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Original Research Article

Chemical Constituents of *Vernonia cinerea* (L.) Less and Their Antioxidant Activity

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ABSTRACT

Vernonia Cinerea (L.) Less was used in traditional herbs as it has potential to wound healing, anti-inflammatory, analgesic, antibacterial, reducing smoking addict and antioxidant. This study focused on extraction of *V. cinerea* by hexane maceration. The crude extract was isolated by column chromatography and biology activity of *V. cinerea* extract was tested using DPPH and ABTS radical scavenging assay. The *V. cinerea* extract yielded two isolated compounds, compound C1 which had an IR spectral data for absorption band in wave number at 2921 cm^{-1}, 1462 cm^{-1}, 700 cm^{-1} corresponding to -CH stretching, -CH bending and C-C stretching, respectively. GC-MS chromatogram revealed that m/z at 380 and the molecular formula was $C_{27}H_{56}$ which similar to heptacosane comparison to NIST library. The IR spectral data of Compound C2 showed the presence of OH at 3450 cm^{-1}, C=C at 1723 cm^{-1} and CH_3 at 2933 cm^{-1}. The m/z Ion peak at 426 was suggested to $C_{30}H_{50}O$ comparison with NIST library, was closely to lupeol. The antioxidant activity of both compounds was calculated as Trolox equivalent antioxidant capacity (TEAC). As a result, Compound C2 gave a better antioxidant activity (93.33±0.38 mM trolox/g DM) than compound C1 (80.00±0.61 mM trolox/g DM) ($p<0.05$). In addition, ABTS assay scavenging activity of compound C1 was 200±0.19 mM trolox/g DM and Compound C2 was 333.33±0.41 mM trolox/g DM ($p<0.05$). From all of the results above, this study suggested that *V. cinerea* can be a novel herb for cosmetic and pharmaceutical industrial application in the future.

INTRODUCTION

Vernonia Cinerea (L.) Less (in Thailand called Yha dok khao, Yha laong or Yha mho noi) is a weed which can be found all over the region in Thailand whether in deserted fields or along roadsides causing *V.cinerea* overlooked and minor study of its constituents and bioactivity. In the preliminary study of the phytochemical composition of *V.cinerea* crude extract, it was found that *V.cinerea* petroleum extract contains interesting constituents including alkaloids: antibacterial antipyretic, terpenoids:

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antioxidant, tannin and saponin: wound healing, steroids: antimicrobial, phenolic compounds: antioxidant and lipid: antioxidant (Prabha, 2015). Optical identification techniques of *V. cinerea* low polarity crude extract showed terpenes and steroids (Misra et al. 1984) which have hydroxyl (-OH) groups in their structure serve to return electrons to unstable free radicals to their more stable form include anti-inflammatory effects of skin proteins (Fadillah & Santoso, 2019), (Kumpunya & Praputbut, 2014). Oth *V.cinerea* extract was also able to fight against the proliferation of gram-positive bacteria (S. aureus and B. subtilis) and gram-negative bacteria. (E. coli, K. pneumonia, P. aeruginosa, and P. vulgaris) (Sonibare et al. 2016). In addition, the distinctive feature that made *V.cinerea* more well-known is that *V.cinerea* is a herb that reduces cravings for cigarettes that have been added to Thai's herbal recipe this is because *V.cinerea* extract contains nitrate, which helps the tongue to numb and less smell the taste of cigarettes (Monton & Luprasong, 2019) and nicotine, which can replace the nicotine that the body needs from cigarettes, thereby reducing the craving for cigarettes eventually (Promputta et al. 2020). However, bioavailability testing of the purified *V.cinerea* hexane extract is still limited and still needs to study further. Therefore, this research aimed to study the low polarity chemical constituent of *V.cinerea* extracted with hexane maceration to extract oil parts as hexane was less harmful, commercial favor and low price isolated by silica gel chromatography to purify the crude extract. The structure of the purified substance of *V.cinerea* was identified by IR, UV-vis and GC-MS along with the antioxidant activity using DPPH and ABTS assays to open a brand new super beneficial Thai herb to the international medical cosmetic industry.

MATERIALS AND METHODS

Plant material

All parts of fresh *V. cinerea* were collected from Ubon Ratchathani in Thailand. The plant was washed and dried under hot air and then ground into powder. Powder of *V.cinerea* was kept in a sealed bag waiting for the extraction process.

Extraction and isolation

Powder of *V. cinerea* (10 kg) was macerated in hexane for 10 days at room temperature. The resulting solution was filtered using Whatman filter paper. The liquid part was evaporated by a rotary evaporator at a reduced pressure of 50 °C. The weight of crude extracts was recorded and yields of all crude extracts were calculated. The *V.cinerea* hexane crude extract was subjected to silica gel column chromatography using hexane: ethyl acetate as elute solvent with gradually increased polarity starting from hexane (100:0 to 0:100). A total of 3 fractions: fraction 1 (1.8 g), fraction 2 (11.6 g) and fraction 3 (5.8 g) were collected and analyzed by TLC. Fraction 1 was recrystallized by ethyl acetate afforded compound C1 as white powder (1.6 g) and fraction 2 was subjected to silica gel column chromatography with the same eluent giving 13 subfractions. Fractions 8 (4.3 g) and 9 (4.1 g) were combined and then subjected to a small column using hexane as eluent afforded compound C2.

Thin layer chromatography (TLC)

3 x 5 cm of TLC plate was performed to identify the components present in each of the isolated compounds of *V.cinerea* hexane extracts. Small spots of components were developed in a TLC chamber using hexane as a solvent system and clarified under UV light at 254 nm.

Fourier-transform infrared spectroscopy (FTIR)

FTIR was used to analyze the functional groups of natural compounds. It helps to confirm the structure of natural substances to be more accurate. *V. cinerea* extract powder was mixed with KBr salt, using a mortar and pestle, and compressed into a thin pellet. Small drops of *V.cinerea* oil extract were placed on the KBr disc. Infrared spectra were recorded as KBr pellets on a Perkin Elmer Spectrum 2000 transmission, Waltham, the USA between 4000 – 400 cm^{-1} according to Abera et al. (2018).

Ultraviolet-visible spectroscopy (UV-vis)

UV absorbing behaviour of *V.cinerea* extract was analyzed by Specord 210 plus, Jena, Germany according to Jain et al. (2016). 2 ml of the extract was added to cuvette as a sample, hexane was added to another cuvette as a reference to find λmax of extract.

Gas chromatography-mass spectrometry (GC-MS)

1 ml of *V.cinerea* hexane extract was injected into Agilent Technologies 7890B, Santa Clara, US. Column width 30 m x 0.25 mm x 0.25 μm of a capillary column, the oven temperature was programmed to raise to 100 °C (isothermal 2 min) at 4 °C/min then raised to 220 °C (isothermal 2 min) at 15 °C/min and increased to 300 °C (isothermal 2 min), operating in electron impact mode at 70 eV; helium was used as carrier gas with a constant flow rate of 1 ml/min according to Abirami & Rajendran (2012).

Determination of antioxidant activity

DPPH assay free radical scavenging activity (DPPH)

DPPH assay was modified according to the method of Olamide et al. (2017). A solution of 0.1 mM 2,2-Diphenyl-1-picrylhydrazyl (DPPH) and methanol was prepared. And then 2 ml of DPPH solution was added and mixed quickly with 150 μl of testing sample solution. The mixture was kept at room temperature for 30 min. The absorbance of the solution at 517 nm was recorded. A mixed solution of 1350 μl of DPPH and 150 μl of DMSO was used as a control. % Radical scavenging activity (%RSA) was obtained from the following equation:

$$\%RSA = (Abs.control-Abs.sample)/(Abs.control) \times 100 \quad (1)$$

where Abs. control was the absorbance of the addition of methanol instead of the testing sample and Abs. the sample was the absorbance of the testing sample solution. The %RSA of extract values were compared with antioxidant activity of Trolox standard curve, y = -5.8822x + 0.9885 R2 = 0.9949. The slope values were calculated for the Trolox Equivalent Antioxidant Capacity (TEAC) of the extract which reported as mM trolox/g DM

ABTS assay free radical scavenging activity (ABTS)

ABTS assay was modified according to the method of Olamide et al. (2017). The reagent was prepared by mixing 10 ml of (3-ethylbenzothiazoline-6-sulphonic acid) ABTS (7 mM) and 5 ml potassium persulphate (2.45 mM). The mixture was kept in the dark for 12 h. The absorbance of the reagent was adjusted with distilled water to 0.700 ± 0.030 at 734 nm and then used for the assay. A stable ABTS radical cation is produced by oxidation of ABTS by potassium persulphate. 150 µl of plant extract was added to the 1350 µl reagent mixture. After incubation for 10 minutes, the reduction of radical cation was measured at 734 nm. The potential to scavenge ABTS radical was calculated by equation (1). The %RSA of extract values were compared with antioxidant activity of Trolox standard curve, y = -2.8809x + 0.5544 R2 = 0.9993. The slope values were calculated for the Trolox Equivalent Antioxidant Capacity (TEAC) of the extract which reported as mM trolox/g DM

Statistical Analysis

Results of antioxidants activity of *V.cinerea* extracts were using one-way analysis of variance (ANOVA). Statistical analysis was performed using Statistical Package for Social Science (SPSS) with a significance of $p < 0.05$ used throughout the study. All the experiments were performed in triplicates and expressed as mean ± standard deviation.

RESULTS AND DISCUSSION

The all part of *V.cinerea* (10 kg) was collected from Ubon Ratchathani, Thailand grounded and extracted using hexane maceration. The organic phase was separated and concentrated yielding deep brown crude extract (143 g, %yield = 1.43%) which was isolated by silica gel column chromatography using hexane: ethyl acetate as diluent with gradually increased polarity to afford 3 fractions. Compound C1 (1.6 g, %yield = 0.016%) was obtained from fraction 1 hexane isolation as white powder melting point was 60 °C appeared one spot on TLC plate when placed under UV light 254 nm with R_f value of 0.4 in hexane solvent system. Compound C2 was isolated from silica gel column chromatography eluted by hexane as a clear oil. Compound C2 (1.2 g, %yield = 0.012%) has R_f values of 1.4 in the hexane solvent system. The structure of two isolated compounds was identified and characterized by spectroscopic techniques based on the library and literature that have been studied.

FTIR

FTIR analysis is a technique used to verify the identity of natural extracts from interactions of functional groups when exposed to radiofrequency. The FTIR spectrum of compound C1 was shown in (figure 1.). The absorption oscillated at the 2921 cm^{-1} range due to the radiofrequency response of $-CH_3$ groups. The bands at 1462 cm^{-1} could be CH_2 stretchings. Bands at 1400 cm^{-1} were $-CH_2$ deformation and bands at 700 cm^{-1} were suggested as C-C stretching. Thus, the IR spectrum of compound C1 indicates the presence of functional characteristics associated with alkane. Compound C2 showed an interaction at 3450 cm^{-1} with a weak broad spectrum observed for the hydroxyl group (-OH) bond vibration. The stretching and bending vibrations of CH_3 were noticed at 2933 cm^{-1} and C=C vibrations were shown around 1723 cm^{-1} (Jain & Bari, 2010) as shown in (figure 1.) From the results of the FTIR analysis it was assumed that compound C2 could be a terpenoids, steroids or phenolic compounds.

UV-vis

The UV-vis analysis was performed to identify the absorption behaviour of compounds containing σ-bonds, π-bonds, lone pair of electrons, chromophores and aromatic rings in *V.cinerea* hexane extract. UV-vis spectra of compound C1 (figure 2.) absorbed UV light at the wavelengths 234 and 271 nm. Compound C2 absorbed UV light at a wavelength of 285 nm (figure 2). UV-vis spectra of two compounds were appearance peak in the region from 200 – 400 nm indicating the presence of unsaturated groups and heteroatoms such as S, N and O (Jain et al. 2016) and this confirms the presence of organic chromophores within *V.cinerea* hexane extract.

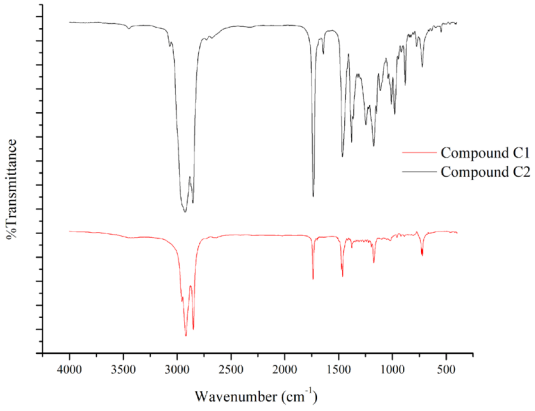

Figure 1. FTIR of compound C1 and Compound C2 from isolated *V.cinerea* extract

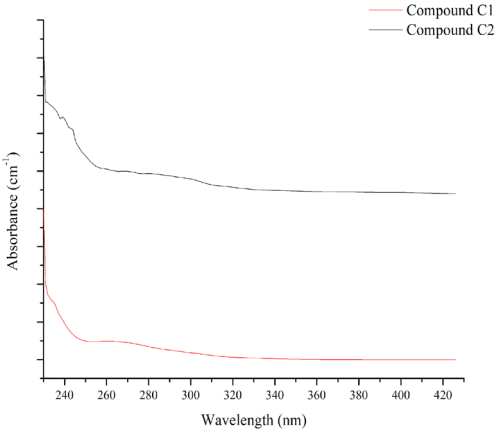

Figure 2. UV-vis spectra of compound C1 and Compound C2 isolated from *V.cinerea* hexane extract

GC-MS

After TLC, IR and UV preliminary structure identified of *V.cinerea* hexane extract. Those isolated compounds were analyzed by GC-MS. Retention time (Rt), %Peak area and mass spectra were compared with those mass spectra of authentic samples from a data library. As shown in (figure 3.) compound C1 has an intensive retention time peak at 39.937 min which had a peak area of 1.94%. From mass spectra (figure 4.) of the compound, C1 found mass molecular $C_{27}H_{56}$ and ion peak 151 m/z that closely to be Heptacosane with %probability was 14.50%. GC chromatogram of Compound C2 (figure 3.) showed strongest Rt peak at 40.687 min with 1.57% % peak area and ion peak was 339 m/z compared mass spectra (figure 4.) with NIST library found Compound C2 was closely to be Lupeol with 83.6% probability (molecular formula $C_{30}H_{50}O$, molecular mass 426).

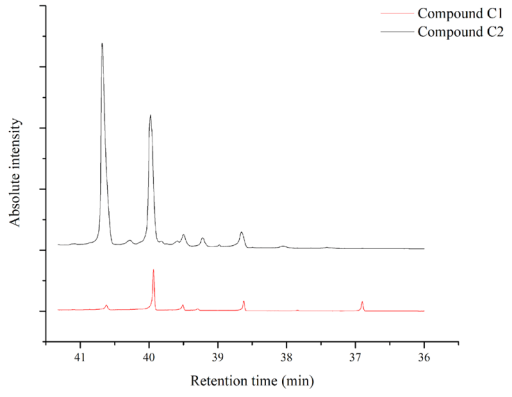

Figure 3. GC chromatogram of compound C1 and Compound C2

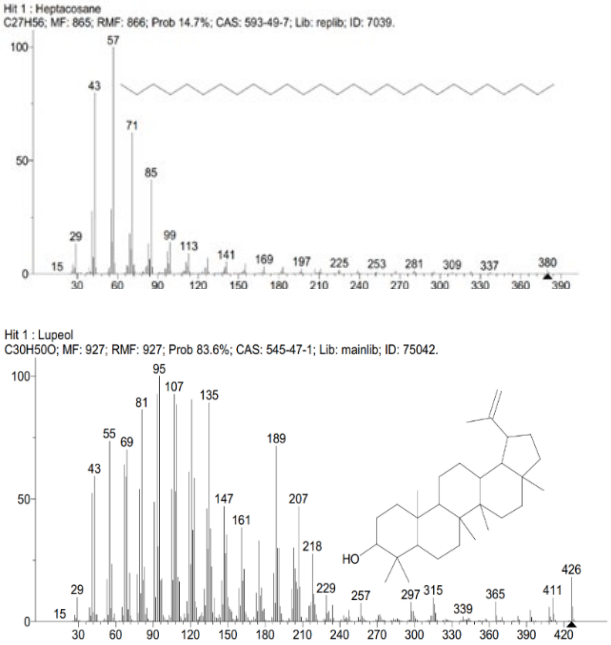

Figure 4. Mass spectra of compound C1 and Compound C2

Determination of antioxidant activity

DPPH assay

DPPH• radical scavenging activity of *V.cinerea* extracted by macerated in hexane and isolated by Silica gel column chromatography is shown in Table 1 and the results were comparable to that of Trolox, a commercial antioxidant agent. Compound C1 showed %RSA of DPPH• radical 15.47%. TEAC of compound C1 was 80.00 mM trolox/g DM when compared with Trolox standard curve (y = -5.8822x + 0.9885 R^2 = 0.9949). Compound C2 was 21.77% DPPH• radical capture and has TEAC 93.33 mM trolox/g DM. The results were observed that Compound C2 had a greater radical scavenging activity than compound C1 due to hydroxyl groups (OH), carbonyl groups (C=O) and double bonds (C=C) in the structure of compound C2 that delocalized radical from DPPH• to make it more stable.

Table 1. Absorbance at 517 nm, %RSA of DPPH• radical and TEAC of an isolated compound of *V.cinerea* hexane extract

Compound	Absorbance (nm)	%RSA of DPPH• radical	TEAC (mM Trolox /g DM)
A	0.6349±0.01[b]	15.47±1.81[b]	80.00±0.61[b]
B	0.5876±0.01[a]	21.77±4.09[a]	93.33±0.38[a]

The concentration of the sample was 1 mM. Data expressed as mean ± SD (n = 3, $p < 0.05$). The absorbance of control was 0.7511±0.02 nm

ABTS assay

Results from table 2 $ABTS^{\bullet+}$ radical scavenging activity of *V.cinerea* hexane extract showed a similar to DPPH radical scavenging activity. Compound C1 has a lower %RSA of $ABTS^{\bullet+}$ radical and TEAC (10.64%, 200.00 mM trolox/g dried DM) than Compound C2 (19.35%, 333.33 mM trolox/g dried DM). This could explain by the electron delocalization potential from the functional groups in the structure of compound 2 such as OH, C=O and C=C that delocalize radical from $ABTS^{\bullet+}$ radical.

Table 2. Absorbance at 734 nm, %RSA of $ABTS^{\bullet+}$ radical and TEAC of an isolated compound of *V.cinerea* hexane extract

Compound	Absorbance (nm)	%RSA of $ABTS^{\bullet+}$ radical	TEAC (mM Trolox /g DM)
C1	0.4696±0.01[b]	10.64±1.55[b]	200.00±0.19[b]
C2	0.4238±0.01[a]	19.35±1.70[a]	333.33±0.41[a]

The concentration of the sample was 1 mM. Data expressed as mean ± SD (n = 3, $p < 0.05$). The absorbance of control was 0.5255±0.01 nm

CONCLUSIONS

The hexane extraction of *V.cinerea* and isolated by Silica gel column chromatography had given one primary metabolite and one secondary metabolite compound which were lipid (compound C1) and Triterpenoids (Compound C2). The results from spectroscopic characterizations identified the structure of two isolated compounds and revealed that compound C1 was

heptacosane and Compound C2 was Lupeol. The antioxidant activity of two isolated compounds showed a good radical scavenging agent that could be applied and used to avoid organs damages, wrinkles, cancer and all possible health problems.

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