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Abstract

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Keywords

acalypha, antioxidant, anticancer, linn, activities, indica, aerial, parts, CMMB

Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

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Antioxidant and Anticancer Activities from Aerial Parts of *Acalypha indica* Linn.

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ABSTRACT

Extracts of Acalypha indica Linn. (aerial parts) were investigated for antioxidant activity, anticancer activity, and cytotoxicity. The extracts showed a non-cytotoxic response against Vero cells (African green monkey kidney). The anticancer activity of the extracts was tested using the Resazurin Microplate Assay (REMA). The methanol extract showed anticancer activity against NCI-H187-Small Cell Lung Cancer with an IC_{50} of 25.00 µgmL^{-1}. In addition, the hexane, chloroform, and methanol extracts also showed significant antioxidant activities with an IC_{50} of 6.19, 5.70, and 7.79 mg/mL, respectively, by means of the DPPH radical scavenging assay. The hexane, chloroform, and methanol extracts also showed significant antioxidant activities with an IC_{50} of 6.13, 6.31, and 6.37 mg/mL, respectively, by means of the ABTS radical scavenging assay. Isolation and purification of the methanolic extract of the aerial part produced substantial amounts of L-quebrachitol, which was characterized by 1D and 2D NMR experiments and the MS data.

Keywords: Antioxidant activities, Anticancer activities, *Acalypha indica* L., Aerial part

INTRODUCTION

Acalypha indica Linn. is a small annual shrub, which generally occurs as a troublesome weed in gardens, roadsides and throughout the plains of India. It is found in tropical Africa and Asia and through to Polynesia (Parveen et al., 2007). The Thai name is Tam Yae Maeo. It is used in traditional medicine for the treatment of scabies (Gurib-Fakim et al., 1993), rheumatoid arthritis, and syphilitic ulcer (Dhar et al., 1968). It is also used for healing wounds (Reddy et al., 2002), as a laxative (Panthong et al., 1991), as an anti-snake venom (Siddiqui and Husain, 1990; Shirwaikar et al., 2004; Mahishi et al., 2005; Samya et al., 2008), and for its anti-implantation and anti-estrogenic activity (Hiremath

et al., 1999). The petroleum ether extract of *A. indica* Linn. showed antimicrobial activity against *Aeromonas hydrophilla, Pseudomonas aeruginosa* (Samy et al., 1999), *Aspergillus niger*, and *Escherichia coli* (Solomon et al., 2005). One study investigated the antibacterial activity of four different extracts (hexane, chloroform, ethyl acetate, and methanol) from the leaves of *A. indica* against *Staphylococcus epidermidis, Bacillus cereus, Streptococcus faecalis, Klebsiella pneumoniae, and Proteus vulgaris* (Govindarajan et al., 2008). The hexane, chloroform, and methanol extracts of *A. indica* aerial parts showed antibacterial activity against *S. aureus* and *P. aeruginosa*. The hexane, chloroform, and methanol extracts of *A. indica* Linn. aerial parts showed antifungal activity against *T. mentagrophyte* and the chloroform and methanol extracts showed inhibition zones against *A. flavus* and *C. albican* (Sanseera et al., 2010). Several chemical constituents have been isolated from *A. indica*, including: tannins, pyranoquinolinone alkaloid flindersin, kaempferol glycosides, mauritianin, clitorin, nicotiflorin, biorobin (Nahrstedt et al., 2006), cyanogenic glucoside acalyphin (Nahrstedt et al., 1982), acalyphamides, aurantiamide, and succinimide (Talapatra et al., 1981).

The present work reports the chemical constituents and biological activities of the extracts of *A. indica*. There is no previously published research on the antioxidant properties, anticancer activities, and cytotoxicity of the aerial parts from this plant.

MATERIALS AND METHODS

Chemicals and Reagents

All chemicals used were of analytical reagent grade. Hexane, chloroform, methanol, and ethanol were purchased from Merck, Germany. ABTS (2,2'-azino-bis(3-ethylbenzothaizoline-6-sulfonic acid)) and DPPH (1,1-diphenyl-2-picrylhydrazyl) were purchased from Sigma (St. Louis, USA). Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) was obtained from Aldrich (Milwaukee, USA). Potassium persulfate was obtained from UNILAB (AU). Ellipticine, Doxorubicin, Resazurin, L-glutamine, and Geneticin were purchased from Sigma (St. Louis, USA).

Plant material

Aerial parts of *Acalypha indica* Linn. were collected from Nakhonsawan, Thailand in June 2010, and identified by J. F. Maxwell, Chiang Mai University, Thailand. A voucher specimen (No. 1) was deposited in the herbarium of the Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand.

Extraction

The aerial parts of *A. indica* were cleaned with running tap water and dried in a hot air oven at 40°C for 24 h. The dried plant was finely ground. Then 1 kg of the dried plant powder was extracted successively with hexane, chloroform, and methanol (2 L each) for three days. For each extraction step, the solvent was

removed in vacuo to give crude extract. After removal of the solvents, three crude extracts were obtained: hexane extract (9.5 g), chloroform extract (10.2 g), and methanol extract (22.5 g), respectively.

Antioxidative Assay

DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate) assay. The antioxidant activity of the extracts was determined by the DPPH radical scavenging assay. This modified method was described by Brand-Williams et al. (1995). The DPPH 6.6 mg/mL (in ethanol) was prepared and stored in the dark before use. Various concentrations of Trolox standard solutions and extract solutions were prepared using ethanol as solvent. This experiment was carried out with samples in the concentrations of 1, 2, 3, 4, 5, 6, 7, and 10 mg/mL. To each well of 96-well microtitre plate, 180 µL of ethanolic DPPH solution and 20 µL of the test sample (the extract in ethanol) were added. The total volume for each reaction mixture in each well was 200 µL. The plates were then incubated at 37ºC for 30 min to check for the colorimetric change (from deep violet to light yellow) when DPPH was reduced. The absorbance of each well was measured at 540 nm. The DPPH solution was used as the negative control. Trolox was used as the reference standard. Radical scavenging capacity was calculated by using the formula:

$$\% \text{ Inhibition} = [(Ac - As) \times 100] / Ac \qquad \text{[Equation 1]},$$

where Ac is the absorbance of the control and As is the absorbance of the test sample after incubation for 30 min. The values of % inhibition were obtained from Equation 1. For the 50% Inhibitory Concentration (IC_{50}) evaluation of the extract, graphs showing the concentration of the test samples (hexane extract, chloroform extract, and methanol extract) versus % Inhibition (% DPPH reduction) were plotted. A linear regression ($R^2 = 0.9984$) of standard Trolox (Figure 1) was also used to calculate the radical scavenging capacity.

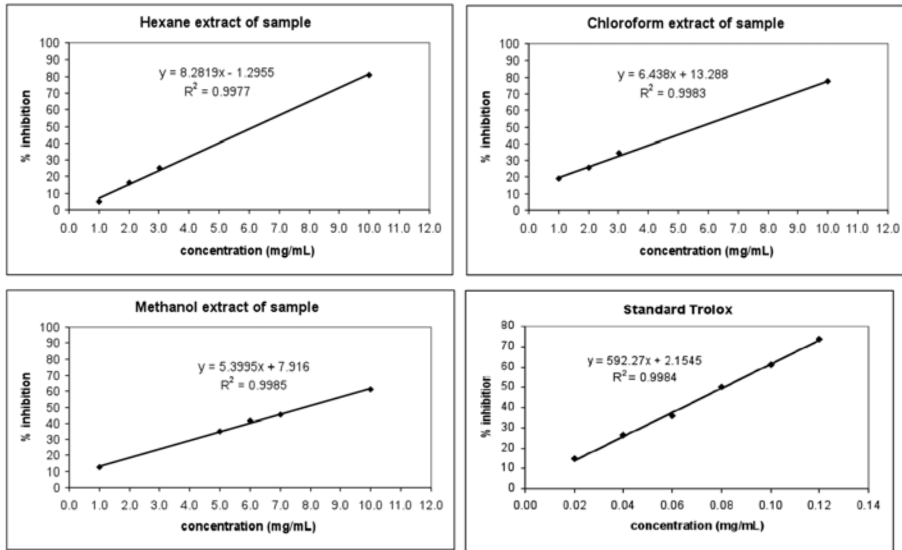

Figure 1. Linear regressions (R^2) of hexane, chloroform, methanol extracts, and Trolox (DPPH assay).

ABTS (2, 2'- azinobis (3 – ethylbenzothiazoline – 6 – sulfonic acid) diammonium salt) assay. The antioxidant activity of the extract was investigated using the ABTS radical cation scavenging assay (Roberta et al., 1999) compared with the Trolox standard. For the ABTS assay, 20 µL of extract (0.1 g mL^{-1}) was mixed with 2.0 mL of diluted ABTS solution (A734nm = 0.700 ± 0.020) and the absorbance was determined at 734 nm after 5 min incubation at room temperature. Appropriate solvent blank was run in each assay. All determinations were carried out at least three times, and in triplicate. Inhibition of free radical by $ABTS^{\bullet+}$ in percent (% Inhibition) was calculated by the following formula:

$$\% \text{ Inhibition} = [(A_{blank} - A_{sample}) / A_{blank}] \times 100 \quad \text{[Equation 2]},$$

where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance of the test compound. The percentage inhibition of the absorbance at 734 nm was calculated and plotted as a function of concentration of antioxidants and of Trolox for the standard reference data (Figure 2).

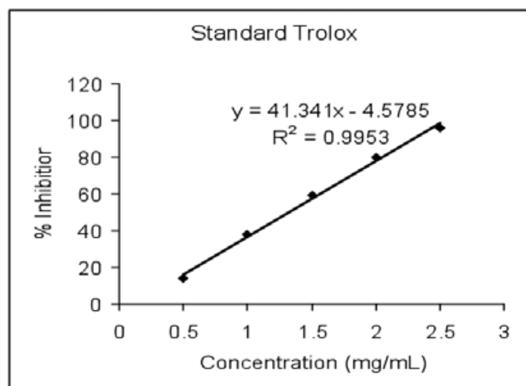

Figure 2. A linear regression (R^2 = 0.9953) of Trolox (ABTS assay)

Statistical Analysis

Each antioxidant activity assay was done three times from the same extract in order to determine their reproducibility. Analysis of variance was used to test any difference in antioxidant activities resulting from these methods.

Anticancer Assay

The anticancer activity of the extracts was assayed by using three cancerous human cell lines: KB cell line (epidermoid carcinoma of oral cavity, ATCC CCL-17), MCF 7 cell line (breast adenocarcinoma, ATCC HTB-22), and NCI-H 187 cell line (small cell lung carcinoma, ATCC CRL-5804). This assay was performed using the method described by Brien et al. (2000). Cells at a logarithmic growth phase were harvested and diluted in fresh medium to 7×10^4 cells/mL for KB and 9×10^4 cells/mL for MCF-7 and NCI-H 187. Then 5 µL of each test sample (the hexane, chloroform, and methanol extracts) was diluted in 5% DMSO, and 45 µL of cell suspension were added to 384-well plates, incubated at 37ºC in 5% CO_2 incubator. After the incubation period (3 days for KB and MCF-7; 5 days for NCI-H187), 12.5 µL of 62.5 µg/mL Resazurin solution was added to each well and the plates were then incubated at 37ºC for 4 hours. Fluorescence signal was measured using a SpectraMax M5 multi-detection microplate reader (Molecular Devices, USA) at the excitation and emission wavelengths of 530 nm and 590 nm. Percent inhibition of cell growth was calculated by the following equation:

$$\% \text{ Inhibition} = [1 - (FU_T / FU_C)] \times 100 \qquad \text{[Equation 3]},$$

where FU_T and FU_C are the mean fluorescent unit from treated and untreated conditions, respectively. Dose response curves were plotted from six concentrations of twofold serially diluted test compounds and the sample concentrations that inhibit cell growth by 50% (IC_{50}) were derived using SOFTMax Pro software (Molecular Devices, USA).

Cytotoxicity Assay

The cytotoxicity of the extract against primate cell line (Vero) was assayed by using the Green Fluorescent Protein (GFP) detection methodology described by Hunt et al. (1999). The GFP-expressing Vero cell line was generated in-house by stably transfecting the African green monkey kidney cell line (Vero, ATCC CCL-81) with pEGFP-N-1 plasmid (Clontech). The cell line was maintained in minimal essential medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 1.5 g/L sodium bicarbonate, and 0.8 mg/mL geneticin at 37ºC in a humidified incubator with 5% CO_2.

The assay was carried out by adding 45 µL of cell suspension at $3.3x10^4$ cells/mL to each well of 384-well plates containing 5 µL of test compounds previously diluted in 0.5% DMSO, and then incubating for 4 days at 37ºC with 5% CO_2. Fluorescence signals were measured by using a SpectralMax M5 multi-detection microplate reader (Molecular Devices, USA) in the bottom reading mode with excitation and emission wavelengths of 485 and 535 nm. The fluorescence signal at day 4 was subtracted from the background flurescence at day 0. The percentage of cytotoxicity was calculated by the following equation:

$$\% \text{ cytotoxicity} = [1 - (FU_T / FU_C)] \times 100 \qquad \text{[Equation 4]}$$

where FU_T and FU_C represent the fluorescence units of cells treated with test compound and untreated cell, respectively. IC_{50} values were derived from dose-response curves, using six concentrations of twofold serially diluted samples, by SOFTMax Pro software (Molecular device). Ellipticine and 0.5% DMSO were used as positive and negative controls, respectively.

Isolation and Purification

The most active extract was the methanol extract of *A. indica,* which was then selected for isolation and purification. The methanol extract (5.0 g) was fractionated by column chromatography eluted with hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH, and MeOH, respectively, to give 12 major fractions (F_1-F_{12}). Fraction F_{12} was purified further. Each fraction of F_{12} was confirmed by TLC. Then all of them were combined together. Fraction F_{12} (867.1 mg) was refractionated by column chromatography, using the same eluents as above, to give 11 subfractions (PF_1-PF_{11}). Subfraction PF_9 (224.1 mg) was taken for analysis. This fraction (PF_9) consisted of white crystals mixed with green-brown sticky liquid. The white crystals were separated from the sticky liquid by washing with methanol, yielding 11 mg of pure white crystals that were subjected to 1H-NMR and 13C-NMR spectroscopic analysis.

The isolated compound was L-Quebrachitol, a white crystalline powder. Table 3 shows the ^{1}H-NMR, ^{13}C-NMR, and 2D-NMR data of L-Quebrachitol; HRESIMS: *m/z* = 195.1270 $[M+1]^+$ (calculated mass 195.1268).

RESULTS AND DISCUSSION

Antioxidant Activity

The antioxidant activities of the hexane, chloroform, and methanol extracts of *A. indica* were performed using DPPH and ABTS assays.

DPPH assay. The antioxidant activity of the extracts was evaluated by using the DPPH radical scavenging assay. Free radical-scavenging capacity of the extracts was measured by the DPPH assay. Determination of the reaction kinetic types DPPH-H is a product of the reaction between DPPH$^{\bullet}$ and an antioxidant (AH) shown in the following equation:

$$DPPH^{\bullet} + AH \leftrightarrow DPPH\text{-}H + A^{\bullet} \qquad \text{[Equation 5]}$$

The reversibility of the reaction is evaluated by adding DPPH-H at the end of the reaction. If there is an increase in the percentage of remaining DPPH$^{\bullet}$ at the plateau, the reaction is reversible; otherwise, it is a complete reaction. In the DPPH assay, the ability of the extracts sample to act as a donor of hydrogen atoms or electrons in transforming of DPPH$^{\bullet}$ into its reduced form DPPH-H was investigated. The examined sample can reduce the stable, purple-colored radical DPPH into yellow-colored DPPH-H. The IC_{50} of the extracts were determined by reference to the calibration curve. The result was compared with the antioxidant standard, Trolox. The hexane, chloroform, and methanol extracts showed significant antioxidant activities with an IC_{50} of 6.19 ± 0.010, 5.70 ± 0.050, and 7.79 ± 0.020 mg/mL, respectively (Table 1).

ABTS assay. The antioxidant activity of the extracts was also evaluated by using the ABTS radical cation scavenging assay. The IC_{50} of the extracts was determined by reference to the calibration curve. The result was compared with antioxidant standard, Trolox. The hexane, chloroform, and methanol extracts showed significant antioxidant activity with the IC_{50} of 6.13 ± 0.010, 6.31 ± 0.020, and 6.37 ± 0.020 mg/mL, respectively (Table 1).

Table 1. Antioxidant activity (DPPH and ABTS) of the extracts of *A. indica* L.

Test Sample	**IC_{50} (mg/mL)**	
	DPPH assay	**ABTS assay**
Hexane extract	6.19 ± 0.010	6.13 ± 0.010
Chloroform extract	5.70 ± 0.050	6.31 ± 0.020
Methanol extract	7.79 ± 0.020	6.37 ± 0.020
Trolox	0.08 ± 0.001	1.32 ± 0.005

Note: Trolox was used as positive control. Values are given as mean ± S.D. of triplicate experiments. Values in each column (DPPH assay and ABTS assay) represent the significantly different results ($p \leq 0.05$).

Anticancer Activity

The anticancer activities of all *A. indica* extracts were investigated. The samples were tested against three cancerous cell lines: KB-Oral Cavity Cancer, MCF7-Breast Cancer, and NCI-H187-Small Cell Lung Cancer, using the Resazurin Microplate Assay (REMA). Triplicated determinations were performed. The methanol extract showed significant anticancer activity against NCI-H187-Small Cell Lung Cancer with an IC_{50} of 25.00 μgmL^{-1}. Results are presented in Table 2.

Table 2. Anticancer activities of the methanol extract of *A. indica* L.

Cancer cell lines	IC_{50} (µg/mL)		
	Methanol extract	**Ellipticine**	**Doxorubicin**
KB-oral cavity cancer	Inactive	0.62	0.16
MCF7-breast cancer	Inactive	-	0.85
NCI-H187-small cell lung cancer	25.00	0.88	0.05

Note: Ellipticine and doxorubicin were used as positive controls; 0.5% DMSO was used as negative control. $IC_{50} > 50$ = Inactive.

Cytotoxicity Test

The cytotoxicity of the aerial part extracts of *A. indica* L was tested against the primate cell line (Vero) using the Green Fluorescent Protein (GFP) detection method. Ellipticine and 0.5% DMSO were used as positive and negative controls, respectively. The extracts were non-cytotoxic to primate cell line.

Isolation and Purification

The results indicated that three extracts of *A. indica* showed different antioxidant activities (Table 1). However, only the methanol extract showed significant anticancer activity against NCI-H187-Small Cell Lung Cancer with the IC_{50} of 25.00 μgmL^{-1} (Table 2). Therefore, methanol extract was used for further isolation and purification.

Isolation and purification of the methanolic extract of *A. indica* yielded L-Quebrachitol. Its structure was established based on spectroscopic evidence.

L-Quebrachitol was isolated as a crystalline white powder (Figure 3), with the molecular formula $C_7H_{14}O_6$ (Figure 4) as deduced from ^{13}C-NMR and HR-ESI-MS data. Analysis of the ^{1}H-NMR, ^{13}C-NMR, and HMBC spectra revealed:

- ^{1}H-NMR (CD_3OD, 400 MHz): δ 4.11 (t, 1H, J 3.2 Hz, H-1); 3.32 (dd, 1H, J 9.2, 3.2 Hz, H-2); 3.61 (t, 1H, J 9.2 Hz, H-3); 3.55 (t, 1H, J 9.2 Hz, H-4); 3.68 (dd, 1H, J 9.2, 2.0 Hz, H-5); 3.94 (t, 1H, J 3.2 Hz, H-6); 3.45 (s, 3H, H–OMe), (Table 3).
- ^{13}C NMR (100 MHz, CD_3OD): δ 69.49 (C1); 82.61 (C2); 73.95 (C3); 74.87 (C4); 72.49 (C5); 73.52 (C6); 58.03 (Me), (Table 3).
- HR-ESI-MS: m/z 195.1270 $[M+1]^+$; calculated mass 195.1268.

The COSY spectrum of these compounds indicated the following spin systems: the hydrogen at δ_H = 4.11 (1H, t, H-1) was coupled to the hydrogen at δ_H = 3.22 (1H, dd, H-2) and the hydrogen at δ_H = 3.94 (1H, t, H-6). The hydrogen at δH = 3.22 (1H, dd, H-2) was coupled to the hydrogen at δH = 4.11 (1H, t, H-1) and the hydrogen at δ_H = 3.61 (1H, t, H-3). The hydrogen at δH = 3.61 (1H, t, H-3) was coupled to the hydrogen at δ_H = 3.22 (1H, dd, H-2) and the hydrogen at δ_H = 3.55 (1H, t, H-4). The hydrogen at δ_H = 3.55 (1H, t, H-4) was coupled to the hydrogen at δ_H = 3.61 (1H, t, H-3) and the hydrogen at δ_H = 3.68 (1H, dd, H-5). The hydrogen at δ_H = 3.68 (1H, t, H-5) was coupled to the hydrogen at δ_H = 3.55 (1H, t, H-4) and the hydrogen at δ_H = 3.94 (1H, t, H-6). The hydrogen at δ_H = 3.94 (1H, t, H-6) was coupled to the hydrogen at δ_H = 4.11 (1H, t, H-1) and the hydrogen at δ_H = 3.68 (1H, dd, H-5) (Table 3).

The HMBC spectrum of this compound indicated the following spin systems: the proton at δ_H = 4.11 correlated with C-2 (δ_C = 82.61) and C-3 (δ_C = 73.52); the proton at δ_H = 3.32 correlated with C-3 (δ_C = 73.52), the proton at δ_H = 3.61 correlated with C-2 (δ_C = 82.61) and C-4 (δ_C = 74.87); the proton at δ_H = 3.55 correlated with C-6 (δ_C = 73.52); the proton at δ_H = 3.68 correlated with C-4 (δ_C = 74.87); the proton at δH = 3.94 correlated with C-1 (δC = 69.49), C-2 (δ_C = 82.61), C-4 (δ_C = 74.87) and C-5 (δ_C = 72.49); and the metroxy proton at δ_H = 3.45 correlated with C-2 (δ_C = 82.61) (Table 3 and Figure 5).

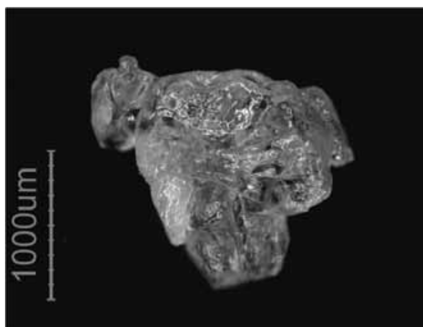

Figure 3. Crystalline white powder of sample.

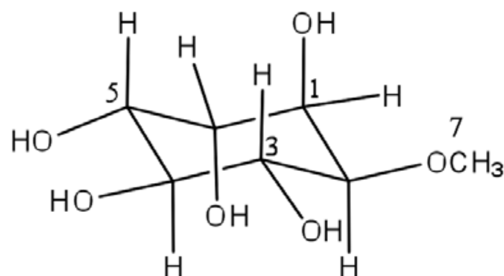

Figure 4. Chemical structure of compound L-quebrachitol.

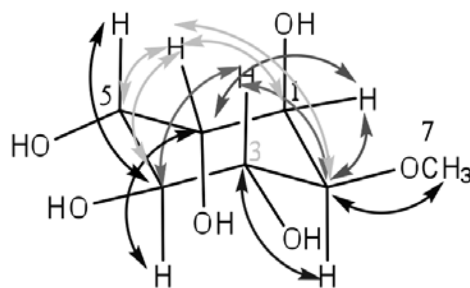

Figure 5. Key HMBC correlations.

Table 3. NMR data of L-quebrachitol in CD_3OD.

Position	^{1}H	^{13}C	COSY	HMBC (H→C)
1	4.11t	69.49	H-2, H-6	C-2, C-6
2	3.32dd	82.61	H-1, H-3	C-3
3	3.61t	73.95	H-2, H-4	C-2, C-4
4	3.55t	74.87	H-3, H-5	C-6
5	3.68dd	72.49	H-4, H-6	C-4
6	3.94t	73.52	H-1, H-5	C-1, C-2, C-4, C-5
7	3.45s	58.03		C-2

L-Quebrachitol is the 2-methyl ether of L-chiro-inositol. L-chiro-inositol is a cyclitol whose isomers occur in various plant sources (i.e., Proteaceae (Bieleski and Briggs, 2005) and Apocynaceae (Nishibe et al., 2001)).

L-Quebrachitol is used as a starting material for the synthesis of a wide variety of bioactive materials, most notably inositol. L-inositol exists in the form of L-(-)-2-*o*-methyl-chiro-inositol in 1% of rubber serum and can be used as starting material for synthesis of optically active organic compounds such as antibiotics and anticancer drug (Sakdapipanich, 2005). Recently, novel antioxidant and anticancer functions of inositol hexaphosphate, a naturally occurring component of plant fiber, have been discovered (Shamsuddin et al., 1997).

CONCLUSION

In this study, the hexane, chloroform, and methanol extracts from the aerial part of *A. indica* showed significant antioxidant activities with the IC_{50} in the ranges of 5.70 ± 0.05 to 7.79 ± 0.02 mg/mL (DPPH assay) and 6.13 ± 0.01 to 6.37 ± 0.02 mg/mL (ABTS assay). This medicinal plant possessed a rich source of antioxidant, indicating its effectiveness in curing disease caused by overproduction of radicals. The methanol extract of this plant also exhibited significant anticancer activity against NCI-H187-Small Cell Lung Cancer with the IC_{50} of 25.00 μgmL^{-1}.

One known compound, L-Quebrachitol, was also isolated from the methanol extract and its structure elucidated. This compound has been used as a starting

material for the synthesis of L-inositol, which could be used for the production of antibiotics and anticancer drugs.

The anticancer activity of this medicinal plant gives hope to the development of potential anticancer drugs.

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