In vitro antioxidant capacity and antiproliferative effects of different parts and extractions from *Ajuga bracteosa* on HepG2 cell line

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The aim of this study was to investigate phenolic content, antioxidant activity and antiproliferative of *Ajuga bracteosa* extracts. These results revealed that *A. bracteosa* flower in water extract contained high amount of phenolic, flavonoids and tannin at 34.23 µg/ml (GAE), 14.23 µg/ml (QE) and 1.606 µg/ml (TE), respectively. Compared to positive control, *A. bracteosa* showed highest result in DPPH radical scavenging activity while lowest result in TEAC assays (ABTS radical scavenging activity) and reducing power. Leaf in water extract of *A. bracteosa* presented the highest activity in DPPH radical scavenging activity, while water, ethanol and methanol extracts of *A. bracteosa* flower showed highest antioxidant activity in TEAC assay compared to other parts. *A. bracteosa* from water extraction and flower part revealed highest activity in antiproliferative effect. From correlation analysis, radical scavenging activity of *A. bracteosa* may be influenced by all phenolic content, while antiproliferative effect may due to flavonoid content. In conclusion, water extract from flower *A. bracteosa* might be used as a potential source of natural antioxidants and antitumor agents.

Keywords: Phenolic content, antioxidant, antiproliferative, HepG2 and *Ajuga bracteosa.*

INTRODUCTION

Estimates from the year 2000 indicate that liver cancer remains the fifth most common malignancy in men and the eighth in women worldwide. The incidence of primary liver cancer is increasing in several developed countries, including the United States and the increase will likely continue for some decades (Bosch et al., 2004). In cases of localized hepatocellular carcinoma, surgical resection is potentially curative. However, many patients with hepatocellular carcinoma confined to the liver, curative surgical resection is frequently not an option due to cirrhosis and other pathologic changes in the liver parenchyma (Trincet et al., 1998). Thus, the development of a new therapeutic approach to hepatocellular carcinoma remains one of the most challenging areas in cancer research. Chemoprevention, a relatively new and promising strategy to prevent cancer, is defined as the use of natural dietary compounds and/or synthetic substances to block, inhibit, reverse, or retard the process of carcinogenesis. Thereafter, a variety of naturally-occurring dietary compounds have been shown to possess significant chemopreventive effects and many experimental attempts have been made to address their underlying mechanisms of action, including antioxidative, anti-inflammatory activity, induction of phase II enzymes, apoptosis, and cell cycle arrest (Pan and Ho, 2008).

There are at least 301 species of the genus *Ajuga* with many variations. Leaves of *A. bracteosa* are used as stimulant, diuretic and in the treatment of various diseases like rheumatism, gout, palsy and amenorrhoea (Kartikar and Basu, 1918). This plant also had been proved for its ability on lipoxigenase inhibition, antipyretic activity, cholineesterase inhibition, antispasmodic action and antifungal activity (Israili and Lyoussi, 2009). For antimalarial properties, the ethanolic leaves extract of *A. bracteosa* (250, 500 and

750 mg/kg/day) demonstrated a dose-dependent chemosuppression during early and in established infections, along with significant ($p<0.05$) repository activity (Chandel and Bagai, 2010). Interestingly, antimalarial natural products might provide a fertile and much needed lead in anticancer drug discovery. In total, 14 out of 15 nature-derived antimalarials (93%) referenced by WHO as well as 146 of 235 antimalarial natural species (62%) from medline search, including *Ajuga decumbens*, were reported as having anticancer activity (Duffy et al., 2010). Two major constituents of *Ajuga decumbens*, cyasterone and 8-acetylharpagide, have potent antitumor-promoting activities on a mouse-skin in vivo two-stage carcinogenesis procedure (Takasaki et al., 1999). There is no clinical efficacy of *Ajuga bracteosa* as an antiproliferative for cancer cell. Therefore, the present study was undertaken to evaluate antioxidant properties and antiproliferative effect of *Ajuga bracteosa* on HepG2 liver hepatocellular carcinoma.

METHODOLOGY

Preparation of plant extracts

Dried *Ajuga bracteosa* was purchased from Pingtung city, Taiwan. Ten grams of the respective part (leaf, flower and stem) of *Ajuga bracteosa* powder were extracted with 200 ml of boiling deionizer water for 5 min under regular stirring. The heated decoction was removed from the hot plate and allowed to infuse for another 30 min under regular stirring. For ethanol and methanol extractions, ten grams of the respective plant powder was added into 200 ml of 80% ethanol or methanol and allowed to macerate for 3 h under regular stirring. The resulting mixture was centrifuged at 3000 rpm for 10 min (Jouan CR 412, Vel, Belgium). After supernatant was filtered (Whatman No.1), the filtrate was concentrated under vacuum (50°C for water extract and 40°C for ethanol and methanol extract) and lyophilized. Lyophilized powders were dissolved and stored at -18°C for further use.

Determination phenolic concent

Determination of total phenolic content

The Folin–Ciocalteau method was conducted for the colorimetric estimation of total phenolic content. Each water extract (200 µL) was oxidised with Folin–Ciocalteau's reagents (2 N, 200 µL) for 4 min, and then the reaction was neutralised with saturated sodium carbonate (75 g/L, 400 µL). The absorbance of the resulting blue color was measured at 725 nm with an ultraviolet– visible spectrophotometer (Shimadzu UV2550, Kyoto, Japan) after incubation for 1 h at room temperature. Quantification was done based on a standard curve using gallic acid as standard. Results were expressed as gallic acid equivalent (GAE) in micrograms per miligram of dry residue (Hwang et al., 2011).

Determination of total flavonoid content

Briefly, 0.5 mL aliquot of appropriately diluted sample solution was mixed with 2 mL of distilled water and subsequently with 0.15 mL of a 5% $NaNO_2$ solution. After 6 min, 0.15 mL of a 10% $AlCl_3$ solution was added and allowed to stand for 6 min, then 2 mL of 4% NaOH solution was added to the mixture. Immediately, water was added to bring the final 3 volume to 5 mL, then the mixture was thoroughly mixed and allowed to stand for another 15 min. Absorbance of the mixture was determined at 510 nm versus a prepared water blank. The total flavonoid content was determined using a standard curve with quercetin (0–50 µg/ml). Result was expressed as microgram of quercetin equivalents (µg QE) per milligram dry weight sample (Zou et al., 2004).

Determination of tannins contents

The presence of tannins was identified using the classic $FeCl_3$ and gelatin tests. Amount of 0.005 g of dry sample was transferred to test tubes; 2.5 ml water was added and boiled for 30 min. After filtration with cotton filter the solution was further transferred to a 50 ml test tubes and water was added ad 25 ml mark. 0.25 ml aliquots were finally transferred to vials, 0.5 ml 1% $K_3Fe(CN)_6$ and 0.5 ml 1% $FeCl_3$ were added, and water was added ad 5 ml volume. After five min time period, the solutions were measured using spectrophotometer at 720 nm. The actual tannin concentrations were calculated on the basis of the optical absorbance values obtained for the standard solutions in range 5-25 µg/ml (Paaver et al., 2010).

Antioxidant capacity

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay

Radical scavenging activity of plant in different extractions and parts were determined according to Luo et al. (2011). Different concentrations of plant powder (100,500 and 1000 µg/ml) in ethanol (2 ml) were mixed with 2 ml of ethanolic solution containing 1 mM DPPH. The mixture was shaken vigorously and then left to stand for 30 min in the dark. The absorbance was measured at 517 nm. The control sample was also prepared as above without any extract and ethanol was used for the baseline correction. Radical scavenging activity was calculated as the inhibition percentage and was calculated using the following formula: (absorbance

control-absorbance sample)/absorbance control × 100%. Ascorbic acid, buthylated hydroxytoluene and gallic acid were used as standards.

TEAC (Trolox Equivalent Antioxidant Capacity) assay

The radical scavenging activity of the isolated compounds against $ABTS^{\bullet+}$ was measured using the methods of Han *et al.* (2008) with some modifications. ABTS was dissolved in PBS (0.01 M, pH 7.4) to a 7 mM concentration. $ABTS^{\bullet+}$ was produced by reacting ABTS stock solution with 2.45 mM potassium persulfate (final concentration) and allowing the mixtures to stand in the dark at room temperature for 16 h before use. The $ABTS^{\bullet+}$ solution was diluted with PBS (0.01 M, pH 7.4) to an absorbance of 0.70 (±0.02) at 734 nm and equilibrated at 30 °C for 30 min. An ethanol solution (2.0 ml) of the samples at various concentrations (100, 500 and 1000 µg/ml) was mixed with 2.0 ml of diluted $ABTS^{\bullet+}$ solution. After reaction at room temperature for 20 min, the absorbance at 734 nm was measured. Lower absorbance of the reaction mixture indicates higher $ABTS^{\bullet+}$ scavenging activity. The capability to scavenge the $ABTS^{\bullet+}$ was calculated using the following formula, where absorbances $_{(blank\ sample)}$ (Abs) was absorbance of 2.0 ml of PBS plus 2 ml of the sample at different concentrations, absorbances $_{(control)}$ (Ac) was absorbance of 2.0 ml of diluted $ABTS^{\bullet+}$ solution plus 2 ml of ethanol and absorbances $_{(blank\ control)}$ (Abc) was absorbance of 2.0 ml of PBS plus 2 ml of ethanol. ABTS radical scavenging activity (%) was calculated by {1-(Asample-Abs)/(Ac-Abc)}x 100%.

Reducing power assay

The reducing power of the samples is determined according to the method described by Zou et al. (2004). One ml of different concentration sample (100, 500 and 1000 µg/ml) in methanol is mixed with 0.2 M phosphate buffer (2.5 ml, pH 6.6) and 1% potassium ferricyanide (2.5 ml), and the mixture is incubated at 50°C for 20 min. Afterward, 2.5 ml of 10% trichloroacetic acid are added to the reaction mixture and centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) is mixed with 2.5 ml distilled water and 1 ml of 1% ferric chloride. The absorbance is measured at 700 nm. A higher absorbance of this mixture indicates a higher reducing activity. Ascorbic acid, buthylated hydroxytoluene and gallic acid were used as standards.

Cell culture

Cancer cell lines, including HepG2, a human hepatocarcinoma cell line, were obtained from reseach center in Hsinchu. The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) heat-inactivated fetal bovine serum (FBS) and 0.05% of penicillin. Cells were maintained in a humidified incubator at 37 °C in a 5% CO_2 atmosphere (Kim *et al.,* 2010). The effect of the extracts on the cell viability of various cancer cell lines was determined by the 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay. Briefly, exponential-phase cells were collected and transferred to a 96-well plate (1×10^5 cells per well). The cells were then incubated for 24 h in the presence of various concentrations of the extracts. After incubation, 0.1 mg of MTT was added to each well and the cells were incubated at 37°C for 4 h. The media was then carefully removed and DMSO (100 µl) were added to each well to dissolve the formazan crystals. The plates were read after 10 min at 570 nm on a microplate reader.

Statistical analysis

Experiments were done in three replication and the data were expressed as mean ± standard deviation (SD). Statistical analysis were performed by SPSS analysis of variance with Duncan's post hoc where $p<0.05$ ($p<0.01$ for antiproliferative assay) indicated that the results have statistically significant differences. Graphics shown were made by SigmaPlot 10.0 graphics software.

RESULTS

Phenolic content of *Ajuga bracteosa*

Determined phenolic contents are including total phenolic content, total flavonoid and total tannin. The revealed results in Table 1 showed that flower part of *A. bracteosa* in water extract contained highest amount of phenolic, flavonoids and tannin at 34.23 µg GAE /mg, 14.28 µg QE/mg and 8.04 µg TAE /mg, respectively. Lowest phenolic and flavonoid content found in *A. bracteosa* stem part of water (11.59 µg GAE /mg) and ethanol (0.65 µg QE/mg) extracts.

Antioxidant capacity of *Ajuga bracteosa*

Antioxidant capacity results were shown in Figure 1-3. Antioxidant activity of *A. bracteosa* extracts are mostly related to its ability in DPPH radical scavenging because it showed highest result. Compared to positive control, TEAC assay (ABTS radical scavenging activity) and reducing power of *A. bracteosa* showed low antioxidant capacity. Leaf in water extract of *A. bracteosa* presented the highest activity in DPPH radical scavenging activity (Figure 1), while water,

Table 1. Total phenolic, flavonoid and tannin content of *Ajuga bracteosa* extracts in different parts and solvent extractions.

Extract	Plant part	Total phenolic content (µg GAE/mg)	Total flavonoid content (µg QE/mg)	Total tannin (µg TAE/mg)
Water	Leaf	21.17±0.97^{c}	7.25 ± 0.05^{e}	6.60 ± 0.04^{b}
	Flower	35.27 ± 1.47^{e}	14.28 ± 0.26^{g}	8.04 ± 0.03^{f}
	Stem	11.59 ± 0.06^{a}	9.52 ± 0.44^{f}	7.32 ± 0.07^{d}
Ethanol	Leaf	18.87 ± 1.27^{b,c}	1.67 ± 0.10^{b}	6.86 ± 0.04^{c}
	Flower	26.96 ± 0.38^{d}	2.80 ± 0.18^{d}	7.67 ± 0.02^{e}
	Stem	13.60 ± 0.93^{a}	0.65 ± 0.04^{a}	6.44 ± 0.08^{a}
Methanol	Leaf	17.13 ± 1.56^{b}	2.18 ± 0.09^{c}	6.95 ± 0.10^{c}
	Flower	24.96 ± 1.64^{d}	3.22 ± 0.18^{d}	8.00 ± 0.03^{f}
	Stem	13.99 ± 0.54^{a}	0.95 ± 0.07^{a}	6.70 ± 0.05^{b}

$^{a-g}$Data with different superscripts are significantly different at P<0.05 (n=3) analyzed by ANOVA with Duncan's post hoc using SPSS 13.

Table 2. Correlation coefficients of phenolic, antioxidant and HepG2 antiproliferative effect

	Phenolic	Flavonoid	Tannin	DPPH	TEAC	Reducing	HepG2
Phenolic	1	.518(*)	.739(**)	.600(**)	.595(**)	.235	-.330
Flavonoid		1	.513(*)	.474(*)	.700(**)	-.405	-.521(*)
Tannin			1	.075	.800(**)	-.134	-.268
DPPH				1	.351	.331	-.332
TEAC					1	-.274	-.395
Reducing						1	.225
HepG2							1

* Correlation is significant at the 0.05 level (2-tailed).
** Correlation is significant at the 0.01 level (2-tailed).

ethanol and methanol extracts of *A. bracteosa* flower showed highest antioxidant activity in TEAC assay compared to other parts (Figure 2). In addition, all extracts of *A. bracteosa* flower also revealed highest antioxidant activity in reducing power assay compared to others (Figure 3).

Antiproliferative effect of *Ajuga bracteosa* on HepG2 cell line

Antiproliferation activities were also studied in vitro using $HepG_2$ human liver hepatocellular carcinoma, and *A. bracteosa* flower extract revealed highest activity. In the concentration of 125 µg/mL, all the extract showed significant antiproliferative activity compared to negative control. Positive control used in this research are 5-Flourouracil (100 µg/mL), and water extract of leaf, flower and stem in the concentration of 500 µg/mL showed highest antiproliferative activity and they have no significant difference to positive control.

Correlation coefficients of phenolic, antioxidant and HepG2 antiproliferative effect

Correlation coefficients of phenolic, antioxidant and HepG2 antiproliferative effect result are shown in Table 2. Result showed that DPPH and TEAC assays closely related to total phenolic content (p<0.01). Total flavonoid and tannin content also closely related with ABTS radical scavenging activity, which mean that all of total phenolic, flavonoid and tannin contents contribute to radical scavenging activity but not for reducing power. On the other hand, HepG2 antiproliferative activity has correlation with total flavonoid content in the lower significance (p<0.05).

DISCUSSION

Phenolic compounds are attracting considerable interest in food and medicine area due to its antioxidant capacity. Antioxidants may act in various ways such as

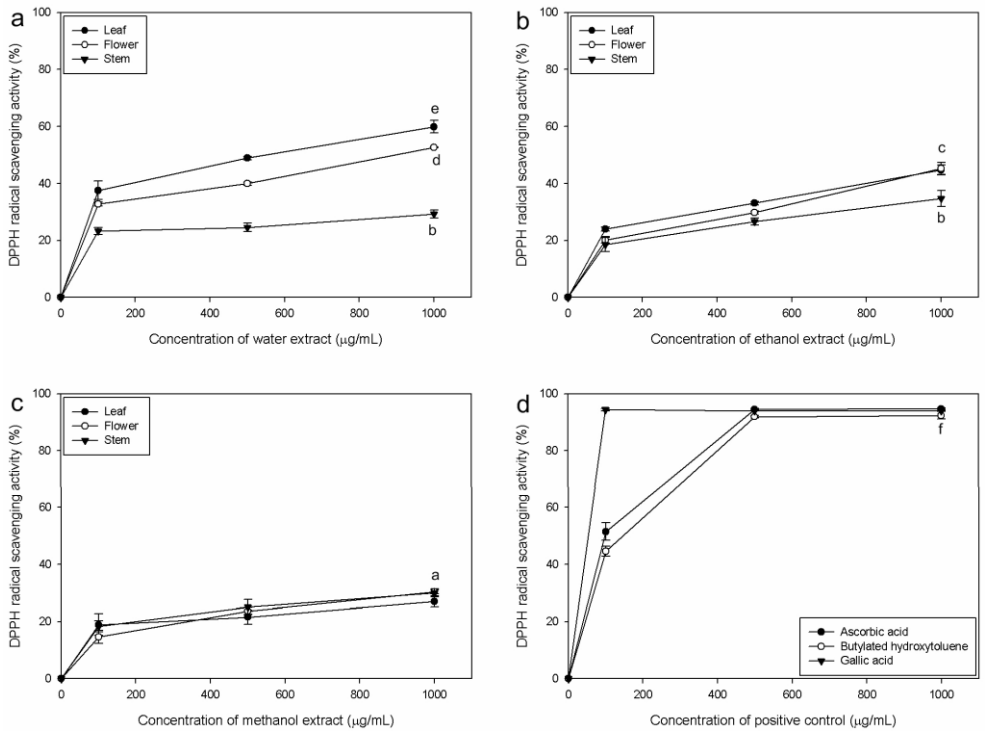

Figure 1. DPPH radical scavenging activities of *Ajuga bracteosa* water extract (a), ethanol extract (b), methanol extract (c) and positive control (d). [a-f]Data with different superscripts are significantly different at P<0.05 (n=3) analyzed by ANOVA with Duncan's post hoc using SPSS 13.

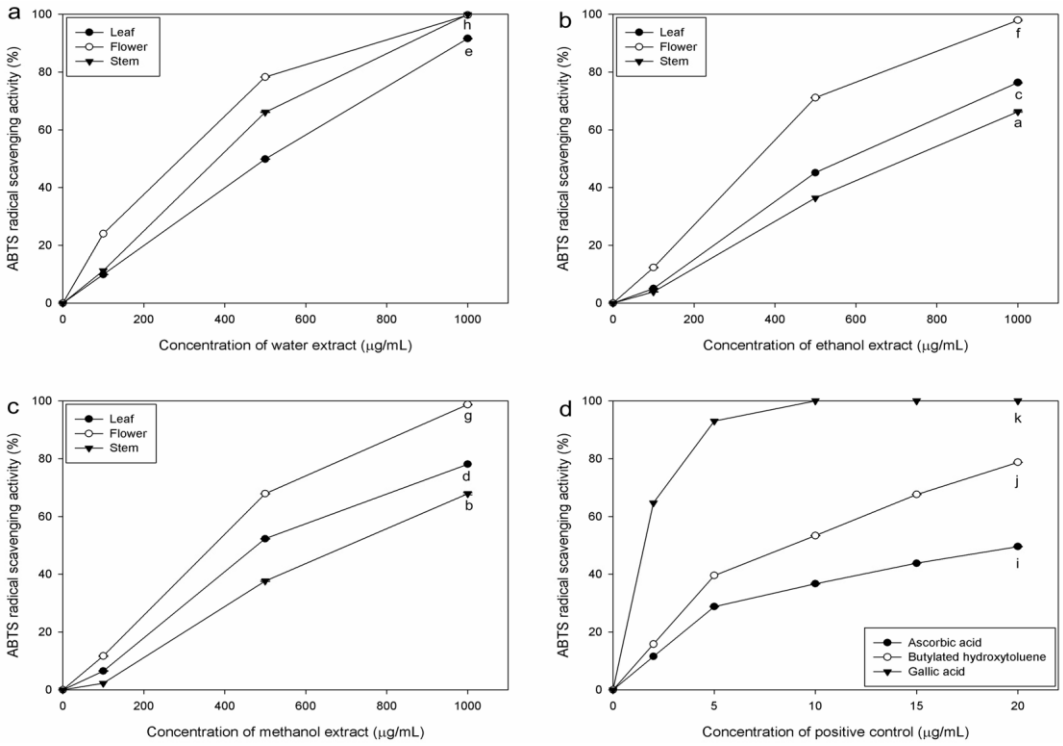

Figure 2. TEAC (Trolox Equivalent Antioxidant Capacity) assay of *Ajuga bracteosa* water extract (a), ethanol extract (b), methanol extract (c) and positive control (d). [a-k]Data with different superscripts are significantly different at P<0.05 (n=3) analyzed by ANOVA with Duncan's post hoc using SPSS 13.

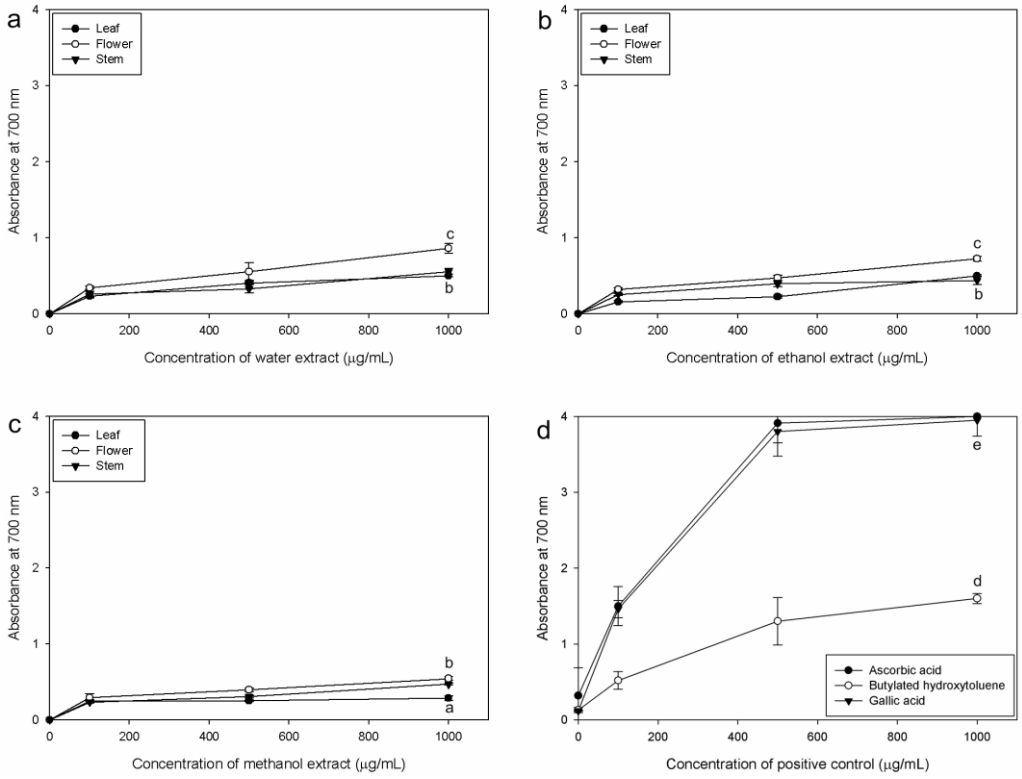

Figure 3. Reducing powers of *Ajuga bracteosa* water extract (a), ethanol extract (b), methanol extract (c) and positive control (d). [a-e]Data with different superscripts are significantly different at P<0.05 (n=3) analyzed by ANOVA with Duncan's post hoc using SPSS 13.

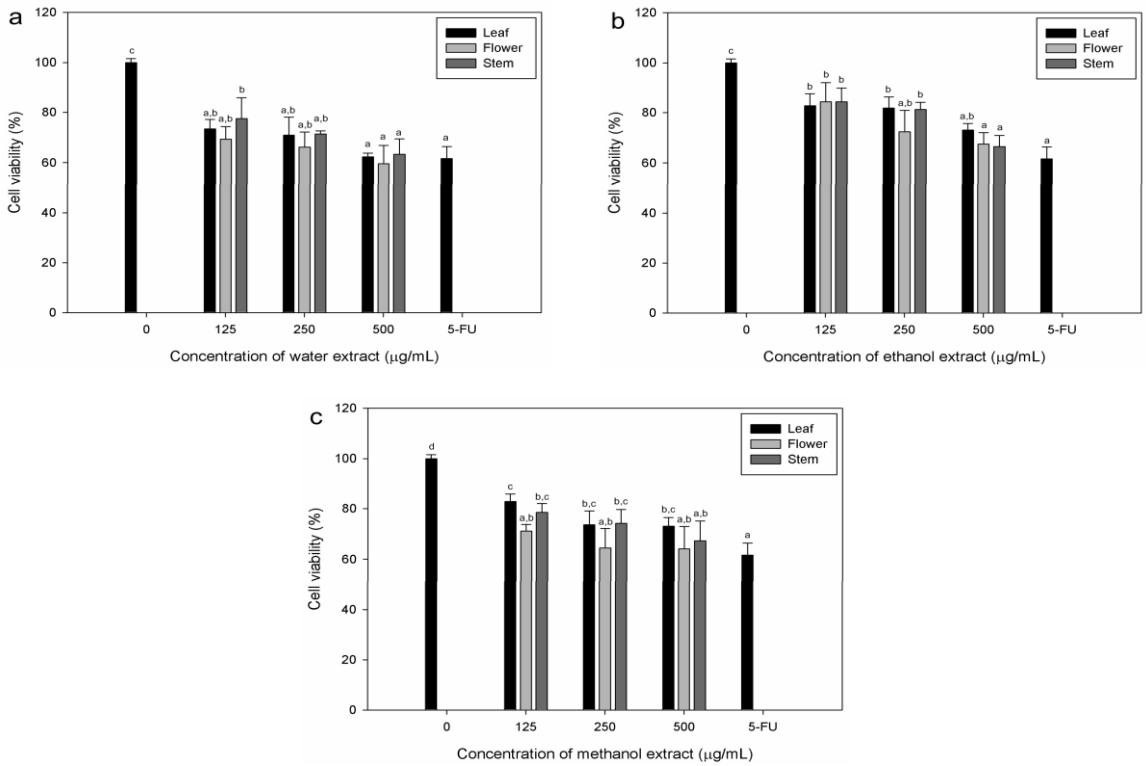

Figure 4. Antiproliferative activity of *Ajuga bracteosa* water extract (a), ethanol extract (b), methanol extract (c) on HepG2 liver hepatocellular carcinoma. [a-d]Data with different superscripts are significantly different at P<0.05 (n=3) analyzed by ANOVA with Duncan's post hoc using SPSS 13.

scavenging the radicals, decomposing the peroxides and chelating the metal ions. Plant polyphenols are multifunctional and can act as reducing agents, hydrogen donating antioxidants, singlet oxygen quenchers and also metal chelation in same case (Rice-Evans et al., 1996). The result of this present study indicated that phenolic compounds of *A. bracteosa* are powerful free radical scavenger of DPPH radical. Rice-Evans *et al.* (1997) has reported that antioxidant properties of phenolic compound relative to Trolox, the water-soluble vitamin E analogue, are related to their structures. The structural arrangements imparting greatest antioxidant activity as determined from these studies are: (1) the *ortho* 3',4'-dihydroxy moiety in the B ring (e.g. in catechin, luteolin and quercetin); (2) the *meta* 5,7-dihydroxy arrangements in the A ring (e.g. in kaempferol, apigenin and chrysin); (3) the 2,3-double bond in combination with both the 4-keto group and the 3-hydroxyl group in the C ring, for electron delocalization (e.g. in quercetin), as long as the o-dihydroxy structure in the B ring is also present. However, alterations in the arrangement of the hydroxyl groups and substitution of contributing hydroxyl groups by glycosylation decrease the antioxidant activity (Rice-Evans et al., 1997). In addition, Hagerman et al. (1998) reported that the high molecular weight phenolics (tannins) have more ability to quench free radicals (ABTS $^{\bullet+}$) and that effectiveness depends on the molecular weight, the number of aromatic rings and nature of hydroxyl groups' substitution than the specific functional groups.

Cancer chemoprevention is currently regarded as promising way for cancer control. The polyphenolic components of higher plants may act as antioxidants or as agents of other mechanisms contributing to anticarcinogenic or cardioprotective action (Rice-Evans et al., 1996). Moderate correlation was observed between antiproliferative effect and total flavonoid content. Great interest has been paid to flavonoids-one of the major classes of natural products with widespread distribution in fruits, vegetables, spices, tea and soy-based foodstuff-for their interesting antiproliferative activities. From its structure–activity relationships, 2′-hydroxychalcones and methoxylated flavanones were found to be potent inhibitors of MCF-7 breast cancer cells growth (Pouget et al., 2001).

eside of phenolic compound, *Ajuga bracteosa* also contains terpenoids and steroids. Withanolides (bracteosin A, B, C), β-sitosterol 3-O-β-D-glucopyranoside, dihydroclerodin-1, cleronidin A, dihydroajugapitin and lupulin A have been isolated from this plant (Riaz, 2004). Other compound such as hexacosanol, tetracosanoic acid (Bhakuni et al., 1978) and betulinic acid (Arfan et al., 1996) also found in *A. bracteosa*. Ichikawa et al. (2006) found that withanolides, biologically active natural steroidal lactones, suppressed NF-κB activation induced by a variety of inflammatory and carcinogenic agents, including tumor necrosis factor (TNF), interleukin-1B, doxorubicin, and cigarette smoke condensate. Suppression was not cell type specific, as both inducible and constitutive NF-κB activation was blocked by withanolides (Ichikawa et al., 2006). In summary, the results of this study indicate that water extract of *A. bracteosa* flower may be considered as good source of natural compounds with significant free radical scavenging and antiproliferative effect and it may due to its phenolic compound.

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