Physicochemical Properties and Antibacterial Activity of Essential Oil of *Ageratum conyzoides* L. Leaves

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Summary

Essential oil (EO) of *Ageratum conyzoides* L. leaves was analyzed by gas chromatography – mass spectrometry (GC-MS) method. Physicochemical properties (acid value, saponification value, ester value, density and freezing point), antioxidant capacity and antibacterial capacity were determined. In general, 34 compounds were identified in EO of this material. Moreover, EO has the antioxidant capacity, and the antimicrobial activity was determined by the paper disc diffusion method for antibiotic susceptibility testing. Essential oil does not affect the growth of *Staphylococcus aureus* - ATCC 25923, *Bacillus subtilis* - ATCC 11774 and *Escherichia coli* - ATCC 25922. However, the result also shows that EO of *A. conyzoides* leaves can inhibit the growth of *Salmonella enteritidis* - ATCC 13076.

Key words

antimicrobial activity, antioxidant capacity, bacteria, GC-MS, plants

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Introduction

Ageratum conyzoides L. (*Asteraceae*) is an annual herb that has a lot of traditional medicinal uses in many different countries and it is also known as billy goat weeds (Okunade, 2002; Singh et al., 2013). It is widely used in various countries, especially tropical countries (Vera, 1993). The stems and leaves are covered with white hairs, the leaves are ovate and up to 7.5 cm in length. The flowers are purple or white color, less than 6 mm across and arranged in close terminal inflorescences. The fruits are achene and are easily dispersed while the seeds are photoblastic and often lost within 12 months (Shekhar and Anju, 2012). *Ageratum conyzoides* is used to treat diseases in central Africa such as pneumonia and burns (Durodola, 1977). The leaves or stems of this plant are utilized against inflammatory stomach or intestine diseases. Additionally, *A. conyzoides* is used in traditional medicine in Asia, South America and Africa (Vera, 1993).

In Vietnam, the local citizens exploited essential oil from the leaves of this plant. According to Osho and Adetunji (2011), the EO of *A. conyzoides* had the strong antibacterial and antifungal activities, for instance, *Bacillus subtilis* (ATCC 6633), *Klebsiella pneumoniae* (ATCC 35657), *Staphylococcus aureus* (ATCC 24213), *Pseudomonas aeruginosa* (ATCC 9027), *Candida albicans* (ATCC 10231), *Candida stellatoidea* (ATCC 20408) and *Candida glabrataz* (ATCC 15126). Besides, the essential oil of this plant also has anti-aflatoxigenic and antioxidant activity (Patil et al., 2010). Some bioactive compounds from this EO can be widely used in the agriculture. They have insecticide effect against *Callosobruchus maculatu* (Gbolade et al., 1999), *Tribolium castaneum* Herbst (Jaya et al., 2014) and *Aedes albopictus* (Liu and Liu, 2014).

Currently, there are many studies related to *A. conyzoides* EO. However, until now there are no studies on components, physicochemical properties, antioxidant activity and antibacterial capacity of this EO in Vietnam. Therefore, the main aim of this study is to determine all above properties of *A. conyzoides* EO.

Materials and methods

Plant material and essential oil extraction

Fresh leaves of *A. conyzoides* were harvested from Quang Tri Province (Vietnam). They were distilled by a Clevenger-type apparatus. Leaves were completely soaked in water and heated to boiling (leaves/water ratio of 1/10). After that, EO was evaporated together with water vapor and finally collected after decantation. The received EO was stored at 4°C until being analyzed.

Bacteria strains

Antibacterial activity was determined against two gram-positive bacteria such as *Bacillus subtilis* (ATCC 11774), *Staphylococcus aureus* (ATCC 25923) and two gram-negative bacteria such as *Salmonella enteritidis* (ATCC 13076) and *Escherichia coli* (ATCC 25922).

Chemicals and reagents

DPPH (1,1-diphenyl-2-picrylhydrazyl) reagent was purchased from Sigma-Aldrich (USA). All organic solvents and other chemicals used in this study were of analytical reagent grade.

Determination of the relative density of EO

According to TCVN 8444:2010 (National technical regulation of Vietnam), the relative density was evaluated by the ratio between the mass of a certain volume of the EO and the mass of the same volume of distilled water taken at the same temperature.

Determination of the freezing point of EO

According to TCVN 8447:2010 (National technical regulation of Vietnam), 5 mL of EO was added into the test-tube. The test-tube was put into the freezing container. The temperature was decreased until the crystallization of EO appeared.

Determination of acid value (AV) of EO

The acid value was determined by the titration method. The EO (1 g) was dissolved in 5 mL of 96% ethanol and about three drops of 1% phenolphthalein were added to the alcoholic solution. The mixture was titrated by 0.1N KOH until the solution turned pink (Ouis and Hariri, 2018).

$$AV=(V_{KOH}\times 0.1\times 56.1)/(Mass\ of\ essential\ oil)$$

Determination of saponification value (SV) of EO

The EO (1.5 g) was put into a glass flask (250 mL), and 25 mL of the ethanol solution of 0.5 mol/L KOH was added. The mixture was heated for 1 hour in the condenser system, then 25 mL of the deionized water and five drops of 1% phenolphthalein were added. The solution was titrated by 0.5N HCl until the solution turned colorless.

$$SV=((V_{blank}-V_{sample})\times 56.1\times 0.5)/(Mass\ of\ essential\ oil)$$

Determination of antioxidant activity of EO

The antioxidant activity of the essential oil to scavenge DPPH free radicals was determined as described by Kirby and Schmidt (1997) with some small modifications. The EO was dissolved in ethanol to achieve the various concentrations (1, 2, 4, 8, 16, 32, 64 mg/mL). The amount of 50 µL of the solution was mixed with 1950 µL of the ethanol solution of DPPH (6×10^{-5} M). The solution was kept in dark for 30 minutes at room temperature (Ouis and Hariri, 2018). Antioxidant activity was recorded by monitoring the decrease in absorbance at 517 nm against a blank consisting of ethanol solution.

$$\%inhibition = (Absorbance\ of\ control - Absorbance\ of\ sample) / (Absorbance\ of\ control) \times 100$$

Determination of antibacterial activity

Antibacterial activity was determined by the paper disc diffusion method for antibiotic susceptibility testing with some slight modifications (Bauer et al., 1966). At first, 100 µL of bacteria suspension (0.5 McFarland standard, approximately 1.5×10^{8} cfu/mL) were spread on MHA media (Mueller-Hinton agar) by a sterile hockey stick. Then, the sterile paper 6 mm in diameter discs were impregnated by the selected EO (10 µL), while gentamicin (10 µg/disc) was used as positive control to compare the antibacterial activity of the EO and 20% dimethylsulfoxide (DMSO) solution (10 µL/disc) was used as negative control. These dishes were

incubated for 24 hours at 37°C and the diameter of the inhibition zones were expressed in mm including disk diameter of 6 mm.

Analysis of essential oil by GC–MS method

The volume of 1 µl of EO was injected into a gas chromatograph (GC, model 6890N, Agilent Technologies, USA) equipped with a quadruple mass analyzer (MS, Agilent 5972) in the electron impact (EI) ionization mode (70 eV), injector split/splitless. A capillary column (HP-5ms, 30 m × 0.25 mm × 0.25 µm, Agilent Technologies, USA) was used with helium as a carrier gas at a constant flow of 1 mL/min. Temperature: injector = 280°C, column = 60°C (5 min), 15°C/min, 300°C (5 min).

Data analysis

Experimental results were analyzed by the one-way analysis of variance (ANOVA) method and significant differences among the means from triplicate analyses at $p < 0.05$ were determined by Fisher's least significant difference (LSD) procedure using Statgraphics software (Centurion XV). The values obtained were expressed in the form of a mean ± standard deviation (SD).

Results and Discussions

Determination of physicochemical properties of EO

The results of physicochemical properties presented in Table 1 show that the pH value of *A. conyzoides* EO was approximately 4.46. The result is similar to that of EO of *Ceratonia siliqua* L. pulp (pH = 4.3) and lower than that of EO of *Ceratonia siliqua* seeds (pH = 5.2) according to study of Ouis and Hariri (2018). This shows that the components of initial material strongly affect pH.

Table 1. Physicochemical properties of *Ageratum conyzoides* leaves essential oil

No	Physicochemical properties	Value
1	pH	4.46±0.058
2	Relative density at 20°C	0.8891±0.0023
3	Freezing point (°C)	-10.33±0.577
4	Acid value (mg KOH/g EO)	1.68±0.000
5	Saponification value (mg KOH/g EO)	3.72±0.006

In general, almost the relative density of EOs is lower than 1 mg/mL and that of *A. conyzoides* EO at 20°C was also 0.8891 mg/mL. However, this relative density is higher than that of coriander EO (0.8737 g/mL; Porter and Lammerink, 1994) and *Ceratonia siliqua* pulp EO (0.833 g/mL; Ouis and Hariri, 2018), but it is lower than that of cumin seeds EO (0.936 g/mL), allspice berries EO (0.978 g/mL) and basil leaves EO (0.912 g/mL) according to results of Martinez-Velazquez et al. (2011) and it is in agreement with kanuka leaves EO (0.8849 g/mL; Porter and Lammerink, 1994). The difference of the relative density of EO depends on many factors such as the temperature and composition of EOs. For all the EOs, the density decreased as the temperature increased (wild thyme, kanuka, lavandin, tarragon, etc.; Porter and Lammerink, 1994).

In fact, EOs will freeze if the temperature is low enough. Essential oils have many different freezing points and it depends on the composition of EO. In this study, freezing point of *A. conyzoides* EO was -10.33°C. It is lower than that of *Eucalyptus camaldulensis* Dehnh. leaves EO (0°C; Abdul-Majeed et al., 2013) and higher than that of cocoa beans EO (-16°C; Bainbridge and Davies, 1912).

The saponification (SV) and acid value (AV) of *A. conyzoides* EO are 3.72 mg KOH/g EO and 1.68 mg KOH/g EO, respectively. Both values in this study were quite low, lower than those of *Ocimum sanctum* L. leaves EO (SV = 35.375 mg KOH/g EO, AV = 2.3 mg KOH/g EO) and *Zingiber officinale* Rosc. rhizome EO (SV = 23.84 mg KOH/g EO, AV = 4.48 mg KOH/g EO) according to results of Tripathi et al. (2008). The SV and AV are two main physical properties to evaluate the quality of EO. However, these parameters depend on distillation techniques, climatic conditions, varieties, regions, genotype, harvest periods, etc.

Determination of antioxidant activity of EO

Figure 1 shows that free radical scavenging activity of the *A. conyzoides* leaves EO was concentration-dependent. It can bleach DPPH's purple color; antioxidant activity increases with the increase of concentration of EO. The IC_{50} value of the *A. conyzoides* leaves EO was approximately 8 mg/mL. However, the IC_{50} of *A. conyzoides* leaves EO was quite higher than those of some previous studies, such as EOs of *Piper betle* L. and *Thymus caramanicus* Jalas whose IC_{50} values were 4 and 263 µg/mL, respectively (Prakash et al., 2010; Safaei-Ghomi et al., 2009). Sharififar et al. (2007) noticed that free radical scavenging activity of EOs depends on the presence of bioactive compounds or synergistic effect of overall compounds. Hence, the *A. conyzoides* leaves EO can be used in the food industry to preserve or improve the quality of food.

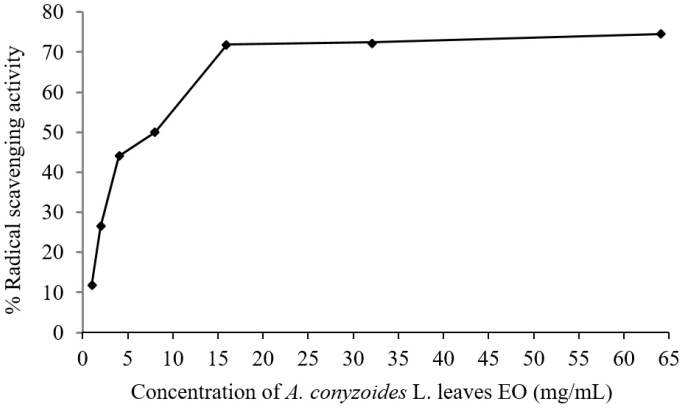

Figure 1. Radical scavenging activity of *Ageratum conyzoides* leaves essential oil

Determination of the chemical compositions of EO

Data analysis of *A. conyzoides* leaves EO led to the identification and quantification of a total of 34 main components accounting for 99.65% of the total components present (Table 2). The major components were Bicyclo [3.1.0] hexane, 6-methylene-, 1,5-Heptadiyne, N-Methyl-7-azabicyclo (2,2,1) hept-2, 4-Oxo-4,5,6,7-tetrahydrobenzofurazan, Tricyclo [3.2.2.0] nonane-2-carboxylic acid, etc. In studies on the chemical composition of *A. conyzoides* aerial parts EO by GC-MS analysis obtained a total

Table 2. Chemical compositions of *Ageratum conyzoides* leaves essential oil

No	Compound	Rt. (min.)	(%)
1	Bicyclo [3.1.0] hexane, 6-methylene-	4.379	12.09
2	Bicyclo [10.1.0] trideca-4,8-diene- 3-carboxamide, N-(3-chlorophenyl)-	4.508	0.34
3	Cyclopropene, 3-methyl-3-vinyl-	4.816	0.26
4	Butanedinitrile	4.856	0.27
5	3-Methyl-1-hexyne	5.054	3.08
6	Bicyclo [2.1.1] hex-2-ene, 2-ethenyl-	5.153	0.38
7	7-Chlorobicyclo [4.1.0] hept-3-ene	5.203	0.17
8	1,6-Heptadiyne	5.342	0.22
9	1,3-Pentadiene	5.520	4.41
10	3-Hexen-1-yne, (Z)-	5.699	1.47
11	2-(2-Hydroxy phenoxy)-1-phenylethanol	5.848	3.05
12	1-Hepten-6-yne	6.145	1.92
13	1,3-Hexadiene, 2,5-dimethyl-	7.832	0.36
14	Spiro [cyclopropane-1,2'-[6.7] diaza bicyclo [3.2.2] non-6-ene]	8.248	2.16
15	1,5-Decadiyne	8.526	0.57
16	Bicyclo [4.1.0] heptane,-3-cyclopropyl,-7-hydroxymethyl, (cis)	8.655	3.74
17	Tricyclo [3.2.2.0] nonane-2-carboxylic acid	8.913	11.04
18	Cyclohexanemethanol, 4-methylene-	8.963	4.25
19	2,3-Hexadiene, 2-methyl-	9.052	1.26
20	4-Oxo-4,5,6,7-tetrahydrobenzofurazan	9.181	11.04
21	1,5-Heptadiyne	9.409	19.53
22	N-Methyl-7-azabicyclo (2,2,1) hept-2-ene	9.488	7.62
23	2,6-Dimethyl-8-oxoocta-2,6-dienoic acid, methyl ester	9.568	1.9
24	Borinic acid, diethyl-, 1-methyl-2-propynyl ester	9.766	0.24
25	Dihydromyrcene	9.835	0.18
26	3,4-Octadiene	9.895	0.78
27	Bicyclo [5.1.0] octane, 8-methylene-	9.945	1.05
28	Dihydromyrcene	9.984	0.77
29	2-Cyclopenten-1-one, 2-methyl-	10.083	0.32
30	3,4-Pentadienal, 2,2-dimethyl-	10.252	1.76
31	9-Azabicyclo [6.1.0] non-4-en-9-amine, (1.alpha.,4Z,8.alpha.)-	10.331	1.42
32	1,1-Dicyanoethane	10.421	1.34
33	Propane, 1,2-dichloro-	10.699	0.3
34	Cyclopentanol, 3-methyl-	12.534	0.36

of 32 components, of which precocene II (45.75%), precocene I (14.09%), β-caryophyllene (12.13%), geracrene D (4.18%) and caryophyllene oxide (4.06%) were the major components (Liu and Liu, 2014). According to Nogueira et al. (2010), the chemical components of GC-MS analysis of *A. conyzoides* aerial parts EO contained precocene II (46.35%), precocene I (42.78%), cumarine compounds (5.01%) and trans-caryophyllene (3.02%). These compounds have important role in the antibacterial mechanism of EOs. Differences in *A. conyzoides* EO can be attributed to parts of plant, extraction method, climatic conditions, varieties, regions, genes and harvest period.

Determination of antimicrobial activity of EO

Gentamicin was used as positive control and inhibited all bacteria in this study. Antibacterial capacity for each bacteria strain was very different. Inhibitor zone of positive control listed in susceptible order: *S. aureus* < *E. coli* < *B. subtilis* < *S. enteritidis*. Table 3 also shows that the *A. conyzoides* leaves EO has the positive antibacterial activity against *S. enteritidis* (inhibitory zone of 15.33 mm). The inhibition zones of *S. enteritidis* are considered "very sensitive" (inhibition diameter was observed in range of 15 to 19 mm; Ponce et al., 2003). It does not inhibit *B. subtilis, E. coli* and *S. aureus*. The achieved results are not similar to those of Patil et al. (2010), where *A. conyzoides* plants EO was recorded against the bacteria *S. aureus, B. subtilis* and *E. coli*. In addition, *A. conyzoides* flowers EO in study of Kouame et al. (2018) inhibited the growth of *S. aureus* and *E. coli*. The difference of antibacterial activity between other studies can be attributed to the difference in chemical composition of EOs.

Table 3. Antibacterial zones of *Ageratum conyzoides* leaves essential oil

No	Microorganisms	Diameter of the inhibitory zones of gentamycin (mm)	Diameter of the inhibitory zones of EO (mm)
1	*S. enteritidis*	24.83 ± 0.29^{Bd}	15.33 ± 0.58^{A}
2	*E. coli*	16.33 ± 0.58^{b}	–
3	*B. subtilis*	21.33 ± 0.58^{c}	–
4	*S. aureus*	15.33 ± 0.58^{a}	–

Different lowercase letters in the same column denote significant differences ($p < 0.05$) with respect to the types of microorganisms

Different capital letters in the same row denote significant differences ($p < 0.05$) with respect to the antibacterial agents

Conclusion

The EO of the leaves of *A. conyzoides* collected in Quang Tri Province shows the antioxidant activity and inhibits the growth of *S. enteritidis*. In addition, some physicochemical properties were also determined. By GC-MS method, 34 main components were identified in *A. conyzoides* leaves EO. This is a cheap source of natural antioxidant substances and could be used in food technology.

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