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Antibacterial, Antioxidant and Anti – Cancerous Activities of *Adiandra megaphylla* Hu Leaf Extracts

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ABSTRACT

Adinandra megaphylla Hu which belongs to *Adinandra genus*, Theaceae family, only narrowly distributed in Vietnam. Biological activities of this plant's secondary compounds have been left open yet. In this study, the antibacterial, antioxidant abilities and inhibiting cancer cell lines activity of leaf extract of *A. megaphylla* collected in Lao Cai province, Vietnam were initially investigated. Poultice extracted from leaves of *Adinandra megaphylla* with three solvents of ethanol, ethyl acetate and dichloromethane were quantified and determined composition of the polyphenol, flavonoid and coumarin groups. The ethanol extract, ethyl acetate extract and dichloromethane extract have been shown to inhibit the growth of *Staphylococcus aureus, Bacillus subtilis, Seratia marcessens, Sarcina lutea, Lactobacillus plantarum* and *Escherichia coli* at concentration of 200 µg mL^{-1}. The ethyl acetate extract and dichloromethane extract have DPPH free-radical activities; the EC50 value reached 30.3 and 33.2 µg mL^{-1}, respectively. In particular, antimicrobial and free-radical activities of the dichloromethane extract were better than ethanol and ethyl acetate extracts. Extracts from *A. megaphylla* showed inhibition of gastric, lung and breast cancer cell lines with values of 67.76, 77.02 and 84.46 µg mL^{-1}, respectively. Research results show that *A. megaphylla* is a potential plant containing many compounds with antibacterial, antioxidant abilities and inhibiting cancer cell lines.

KEY WORDS: *ADINANDRA MEGAPHYLLA*, ANTIOXIDANT, ANTIBACTERIAL, ANTI-CANCER, POULTICE.

INTRODUCTION

Vietnam has high potential for medicinal plants, which their chemical composition and pharmacological activities of some herbaceous species have been studied in previous studies (Hung et al., 2019; Minh et al., 2010; Vu et al., 2019), but there are still many species that have not been assessed their medicinal value, including *Adinandra megaphylla* of the genus *Adinandra*, the tea family (Theaceae). In the world, there are about 85 species in the *Adinandra* genus distributed in Bangladesh, Cambodia, China, India, Indonesia, Southern Japan, Laos, Malaysia, Myanmar, New Guinea, Philippines, Sri Lanka, Thailand, Vietnam and African rainforests. In China, the genus *Adinandra* has 22 species, of which up to 17 are endemic (Min and Bruce, 2007). In "An illustrated flora of Vietnam", Pham Hoang Ho (1999) indicated that the genus *Adinandra* in Vietnam contains about 11 species which scattered throughout the country.

Species of the genus *Adinandra* consist of secondary compounds with antibacterial, anti-inflammatory, antioxidant, anti-free radical and anti-cancer activities. However, studies on the genus *Adinandra* have been

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mainly focused on the species *Adinandra nitida*. In 2008, Liu et al. isolated camellianin A from *A. nitida* Merr. ex Li by column chromatography and determined its content by HPLC. At the same time, their study demonstrated high antioxidant capacity from flavonoids extracted by DPPH and free radical cleaning method (Liu et al., 2008). In addition, Liu et al. (2013) optimized flavonoid extraction method and obtained camellianin A from *A. nitida* leaves; flavonoids were also reported to have antioxidant ability at the concentration of 0.02 mg mL^{-1} (Liu et al., 2013).

Thus, previous studies have shown that flavonoids like epicatechin, apigenin, quercitrin, camellianin A and camellianin B to have biological and antioxidant activities. Chemical composition of *A. nitida* has been isolated and determined by Wang et al. (2008) using column chromatography. The structure of saponins compounds comprise 6 types, including 2alpha, 3alpha, 19alpha- trihydroxy-olean-12-en-28-oic acid-28-O-beta-D-glucopyranoside; arjunetin; sericoside; glucosyl tormentate; nigaichigoside F1 and arjunglucoside I. Among of which, 2alpha, 3alpha, 19alpha-trihydroxy-olean-12-en-28-oic acid-28-O-beta-D-glucopyranoside is a new substance. The remaining substances were first discovered in *A. nitida* (Wang et al., 2008).

Moreover, in the *Adinandra* genus, *Adinandra lienii* was initially studied for its geographical distribution and *matK* sequence to help identify this species in Lao Cai province, Vietnam. Meanwhile, there have been not any studies on chemical composition and biological activity of total extracts from *Adinandra megaphylla* yet. In this study, we present results of qualitative analysis of chemical composition and evaluation of antibacterial, antioxidant, anti-cancer activities of the *A. megaphylla* Hu extracts.

MATERIAL AND METHODS

Figure 1: Morphological characteristics of *A. megaphylla* Hu collected in Lao Cai province, Vietnam. Life form (A), branches with buds (B, C), flowers (D)

***A. megaphylla* samples:** *A. megaphylla* samples was collected from Lao Cai province, Vietnam in the 1200-1800 m altitude at 21°59'15''N; 104°19'28''E. *A. megaphylla* Hu samples (branches with leaves and flowers) were collected to determine the scientific name in laboratory. *A. megaphylla* Hu leaves were used for poultice extraction with ethanol, ethyl acetate and dichloromethane. The scientific name of species is determined by comparative morphological methods according to monograph including "An Illustrate Flora of Vietnam" and "Flora of China" (Figure 1).

Bacterial strains and cancer cell lines: Bacterial strains (*Bacillus subtilis, Serratia marcescens*, *Escherichia coli*, *Sarcina lutea*, and *Lactobacillus plantanum*) were selected for the antibacterial activity assay. They were grown in liquid Luria-Bertani (LB) medium (0.5% (w/v) yeast extract, 1.0% (w/v) peptone, 1.0% (w/v) NaCl, pH 7.0) overnight at 28°C, and the diluted bacterial suspension (106ˆmL^{-1}) was ready for detection. Solid LB medium contained additionally 2.0% (w/v) agar. Cancer cell lines, including the breast cancer cell line (MDA-MB-231), the stomach cancer cell line (AGS) and the lung cancer cell line (A549) were used in cytotoxicity assays.

Materials and chemicals: Yeast extract and peptone were purchased from Bio Basic Inc. (USA); Ethanol, ethyl acetate and dichloromethane were from Fluka (China); TLC silica gel 60 F254 was from Merck (Germany).

Method of sample preparation: The leaves of *A. megaphylla* are washed thoroughly, then cut into pieces and dried at a temperature of 50°C to constant mass. The crushed sample is extracted twice with ethanol in an ultrasonic machine at room temperature. The crude extracts were collected by solvent removal under reduced pressure conditions, at 50°C and extracted with solvents of dichloromethane and ethyl acetate. The residue of ethanol, dichloromethane and ethyl acetate were cleaned solvent and dried at 50°C to collect ethanol extract, dichloromethane extract and ethyl acetate extract, respectively.

Polyphenols were detected by reaction with iron salts (III)/sulfuric acid: Reaction with iron salts (III): 5 mL of ethanol extract were added into two tubes, denoted by I and II, respectively. The tube II was supplemented with 0.5 mL iron salts (III), shake and observe color. Depending on the number and location of hydroxyl groups in polyphenol molecules, results are green, blue or brown.

Reaction with sulfuric acid: 2 mL of ethanol extract were added into two tubes, denoted by I and II, respectively. The tube II was supplemented with 1-2 drops of H_2SO_4, shake and observe color. Add H_2SO_4 concentrate to flavones and flavonols to give a deep yellow; to chalcones and aurones to produce a red, crimson red or bright red solution; to flavanones to give orange red.

Flavonoids were detected by reaction with hydrochloric acid and magnesium powder:The tube contained 0.05g ethanol extract and 10 mL CH_3OH, which were shaken, heated to dissolve and filtered through filter paper. 2 mL of filtrate was added into two tubes, denoted by I and II, then was added a pinch of magnesium powder and shaken. The tube II was supplemented with five drops of HCl and boiled for 3 mins. The solution changed to yellow, red to green colors, as it contained flavonoids. Coumarin was detected by reaction with NaOH solution:

2 mL of ethanol extract were added into two tubes, denoted by I and II. The tube II was supplemented with 0.5 mL of 10% NaOH solution. Two tubes were boiled, cooled down to room temperature and added 4 mL distilled water. The tube II is more transparent or clear than tube I, indicates the presence of coumarin. When the two test tubes were added with a few drops of HCl, the solution turned a dull yellow, so the coumarin was determined following this method (Nguyen and Hung, 2008).

Thin layer chromatographic method (TLC): Ethanol extract from *A. megaphylla* leaves were detected by TLC (3.5 × 10 cm layer of silica gel 60), performed with two mobile phases of n-hecxane/acetone (1:1, v/v) and dichlomethane/n-hexane (3:1, v/v). The products were visualized by spraying the TLC plate with 10% (v/v) sulfuric acid in ethanol and incubating at 100°C until color appeared.

Determination of antibacterial activity of extracts: Antibacterial activity of extracts was performed according to the method of Mahesh and Satish (2008). In order to determine antibacterial activity of the extracts, 70 mL of diluted bacterial suspension (106^mL^{-1}) was brushed on 0.5-cm-thick LB plates. The LB plates were perforated with 0.5-cm-diameter holes, and each hole was supplemented with 100 mL of each ethanol, ethyl acetate and dichloromethane extract from *A. megaphylla* Hu leaves with different concentrations (20, 60 and 200 µg mL^{-1}) or with DMSO for the control. The inhibition activity of extracts against bacterial growth was observed after incubation at 30°C for 18-40 hours. The antibacterial levels were determined by diameter of inhibition zones (in millimeters) around the holes. The diameter of antibacterial ring was determined by the formula: H = D-d (mm). In which: D is the diameter of the antibacterial ring from the center of perforations (mm); d is the diameter of perforated agar (mm).

Determination of oxidation activity of extracts: Antioxidant activity of *A. megaphylla* extracts was determined by Tabart et al. (2009) using DPPH radical scavenging. 100 µL of each extract at five concentrations, including 0.5, 2, 8, 32 and 64 µg mL^{-1} were added with 2.9 mL of 0.1 mM DPPH solution mixed in methanol solution, shook and left in the dark for 30 min at room temperature, the absorbance was measured at 517 nm. The inhibition of DPPH radical by the samples was calculated by following formula: DPPH activity (%) = 100 × (Ac - As)/Ac, in which Ac: the absorbance of control, As: the absorbance of sample. Antioxidant activity was determined based on EC_{50} values (the concentration of DPPH free radical scavenging samples is 50%) (Tabart et al., 2009).

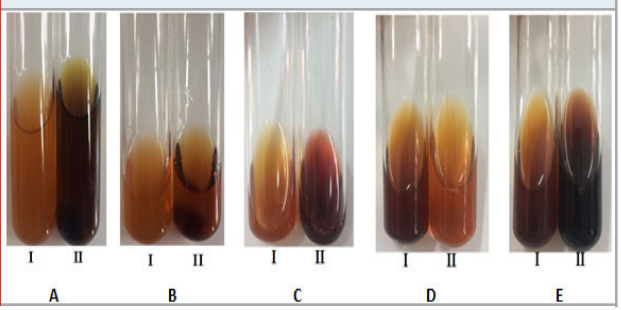
Cytotoxic assays: Cancer cytotoxicity was determined using the method of Monks et al. (1991) (Monks et al., 1991). Poultice extracted from *A. megaphylla* leaves was prepared and tested at four concentrations, including 100, 20, 4 and 0.8 µg mL^{-1}. Ellipticine was used as a reference at four concentrations: 10, 2, 0.4 and 0.08 µg mL^{-1}. Dimethyl sulfoxide (DMSO) at 10% concentration was used as a negative control. Total protein content of a cell is determined based on the optical density (OD) when proteins of the cell are stained with sulforhodamine B (SRB). The OD results were read on a wave step of 515 nm in an ELISA Plate Reader. OD values are proportional to the amount of SRB, which is attached to protein molecules. The larger the OD value, higher the amount of protein and higher the amount of cells. Cytotoxicity was expressed as the concentration of drug that inhibited cell growth by 50%. Inhibitory concentration 50% (IC_{50}) is the concentration of the sample at which it can inhibit 50% of cells. The substance is considered to have good activity when IC_{50} = 5 mM (Hughes et al., 2011).

RESULTS AND DISCUSSION

Results of thin layer chromatographic analysis show that, there were 7 marks in the ethanol extract and 6 marks in the ethyl acetate extract for the n-hexane/acetone at a 1:1 ratio (Figure 2A). There were 3 marks in the ethanol extract and 6 marks in the ethyl acetate extract for the dichloromethane/n-hexane solvent system at a 3:1 ratio (Figure 2B). Thus, on the thin layer chromatographic analysis with two different solvent systems showed that the extract from A. megaphylla Hu has many bands with different colors.

Figure 2: Results of thin layer chromatographic analysis of ethanol extract (A) and ethyl acetate extract (B) in the n-hexane/acetone at a 1:1 ratio (I) and dichloromethane/n-hexane solvent system at a 3:1 ratio (II)

Figure 3: Color reactions for detection of polyphenols (A, B), flavonoids (C) and coumarin (D, E) in the ethanol extract from leaves of *A. megaphylla*. The ethanol extract before (I) and after (II) reacting with iron salts (III) (3A); with sulfuric acid (3B); with Mg in HCl solution (3C); with NaOH (3D); with HCl solution (3E).



The extract of *A. megaphylla* was quantified polyphenols, flavonoids and coumarin by different reagents. The results were shown in Figure 3.

Polyphenols in the ethanol extract was detected by reacting with iron salts (III), the solution in the tube II turned dark green due to reaction between polyphenols and iron salts (III). The solution in the tube II turned dark yellow (Figure 3B), if flavonoid reacted with sulfuric acid. According to flavonoids qualitative, the solution in the tube II changed color from yellow to dark red (Figure 3C). Coumarin was detected by reaction with 10% NaOH solution, the results showed in tube II is more transparent than tube I (Figure 3D). Then, when a few drops of HCl were added to both tubes, the tube II changed from dark opaque yellow to light transparent yellow color (Figure 3E). Therefore, it is clear from these results that the extract of *A. megaphylla* leaves contained polyphenols, flavonoids and coumarin. Previous studies have also demonstrated that leaves of *Adinadra nitida* contained total flavonoids Gao et al., 2010; Chen et al., 2017. Bioactive compounds in these plants have an important role in producing medicinal products as well as a basis for further research.

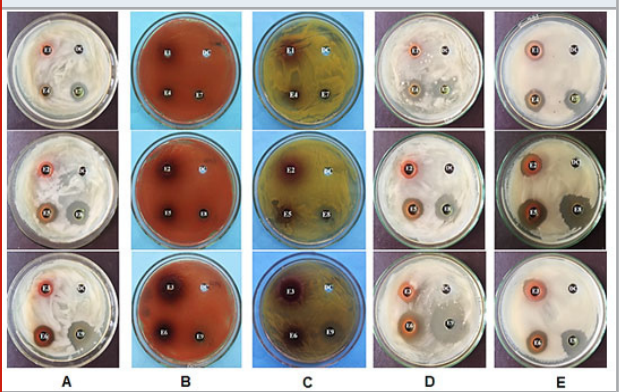
Antibacterial activity of ethanol, ethyl acetate and dichloromethane extracts from leaves of *A. megaphylla* Hu was tested at different concentrations using bacteria via the agar diffusion method (Table 1 and figure 4). The ethanol extract had no bactericidal effects at a concentration of 20 µg mL^{-1}, and had low activity at 60 and 200 µg mL^{-1} concentrations on *B. subtilis*. Whereas, the ethyl acetate and dichloromethane extract showed antibacterial activity against *B. subtilis* at all concentrations tested, and the dichloromethane extract at 200 µg mL^{-1} had the strongest antibacterial activity (Figure 4A).

Table 1. Antibacterial activities of extracts from leaves of *A. megaphylla* Hu

No.	Bacterial	Experimental concentrations of the extracts								
		E1	E2	E3	E4	E5	E6	E7	E8	E9
1	*B. subtilis*	-	+	+	+	++	++	++	+++	+++
2	*S. macescens*	-	-	-	-	-	-	-	-	+
3	*S. lutea*	-	-	-	-	-	-	-	++	+++
4	*L. plantarum*	+	++	++	++	++	+	+++	+++	+++
5	*E. coli*	+	+++	+++	++	+++	++	++	+++	+++

Note: (-) No inhibition (no antibacterial zones); (+) Weak inhibition (the diameters of inhibition zones are from 1 to 5 mm); (++) Inhibition (the diameters of inhibition zones are from 6-10 mm); (+++) Strong inhibition (the diameters of inhibition zones are >10mm).
The ethanol extract at concentration of 20 (E1); 60 (E2) and 200 (E3) µg mL^{-1}; the ethyl acetate extract at concentration of 20 (E4); 60 (E5) and 200 (E6) µg mL^{-1}; the dichloromethane extract at concentration of 20 (E7); 60 (E8) and 200 (E9) µg mL^{-1}.

Figure 4: Antibacterial activities against *B. subtilis* (A), *S. marcescens* (B), *S. lutea* (C), *L. plantarum* (D) and *E. coli* (E) of *A. megaphylla* Hu extracts; DC: Dimethyl sulfoxide (DMSO) served as a control.



The ethanol, ethyl acetate and dichloromethane extracts had no antibacterial activity against *S. macescens* at all concentrations (Figure 4B). For *S. lutea* bacteria, the ethanol extract, the ethyl acetate extracts at concentration of 20, 60 and 200 µg mL^{-1} and the dichloromethane extract at concentration of 20 µg mL^{-1} had no antibacterial activity. While, the dichloromethane extract at 60 and 200 µg mL^{-1} concentrations had strong antibacterial activity against *S. lutea* (Figure 4C). The ethyl acetate and dichloromethane extract had antibacterial activity against *L. plantarum* at all concentrations tested, and the strongest antibacterial activity was observed with the dichloromethane extract at 200 µg mL^{-1} (Figure 4D). The ethanol, ethyl acetate and dichloromethane extracts had antibacterial activity against *E. coli* at all concentrations; The strongest resistance activity was at 60 µg mL^{-1} for the dichloromethane extract, followed by the ethyl acetate (Figure 4E).

Antibacterial activities of extracts from leaves of *A. megaphylla* Hu on five bacterial strains showed that: (1) The ethanol and ethyl acetate extracts were able to inhibit *B. subtilis, L. plantarum, E. coli*, and no inhibit *S. lutea, S. macescens*. (2) The dichloromethane extract had inhibited *B. subtilis, S. marcessens, S. lutea, L. plantarum*, *E. coli*. (3) Antimicrobial activities of dichloromethane extract is better than the ethanol and ethyl acetate extracts.

Table 2. Antioxidant activities of extracts from leaves of *A. megaphylla*

Concentrations (µg mL^{-1})	DPPH free radical scavenging activity (%)		
	Ethanol extract	Dichloromethane extract	Ethyl acetate extract
0.5	0	0	25.4 ± 0.54
2.0	0	0	28.6 ± 2.02
8.0	0	0	29.2 ± 4.04
32	48.2 ± 4.45	0	52.7 ± 5.52
128	71.8 ± 5.28	40.5 ± 0.35	75.7 ± 0.96
EC50	33.2 ± 0.42	> 128 ± 0.98	30.3 ± 3.26

Antioxidant activities of extracts from leaves of *A. megaphylla* Hu: The results showed that the DPPH free radical removal efficiency of the ethanol, dichloromethane and ethyl acetate extracts were directly proportional to the extract concentrations. The free radical removal efficiency increased from 0 to 75.7% with the increase of extract concentration from 0.5 to 128 µg mL^{-1}. The ethyl acetate extract proved to have the strongest DPPH free radical activity with an EC50 value of 30.3 µg mL^{-1}. The ethanol extract showed DPPH free radical activity with an EC_{50} value of 33.2 µg mL^{-1}. By contrast, the DPPH free radical activity of dichloromethane extract was very weak with the EC_{50} value >128 µg mL^{-1} (Table 2).

Cytotoxic activities of the ethanol extract from leaves of *A. megaphylla* Hu against cancer cell lines: According to Gao et al. (2010), camellianin A, a flavonoid from leaves of *Adinandra nitida*, was determined to inhibit proliferation and apoptosis of liver cancer cells (Hep G2) and breast cancer (MCF-7) (Gao et al., 2010). Some heterocyclic compounds containing coumarin-related properties such as anti-inflammatory (El-Haggar and Al-Wabli, 2015), antibacterial (Shi and Zhou, 2011), antiviral (Tsay et al., 2014) and anti-cancer (Jacquot et al., 2007). Moreover, coumarin inhibited Hep2 cell growth and showed typical characteristics of apoptosis including the morphological changes and DNA fragmentation (Mirunalini et al., 2014).

In our study, the *A. megaphylla* extracts was found to contain flavonoids and coumarin compound, however it is necessary to identify whether or not they are resistant to cancer cells. Therefore, we preliminarily determined the anti-cancer activities of ethanol extract from leaves of *A. megaphylla* as a basis for efficient purification of flavonoid and coumarin compounds. The cytotoxic activity of ethanol extract against three cancer cell lines, including MDA-MB-231 (breast cancer cell line), AGS (stomach cancer cell line) and A549 (lung cancer cell line) was investigates in this study. The results showed that the extract of *A. megaphylla* has strong cytotoxic activity against MDA-MB-231, AGS and A549 with IC_{50} values are 84.46, 67.76 and 77.02 µg mL^{-1}, respectively (Table 3).

Table 3. Cytotoxicity of extract from leaves of *A. megaphylla* against human cancer cell lines in vitro (IC_{50}, µmol L^{-1})

Concentrations (µg mL^{-1})	Inhibition of human cancer cell line growth (%)		
	A549	AGS	MDA-MB-231
100	73.07 ± 0.66	84.61 ± 3.20	65.06 ± 0.41
20	5.96 ± 3.06	8.20 ± 2.62	4.64 ± 0.66
4	1.25 ± 1.59	3.44 ± 2.13	0.90 ± 0.08
0.8	-2.63 ± 0.80	-3.97 ± 0.29	-1.66 ± 0.74
IC50	77.02 ± 5.27	67.76 ± 3.31	84.46 ± 9.09

Note: IC_{50} values are means from three independent experiments (average ± SD) in which each compound concentration was tested in three replicate wells. Ellipticine (as a reference compound) was the positive control and assayed at concentrations of 10, 2, 0.4 and 0.08 µg mL^{-1}.

In particular, the inhibitory effect of the extract against stomach cancer cell line is highest, followed by lung cancer cell line and the lowest is the breast cancer cell line. Thus, the extract from *A. megaphylla* has activity against cancer cell lines, including the breast cancer cell line, the stomach cancer cell line and the lung cancer cell line. This result is the basis for the isolation of pure compounds with anti-cancer properties.

CONCLUSION

Results of thin layer chromatographic analysis using n-hexane/acetone at a 1:1 ratio and dichloromethane/n-hexane solvent system at a 3:1 ratio had been identified to contain polyphenols, coumarin in the extracts from leaves of *A. megaphylla* Hu. The ethanol and ethyl acetate extracts were able to inhibit *B. subtilis, L. plantarum*, *E. coli*, but not inhibit *S. lutea, S. macescens*. The dichloromethane extract had inhibited *B. subtilis, S. marcessens, S. lutea, L. plantarum, E. coli*. DPPH free radical activity of the dichloromethane extract is strongest in comparison to the ethanol and ethyl acetate extracts, the EC_{50} value of dichloromethane extract is 30.3 µg mL^{-1}. The ethanol extract from leaves of *A. megaphylla* Hu has activity against breast, stomach and lung cancer, the IC_{50} values reached to 84.46, 67.76 and 77.02 µg mL^{-1}, respectively. Thus, the dichloromethane

extract showed stronger biological activities comparing to the ethanol and ethyl acetate extracts. These research results have demonstrated that *A. megaphylla* contains bioactive and pharmacological compounds, which is a new potential source for isolating these compounds to produce medicine.

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