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EVALUATING SOME *IN VITRO* BIOACTIVITIES OF ETHANOL EXTRACT FROM *AGERATUM CONYZOIDES* L. LEAVES COLLECTED IN VIETNAM IN SUPPORTING SKIN WOUND TREATMENT

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ABSTRACT

Ageratum conyzoides L. is widely used for the treatment of skin wound in some communities in Asia, Africa, and South America, including in Vietnam. In this study, we demonstrated that the 70% ethanol extract of *A. conyzoides* L. leaves collected in Bidoup National Park, Nui Ba, Lam Dong, Vietnam had some properties that would be advantageous for the treatment of skin wounds. Firstly, we found that the extract contained 64.9±2.58 mgGAE/gE polyphenols and 79.33±1.03 mgQE/gE flavonoids, and had antioxidant activity with the IC_{50} of 131.74±2.67 µg/mL. This extract was also proven to have antimicrobial activities against some pathogenic bacteria, including *S. aureus*, *P. aeruginosa*, *E. faecalis*, and *E. coli*. We also demonstrated that this extract could inhibit the generation of nitric oxide in LPS-activated Raw 264.7 cells, indicating its *in vitro* anti-inflammatory activity. And finally, for the first time, we found that the ethanol extract of *A. conyzoides* leaves could promote the proliferation of fibroblast NIH-3T3 cell line. All together, these findings support the traditional use of this plant in skin wound treatment.

Keywords: skin wound treatment, *Ageratum conyzoides* L., antioxidant activity, antimicrobial activity, anti-inflammatory activity, proliferation effect.

INTRODUCTION

Ageratum conyzoides L. is an aromatic herb belonging to the Asteraceae family. This plant is native to Central and South America but it has now spread to various countries across Africa, America, Australia, and Asia with the common name “Billy goat weed” (Kaur *et al.*, 2023).

A. conyzoides L. has been widely used as a traditional medicinal herb for the treatment

of skin inflammation and wounds in Asia, Africa, and South America (Kamboj *et al.*, 2008a; Okunade, 2002; Singh *et al.*, 2013). In Vietnam, the leaves of this plant were traditionally used to treat skin wounds (Đỗ Huy Bích *et al.*, 2006; Đỗ Tất Lợi, 2004). Various previous studies also reported that *A. conyzoides* leaves collected from some Asian and African countries have the antioxidant and antimicrobial activities against some skin pathogens as well as wound healing activity (Adebayo *et al.*, 2010; Adegrave *et al.*, 2009; Durodola, 1977; Galati *et al.*, 2001; Hossain *et al.*, 2013b; Kamboj *et al.*, 2008a; Patil *et al.*, 2010). Polymethoxyflavone extract of *A. conyzoides* L. leaves collected in Brazil was reported to have anti-inflammatory activities through reducing the expression of inflammation-related genes IL-1β, PEG2, and TNF-α (Faqueti *et al.*, 2016). The extracts of *A. conyzoides* L. leaves collected in India and Nigeria were shown to promote wound healing on mouse model (Arulprakash *et al.*, 2012; Chah *et al.*, 2006). More than 20 compounds isolated from *A. conyzoides* L. have been reported so far (Yadav *et al.*, 2019). Among them, some compounds such as kaempferol, *β*-phellendrene, *β*-sitosterol, precocene II, and stigmasterol were demonstrated to have wound healing, antimicrobial and anti-inflammatory activities (Aherne *et al.*, 2008; de Christo Scherer *et al.*, 2019; Periferakis *et al.*, 2022; Prieto *et al.*, 2006; Simic *et al.*, 2002; Wang *et al.*, 2023; Zulkefli *et al.*, 2023).

However, the bioactivities of a plant vary depending on the geographical area and climatic conditions. Therefore, this study was carried out with the aim of examining some *in vitro* bioactivities, such as antioxidant, antimicrobial, anti-inflammatory and fibroblast proliferation activities of the ethanol extract of *A. conyzoides* L. leaves (AcEE) collected in Vietnam, which could provide some science-based evidence for the traditional usage of this plant in skin wound treatment.

MATERIALS AND METHODS

Plant material

Ageratum conyzoides (L.) leaves were collected in Bidoup-Nui Bà National Park, Lam Dong Province, Vietnam. The scientific name was identified by Dr. Nguyen Xuan Minh Ai, at the Laboratory of Plants, University of Science, Vietnam National University, Ho Chi Minh City, Vietnam (voucher specimen: PHH0004877).

Extract preparation

The dried leaves were ground into powder and percolated with 70% ethanol (1:10, w/v) at room temperature for 24 hours. This process was repeated 3 times to maximally extract bioactive compounds. The solution was then collected, filtered and concentrated by an evaporator (Hahnvapor, Korea). Finally, the solution was dried by a freeze dryer (Benchtop K Virtis, USA) to obtain the extract powder. The extract was dissolved in 99.9% DMSO (Merck, Germany) to the final concentration of 1 mg/mL and stored at -30˚C. Before use, the extract was diluted in a phosphate buffered saline (PBS) solution and filter-sterilized through a 0.22 µM syringe filter (Sartorius, Germany).

Determination of the total phenolic and flavonoid contents

Total phenolic content (TPC) was determined following the protocol of Do et al. (2014) with modifications. Briefly, 80 µL

of diluted extract sample was added into a tube containing 80 µL Foline-Ciocalteu reagent (Merck, Germany) and 720 µL distilled water (dH_2O) and mixed thoroughly for 3 minutes. After that, 800 µL Na_2CO_3 (7,5%), and 320 µL dH_2O were added and the mixture was incubated in the dark at room temperature for 30 minutes. The result was read at 760 nm wavelength (OD_{760}). TPC of the extract was deduced from a standard curve of gallic acid (25-400 µg/mL) using the following equation: $Y=0.04382+0.01975$ ($R^2 = 0.9919$); wherein X is the concentration of gallic acid (µg/mL) and Y is the OD_{760} value of the sample (Figure 1A).

Total flavonoid content (TFC) was determined following the protocol of Eghdami et al. (2010) with modification. Accordingly, 200 µL of diluted extract sample was mixed with 120 µL $NaNO_2$ (5%) solution. After 5 minutes, 120 µL of 10% $AlCl_3$ solution, 800 µL of 1 M NaOH solution, and 760 µL dH_2O were added and the mixture was vortexed for 10 seconds. The result was read at 415 nm wavelength (OD_{415}). TFC of the extract was deduced from a standard curve of quercetin (31.25-1000 µg/mL) using the following equation: $Y=0.001039X+0.1251$ ($R^2 = 0.9989$); wherein, X is the concentration of quercetin (µg/mL) and Y is the OD_{415} value of the sample (Figure 1B).

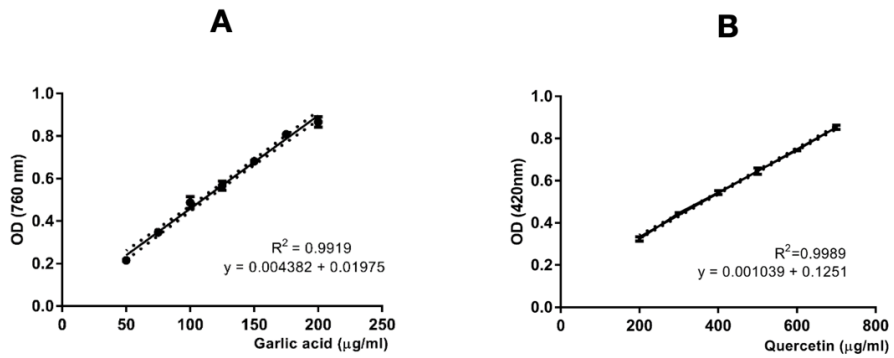

Figure 1. Standard curves of gallic acid (A) and quercetin (B).

Antioxidant activity evaluation using a DPPH scavenging assay

A reaction containing 500 µL of extract at each concentration (200, 100, 50, 25 or 12.5 µg/mL in 70% ethanol solvent) and 2000 µL of 2,2-diphenyl-1-picrylhydrazyl (DPPH) solution (100 µM DPPH in 70% ethanol) was prepared. The reaction was incubated at room temperature for 30 minutes and the absorbance at 517 nm (OD_{517}) was measured. The same reaction containing 500 µL ethanol (70%) and 2000 µL of DPPH solution was also prepared as the negative control.

The percentage of radical scavenging activity (%RSA) of the extract at each concentration was calculated as follows: $\%RSA=(A_o-A_j)/A_o*100\%$, in which A_j is the OD_{517} value of the reaction containing extract and A_o is the OD_{517} value of the negative control reaction. Gallic acid was used as the standard. The antioxidant activity of the extract was calculated by dividing the IC_{50} value of gallic acid by that of the extract, and expressed as mg gallic

acid equivalent antioxidant capacity per gram extract (mgGAEAC/gE) or per gram leaf dry weight (mgGAEAC/gdw). The IC_{50} values of the extract and gallic acid were deduced from the dose-response curves.

Antimicrobial analysis

The extract was dissolved in a 35% ethanol solvent at the highest concentration of 400 mg/mL and used for this experiment. The antimicrobial activity of the extract was examined using the agar diffusion assay. Briefly, 100 µL culture of tested bacteria at OD_{600}=0.1 was mixed well with 5 mL of soft TSB medium (0.8% agar) and the mixture was overlaid onto a TSB agar (2%) plate. After the medium was completely solidified, 8-mm diameter wells were created on the plate and filled with 100 µL of the extract at a concentration of 400 or 200 mg/mL. Besides, 100 µL of 35% ethanol solvent was loaded into the negative control well and 100 µL ampicillin solution (100 µg/mL) or tetracycline solution (300 µg/mL) was loaded into the positive well. Diameter of the inhibition zone (total diameter of the inhibition zone minus the diameter of the well) was measured after 16 hours of incubation.

Evaluation of anti-inflammatory activity on the RAW 264.7 cell line

RAW 264.7 cells (ATCC-TIB-71) were seeded into wells of a 96-well plate at a density of 10^4 cells/well in 100 µL DMEM-F12 medium supplemented with 10% FBS (Sigma, USA). The plate was incubated at 37˚C, 5% CO_2 for 24 hours. After that, the medium was replaced by 100 µL of fresh DMEM-F12 medium supplemented with 1% FBS and the plate was further incubated at 37˚C, 5% CO_2 for 6 hours. The medium was then replaced by 100 µL of fresh DMEM-F12 medium supplemented with 10% FBS, 0.1% DMSO, 1% LPS, and the extract at the tested concentration. A well containing cells without extract treatment and a well containing cells treated with 50 µg/mL dexamethasone were also prepared as the negative and possitive controls, respectively. The plate was incubated at 37˚C, 5% CO_2 for 24 hours. Finally, 50 µL of medium was removed and 50 µL of Griess reagent was added to the wells. The plate was incubated for 10 minutes and the absorbance at 550 nm (OD_{550}) was measured.

Examining the effect of *A. conyzoides* extract on the proliferation of NIH-3T3 cells

NIH-3T3 cells (ATCC CRL-1658) were seeded into wells of a 96-well plate at the density of 10^4 cells/well in 100 µL DMEM-F12 medium supplemented with 10% FBS (Sigma, USA). The plate was incubated at 37˚C, 5% CO_2 for 24 hours. After that, the medium was replaced by 100 µL of fresh DMEM-F12 medium supplemented with 2% FBS and the extract at tested concentrations, and the plate was further incubated at 37˚C, 5% CO_2 for 48 hours.

A well containing cells cultured in DMEM-F12 medium supplemented with 2% FBS and 0.1% DMSO and a well containing cells cultured in DMEM-F12 medium supplemented with 10% FBS were also prepared as the negative and positive controls, respectively. The cell density was measured using the MTT assay following the protocol previously described by Nguyen *et al.* (2020).

Statistical analysis

In our study, all experiments were repeated at least 3 times and the statistical results were analyzed by GraphPad Prism 8 software and presented as mean ± SD. The differences between mean values were tested by an unpaired Student's t-test (two-tailed).

RESULTS AND DISCUSSION

Phenolic and flavonoid contents of *A. conyzoides* ethanol extract

Ethanol-water mixture is one of the most popular solvents for plant extraction because of its high efficiency and safety for human consumption. Therefore, in this study, the extract of *A. conyzoides* leaves was prepared using 70% ethanol with an extraction yield of 21.4%.

The AcEE was then tested for total polyphenolic content (TPC) and total flavonoid content (TFC). The TPC was expressed as mg quercetin equivalent per gram extract (mgQE/gE) or per gram leaf dry weight (mgQE/gdw), whereas the TFC was expressed as mg gallic acid equivalent per gram extract (mgGAE/gE) or per gram leaf dry weight (mgGAE/gdw) (Table 1). The results showed that the AcEE contained 64.9±2.58 mgGAE/gE polyphenols and 79.33±1.03 mgQE/gE flavonoids.

It has been previously reported that the wound healing activities of some herbal plants come from the (poly)phenolic and flavonoid components (Budovsky *et al.*, 2015; Fraga *et al.*, 1987; Ghosh *et al.*, 2013; Sharma *et al.*, 2013). Therefore, various studies evaluated the TPC and TFC of plants for their wound healing activities. Amadi *et al.* (2012) demonstrated that *A. conyzoides* leaves contained a high amount of flavonoids and a medium amount of phenolic compounds. According to Kamboj *et al.* (2008b), at least 21 flavonoid compounds were detected in *A. conyzoides*. In another study, Hossain et al. reported that the 80% ethanol extract of *A. conyzoides* leaves collected from Bangladesh (2013a) contained a high amount of polyphenol components, up to 378.37 mg GAE/gE, much higher than what we detected in this study. Although the different climatic conditions, edaphic factors, and extraction methods might affect the TPC and TFC in these studies, this finding verified the presence of flavonoids and polyphenols in *A. conyzoides* leaves.

Table 1. Total polyphenol and flavonoid contents of *A. conyzoides* ethanol extract.

Ethanol extract	Amount
Total polyphenol content (mgGAE/gE)	64.9±2.58
Total polyphenol content (mgGAE/gdw)	13.9±0.19
Total flavonoid content (mgQE/gE)	79.33±1.03
Total flavonoid content (mgQE/gdw)	16.99±0.06

Antioxidant activity of *A. conyzoides* ethanol extract

Antioxidants are important to regulate the inflammation, control wound oxidative stress, prevent excessive cell activation, and thereby fasten wound healing (Comino-Sanz *et al.*, 2021; Fitzmaurice *et al.*, 2011).

Therefore, we evaluated the antioxidant activity of *A. conyzoides* leaves using a DPPH assay, which resulted in an IC_{50} of 131.74 µg/mL, equivalent to 22.01±0.45 mgGAEAC/gE (Table 2).

Some previous studies reported the antioxidant activity of *A. conyzoides in vitro* using DPPH or FRAP assays, and *in vivo* in mice (Dewan *et al.*, 2013; Faqueti *et al.*, 2016; Hossain *et al.*, 2013b). According to Patil *et al.* (2010), the methanol extract of *A. conyzoides* had a higher DPPH-scavenging capacity than its essential oil, with an IC_{50} of 22.5 µg/mL vs. 570 µg/mL; but the essential oil had a higher ability to prevent the formation of lipid peroxide than the methanol extract, with an IC_{50} of 15.50 µg/mL vs. 1290 µg/mL. According to Galati *et al.* (2001), its flavonoid fraction showed a DPPH-scavenging capacity with an IC_{50} of 93.90 µg/mL. In this study, we demonstrated that the ethanol extract of *A. conyzoides* also had antioxidant activity. However, the IC_{50} of AcEE was much higher than that of gallic acid (Table 2), suggesting that the antioxidant effect of this extract is relatively weak.

Table 2. DPPH-scavenging activity of *A. conyzoides* ethanol extract

Value	Gallic acid	Extract
IC_{50} (µg/mL)	2.9±0.05	131.74±2.67
mg GAEAC/gE		22.01±0.45
mg GAEAC/gdw		4.71±0.10

Antimicrobial properties of *A. conyzoides* extract

We next examined the antimicrobial activity of AcEE against some common bacteria causing skin infections. We found that AcEE at a high concentration (400 mg/mL) could inhibit the growth of all four tested bacteria, including *S. aureus*, *P. aeruginosa*, *E. faecalis*, and *E. coli* but the lower concentration (200 mg/mL) did not show an inhibitory effect on the growth of *E. coli*. This result is consistent with previous studies of Adetutu *et al*. and Namuga *et al.*, which also reported antimicrobial activity of *A. conyzoides* against these pathogens (Adetutu *et al.*, 2011; Namuga *et al.*, 2022).

Table 3. The antimicrobial activity of *A. conyzoides* ethanol extract.

Sample	The diameter of inhibition zone			
	S. aureus	*P. aeruginosa*	*E. faecalis*	*E. coli*
400 mg/mL extract	6,8±0,3	9,8±0,3	8,3±0,6	2,5±0,5
200 mg/mL extract	2,8±0,3	2,8±0,3	4,7±0,6	0
Positive control*	13±0,5	8,7±0,6	5,2±0,8	14,7±0,6
Negative control (35% Ethanol)	-	-	-	-

* Possitive control: 100 µg/mL ampicillin for *S. aureus*, *E. faecalis, E. coli*; and 300 µg/mL tetracycline for *P. aeruginosa.*

Anti-inflammatory effect of AcEE on the RAW 264.7 cell line

Inflammation occurs at the early phase of wound healing and is crucial for removal of pathogen and wound debris (MacLeod *et al.*, 2018). However, excessive and prolonged inflammation can lead to delayed healing (Pierce, 2001). Therefore, many plants have been used since antiquity for wound healing due to their anti-inflammatory activities (Shukla *et al.*, 2019). In this study, we also assessed the anti-inflammatory capacity of AcEE on the macrophage cell line RAW 264.7 activated by lipopolysaccharide (LPS), which has been widely used as an *in vitro* model to examine this effect. LPS can activate macrophages, an important cell type in inflammation, resulting in the production of nitrite oxide (NO) by inducing the expression of oxide nitrite synthase (Seminara, 2005). We found that LPS caused the elevated NO level in the Raw 264.7 cells and the treatment with AcEE significantly reduced the NO level of LPS-activated cells as well as did the control drug dexamethasone (Figure 2). In previous studies, Jonville et al. (2011) demonstrated that the dichloromethane extract of *A. conyzoides* inhibited the production of NO in LPS-stimulated Raw 264.7 and Galati *et al.* (2001) reported that the methanol extract reduced the paw edema in a carrageenan-induced rat. Taken together, these findings suggested the anti-inflammatory activities of this plant.

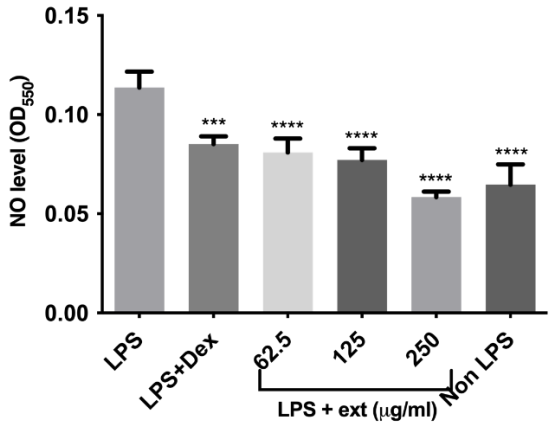

Figure 2. *A. conyzoides* ethanol extract inhibited the production of NO in LPS-activated Raw 264.7 cells. Samples included: LPS, cells treated with 1 µg/mL LPS; LPS+dex, cells treated with 1 µg/mL LPS and 50 µg/mL dexamethasone (dex); LPS+ext, cells treated with 1 µg/mL LPS and extract (ext) at tested concentration; Non LPS, untreated cells. The differences between the LPS-treated sample and the other samples were analyzed using an unpaired T-test ($p < 0,05$; ** $p < 0,01$; *** $p < 0,001$; **** $p < 0,0001$).

***A. conyzoides* ethanol extract improved the proliferation of mouse fibroblasts**

Fibroblast cells are extremely important for all phases of wound healing. During the inflammatory phase, fibroblasts can promote the recruitment of immune cells to the wound site and particularly regulate the transition from the inflammatory phase to the subsequent proliferation phase, which determines the correct progression of the healing process (Mescher, 2017). During the proliferation phase, fibroblasts become even more important since they secrete numerous

growth factors and enzymes to contribute to angiogenesis and granulation tissue formation, as well as to degrade and replace the fibrin clot with a new provisional matrix supporting keratinocyte migration (Cialdai et al., 2022). In the final phase of the healing process, myofibroblast, an activated form of fibroblast, can regulate wound contraction and tissue remodeling by synthesizing various extracellular proteins (Tomasek et al., 2002). Therefore, fibroblast proliferation has been used as an in vitro indicator for wound healing assessment in many previous studies. We here showed that AcEE at concentrations of 31.25-500 µg/mL significantly increased the growth of the mouse fibroblast NIH-3T3 cells (Figure 3). To the best of our knowledge, this is the first study to report the effect of *A. conyzoides* on the growth of fibroblasts *in vitro*, which suggest the potential of this extract to speed up the wound healing process.

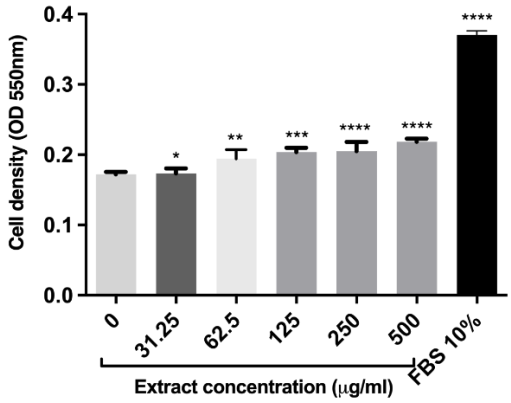

Figure 3. Effect of *A. conyzoides* ethanol extract on the proliferation of NIH-3T3. The differences between the non-treated sample (0 µg/mL) and the treated samples were analyzed using an unpaired T-test ($p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$).

CONCLUSION

In this study, we demonstrated that the ethanol extract of *A. conyzoides* leaves collected from Bidoup National Park, Nui Ba, Lam Dong, Vietnam had antioxidant activity with an IC_{50} of 131.74±2.67 µg/mL; antimicrobial activities against *S. aureus, P. aeruginosa, E. faecalis*, and *E. coli;* anti-inflammatory activity on the Raw 264.7 cell line; and a proliferation effect on the fibroblast NIH-3T3 cell line. Since these activities are important for skin recovery and infection prevention after the injury, this study provided some *in vitro* evidence for the traditional use of this plant in wound treatment.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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