**ФАРМАКОЛОГИЯ, КЛИНИЧЕСКАЯ ФАРМАКОЛОГИЯ**
**PHARMACOLOGY**

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**DOI: 10.18413/2658-6533-2024-10-4-0-5** **УДК 615.2**

# Study on biological activities of herbs' decoction combining *Scutellaria barbata, Hedyotis diffusa* and *Ehretia asperula*

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**Abstract**
**Background:** Cancer is a leading cause of death worldwide**.** Nowadays, cancer is often treated with different methods, such as surgery, radiation therapy, and chemotherapy. However, these methods have lots of side effects for patients, so natural products are often combined with them to diminish those side effects. According to oriental medicine, there are many folk remedies to support cancer treatment. Each different type of cancer has corresponding remedies. In particular, to support the liver cancer treatment, some medicinal herbs were used, such as *Scutellaria barbata, Hedyotis diffusa, Ehretia asperula*. **The aim of the study:** Determining the biological activities of herbal decoctions combining *Scutellaria barbata, Hedyotis diffusa,* and *Ehretia asperula,* which were tested on two different weight ratio recipes (denoted R1 and R2). **Materials and methods:** Mixing *Scutellaria barbata, Hedyotis diffusa,* and *Ehretia asperula* into dry mixtures and then collecting the decoction. Testing antioxidant activity by the reducing power assay and the DPPH free radical scavenging assay. Evaluating antibacterial activity by the agar well diffusion assay and cytotoxic ability by the sulforhodamine B assay. **Results:** R1 decoction, which had a higher ratio of *E. asperula,* showed greater antioxidant, antibacterial, and cytotoxic capacity than R2 decoction. In the reducing power assay, R1 decoction's optical density value was 2.80, while the other was 2.23 at 0.7% concentration. In the DPPH free radical scavenging assay, the $EC_{50}$ values of R1 and R2 decoctions were 0.022% and 0.035%, respectively. Both R1 and R2 decoctions had antimicrobial activities against *Bacillus subtilis* at 100% concentration, as shown by the diameters of inhibition growth zones of 11.33 mm and 7.40 mm, respectively. In the Sulforhodamine B assay, R1 decoction's $IC_{50}$ was 0.47%, and R2 decoction's $IC_{50}$ was 0.66%. **Conclusion:** In the same condition, R1 decoction had better tested biological activities than R2 decoction.
**Keywords:** DPPH free radical scavenging; reducing power; cytotoxicity

**For citation:** Thi HN, Anh TP, Khanh DTD, et al. Study on biological activities of herbs' decoction combining *Scutellaria barbata, Hedyotis diffusa* and *Ehretia asperula* Research Results in Biomedicine. 2024;10(4):553-562. Russian. DOI: 10.18413/2658-6533-2024-10-4-0-5

**Introduction.** Nowadays, natural products are widely used in disease treatments all over the world for various reasons, such as solving side effects in chemotherapy, lowering medical service fees, and so on. Vietnam has had extremely rich sources of medicinal herbs for a long time. These herbs have been mixed into various remedies to cure diseases such as diabetes, diarrhea, and so on, especially cancer. This shows the potential of herbs in the pharmaceutical industry. *S. barbata* has been proven to have antioxidant capacity [1], anti-inflammatory activity [2], and cytotoxicity in four different cell lines [3]. Similarity, *H. diffusa* has also been shown to have antioxidant and hepatoprotective capacity against $H_2O_2$ toxicity [4], cytotoxicity on six different cell lines [5], and its ability to enhance the activity of CIK cells – natural anti-cancer cells [6]. In 2020, Chinese scientists demonstrated the ability of the combination of *S. barbata* and *H. diffusa* to inhibit the proliferation and migration of tumor cells [7]. *E. asperula* has received much attention from domestic researchers. Rosmarinic acid, an acid with antimicrobial, anti-inflammatory, antioxidant, and anti-tumor capacities, has been extracted from this herb [8]. In addition, its stems also showed cytotoxicity on three test cell lines [9], and its leaves demonstrated cytotoxicity on three cell lines and antioxidant capacity [10]. The potency of oriental remedies comes not only from the active ingredients of each herb but also from their effects when combined with other herbs. For example, to treat cancer, a remedy combines a host herb that has cancer cytotoxic ability with an increasing immunity or antioxidant herb. In this study, *S. barbata*, *H. diffusa*, and *E. asperula* were mixed into remedies, and then their biological activities were determined. The results provide the basic data for later clinical trials.

**Materials and methods**

*Decoction preparation.*

Herbs were purchased at the Research Center for planting and processing medicinal plants, Saigon Medicinal Plants, 35/21B5 Tran Dinh Xu, District 1, Ho Chi Minh City. The decoctions were mixed based on two recipes with different weight ratios. The ratios of *S. barbata* : *H. diffusa* : *E. asperula* were 2 : 2 : 5 (R1) and 2 : 3 : 4 (R2), respectively. 9 g of each recipe was cut into small pieces, then added to 500 mL of distilled water and boiled for 3 hours at 70-80 ºC. This primary decoction was centrifuged at 3000 rpm for 10 minutes, after which sediment was removed. Then, the decoction was boiled down at 50-60 ºC until its volume reached 9 mL. The final concentration was 100%. It was diluted to various concentrations to conduct the biological activity assays.

*Reducing power assay.*

The principle of this assay is based on the antioxidants' capacity for reducing potassium hexacyanoferrate (III) to potassium hexacyanoferrate (II). The reaction of ferric chloride and potassium hexacyanoferrate (II) forms a colored complex. It was measured at 700 nm. The higher measured optical density value shows better antioxidant activity. The procedure was carried out according to the report by Oyaizu [11] and modified [12].

2.5 mL of phosphate buffer and 2.5 mL of potassium ferricyanide (1% w/v) were added to 1 mL of various concentrations of the decoction. This mixture was kept at 50 ºC in a water bath for 20 minutes. Then, 2.5 mL of trichloroacetic acid (TCA) (10% w/v) was added and centrifuged at 3000 rpm for 10 minutes if necessary. 2.5 mL of the upper layer of solution was mixed with 2.5 mL of distilled water and 0.5 mL of ferric chloride solution (0.1% w/v). The optical density value was measured at 700 nm. The higher the optical density (OD) value of the mixture, the higher the decoction's reducing power.

*DPPH free radical scavenging assay.*

2,2-diphenyl-1-picrylhydrazyl (DPPH) is a stable free radical that shows maximum absorbance at 517 nm. The antioxidant activity of the decoction was determined by free radical scavenging capacity following the standard protocol of Brand-Williams [13] with some modifications [14].

5 mL of DPPH methanol solution (0.08 mM) was added to 1 mL of various concentrations of the decoction and kept in the dark for 30 minutes. Then, the absorbance was measured at 517 nm. The free radical scavenging capacity (EC%) was calculated by the following

equation: EC% = ($A_{control}$ – $A_{sample}$)/$A_{control}$ x 100%. $A_{control}$: absorbance value of DPPH solution with distilled water; $A_{sample}$: absorbance value of DPPH solution with decoction. Finally, the linear correlation equation was built, and the $EC_{50}$ value was determined. The lower the $EC_{50}$ value, the higher the scavenging capacity.

*Agar well diffusion assay.*

*Escherichia coli* and *Bacillus subtilis* were supplied by the Microbiology and Biochemistry Laboratory, Biology Department, Ho Chi Minh City University of Education.

The antimicrobial activity of the decoction was determined following the standard protocol of Hadacek [15] with modifications [16]. The agar plate surface was inoculated by spreading 100 µL of microbial inoculum (4-5 x $10^8$ CFU/mL) over the entire agar surface. A 6 mm diameter hole was aseptically punched with a sterile cork borer and filled with 100 µL of various concentrations of the decoction. After 4 hours of keeping in a cool refrigerator compartment, the plates were incubated at 37 ºC for 24 hours. Finally, the diameters of the growth inhibition zones were measured.

*Sulforhodamine B (SRB) assay*.

The principle of this method is based on the property of Sulforhodamine B (SRB) to bind cellular proteins under mild acidic conditions. Thus, colorimetric evaluation provides an estimate of total protein mass, which is related to the number of survival cells. The procedure was carried out according to the report of Monks [17] with some modifications [18].

190 µL of cell cultures were incubated with 10 µL of various concentrations of the decoction in 10% dimethylsulfoxide (DMSO) for 3 days on 96-well plates. In contrast, 190 µL of cell cultures were incubated with 10 µL of $H_2O$, DMSO, or medium as a negative control. Cells were fixed in 10% TCA for 30 minutes, then stained for 1 hour with SRB at 37 ºC. Then, the plates were washed three times with 5% acetic acid and dried at room temperature. Finally, the pellets were solubilized with 10 mM Tris base and shaken for 10 minutes. The absorbance was measured at 490 nm. The cytotoxic capacity (IC%) was calculated by the following equation: IC% = 100% – ($A_{sample}$ – $A_{blank}$)/($A_{control}$ – $A_{blank}$) x 100%. $A_{control}$: absorbance value of solution containing cells and medium. $A_{sample}$: absorbance value of solution containing cells and sample. $A_{blank}$: absorbance value of the solution containing only culture medium. Then, the correlation equation was built, and the $IC_{50}$ was determined. The lower the $IC_{50}$, the higher the cytotoxic capacity.

*Statical analysis.* The data of the study were analyzed in the GraphPad Prism 9 statistical program. The values were presented as means ± SD. The correlation equation was determined by the equation with a p-value < 0.05.

**Results**

*Result of antioxidant activity*.

In this study, the antioxidant abilities of both R1 and R2 decoctions were determined through reducing power and DPPH free radical scavenging assays. Based on the measured optical density value of the samples, the ferrous ion reducing capacity of herbal decoction at 0.1% concentration was compared in Fig. 1. The absorbance of EA decoction at 700 nm (0.581 ± 0.039) was 3.2 times that of SB decoction (0.180 ± 0.009) as well as the positive control (0.180 ± 0.003), and 6 times that of HD decoction (0.096 ± 0.004). The ferrous ion reducing capacity of R1 and R2 decoctions increases with increasing concentration (p < 0.04) (Fig. 2). The optical density value of R1 decoction is always higher than that of R2 decoction, about 2 times higher, from 0.01% to 0.1% concentration. When the concentrations of R1 and R2 range from 0.5% to 0.7%, the OD value of R1 is 20% higher than that of R2. The positive control (ascorbic acid) regression equation is: y = 0.0123x – 0.0001 ($R^2$ = 0.998). At 0.7% concentration, the capacities of R1 and R2 decoctions are equivalent to 226.024 µg/mL and 182.772 µg/mL ascorbic acid, respectively. So R1 had a higher capacity in reducing power assay.

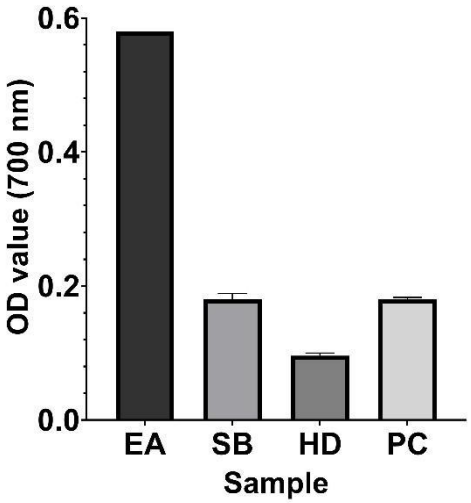


Fig. 1. Ferrous ion reducing power of herbal decoctions at 0.1% concentration
Note: *Ehretia asperula* decoction (EA); *Scutellaria barbata* decoction (SB); *Hedyotis diffusa* decoction (HD); positive control (PC) – 16 µg/mL ascorbic acid

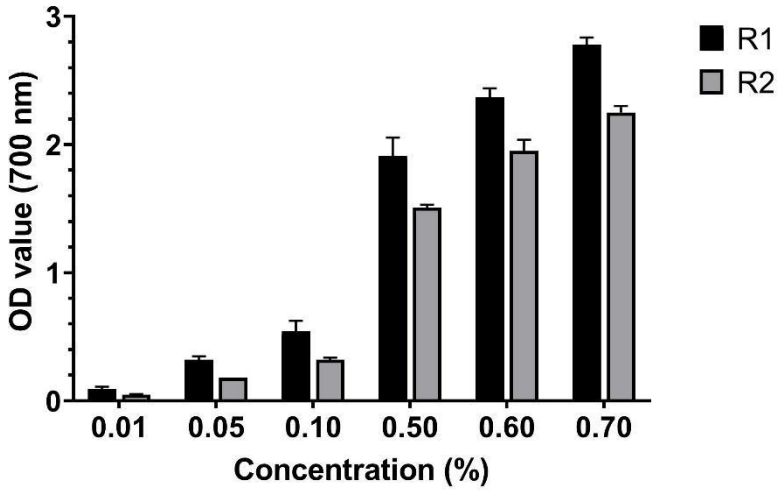


Fig. 2. Ferrous ion reducing power of R1 and R2 decoctions
Note: R1: recipe 1 decoction; R2: recipe 2 decoction

At 0.1% concentration, the DPPH free radical scavenging capacity of EA, SB, HD decoctions, and positive control was 88.02 ± 1.366%, 49.52 ± 1.049%, 11.27 ± 0.368%, and 48.22 ± 0.303%, respectively (Fig. 3). The correlation equations' graphs of herbal decoctions were described in Fig. 4. The slope of R1 decoction's graph is higher than that of R2 decoction. From the correlation equations ($p < 0.0001$), the $EC_{50}$ values of EA, SB, HD, R1, and R2 decoctions were calculated (Table 1). The correlation equation and $EC_{50}$ value of the positive control (ascorbic acid) were estimated as $y = 3.1147x + 0.1909$ ($R^2 = 0.998$) and 16 µg/mL, respectively.

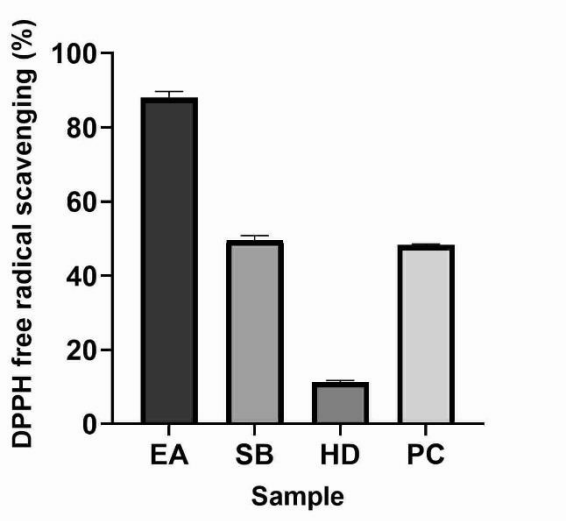


Fig. 3. Percentage of DPPH free radical scavenging of herbal decoctions at 0.1% concentration
Note: *Ehretia asperula* decoction (EA); *Scutellaria barbata* decoction (SB); *Hedyotis diffusa* decoction (HD); positive control (PC) – 16 µg/mL ascorbic acid

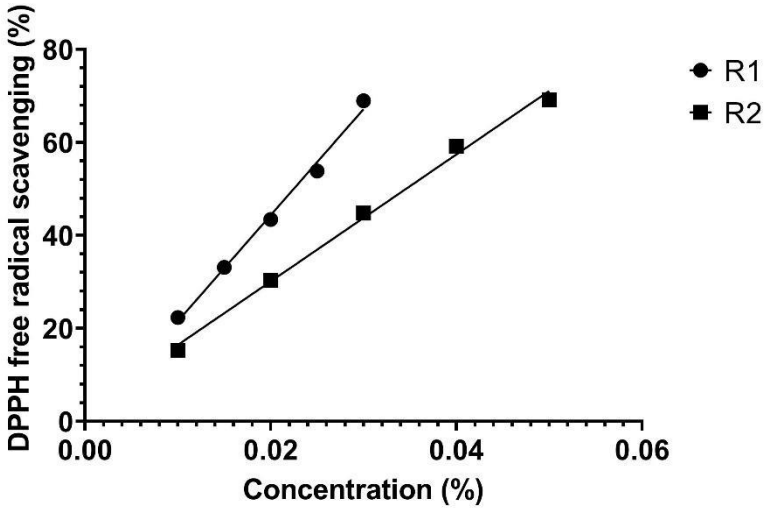


Fig. 4. Percentage of DPPH free radical scavenging of R1 and R2 decoctions
Note: recipe 1 decoction (R1); recipe 2 decoction (R2)

*Table 1*

**Regression equations, R-squared, and $EC_{50}$ of decoctions in DPPH assay**

| Decoction | Regression equations | R-squared | $EC_{50}$ |
|---|---|---|---|
| EA | Y = 3210*X + 9.823 | 0.923 | 0,013% |
| SB | Y = 460.1*X + 2.931 | 0.984 | 0.102% |
| HD | Y= 53.78*X + 25.38 | 0.984 | 0.458% |
| R1 | Y = 2280*X – 1.270 | 0.985 | 0.022% |
| R2 | Y = 1365*X + 2.775 | 0.991 | 0.035% |

Note: *Ehretia asperula* decoction (EA); *Scutellaria barbata* decoction (SB); *Hedyotis diffusa* decoction (HD); recipe 1 decoction (R1); recipe 2 decoction (R2)

*Result of antibacterial activity.*

Both recipes showed antibacterial capacity against *B. subtilis* at 100% concentration, but incapacity against *E. coli* (Table 2). R1 decoction has a larger inhibition zone when compared to R2 decoction.

*Table 2*

**Inhibition growth zones (mm) of R1 and R2 decoctions**

| Bacteria | Recipe 1 | | | Recipe 2 | | |
|---|---|---|---|---|---|---|
| | 1% | 10% | 100% | 1% | 10% | 100% |
| ***Eschirichia coli*** | – | – | – | – | – | – |
| ***Bacillus subtilis*** | – | – | 11.33 ± 1.76 | – | – | 7.40 ± 0.92 |

*Result of cytotoxic activity.*

The cytotoxic activity of both recipes decoctions at various concentrations was demonstrated through an SRB assay on a Hep-G2 cell line (Fig. 5). The correlation equation was built, and the $IC_{50}$ values of R1 and R2 decoctions were calculated. The results were 0.47% and 0.66%, respectively (Table 3). The presented data show that the cytotoxic capacity of the R1 decoction was stronger than the other.

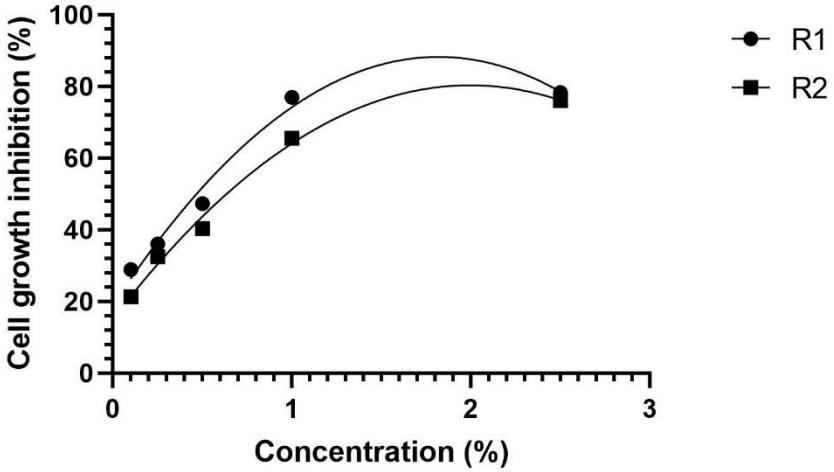


Fig. 5. Percentage of cell growth inhibition of R1 and R2 decoctions

Note: R1: recipe 1 decoction; R2: recipe 2 decoction

*Table 3*

**Regression equations, R-squared, and $IC_{50}$ of decoctions in SRB assay**

| Decoction | Regression equations | R-squared | $IC_{50}$ |
|---|---|---|---|
| R1 | Y = 18.89 + 76.25*X – 20.96*X^2 | 0.973 | 0.470% |
| R2 | Y = 15.02 + 65.44*X – 16.40*X^2 | 0.958 | 0.661% |

Note: R1: recipe 1 decoction; R2: recipe 2 decoction

Moreover, the morphological changes of HepG2 cells were illustrated in Fig. 7. HepG2 cells have an epithelial-like morphology. In negative controls, the cells that were treated with medium or $H_2O$ (Fig. 6a and b), after 3 days, the growth extended outward from the adherent cell colonies. While in Fig. 6c and d, cells attached in small patches of cells or single cells, there were many round cells floating. In Fig. 7e, the cell colonies were smaller than in Fig. 7a and b, which meant the density of cells decreased.

Based on the morphology, the density of cells in R1 and R2 was lower than the positive control because in R1 and R2 there were many round cells floating in the medium, they were dead cells, and the colonies attached to the well surface were smaller than the positive control. While in positive control (0.07 µg/mL camptothecin), the cells growth extended wider than R1 and R2, which were alive cells. In suspension in the medium, there were little round cells floating. In conclusion, at 1% concentration, R1 and R2 had better inhibition capacities than the positive control.

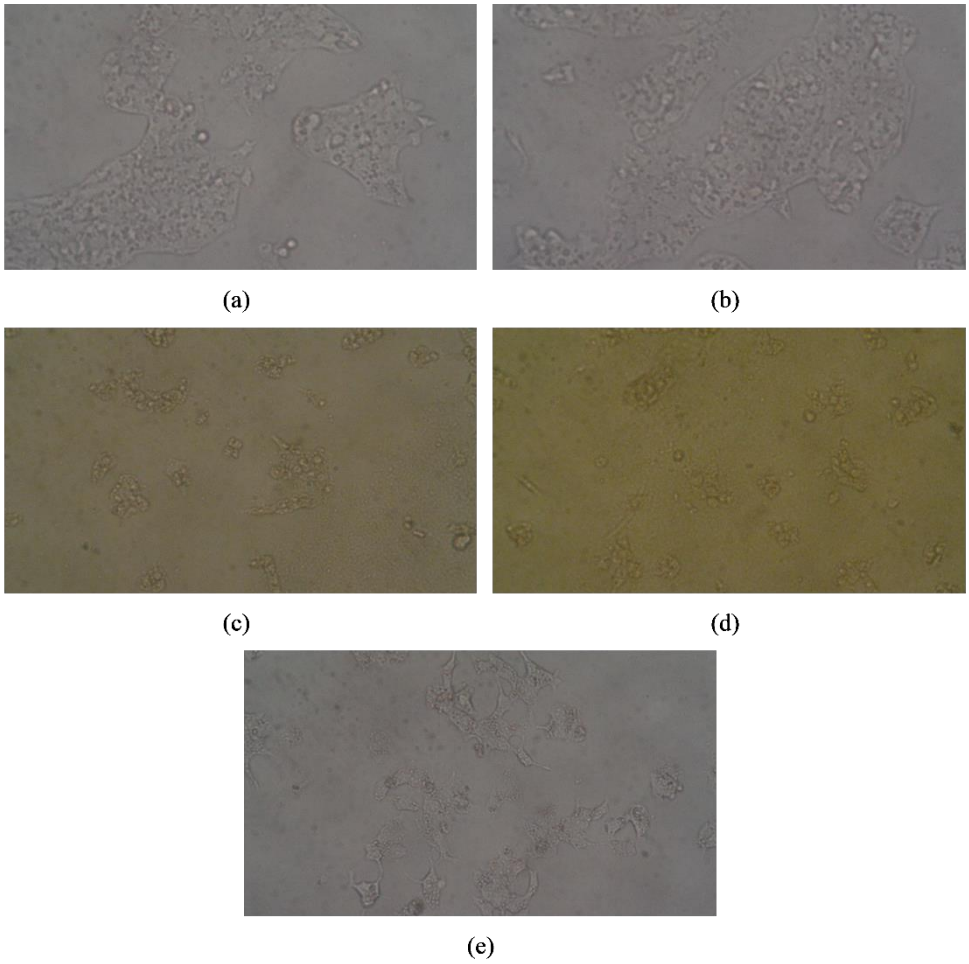


Fig. 6. HepG2 cells incubated with samples
Note: (a) no samples; (b) $H_2O$ 5%; (c) 1% R1 decoction; (d) 1% R2 decoction; (e) 0.07 μg/mL camptothecin

**Discussion.** The antioxidant capacity of R1 and R2 decoctions was proven through ferrous ion reducing power and DPPH free radical scavenging capacity due to the antioxidant activity of each herb. In fact, *S. barbata* has been proven to contain polysaccharides, and these components have antioxidant capacity through the total antioxidant activity test and the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) free radical scavenging assay. From the concentrations of 0.24 to 0.48 mg/mL, it had similar effects as the positive control in the total antioxidant activity test. In the ABTS assay, its effects increased and reached the positive control's value at a concentration of 1.2 mg/mL [1]. In 2015, Xin Gao et al. demonstrated *H. diffusa* water extract's antioxidant capacity by DPPH assay with a calculated $EC_{50}$ value of 0.15 mg/mL [4]. *E. asperula* total extract (ethanol 90%) and ethyl acetate extract's antioxidant activity was determined through the DPPH free radical scavenging assay with $EC_{50}$ values of 48.5 μg/mL and 46.9 μg/mL respectively [9]. The R1 and R2 decoction's $EC_{50}$ values in the DPPH assay and OD values at 0.1% concentration in the reducing power assay were compared to the determined component herbs' values in the same assay and condition (Fig. 1 and Table 1). From these data, it could be concluded that the antioxidant capacity was ranked in ascending order: *H. diffusa*, *S. barbata*, R2, R1, and *E. asperula*. This could be due to the ratio of EA in these decoctions: R1 decoction (the ratio of SA:HD:EA was 2:2:5, w/w/w) and R2 decoction (the ratio of SA:HD:EA was 2:3:4, w/w/w). According to the oriental medicine formulation, *E. asperula* can be considered the main herb in this remedy, while *H. diffusa* and

*S. barbata* can be considered adjuvant herbs with side functions.

In the antibacterial activity test, in the same condition, R1 decoction showed better antibacterial capacity than R2 decoction against *B. subtilis* (11.33 mm and 7.40 mm, respectively). *B. subtilis* grows in the digestive tract of humans and many animals, especially ruminants. It's beneficial to humans, so they are also called probiotic bacteria [19]. Which means this decoction is safe for users' digestive tracts if they use it in moderation; if not, some unexpected digestive problems may appear. According to the formulation of oriental medicine, the remedy needs one or more additional herbs in the recipe to support the digestive tract.

In comparison between R1 and R2 decoction's HepG2 cytotoxicity activity, R1 decoction has an $IC_{50}$ value lower than R2 decoction (0.47% < 0.66%). This means that the cytotoxic capacity of R1 decoction on HepG2 cells was better than that of R2. This can be explained by the higher ratio of *E. asperula* in recipe 1. *E. asperula* was demonstrated to have strong cytotoxicity capacity on HepG2 cells, with an $IC_{50}$ value of n-hexane extract of 28.30 µg/mL through the SRB assay [9]. The concentration of *H. diffusa* inhibiting 50% cell growth ($IC_{50}$) was estimated to be 4.62 mg/mL. This led to the R2 decoction (which has a higher rate of HD in the recipe) not being as effective as R1. However, the water extract of this herb was proven to have the capacity to enhance the antitumor activities of cytokine-induced killer cells (natural anti-cancer cells) [6] as well as normal liver cells protection ability [4]. These are reasons to consider *H. diffusa* as an adjuvant herb.

There is a close relationship between oxidative stress and liver cancer. When free radical content increases, cancer can form [20]. This remedy has strong free radical scavenging activity, so it can be used as a cancer prevention remedy. In addition, the ability to cause cytotoxicity in liver cancer cells was also strong. Thus, the remedy in this study can be used as a preventive, curable liver cancer treatment. Moreover, further studies need to be carried out to demonstrate the molecular mechanism of hepatocellular cytotoxicity as well as its non-toxicity on normal cells to be able to apply it in medicine.

**Conclusion.** Based on the data and results obtained above, the remedy decoction combining *Scutellaria barbata, Hedyotis diffusa*, and *Ehretia asperula* (two different recipes in weight ratio), in which *E. asperula* accounts for the most proportion, showed outstanding biological activities such as antioxidant activity, cytotoxicity activity against HepG2, and antibacterial activity against *B. subtilis*. The R1 decoction (the ratio of SA:HD:EA was 2:2:5, w/w/w) has better biological activities than the R2 decoction (the ratio of SA:HD:EA was 2:3:4, w/w/w).

**Financial support**

*No financial support has been provided for this work.*

**Conflict of interests**

*The authors have no conflict of interest to declare.*

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Received 3 July 2023
Revised 13 September 2023
Accepted 19 October 2023

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