

## Original Article

# Phenolic Composition, in Vitro Antioxidant and Anticancer Activities of *Hypericum Japonicum* Thunb and *Scoparia Dulcis* L

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## Abstract

**Background and Aim:** *Hypericum Japonicum* Thunb (HT) and *Scoparia Dulcis* L (SL) have received considerable attentions as natural products for their applications with health benefits. This study aimed at investigating the *in vitro* antioxidant and HepG2 (human liver cancer cell line) and A549 (adenocarcinomic human alveolar basal epithelial cell line) growth inhibitory effect of the ethanol extracts from the HT and SL.

**Materials and Methods:** Total phenolic and flavonoid contents were calculated. Antioxidant effect was determined by measuring hydroxyl radical scavenging activity, 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS<sup>+</sup>) and 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activities. Phenolic compounds were identified by high performance liquid chromatography (HPLC) method. The activity of the apoptotic proteins Bcl-2, Bad, Bax, Caspase-3, -9 were measured by Western blotting.

**Results:** The rosmarinic acid and chlorogenic acid were isolated from the HT. The rutin and rosmarinic acid were newly identified compounds from the SL. Moreover, results revealed that the HT extract possesses the higher levels of ABTS<sup>+</sup>, DPPH and hydroxyl radical scavenging capacity with IC<sub>50</sub> of 45.13±0.89, 89.29±0.78 and 38.32±0.17µg/mL, respectively. The HT and SL extracts induce HepG2 growth inhibition with IC<sub>50</sub>: respectively 245.755 ± 2.23<sup>b</sup> µg/mL and 553.32 ± 14.46<sup>a</sup> µg/mL and induce A549 growth inhibition with IC<sub>50</sub> of the HT and SL: 314.15± 5.96<sup>b</sup> µg/mL and 446.35± 0.9<sup>a</sup> µg/mL at 48 hr, respectively. The HT and SL extracts activated the apoptotic proteins including bcl-2, bad, caspase-3, -9 in HepG2; p53, caspase-3, bax and bad in A549.

**Conclusion:** The findings strongly suggest that the HT and SL extracts could be excellent sources of antioxidants as functional food ingredients in cancer prevention and treatment.

**Keywords:** Antioxidant, Anticancer, Apoptosis, *Hypericum Japonicum* Thunb, *Scoparia Dulcis*

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## Introduction

In recent decades, researchers have been concerned with the investigation of antioxidative molecules widely distributed in the natural products, as they prevent oxidative stress by scavenging free radicals (1). Hence, increased consumption of the herb medicines that are potential natural sources of anti-cancer compounds has been recommended. *Hypericum japonicum* Thunb (HT), a member of *Hypericaceae* family, contains more than 50 of isolated compounds (2). Some of the major compounds include quercetin and quercitrin (3); isoquercitrin, quercetin-7-O- $\alpha$ -L-rhamnoside, kaempferol and kaempferol-7-O- $\alpha$ -L-rhamnoside (4); rutin (5); chlorogenic acid (6); 3,4-dihydroxybenzoic acid (7). It has been used in ethnic medicine for relieving internal heat or fever, hemostasis and detumescence. Modern pharmacological studies have indicated that the HT could be used to treat bacterial diseases, hepatic diseases, gastrointestinal disorders and tumors. So far, no study has been reported so far on the toxicity of the HT (2). This species has been exploited for decades, hence and its phytochemical characteristics have been sufficiently described also. However, this study is the first research conducted on the cytotoxicity against A549 tumor cell line (human alveolar basal epithelial cells).

*Scoparia Dulcis* L (SL), a member of *plantaginaceae* family, is widely consumed in Indian traditional medicine to treat diabetes mellitus and used for ailments like diarrhea, stomach-ache and renal problems (8). It is a rich source of flavones, terpenes and steroids (9); scopadulcic B (10); glutinol (11); dulcinodal and scopadiol decanoate (12). This study aimed at clarifying the total phenolic and flavonoid content, antioxidant effect and HepG2 (human liver cancer cell line) and A549 growth inhibitory effect from whole plant ethanol extracts (*Hypericum Japonicum* Thunb and *Scoparia Dulcis* L).

## Materials and Methods

### Chemicals and reagents

Folin-Ciocalteu reagent, gallic acid, quercetin, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2'-azino-bis (3-

ethylbenzothiazoline-6-sulphonic acid (ABTS), butylated hydroxytoluene (BHT), phosphate buffer, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), tris-HCl, NaCl, tween 20, nonfat dry milk, and Kaighn's modification of Ham's F12 medium with L-glutamine (F-12K) medium were procured from Sigma-Aldrich (St. Louis, MO, USA). Sodium carbonate ( $\text{Na}_2\text{CO}_3$ ), aluminum chloride ( $\text{AlCl}_3$ ) and potassium acetate ( $\text{CH}_3\text{COOK}$ ) were purchased from Showa Chemical Co., LTD. (Japan). Dulbecco's Modified Eagle Medium (DMEM), 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 Chemical Co., LTD. (Taiwan). Nitrocellulose filters were purchased from Scheicher & Schnell BioScience, Dassel, Germany. 1xRIPA buffer and all antibodies; horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG) and anti-rabbit IgG were purchased from Cell Signaling Technology, Beverly, MA, USA.

### Preparation of samples

Sieved dry powders of samples were separately extracted twice with 70 % (v/v) ethanol at 30°C for 2 hr in an ultrasonic bath. The extracts were filtered and filtrates were evaporated at 45°C to dryness in a rotary evaporator. The dehydrated fractionation was dissolved in dimethyl sulfoxide to a regular concentration.

### Total Phenolic Content (TPC)

The TPC was determined according to Djeridane et al. using gallic acid as a standard (13). 500  $\mu\text{L}$  of sample (1mg/mL) was reacted in 1.5 mL of Folin Ciocalteu's diluted with distilled water (1:10 v/v) and incubated at room temperature. After 1min 2 mL of 7.5%  $\text{Na}_2\text{CO}_3$  was added and the absorbance was recorded at 765 nm after incubating for 1.5 hr in the dark with intermittent shaking. The results were exhibited as a gallic acid equivalent (mg of GAE/g of dry extract).

### Total Flavonoid Content (TFC)

The TFC was determined according to Meda et al. using quercetin as a standard (14). Briefly, 0.5 mL of appropriately diluted sample was combined with 1.5

mL of 95% ethanol, 2.3 mL of distilled water, 0.1 mL of 1M CH<sub>3</sub>COOK, 0.1 mL of 10% AlCl<sub>3</sub>, and 1mL of 1M NaOH. The mixture solution was incubated at the room temperature for 1.5 hr and the absorbance was recorded at 415nm. The results were expressed as quercetin equivalent (mg of QUE/g of dry extract).

#### High Performance Liquid Chromatography (HPLC) Analysis

The samples were dissolved in 100% methanol and filtrated through a 0.45 µm Whatman filter unit and 20 µL of sample was injected into HPLC (Hitachi, Tokyo, Japan) with a Mightysil RP-18 GP column (4.6 x 250 mm, 5µm) (Japan). The mobile phase was a mixture of (A)-distilled water ragulated at pH 2.8 and (B)-methanol was at appropriate pH by phosphoric acid and was measured by pH meter SP-2100. A linear gradient was used: 0-2 min, 3% B; 2-6 min, 6 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 30 % B; 30-35 min, 30 to 32 % B; 35-40 min, 32to 40 % B; 40-45 min, 40 to 45 % B; 45-50 min, 45 to 48 % B; 50-60 min, 48 to 30 % B; 60-68 min, 30 to 35 % B. The flow rate, UV detector and column temperature were 1.0 mL/min, 280 nm and 40°C respectively. The results were obtained by interpolation using the linear regression plot from the standard component solution

#### ABTS<sup>+</sup> Radical Scavenging Ability

ABTS<sup>+</sup> radical scavenging assay was performed according to Re *et al.* (15). ABTS<sup>+</sup> cationic was prepared by mixing 7 mM azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) solution with 2.45 mM potassium persulfate (1/1, v/v) until the absorbance at 734 nm to 0.8 ± 0.05 with methanol. 0.1 mL of diluted solutions of the methanol extracts of butylated hydroxytoluene as a positive control and of the methanol extracts of the HT and SL (5 to 160 µg/mL) was mixed with 0.9 mL of ABTS<sup>+</sup> solution. The absorbance was recorded at 734 nm after 30 min at 25°C. ABTS<sup>+</sup> radical-scavenging activity (%) =  $(1-A_s/A_c) \times 100$ . A<sub>c</sub> = absorbance of blank without sample, A<sub>s</sub> = absorbance of sample.

#### DPPH Free Radical Scavenging Ability

1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was carried out according to Duan *et al.* (16). A mixture

including 100 µL of samples (5 to 160µg/mL) and 0.9 mL of 0.1 mM DPPH solution was formed and it was incubated at 37°C for 30 min, then the absorbance was recorded at 517 nm. The BHT was used as a positive control. The percentage of DPPH inhibition =  $(1-A_s/A_o) \times 100$  A<sub>s</sub> = absorbance of sample, A<sub>o</sub> = absorbance of blank without sample.

#### Hydroxyl Radical Scavenging Activity

This assay was carried out according to De Avellar *et al.* (17). 0.75 mM 1,10-phenanthroline and 0.75 mM FeSO<sub>4</sub> were prepared in 0.05M phosphate buffer (pH 7.4). 100 µL of sample was added 0.9 mL of above solution and incubated at 37 °C. After 30 min 20 µL of H<sub>2</sub>O<sub>2</sub> (0.01%) were added and absorbance was measured at 536 nm for 60 min at 37 °C. The BHT was used as a positive control. Inhibition (%) =  $[(A_s-A_c)/(A_o-A_c)] \times 100$  A<sub>s</sub> = absorbance of sample; A<sub>c</sub> = absorbance of control solution containing 1,10-phenanthroline, FeSO<sub>4</sub> and H<sub>2</sub>O<sub>2</sub> without sample; A<sub>o</sub>= absorbance of blank solution containing 1,10-phenanthroline and FeSO<sub>4</sub> without H<sub>2</sub>O<sub>2</sub> and sample.

#### Effect of the Extracts on Cancer Cell Growth

##### Cell Culture

HepG2 cells and A549 lung cancer cells were cultured in DMEM Kaighn's modification of Ham's F12 medium, respectively. Both of the media were supplemented with 10% fetal bovine serum (FBS), 100U/mL penicillin and 100 µg/mL streptomycin with a change of medium every 72 hr in a 5% CO<sub>2</sub> incubator.

##### Cell Viability

HepG<sub>2</sub> and A549 (5x10<sup>3</sup> cells/well) were seeded onto 96-well plates. After incubating in a CO<sub>2</sub> incubator for 24 hr, the culture medium was removed and changed with fresh medium containing samples at various concentrations (62.5 to 750 µg/mL) and cells with 0.2% DMSO were used as control and then incubated for 24 and 48hr. The media were replaced by fresh medium containing 10 µL of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazoliumbromide] (MTT) solution in PBS (5mg/mL) and 100 µL fresh medium. Then the plates were wrapped by aluminum foil and incubated at 37°C for 2 hr. MTT-containing media were taken out and the reduced formazan dye was solubilized by adding 100 µL of DMSO to each well. The plates were wrapped and incubated at 37°C. After 20 min, the plates were shacked lightly for 5 minutes.

The absorbance was measured at 570 nm. Viable cells (%) =  $[(A_s - A_o) / (A_c - A_o)] \times 100\%$ . Where  $A_s$  was absorbance of extraction sample treated cells,  $A_o$  was absorbance of blank without cells and  $A_c$  was absorbance of control group (treated with DMSO).

#### Western Blot Analysis

The HT extract having higher anticancer effect (Figure 5) was used to induce apoptosis in HepG2 and A549 cells. This assay was conducted according to Nho *et al.* (18). Briefly, cells were collected by 1X RIPA buffer including complete protease inhibitor cocktail. Purity proteins were mixed with 2x sample buffer and loaded onto 12% polyacrylamide gel electrophoresis at 80 V, 50 AM, 1.5 hr. Separated proteins were transferred onto polyvinylidene difluoride membranes, and then blocked for 2 hr using blocking buffer (5% nonfat dry milk solved in TBST including tris-base, NaCl, Tween-20, distilled water) and incubated with appropriate primary antibodies (beta-actin (1:3000); bcl-2, bad, bax and P53 (1:1000); caspase-3, caspase-9 (1:500) ) in blocked buffer overnight at 4°C. After washing thrice for 30 min, membranes were incubated with solution containing secondary antibody for 1 hr and then were washed thrice with TBST and visualized with enhanced chemiluminescence Kit using an image analyzer. The intensities of the bands were analyzed using a software provided with the system.

#### Statistical Analysis

Experiments were done in triplicates and the data were recorded as means  $\pm$  SD and analyzed using one-way ANOVA by SAS 9.2 version. The level of Least significance difference was determined using Duncan's multiple range test at  $p < .01$  for comparing the means of the treatments.

## Results and Discussion

#### Total Phenolic and Flavonoid Content

There were significant differences ( $p < 0.01$ ) in the TPC and TFC between two kinds of the extract. The higher levels of TPC and TFC were found in the HT extract ( $190.23 \pm 0.43^a$  mg GAE/g of dry extract for phenolics and  $81.04 \pm 0.51^a$  mg QUE/g of dry extract for flavonoids), while TPC and TFC of the SL extract were lower,  $61.33 \pm 0.14^b$  mg GAE/g of dry extract and  $50.33 \pm 0.1^b$  mg QUE/g of dry, respectively (Table 1).

#### Phenolic Identification by High Performance Liquid Chromatography

The chlorogenic acid and rosmarinic acid were quantified from the HT ( $17.68 \pm 0.02$   $\mu$ g/g of dry extract and  $13739 \pm 0.01$   $\mu$ g/g of dry extract, respectively) in Figure 1(A). The antitumor activity of chlorogenic acid was reported by Hou *et al.* (19). Rutin and rosmarinic acid were newly identified compounds from the SL ( $22330 \pm 0.02$   $\mu$ g/g of dry extract,  $9528 \pm 0.02$   $\mu$ g/g of dry extract, respectively) of which rutin is a major compound in Figure 1(B). The rutin antioxidant property was reported by Yang *et al.* (20). Furthermore, the anticancer effect of rutin was also demonstrated by Perk *et al.* (21). Hossan *et al.* (22) reported that rosmarinic acid could have anticancer capacity. The findings helped to confirm that the antioxidant and anticancer effects of the HT and SL were contributed by these compounds. Moreover, the different levels of antioxidant capacities between the HT and SL may be explained that the antioxidant capacity of phenolics coherently related to the number and the position of hydrogen-donating hydroxyl groups on the aromatic cycles of the phenolic molecules (13).

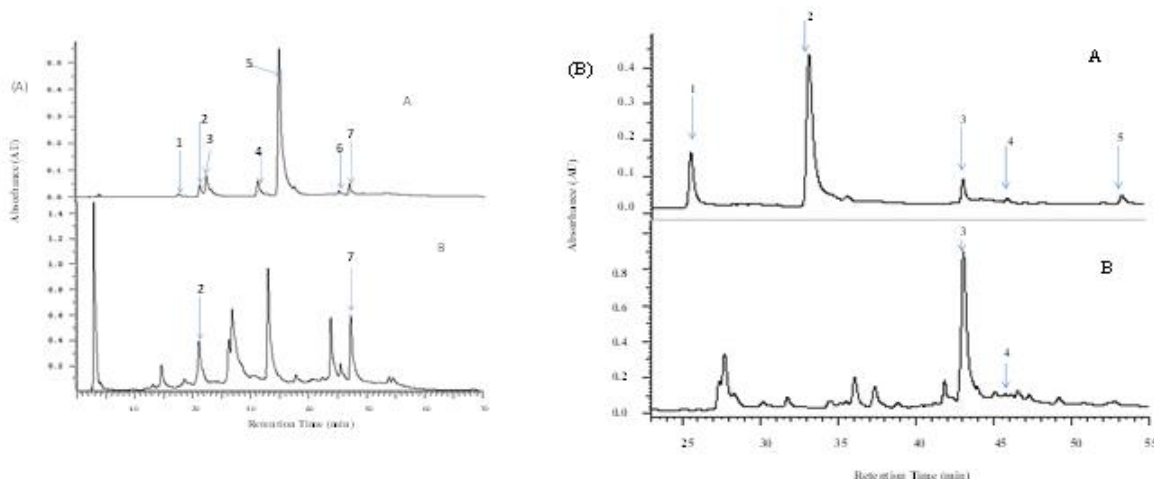
**Table 1:** Phenolic content, flavonoid content and antioxidant activities of the HT and SL extracts.

	<i>Hypericum Thunb</i>	<i>Japonicum</i>	<i>Scoparia Dulcis L</i>	Butylated hydroxytoluene
Total phenolic content <sup>1</sup>	$190.23 \pm 0.43^a$		$61.33 \pm 0.14^b$	
Total flavonoid content <sup>2</sup>	$81.04 \pm 0.51^a$		$50.33 \pm 0.1^b$	
ABTS <sup>+</sup> (IC <sub>50</sub> $\mu$ g/mL)	$45.13 \pm 0.89^b$		$168.72 \pm 7.12^a$	$11.53 \pm 0.05^c$
DPPH (IC <sub>50</sub> $\mu$ g/mL)	$89.29 \pm 0.87^b$		$140.61 \pm 0.93^a$	$9.09 \pm 0.07^c$
Hydroxyl radical scavenging (IC <sub>50</sub> $\mu$ g/mL)	$38.32 \pm 0.17^b$		$134.78 \pm 0.22^a$	$10.98 \pm 0.23^c$

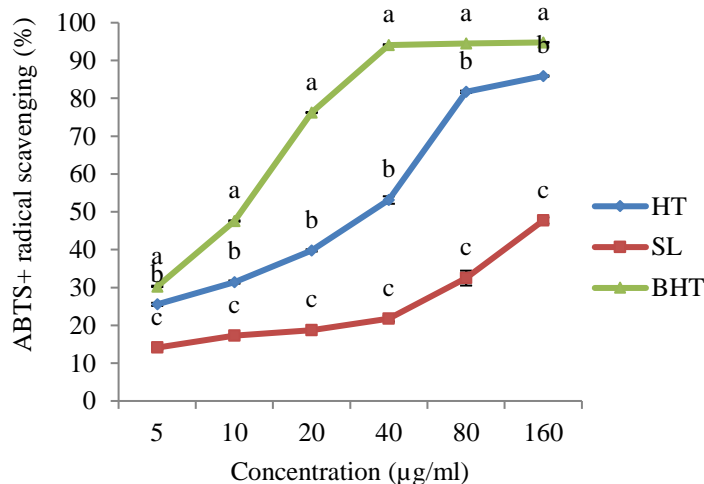
<sup>a-b</sup>values (mean  $\pm$  SD) with different superscripts were significantly different at  $p < .01$  ( $n=3$ )

<sup>1</sup>Total phenolic content is expressed as a gallic acid equivalent (mg of GAE/g of dry extract)

<sup>2</sup>Total flavonoid is expressed as quercetin equivalent (mg of QUE/g of dry extract)



**Figure 1. (A) HPLC chromatogram of phenolic composition of the HT.** A- Standard phenolic peaks: (1), 4-hydroxybenzoic acid; (2), chlorogenic acid; (3), benzoic acid; (4), p-coumaric acid; (5), caffeic acid; (6), ferulic acid; (7), rosmarinic acid; B- Sample phenolic peaks. **(B) HPLC chromatogram of phenolic composition of the SL.** A-Standard phenolic peaks: (1), epicatechin; (2), ferulic acid; (3), rutin; (4), rosmarinic acid; (5), quercetin; B- Sample phenolic peaks.



**Figure 2.** ABTS<sup>+</sup> radical scavenging capacity of the HT, SL, and BHT (%).

**Antioxidant Properties**

The ABTS<sup>+</sup> radical scavenging capacity is exhibited in Figure 2. The IC<sub>50</sub> values of the HT, SL and BHT were respectively 45.13 ± 0.89<sup>b</sup>, 168.72 ± 7.12<sup>a</sup> and 11.53 ± 0.05<sup>c</sup> µg/mL (Table 1). The results of DPPH scavenging activity showed significant differences (p<0.01) (Figure 3). The IC<sub>50</sub> values of SL, HT and BHT were respectively 140.61 ± 0.93<sup>a</sup>, 89.29 ± 0.78<sup>b</sup> and 9.09 ± 0.07<sup>c</sup> µg/mL (Table 1). The scavenged hydroxyl radical effect showed significant differences (p<0.01) (Figure 4). The IC<sub>50</sub> values of SL, HT and BHT were respectively 134.78 ± 0.22<sup>a</sup>, 38.32 ± 0.17<sup>b</sup> and 10.98 ± 0.23<sup>c</sup> µg/mL (Table 1).

The higher IC<sub>50</sub> value indicates the lower anti-radical capacity, so the HL extract exhibited higher *in-vitro* ABTS<sup>+</sup>, DPPH, hydroxyl radical scavenging potential compared to the SL extract.

The different bioavailability of phenolic and flavonoid compounds may be related to differences observed antioxidant activity by the aforementioned method. It was reported in a study that antioxidant activity is very coherently correlated with the content of phenolic compounds (23). The results indicate that the extract with higher total phenolic and flavonoid contents has higher antioxidant capacity. Le *et al* (24) reported that higher antioxidant capacity would be associated with

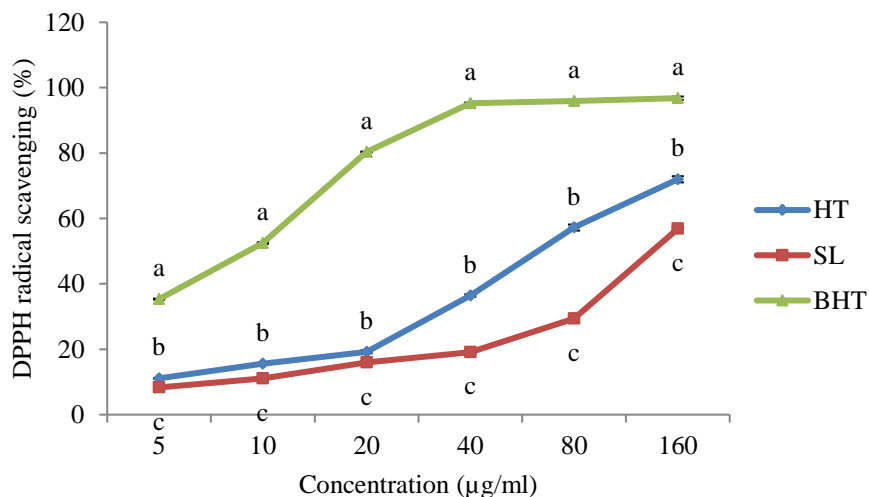


Figure 3. DPPH radical scavenging capacity of the HT, SL, and BHT (%).

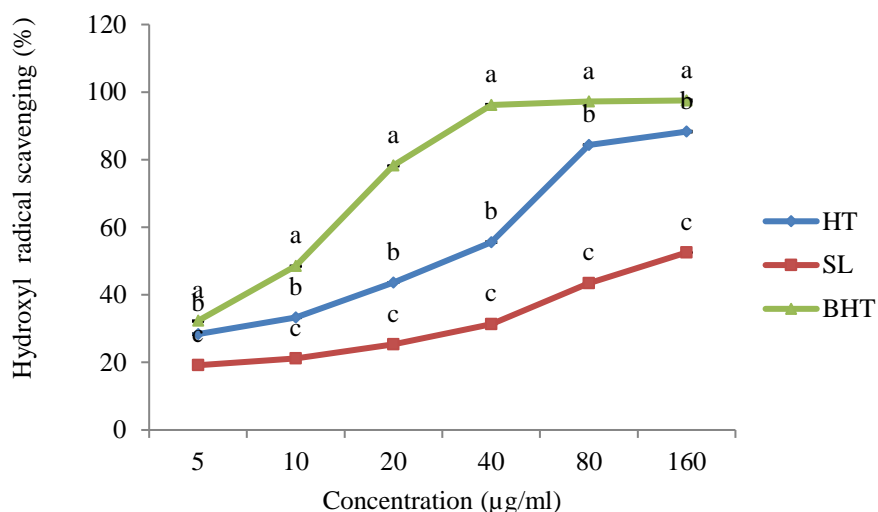


Figure 4. Hydroxyl radical scavenging capacity of the HT, SL, and BHT (%).

the presence of compounds exerting their action by breaking the free radicals and their stronger affinities for the cationic radical molecules. Phenolic compounds were considered as the main contributors in the mechanisms of the overall antioxidant activities (25). These mechanisms include scavenging and neutralizing free radicals, quenching singlet and triplet oxygen (26).

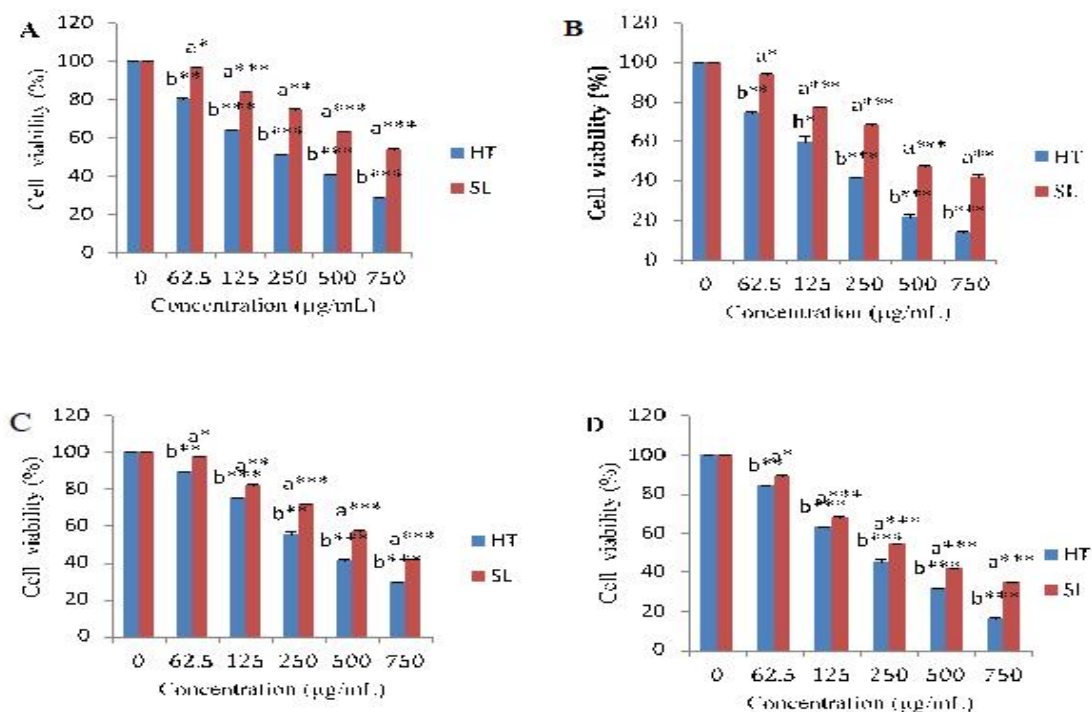
**The HT and SL Extracts Affecting Growth in HepG2 and A549 Cells**

**MTT Assay**

Increasing concentrations of the HT and SL extracts lowered cell viability (Figure 5). The HT and SL extracts induce HepG2 growth inhibition with IC<sub>50</sub>: respectively 384.99 ± 3.22<sup>b</sup> µg/mL and 772.49 ± 7.4<sup>a</sup>

µg/mL at 24 hr; 245.755 ± 2.23<sup>b</sup>µg/mL and 553.32 ± 14.46<sup>a</sup> µg/mL at 48hr. These extracts also induce A549 growth inhibition with IC<sub>50</sub> of the HT and SL: respectively 436.0 ± 1.29<sup>b</sup> µg/mL and 629.8 ± 2.02<sup>a</sup> µg/mL at 24 hr; 314.15 ± 5.96<sup>b</sup> µg/mL and 446.35 ± 0.9<sup>a</sup> µg/mL at 48 hr. The higher cell viability and IC<sub>50</sub> values mean the lower inhibitory capacity and anticancer effect. The HT extract exhibited higher cell growth inhibitory effect compared to the SL extract, which could be explained that it has higher phenolic and flavonoid contents. The phenolic compounds may possess positive effects against cancer because they act as antioxidants by neutralizing reactive oxygen species (27). Our results confirm the findings of earlier researches that have approved the anticancer capacity





**Figure 5.** (A) The HT and SL extracts induced growth inhibition in HepG2 after 24hr; (B) The HT and SL extracts induced growth inhibition in HepG2 after 48hr; (C) The HT and SL extracts induced growth inhibition in A549 after 24hr; (D) The HT and SL extracts induced growth inhibition in A549 after 48hr. <sup>a-b</sup>values (mean  $\pm$  SD) with different superscripts were significantly different at  $p < .01$  ( $n=3$ ) at the same time and concentration; \* $p < .05$  versus untreated sample, \*\* $p < .001$  versus untreated sample; \*\*\* $p < .0001$  versus untreated sample.

of the HT (2)

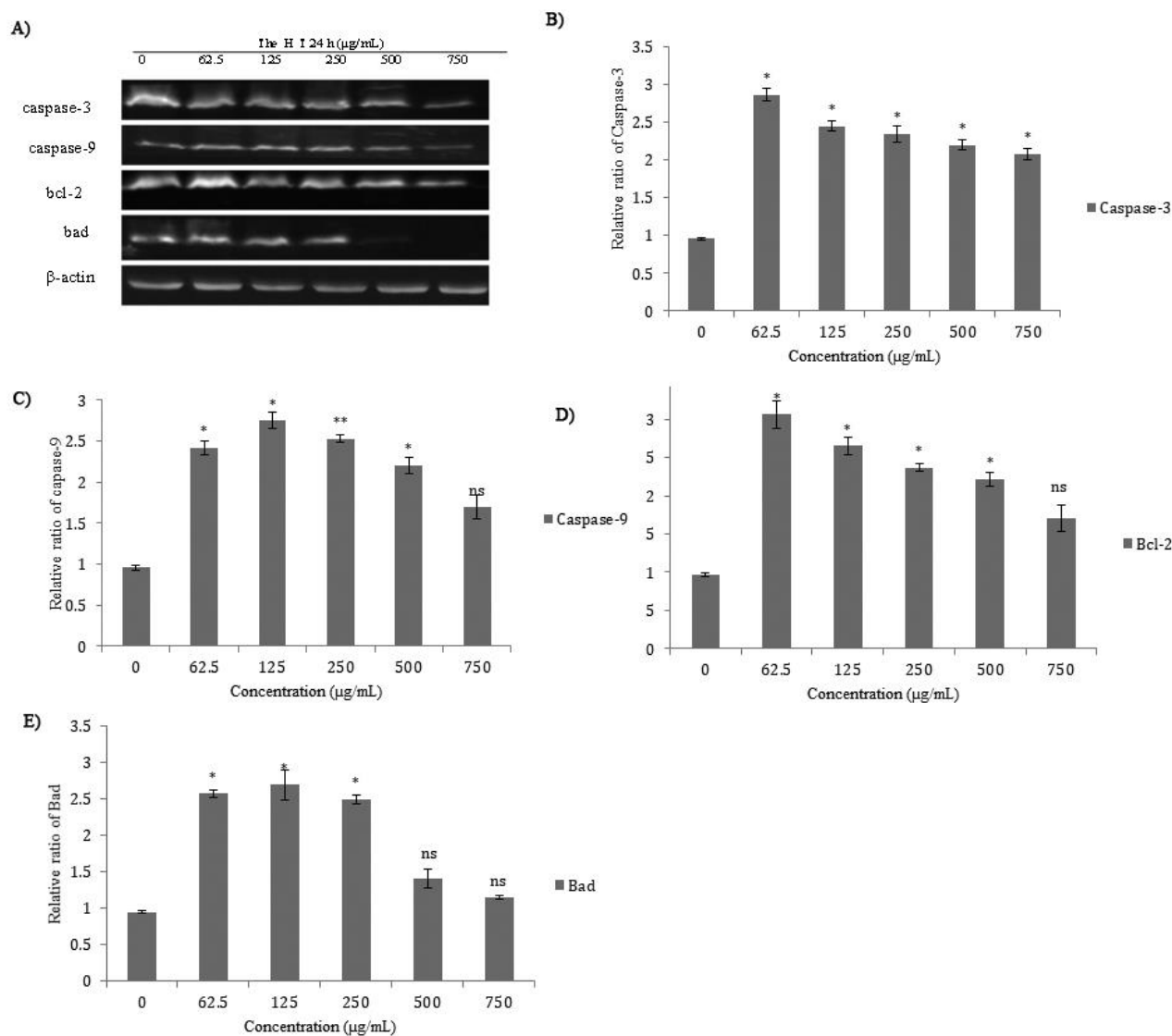
#### Alteration of the Expression of Apoptosis-Related Proteins in HepG2 and A549 Cells by the HT

The HT extract down-regulated pro-caspase-3 and pro-caspase-9 in HepG2 cells (Figure 6). Caspase-9 being an initiator is closely coupled to pro-apoptotic signals (28). Caspase-3 plays a crucial role in the terminal and execution phases of apoptosis induced by varied stimuli (29). The expressions of Bcl-2 and Bad protein in HepG2 cells were also observed. Members of the Bcl-2 family are vital regulators of the apoptotic pathway, either as an inhibitor or as an activator (30). They also regulate autophagy (31). The HT extract down-regulated pro-caspase-3, Bad and Bax and cleavage of caspase-3 and p53 protein was up-regulated in A549 (Figure 7). The product of the p53 protein, the tumor suppressor gene, is

involved in many cellular processes including gene transcription, DNA repair, genomic stability, cell cycle control, and apoptosis (32). A couple of studies have confirmed that the up-regulation of p53 plays a critical role in apoptosis in A549 cells (33). The aforementioned findings strongly indicate that the HT could induce apoptotic cell death in HepG2 and A549 cells through the stimuli of apoptotic factors.

## Conclusion

The *Hypericum Japonicum Thunb* had higher levels of total phenolic and flavonoid contents, *in vitro* antioxidant effect and HepG2 and A549 cell growth inhibitory capacity compared to the *Scoparia Dulcis L*. These herbs and their natural compounds may emerge as cheap antioxidants and could be used to support the body in areas relating to cancer. In this regard,



**Figure 6.** The HT altered the expression of apoptosis-related proteins in HepG2 cells after 24hr. Data in the histograms represent mean  $\pm$  SD. The band intensity relative to land of untreated sample was determined after normalization \* $p < 0.05$  versus untreated sample, \*\* $p < 0.001$  versus untreated sample; versus untreated sample, ns-non significant.

molecular mechanisms by which their extracts *in vivo* experiments need to be evaluated to have a more complete assessment of the antioxidant and anticancer properties.

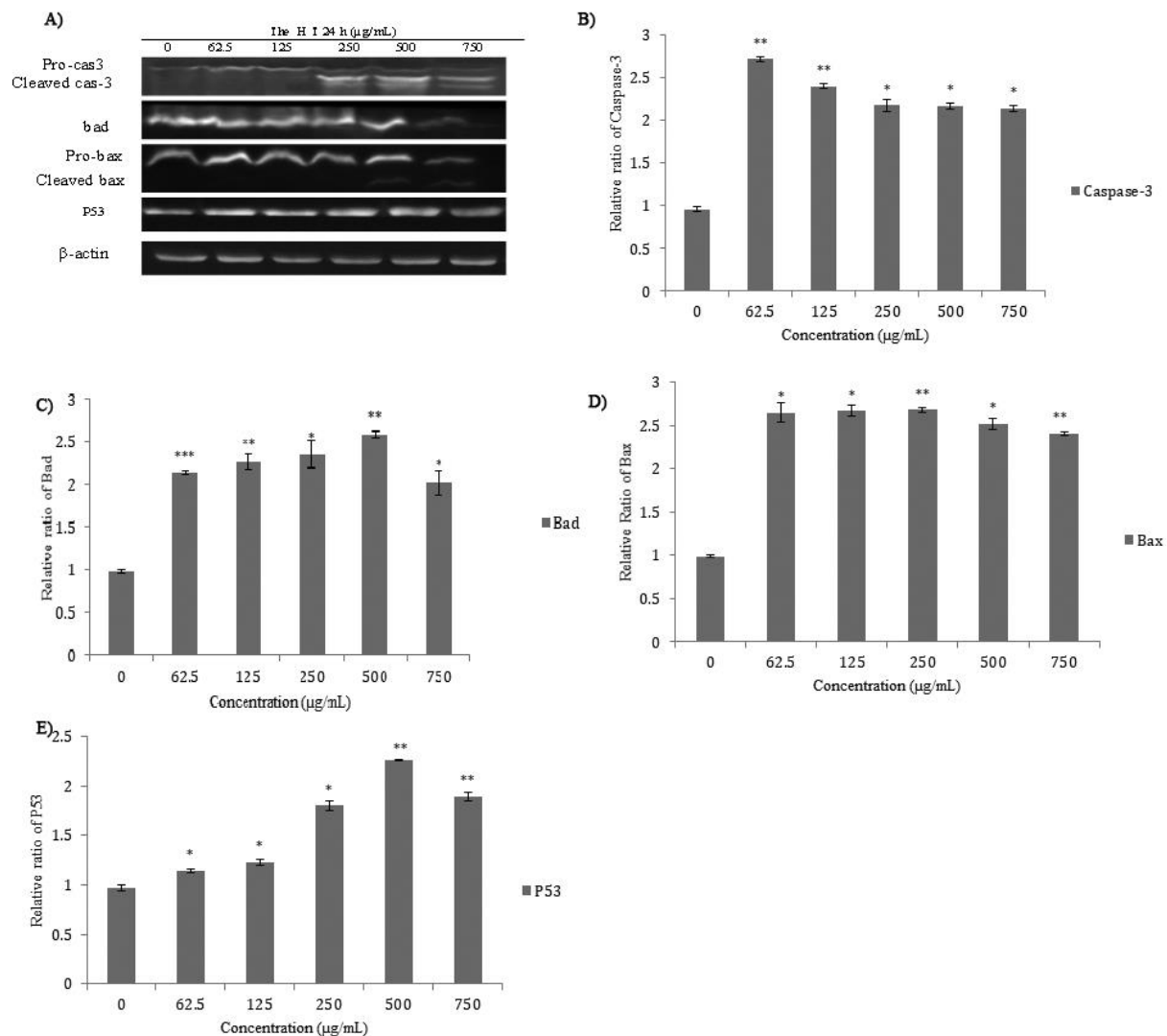
### Acknowledgment

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### Conflict of Interest

The authors declare that they have no conflict of interest.





**Figure 7. The HT altered the expression of apoptosis-related proteins in A549 cells after 24hr**

Data in the histograms represent mean  $\pm$  SD. The band intensity relative to land of untreated sample was determined after normalization \* $p < 0.05$  versus untreated sample, \*\* $p < 0.001$  versus untreated sample; \*\*\* $p < 0.0001$  versus untreated sample.

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