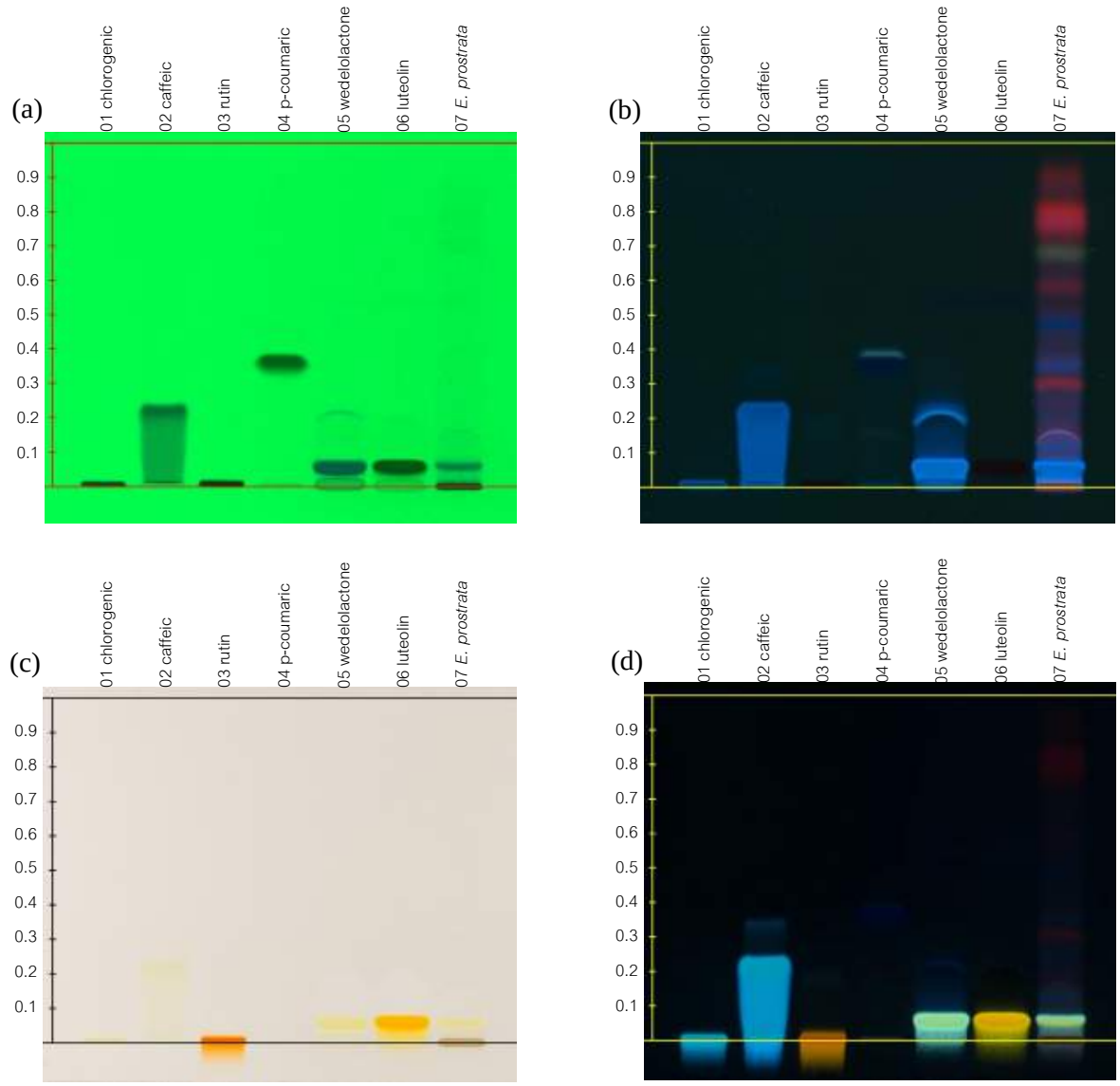

Fig. 1 HPTLC profiles of *E. prostrata* crude extracts from freeze-dried leaves and soxhlet extraction and phenolic standards of chlorogenic acid, caffeic acid, rutin, p-coumaric acid, wedelolactone and luteolin: (a) image at 254 nm, (b) image at 366 nm, and (c,d) images in white light and at 366 nm after derivatized with natural product reagents.

the TNF-α stimulated cells when compared to that on the non-stimulated cells. As the IC_{50} values exceeded 200 μg/ml of the *E. prostrata* extract for the anti-proliferation of HaCaT, they might indicate that it is an ineffective psoriasis treatment[27]. However, an appropriate extraction of *E. prostrata* leaves to obtain high luteolin and wedelolactone levels may be a promising candidate to be developed into local and systemic treatments for psoriasis and other inflammatory skin diseases. The information indicated that luteolin significantly decreased the TNF-induced phosphorylation, nuclear translocation and DNA binding of the nuclear factor-kappa B (NF-κB), which is typically involved in inflammatory mediator transcription[9]. In addition, small-molecule inhibitors of IkB kinase (IKK) inhibition following UVB exposure by wedelolactone prevented the induction of NF-κB, limited inflammation and modulated the cell environment to a non-persuasive state for neoplastic transformation following UVB exposure[10]. The anti-proliferation efficiency of the HaCaT keratinocyte cell line to wedelolactone and

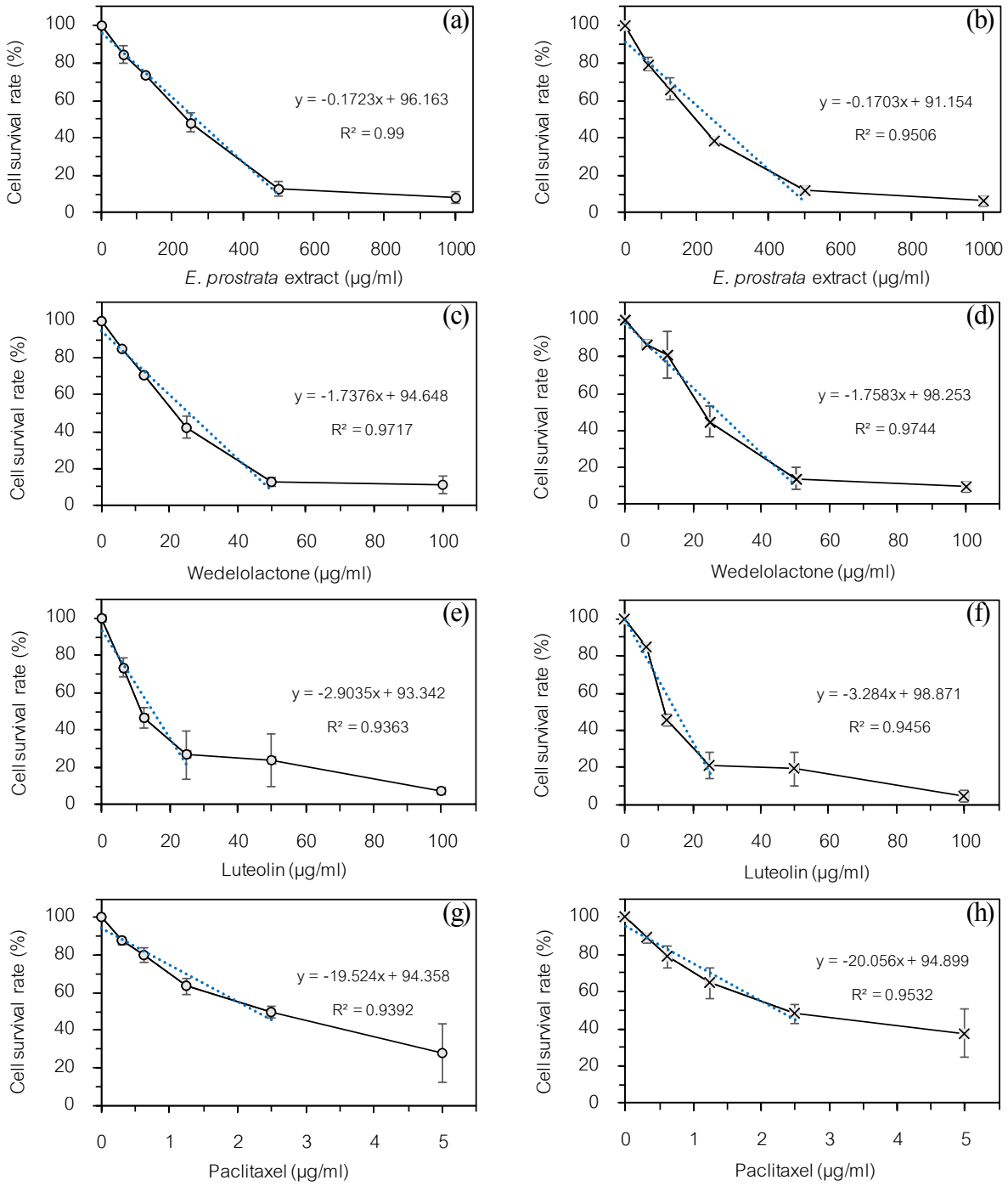

Fig. 2 Cell survival rates of HaCaT cells in the presence of the *E. prostrata extract*, wedelolactone, luteolin, and paclitaxel; (a, c, e, and g) are cell survival rates as affected by presence of the extract and chemicals; (b, d, f, and h) are survival rates due to TNF-α treatment following addition of the extract and chemicals.

luteolin was also reported in this study.

CONCLUSIONS

The variability in phenolic compounds and antioxidant activity of herbal leaves depended on the dry-

ing method. The effect of different drying methods (oven-drying at 60 °C, freeze drying, and microwave drying) on polyphenols and antioxidant activities of *E. prostrata* was investigated in this study. Under ethanolic solvent extraction, the results and correlations among the extraction methods, the drying methods, and the values of TPC, TFC, and FRSA clearly showed that phenolic and flavonoid compounds would be the major antioxidants in *E. prostrata* extracts. The extraction methods seem to have no correlation to the phenolic and antioxidant activities in the extracts, but the drying methods were considered to be a critical factor following the order of freeze-dried, microwave dried, oven-dried leaf extracts. The *E. prostrata* extracts contained luteolin, wedelolactone, and their derivatives, in which the compounds were reduced by thermal oven-drying to very small levels. HPTLC showed mainly wedelolactone, chlorophyll, and some non-phenolic compounds in the crude extract, which was obtained from freeze-dried leaves and soxhlet extraction. The IC_{50} values of the crude extract for anti-proliferation of HaCaT indicated the low efficiency for psoriasis treatment. However, luteolin and wedelolactone, which are the marker compounds of the *E. prostrata* extract, could be candidates to be developed into local and systemic treatments for psoriasis and other inflammatory skin diseases.

The abilities of different ratios of ethanol/water on extraction of phenolics, flavonoids, and their glycosides should be further studied to obtain larger amounts of interesting compounds[6,33], but a too high water content could bring an increased concomitant extraction of other compounds that would lower the phenol concentrations in the extracts[36]. A high yield of phenolic components in a short time and use of less ethanol and energy should be investigated further to provide a valuable and green method.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found at http://dx.doi.org/10.2306/scienceasia1513-1874.2019.45.127.

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Appendix A. Supplementary data

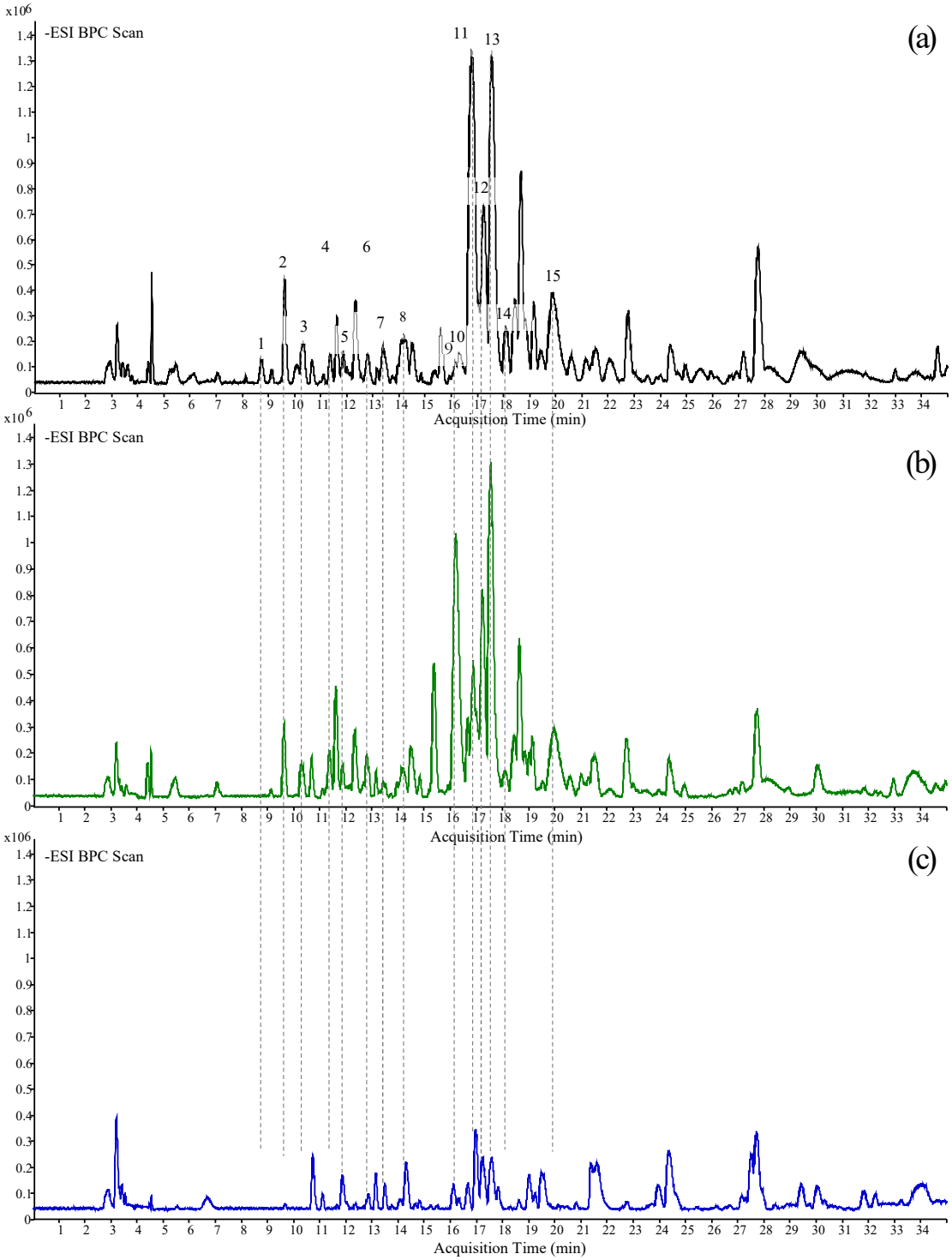

Fig. 1S LC-ESI base peak chromatogram (BPC) of *E. prostrata* extracts obtained from soxhlet extraction with 99.9% (v/v) ethanol of drying leaves by three-drying methods: (a) freeze drying, (b) microwave drying, and (c) oven drying. For main peak assignments, see Table 5.