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***Corresponding author:**
Ming-Chang Wu
Department of Food Science, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan
Tel: +886-932775187
Fax: +886-8-7740378
Email: mcwu@mail.npust.edu.tw

Antioxidant activities and A549 lung adenocarcinomic cells against capacity from various extracts of *Agrimonia pilosa* Ledeb

Quang-Ung Le[1], Horng-Liang Lay[2], Ming-Chang Wu[3]

1 *Department of Tropical Agriculture and international Cooperation, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan*

2 *Department of Plant Industry, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan*

3 *Department of Food Science, National Pingtung University of Science and Technology, Pingtung 91201, Taiwan*

ABSTRACT

Agrimonia pilosa Ledeb (AL) has received considerable attention as a herbal medicine for its applications in ethnopharmacology with heath benefits. This study aimed to investigate antioxidant activities and A549 growth inhibitory capacity from its root extract (RE) and aerial parts extract (AE). The 50% ethanol extracts were used for the tests. The total polyphenol content and the antioxidant effects comprising ABTS+ and DPPH free radical scavenging activities were evaluated. Phenolic compounds in the extracts were isolated using high performance liquid chromatography (HPLC). Lactate dehydrogenase released in medium was also evaluated. Total phenolic and flavonoid content, and *in vitro* antioxidant potential of the RE were higher ($p<0.01$) than that of the AE. Two individual phenolic compounds consisting of 4-hydroxybenzoic acid and p-coumaric acid were firstly identified in both by HPLC. The RE exhibited higher A549 inhibitory capacity compared to the AE and activated the apoptotic proteins of bcl-2, bax, bad, caspase-3 and caspase-9 in A549. In conclusion, the AL extracts were more effective in antioxidant and A549 cells inhibitory capacity.

Keywords: *Agrimonia pilosa*, Antioxidant, Anticancer, 4-hydroxybenzoic acid, p-coumaric acid.

INTRODUCTION

Antioxidant results have an outstanding function in many human diseases comprising cancer, diabetic complications, heart disease, liver damage, autism, and Alzheimer's disease [1]. Oxidants such as reactive oxygen species generated from activated neutrophils and macrophages have been reported to have a substantial function in the pathogenesis of diverse pain-related diseases, containing neurodegenerative disorders and cancer [2, 3]. Applying herbal medicine is universal and plants are still considered an interesting source of antioxidants that might contribute as leads for the advancement of new drugs. Several drugs such as anti-inflammatory digestive, neuro-protection, hepato-protection and antinecrotic have recently been exhibited to have mechanism of adsorbing and neutralizing free radicals as a part of their activity [4, 5]. During the past decades many herbal medicines have been broadly advanced for their antioxidant evidences and free radical inhibitory activity [6-9]. Herbal medicines possessing anticancer properties have been identified and are the source of substitutive medicine for cancer remedy [10]. Hence, they can be

consumed as a continuous source of novel medicines for present and future health problems of humans including cancer.

Agrimonia Pilosa Ledeb**,** a herbal medicine of the *rosaceae* family, has been recorded to have potential biological activities such as anti-tumor, anti-inflammatory, diuretic, and anti-diabetic properties [11,12]. It, one of the economically important medicinal herbs, is used as a tea being good for human heath in many countries. According to scientific works, its overground part contains phenol compounds: flavonoids and their glycosides: apigenin, kaempferol, quercetin, rutin, and luteolin [13]; hydroxy-cinnamonic acids: caffeic and chlorogenic acid [14]. Although there are many investigations of antioxidant capacity of the AL. and the results demonstrated that this plant was an excellent antioxidant source, its characteristics have not been detail and specific for its various parts. Thus, this study was designed to investigate *in vitro* for antioxidant and A549 cells growth inhibitory activities from underground and aerial parts of the AL.

MATERIALS AND METHODS

Chemicals and Reagents

Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), butylated hydroxytoluene (BHT), gallic acid, quercetin, phosphate buffer, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), lactate dehydrogenase (LDH) cytotoxicity assay kit, tris-HCl, NaCl, tween 20 and nonfat dry milk were obtained from Sigma-Aldrich (St. Louis, MO, USA). Na_2CO_3, $AlCl_3$ and CH_3COOK were obtained from Showa Chemical Co., LTD. (Japan). Kaighn's modification of Ham's F12 medium with L-glutamine (F-12K), fetal bovine serum (FBS), phosphate buffered saline (PBS) and penicillin G-streptomycin were obtained from GIBCO Laboratories (Grand Island, NY, USA). Dimethyl sulfoxide (DMSO) was purchased from J.T. Baker (Avantor Performance Inc, USA). Ethanol (95%) was purchased from Echo ChemicalCo., LTD. (Taiwan), and other chemicals and reagents.

Preparation of samples

The dried powder of root parts or aerial parts of *Agrimonia pilosa* (2 gams) was extracted twice with 50% ethanol at 30 ^oC for 2 hr by ultrasonic. Then, the decoction was filtered and removed of ethanol under reduced pressure in a vacuum evaporator at 45^oC. The yield of dried extract from dehydrated fractionation was calculated. The lyophilized fractionation was dissolved in dimethyl sulfoxide and then filtered to have a regular stock solution.

Determination of total phenolic and flavonoid content

The amount of total phenolics was determined by using the Folin-Ciocalteu method [15]. Gallic acid was used as standard to plot a standard curve. 500 µL of sample (1mg/mL) was dissolved in 1500 µL (1:10 v/v diluted with distilled water) of Folin-Ciocalteu's reagent, and 2 mL of 7.5% Na_2CO_3. The mixture was shaken and incubated for 1.5 hr in the dark at room temperature. The absorbance was recorded at 765 nm and the total phenolic contents are expressed in gallic acid equivalent per g of dry extract (mg of GAE/g of dry extract).

The amount of total flavonoid in extracts was determined spectrophotometrically according to method of Arvouet–Grand et al. (1994) [16] with slight modifications. Quercetin was used to creat the calibration curve. 500 µL of diluted sample (1 mg/mL) was dissolved in 1.5 mL of 95% ethanol and 2.3 mL of distilled water. Then 0.1 mL of 1M CH_3COOK, 0.1 mL of 10% $AlCl_3$, and 1mL of 1M NaOH were added. The mixture was incubated at room temperature for 1.5 hr and the absorbance was recorded at 415 nm. The total flavonoid contents are expressed in quercetin equivalent per g of dry extract (mg of QUE/g of dry extract). All samples were analyzed thrice and results averaged.

Antioxidant properties

$ABTS^+$ radical-scavenging activity

$ABTS^+$ radical-scavenging activity was determined by using procedure reported by Re [17]. This assay was based on the ability of different substances to scavenge 2,2'- azino-bis (ethybenzthiazoline-6-sulfonic acid). $ABTS^+$ was formed by reacting 7 mM ABTS stock solution with 2.45 mM potasium persulfate (1/1, vol/vol) for 12-16 hr in the dark. The $ABTS^+$ solution was adjusted to get the absorbance at 734 nm to 0.8 ± 0.05. 0.1 mL of BHT solution, as a positive control, diluted with methanol (2.5 to 160 µg/mL) and of the methanol extracts of root and aerial parts (5 to 160 µg/mL) was dissolved in 0.9 mL of $ABTS^+$ solution. Then the mixture was incubated for 30 min at 25^oC. The absorbance of all extracts was recorded at 734 nm. All tests were run in triplicate. The IC_{50} value was determined by exponential regression of the plotted points for each extract, revealing the concentration required to reduce the initial concentration of $ABTS^+$ radicals by 50%. The percentage scavenging values were calculated by following formula: $ABTS^+$ radical-scavenging activity (%) = $(1-A_s/A_c) \times 100$. Where A_c was the absorbance of the blank without extract and A_s was the absorbance of the sample.

DPPH radical-scavenging activity

Scavenging of free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was measured according to earlier report earlier with slight modifications [18]. The 0.1mM DPPH solution was prepared with ethanol 95%. At this concentration, an absorbance of 0.8 ± 0.025 at 517 nm is for all measurements. 0.1 mL of BHT solution, as a positive control, diluted with methanol (2.5 to 160 µg/mL) and of the methanol extracts of root and aerial parts at different concentrations of 10 to 160 µg/mL was dissolved in 0.9 mL of DPPH solution. The solutions were incubated at 37^oC for 30 min in the dark and the absorbance was recorded at 517 nm. All tests were run in triplicate. The IC_{50} was also determined. DPPH scavenging activity (%) = $(1-A_s/A_o) \times 100$. Where A_s was the absorbance of the sample and A_o was the absorbance of the blank without addition of extract.

High performance liquid chromatography (HPLC) analysis

Phenolic compounds was analyzed by the HPLC system (Hitachi, Tokyo, Japan) with a Chromaster 5110 pump, a chromaster 5210 autosampler, a chromaster 5430 diode array detector and a Mightysil RP-18 GP column (4.6 x 250 mm). Samples were centrifuged at 3000 x g for 10 min and filtered by 22 µm membrane prior to HPLC injection. Running program was carried out at a flow rate of 1.0 mL/min with following gradients: A = phosphoric acid/distilled water (pH 2.8); B = methanol with pH 2.8 adjusted by phosphoric acid; 0-3 min, 5% B, 3-6 min, 7 to 10% B; 6-10 min, 10 to 15 % B; 10-20 min, 15 to 20 % B, 20-25 min, 20 to 25% B; 25-30 min, 25 to 28 % B; 30-35min, 28 to 30 % B; 35-40 min, 30 to 40 % B; 40-45 min, 40 to 42 % B; 45-50 min, 42 to 45 % B; 50-60 min, 45 to 30 % B; 60-65 min, 30 to 35 % B; 65-70 min, 35% B. The injection volume was 15 µL under UV detector at 280 nm and temperature of 40^oC. The results were obtained by interpolation using the linear regression plot from the standard component solution.

Effect of extracts on tumor growth

Cell culture

A549 cells were cultured using F-12K added with 10% FBS, 100U/mL penicillin and 100 µg/mL streptomycin and incubated in a humidified atmosphere of 5% CO_2 at 37^oC.

Cell Viability Assay

A549 cells were cultured in 96-well plates at a density of $5x10^3$ cells/well. The cells were treated with samples at specified concentrations (62.5 to 750 µg/mL) and with 0.2% DMSO (as control) for 24 and 48 hr, followed by MTT assays. The cell viability was assessed by adding 10 µL of MTT solution in PBS (5mg/mL) and 100 µL medium into media-removed cell. The plates were avoided light by wrapping aluminum foil and incubated for 2 at 37^oC in a humidified incubator. MTT-containing media were discarded and the reduced formazan dye was solubilized by adding 100 µL of DMSO to each well. The plates were wrapped by aluminum foil again and incubated at 37^oC for another 20 minutes and then were shacked gently for 5 minutes. Absorbance was measured at 570 nm. Viable cells (%) = $[(A_s - A_o)/(A_c - A_o)] \times 100$. Where A_s - absorbance of extraction sample treated cells, A_o - absorbance of blank without cells and A_c - absorbance of control group (treated with DMSO).

Lactate dehydrogenase (LDH) released determination

Cell suspension (200 µL) was added to each well of a flat-bottom 96 well plate at a density of 5x10^4 cells/mL. After incubation for 24 hr, the culture medium was removed and changed with medium containing samples at specified concentrations (0 to 750 µg/mL) and then incubated for 48 hr. After injury, 25 µL of cell media were transfered into other 96- well plates and mixed with 75 µL of PBS and 100 µL of the LDH assay reagent, followed by incubating at room temperature for 30 min. Spectrophotometric absorbacne of the colored formazan was measured at 490 nm and referenced- 690 nm. Cells without treatment or lysed with 0.5 % (v/v) of triton X-100 were used as negative and positive controls, respectively. All assays were performed in triplicates. The LDH activity was determined as the percentage of experimental group and positive control, after subtraction of negative control respectively. The p-coumaric acid was used as a control reference [19].

Western blot analysis

Western blot analysis was performed by modifying the method described by Nho [20]. The RE was chosen to induce apoptotic cell death in A549 cells. Proteins were collected from the cells using 1xRIPA buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxyclolate, 0.1% SDS and protease inhibitor cocktail). Lysates were purified by centrifugation (12000 xg, 4^{o}C, 20 min) to collect and supernatants. The amount of protein content was quantified by the Bradford method. Proteins were added with 2x sample buffer, boiled at 95 ^{o}C for 5 min and separated on 12% polyacrylamade gels using the Mini Protean 3 Cell (Bio-Rad) and electrophoretically transferred onto nitrocellulose membranes. After blocking for 2 hr by blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 3% nonfat dry milk), the membrances were incubated with primary antibody at 4 ^{o}C overnight, then washed and probed with horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) and anti-rabbit IgG. Blots were developed with ECL Advance Western Blotting Detection Kit using a LAS-3000 luminescent image analyzer. The antibodies were diluted with specified concentrations: beta-actin (1:1000), bcl-2 (1:1000), bax (1:1000), bad (1:1000) and caspase-3 and caspase-9 (1:1000).

Statistical analysis

The data were analyzed using one-way ANOVA, P value <0.01 indicates statistically significant differences. All data were expressed as mean ± SD. All analyses were performed using the SAS statistical package (SAS Institute 1990).

RERUSTS AND DISCUSSION

Total phenolic and flavonoid content

The contents of phenolics and flavonoids from various parts of the AL are determined. There is a significant difference (p<0.01) in the amount of phenolic content (155.25±0.4^a mg GAE/g of dry extract in the RE and 136.02±1.24^b mg GAE/g of dry extract in the AE). The amount of flavonoid content also exhibited significant difference (p<0.01) (112.44±0.51^a mg QUE/g of dry extract in the RE and 101.67±0.22^b mg QUE/g of dry extract in the AE). The results showed that the amount of phenolic and flavonoid content of the RE are higher than that of the AE.

Antioxidant properties

The ABTS$^+$ scavenging capacity showed in Fig. 1. The comparative IC$_{50}$ values of the BHT, RE and AE were respectively 13.95±0.22^c, 114.27±2.45^b and 152.30±0.14^a µg/mL. So the ABTS$^+$ radical scavenging effect was ranged: BHT>RE>AE. Phenolic compounds have an important role in scavenging and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [21]. The high anti-oxidant activities of extracts may be because they possess the high amount of phenolics and flavonoids [22]. The evidences of antioxidant potential of many phenolic compounds were recorded because of their ability to scavenge free radicals and active oxygen species such as singlet oxygen, superoxide anion radical, and hydroxyl radicals [23]. A highly positive linear relationship exists between antioxidant capacity and the amount of phenolic content in many herbal medicines [24]. The present results confirmed that extract with the higher phenolic content has higher ABTS$^+$ radical scavenging capacity, which agrees with many former researches of positive correlations between the amount of phenol content and antioxidant capacity [25].

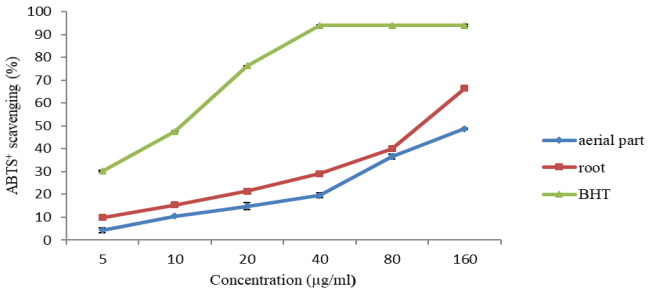

Figure 1: ABTS$^+$ radical scavenging capacity of extracts

The free radical scavenging ability of the herb was subsequently assessed using DPPH radical and presented in Fig. 2. The IC$_{50}$ values of aerial parts and root were respectively 62.38±4.7^a and 38.74±4.1^b µg/mL. These values were higher than those found with the antioxidant standard BHT, which exhibited an IC$_{50}$ value of 10.72±0.2^c µg/mL. Therefore, the scavenging effect of the tested samples was ranked as follows: BHT>RE>AE. The findings revealed that the RE exhibited excellent scavenging activity toward the antiradical and antioxidant. The RE possesses higher DPPH radical scavenging potential compared to the AE, which could be due to it has higher phenolic and flavonoid content. DPPH capacity highly exhibited in aqueous extract of the AL with IC$_{50}$ value of 13.0 µg/mL Zhu et al. (2009) [26]. Chunhuan et al. (2010) have reported that its ultrasonic extracts showed higher antioxidant effect than soxhlet extracts, which correlated to their polyphenolic content [27]. At 25 µg/mL concentration, methanol extract exhibited an outstanding ABTS$^+$ scavenging value reaching 93.1% [28]. The antioxidant properties have coherent relation with the presence of compounds acting to breaking the free radical. The amount of phenolic content of a spice has been usually found to correlate highly with their ABTS$^+$, DPPH scavenging activities, which emphasized that phenolics are main contributors responsible for free-radical scavenging of a spice [29, 30]. Our results are in agreement with the conclusions of these reports. In addition, the synergism between the antioxidants creates the difference of antioxidant activities not only dependent on the amount of phenolic compouds, but also on the structure and the interaction between the antioxidants [31].

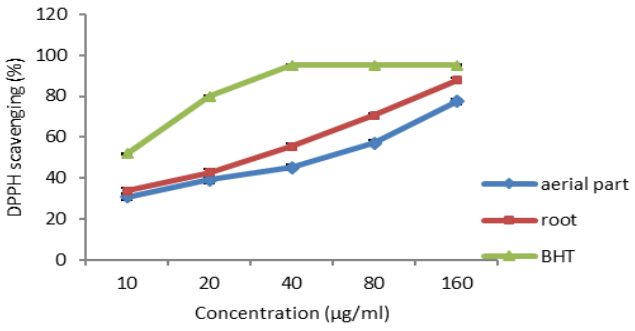

Figure 2: DPPH radical scavenging capacity of extracts

High performance liquid chromatography (HPLC) analysis

Three phenolics were identified, of which two new phenolics were identified and quantified in the AE and RE: 4-hydroxybenzoic acid (251.33±0.01[a] µg/g of dry extract and 144.18±0.02[b] µg/g of dry extract), and p-coumaric acid (2449.39±0.01[b] µg/g of dry extract, 7248.02[a]±0.02 µg/g of dry extract), respectively. The result is in agreement with previous reported works that the aerial parts of *Agrimonia Pilosa* contain chlorogenic acid [14] showed in Fig. 3. These compounds were reported to have antitumor activities: chlorogenic acid [32, 33]; 4- hydroxybenzoic acid [34]; p-coumaric acid [35, 36]. These compounds contribute in bio-activities of *Agrimonia pilosa*. We could not identify all of the reported compounds.

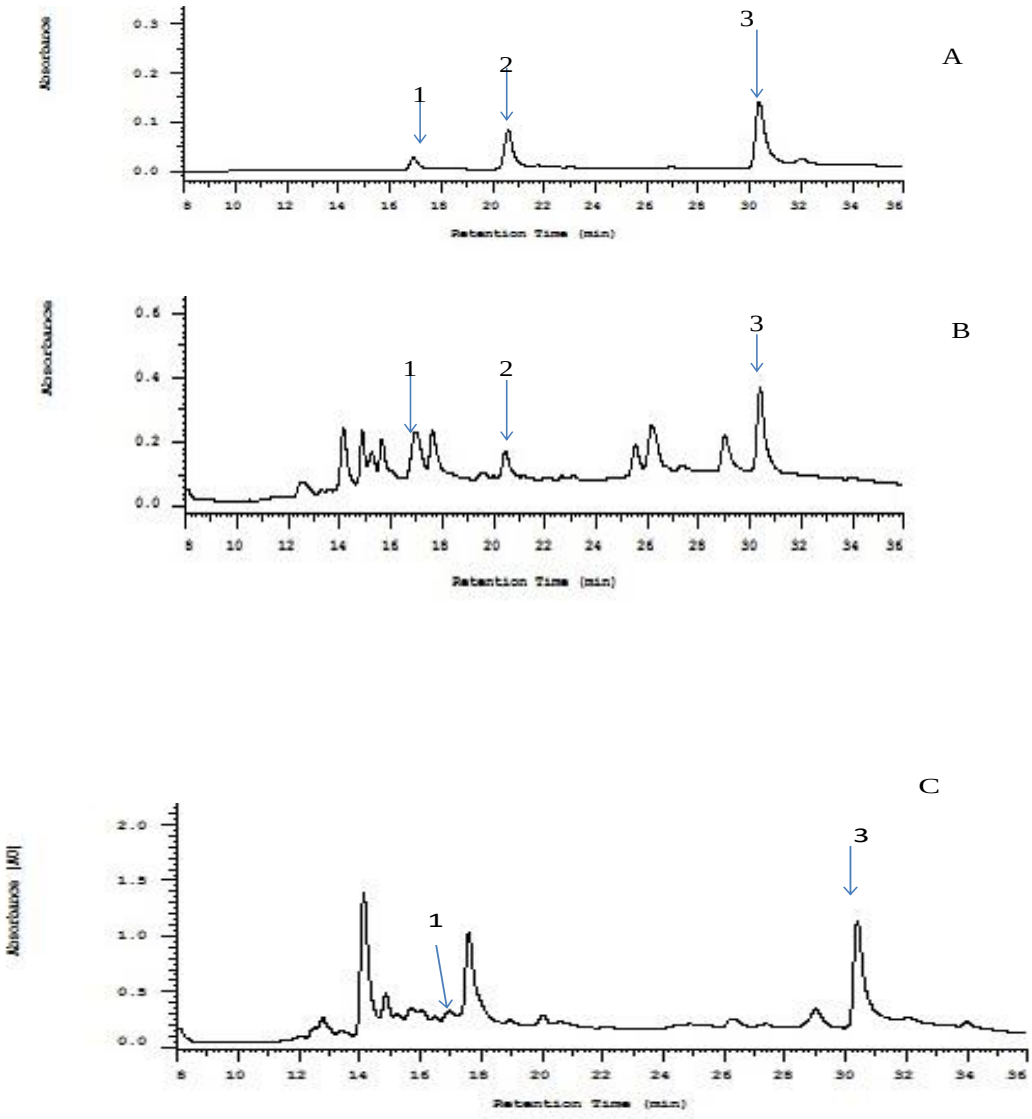

Figure 3: HPLC chromatogram of phenolic composition, (A) standard phenolic; (B) peaks of aerial parts, (C) peaks of root: (1), 4-hydroxybenzoic acid; (2), chlorogenic acid; (3), p-coumaric acid

Table 1: Various extracts inhibit growth of A549 cells

Concentration (µg/ml)	Cell viability (% of control)			
	after 24 hr		after 48 hr	
	aerial parts	root	aerial parts	root
control	100	100	100	100
62.5	91.51±0.31a*	80.10±0.61b*	63.00±0.08a**	59.24±0.86b**
125	84.74±0.23a**	71.51±0.22b**	56.10±0.74a**	52.84±0.16b**
250	66.73±0.46a**	62.06±0.94b**	47.58±0.27a**	45.44±0.40b**
500	36.60±0.70a**	34.76±0.17b**	29.06±0.2ab**	26.77±0.66b**
750	28.00±0.53a**	24.84±0.30b**	22.67±0.81a**	17.73±0.48b**

[a-b]values (mean ± SD) with different superscripts were significantly different at p less than 0.01 (n=3) at the same time and concentration; *p<0.001 versus untreated sample; **p<0.0001 versus untreated sample

Cell viability assay

Cell viability lowered when concentration of the RE and AE increase (Table 1). The RE was effective in lowering cell viability compared to the AE. At the highest concentrations of the RE and AE (750 µg/mL), 22.67% and 17.73% were viable after 48 hr. The higher viability means the lower inhibit capacity. At higher specified concentrations of the *Agrimonia Pilosa* extracts, the cell membrane might be damaged and cell viability lowered. The IC_{50} was 453.44 µg/mL and 394.19 µg/mL at 24 h; 231.29 µg/mL and 180.311 µg/mL at 48 h for aerial parts and root, respectively. Our results are in agreement with previous works that the AL had anticancer capacity [20, 37]. The findings revealed that the RE exhibited higher A549 inhibitory capacity compared to the AE.

Lactate dehydrogenase (LDH) released determination

The measuring released LDH helps to determine the loss of membrance integrity as a function of the amount of cytoplasmic lactate dehydrogenase release into medium by various extract on A549 (Fig. 4). It is obvious that aerial part and root extracts induced increases in the values of LDH in a concentration-dependent manner and LDH values are significant difference ($p<0.01$) between aerial parts extract and root extract at the same concentration. The funding showed that LDH released value increases while there is a fall in MTT cell viability. The previous works reported that LDH is a more precise marker of cytotoxicity, since injured cells are entirely fragmented during the progression of prolonged incubation with substances [38]. The intracellular LDH release to the medium is a determinable method of irreversible cell death when damaged cell membrane, where it is directly up regulate the subsequent induction of apoptosis [39].

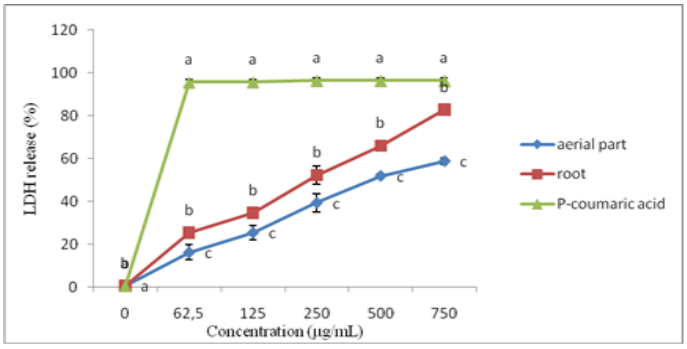

Figure 4: LDH release in cell culture supernatant of different concentration from various extracts of A. *pilosa* on A549 after 24 h treatment. [a-c]values (mean±SD) with different small letter being significantly different at the same concentration (62.5 to 750 µg/ml).

RE altered the expression of apoptosis-related proteins in A549 cells

Western blotting results showed that the RE down-regulated full-length (FL) caspase-3, bcl-2, bax, and bad, and up-regulated cleaved (CL) caspase-3. Especially, caspase-9 was obviousest expression at concentrations of 125 and 250 µg/mL. It could be explained that for 24 h at these concentrations, caspase-9 initiator strongliest activated. The RE induced degradation of caspase-9 (46 kDa) into a 37 kDa fragment (Fig. 5). The caspase-9, an essential initiator, is activated on the apoptosome complex and its pro-caspases exists as monomers and possess long-pro-domains containing specific protein-protein interaction sites that are crucial for initiator caspase activation [40]. The caspase-3 has an essential role in the terminal and execution phases of apoptosis which is induced by diverse stimuli [41]. The Bcl-2 family plays a crucial regulatory role, either as an inhibitor or activator in apoptosis [42]. The present results might be fundamental evidences that the RE inhibits growth of A549 by apoptosis mechanism.

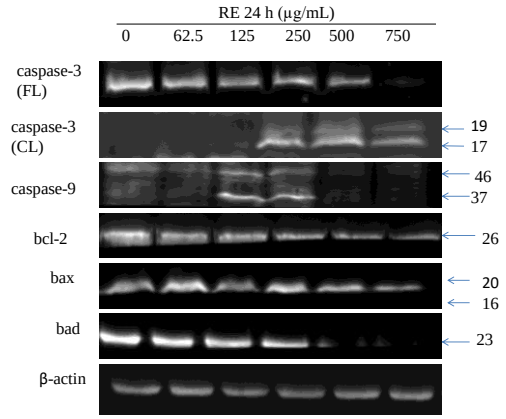

Figure 5: The RE activated apoptosis-related proteins (caspase-3, caspase-9, bcl-2,bax and bad) A549 cells after 24h.

CONCLUSION

This study provided detailed information on antioxidant activities and A549 cells growth inhibitory capacity from various parts of *Agrimonia pilosa* Ledeb. The RE has higher levels of the total phenolic and flavonoid content, and *in vitro* antioxidant capacities compared to the AE. In addition, the RE also showed higher A549 growth inhibitory capacity than the AE. The characteristics of the antioxidant mechanisms of *Agrimonia pilosa* Ledeb extracts should be evaluated in the future.

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