Evaluation of the Antioxidant and Anticancer Activity of *Scutellaria barbata*, *Hedyotis diffusa*, and *Celastrus hindsii*

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ABSTRACT: *Scutellaria barbata, Hedyotis diffusa*, and *Celastrus hindsii* (SHC) each contain abundant medicinal properties and have been used by the Vietnamese for decades as traditional medicines. Nonetheless, there is no scientific research to prove the efficacy these usages. The purpose of this research project is to examine the chemical composition and biological activity of the three medicinal plants. Chemical composition analysis utilizing GC/MS determined 48 chemical compounds in extracts of the plants. Furthermore, a DPPH radical scavenging method was applied to examine the antioxidant activity of SHC extract which was found to have an IC_{50} value of 311.90 ± 20.89 μg/ml. Monks method verifies in vitro viral toxicity and yielded the following results: breast cancer cell line MCF7 was 77.16 ± 1.46%; liver cancer cell line HepG2 was 54.36 ± 1.32%; and lastly, lung cancer cell line NCI H460 resulted in 27.76 ± 1.50%. The extracts did not affect fibroblast cell line, -24.27 ± 1.40%.

KEYWORDS: Antioxidant; GC/MS; DPPH; Anticancer; Medical; *Hedyotis diffusa*; *Scutellaria barbata*; *Celastrus hindsii*

INTRODUCTION

Purpose statement

Oxidative stress is an imbalance attributed to the increase in the concentration of reactive oxygen species (ROS) in cells and the body's oxidative resistance system. Studies showed incurable diseases such as Atherosclerosis, Glycosuria, and many types of cancer are all related to oxidative stress. [1,2]

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body.[3] According to the World Health Organization (W.H.O), in 2018 approximately 9.6 million deaths were caused by cancer worldwide. Of this amount, 2.09 million cases of lung cancer, followed by 2 million cases of breast cancer and over 782,000 cases of liver cancer led to mortality.[4] Further investigations of oxidative and cancer resistance are required for improved cancer outcomes through a transdisciplinary approach involving biochemistry, biomedical, and health sciences.

Vietnam has diverse vegetation with many medicinal herbs such as *Scutellaria barbata*, *Hedyotis diffusa*, and *Celastrus hindsii* (SHC), all of which contain valuable medical properties and are utilized by the Vietnamese in the belief that they can heal diseases. *Hedyotis diffusa* is a type of herb in the *Rubiaceae* family, found primarily in the tropics of Asia and typically grows on moist meadows and farmland. Visually, it has a flat body, scabrous and symmetrical leaves, flowers in pairs with white corollas. The plant is commonly used in treating *Hepatitis, Urethritis*, and *Appendicitis*.[5-6] *Scutellaria Barbara* of the *Labiatae* family is often seen at the edge of rice-fields and moist meadows. This herb has a straight body, alternate leaves, rough petiole, purple-blue corollas, and flat brown nuts. It is a medicinal plant used for curing Hepatitis and Enterocolitis.[8,9] *Celastrus hindsii* from the *Celastraceae* family grows primarily in China and Thailand. In Vietnam, *Celastrus hindsii* originates in Hoa Binh and Lao Cai forests. It has delicate green leaves, black sap, white flowers grow on top of the limbs, yellow-orange nuts when ripe. For traditional medication, these three herbs are used for anti-inflammation.[10,11] In addition, they possess the potential for promoting oxidative activity and cancer treatment. However, there is no scientific proof of the efficacy of the usages of these folk remedies.

Thus, we launched our research with the hope of developing a foundation in the methods for supporting cancer treatments. Further, we hope the results will be an important factor in the application of health and biomedicine.

Theory

We made an extract from three medicinal herbs *Hedyotis diffusa*, *Scutellaria barbata*, and *Celastrus hindsii* using methanol solvent. We proceeded to examine the chemical compositions and compound mass percentages through GC/MS, followed by a free radical scavenging DPPH method which determines antioxidant activity. The in vitro cell toxicity test method was implemented to scrutinize the effect of the extract on cancer cells as well as common wild type - fibroblast cells.

The novelty of the research

This is the first research project to examine methanolic extracts from the medicinal herbs *Hedyotis diffusa*, *Scutellaria barbata*, and *Celastrus hindsii*. The combination of these three herbs has been used as a medicine by Vietnamese people for years; however, our research furthers this traditional medicine application and develops new methods for cancer treatments. The SHC extract is scrutinized to record chemical composition and substance concentration as well as tested for antioxidant activity and cancer cell inhibition.

RESULTS AND DISCUSSION

Methanolic extraction of SHC

By using immersion and rotary evaporation method, the weight obtained for the extract was 7.7 grams per 100 grams of dry specimen. SHC extract was acquired with the mass percentages shown in Table 1.

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Table 1. Methanolic extract of SHC.

	Extract of SHC
Percentage (%)	7.7

As seen in Table 1. The mass percentage recorded from the SHC methanolic extract was 7.7%. The number acquired was higher than the average mass percentage which is 4% to 6%, and the result might be affected by the strong polarity of methanol which can diffuse high polarized materials. As a result, a high mass percentage of SHC extract was obtained.

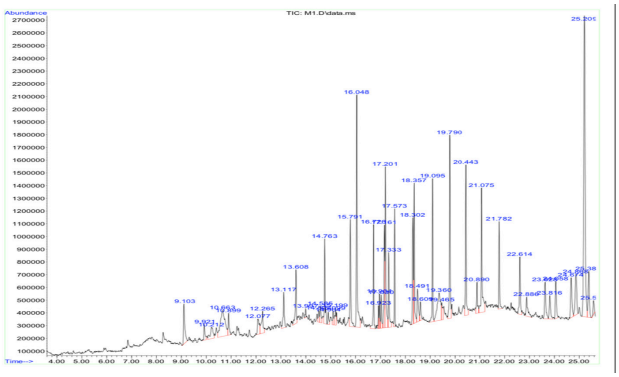

Figure 1. GC/MS spectrum of SHC extract.

Chemical composition of the methanolic extract

After examining the results from the GC/MS spectrum, 48 chemical compounds were identified. Among these chemical compounds, 10 main compounds that hold the highest percentage of SHC extract are shown in Table 2.

These 10 chemical compounds make up approximately 50% of the total compounds in SHC extract and contain the antioxidant activity and cancer resistance.[12]

Among the 10 highest percentage compounds, n-hexadecanoic acid 6.44% holds high viral and antioxidant activity, and this compound is also widely implicated to inhibit cancer cells.[17] Additionally, methylpropionphenone, 2-ethylacridine, and 9,12,15 octadecatrienoic acid were all contained in the extract and each possess activity such as antitumor properties, mimicking that of modern-day chemotherapeutics.

As a result, we continued our research by examining antioxidant activity DPPH free radical scavenging percentage with 3 cancer cell lines and fibroblast cell control.

Percentage DPPH free radical scavenging of methanolic extract of SHC

In order to acquire the most accurate result possible, we analyzed the extraction activity due to its concentration and repeated the process in triplicate to obtain the average DPPH radical scavenging percentage. The statistical analyses from the process are shown in Table 3.

Table 2. Chemical composition of the methanolic extract of SHC.

	CHEMICAL COMPOUND OF SHC EXTRACT	CONCENTRATION (%)	CHEMICAL STRUCTURE
1	Methylpropiophenone	4.1	$C_{10}H_{12}O$
2	Cyclononasiloxane, tetradecamethyl-	2.94	$C_{14}H_{42}O_7Si_7$
3	n-Hexadecanoic acid	6.44	$C_{16}H_{32}O_2$
4	9,12,15-Octadecatrienoic acid	4.34	$C_{18}H_{30}O_2$
5	Cyclononasiloxane, octadecamethyl-	3.14	$C_{18}H_{54}O_9Si_9$
6	2-Ethylacridine	3.07	$C_{15}H_{13}N$
7	Hepta siloxane, hexadecamethyl-	4.51	$C_{16}H_{48}O_6Si_7$
8	2,5-Dihydroxybenzoic acid, 3TMS	3.45	$C_{16}H_{30}O_4Si_3$
9	3,6-Dioxa-2,4,5,7-tetrasil octane	3.85	$C_{16}H_{30}O_2Si_4$
10	beta-Amyrin	14.15	$C_{30}H_{50}O$

Table 3. Percentage DPPH free radical scavenging of methanolic extract of SHC.

T (µg/mL)	Trial 1	Trial 2	Trial 3	Q_{ave} of methanolic extract
500.0	91.17	91.78	91.76	**91.57 ± 0.35**
250.0	78.80	77.67	78.28	**78.25 ± 0.57**
125.0	47.96	49.45	47.32	**48.24 ± 1.09**
62.5	21.74	30.06	27.84	**26.55 ± 4.31**
31.25	10.33	18.40	15.48	**14.74 ± 4.09**

The increase in concentration from 31.25 - 500 µg/mL resulted in a proportional DPPH free radical scavenging percentage growth. It can be concluded that the antioxidant activity of SHC extract is in direct proportion to the increase of concentration (see Table 3). With the highest concentration at 500 µg/mL, the percentage of DPPH free radical scavenging activity of SHC methanolic extract reached the highest value at 91.57%. We implemented the results above to construct a linear regression equation: y = 20.536x - 9.738 (R^2 = 0.97964). Utilizing the results above, we constructed a linear regression equation and the value of IC50 was calculated after three experiments as shown in Table 4.

Table 4. The IC^{50} value of the methanol extract of SHC.

	Trial 1	Trial 2	Trial 3	*Average + Standard deviation*
IC_{50} value	126.60	112.30	118.80	**119.23 ± 7.16**

After three trials to determine the IC50 value, the results yielded the following: 126.60 from the first trial, 112.30, and 118.80 respectively for the second and third trials. The numerical data were aggregated to calculate an average value of 119.23 and a standard deviation of 7.16. The IC_{50} value of SHC extract holds antioxidant activity and DPPH free radical scavenging capacity is compared to other herbal extractions as detailed in Table 5.

Table 5. Comparison table of IC^{50} values of the methanolic extracts.

	Trial 1	Trial 2	Trial 3	*Average + Standard deviation*
IC_{50} value	126.60	112.30	118.80	**119.23 ± 7.16**

The four methanolic extract samples chosen were *Streptocaulon juventas* (349.35), *Solanum hainanense* (1,734), *Imperata cylindrica* (313.76), and *Sophora japonica* (185.2), all of which had high antioxidant and anticancer activity. The selected samples also featured a wide range of capabilities to be implemented in the medical field and are highly regarded as potential medical products.

As observed from Table 5, it is noticeable that the IC_{50} value of SHC extract is lower than the values of other herbal extractions such as *Streptocaulon juventas*, *Solanum hainanense, Imperata cylindrica*, and *Sophora japonica*. This shows the highly effective antioxidant activity and free radical inhibition of the SHC extract which was examined in this research.

Evaluating Monks method of the methanolic extract

We proceeded to scrutinize cancer cell toxicity activity by utilizing Monks method on three cancer cell lines: breast cancer cell (MCF-7), lung cancer cell (NCI H460) and liver cancer cell (Hep G2), while simultaneously examining a wild type cell line - fibroblast cell to evaluate the effect SHC extract has on this wild type cell of connective tissue. Cancer cell toxicity at the concentration of 100 µg/mL is detailed in Table 6.

Table 6. Percentage of cytotoxic cancer resistance of methanolic extract of SHC determined by Monks method at a 100 µg/mL concentration.

Cell line	Percentage of cytotoxic cancer resistance (%) of methanolic extract			
	Trial 1	Trial 2	Trial 3	*Average + Standard deviation*
MCF-7	68.87	67.98	66.53	**67.79 ± 1.18**
Hep G2	45.58	48.62	42.43	**45.54 ± 2.53**
NCI H460	34.56	35.87	33.32	**34.58 ± 1.28**
Fibroblast	-25.57	-22.78	-24.45	**-24.27±1.40**

(Positive values represent intoxication ability, while negative values represent growth ability)

As seen from the results, SHC extract most effectively inhibited the breast cancer cell line MCF7 with 67.79 ± 1.18%, next in line was liver cancer cell line, Hep G2 with 45.54 ± 2.53%, and finally lung cancer cell line NCI H460 with 34.58 ± 1.28%. Besides cancer cell resistance ability, the extraction is completely innocuous to the growth of normal cells with the fibroblast cell developing -24.27±1.40%.

CONCLUSION

The mass percentage of the methanolic extract is 7.7%.

The chemical composition of the methanolic extract: 48 chemical compounds were examined from SHC extract including 10 main compounds.

The antioxidant activity, detected by DPPH free radical scavenging IC50 value of SHC methanolic extract was determined to be 119.23 ± 7.16 µg/ml.

SHC methanolic extract most effectively reduced breast cancer cell line MCF7 with 67.79 ± 1.18%, the liver cancer cell line HepG2 with 45.54 ± 2.53%, and finally the lung cancer cell line NCI H460 with 34.58 ± 1.28%. However, the extract was completely innocuous to the growth of a normal cells with the fibroblast cell developing -24.27±1.40%.

According to the results of this research, SHC extract which comprises *Scutellaria barbata, Hedyotis diffusa*, and *Celastrus hindsii* holds multiple potentials in treating cancer, possessing high antioxidant ability as well as being a practical application in the field of health science.

METHODS

Research material

Hedyotis diffusa, Scutellaria barbata, Celastrus hindsii were harvested in Sa Pa, Lao Cai Province, Vietnam.

Cancer cell lines were provided by American Type Culture Collection-ATCC (US).

Common cell-Fibroblast was provided by the Department of Biotechnology-Faculty of Medicine, National University of Ho Chi Minh City.

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Immersion method

Scutellaria barbata, Hedyotis diffusa, and Celastrus hindsii with a ratio of 2:1:1 in 100 grams were washed and dried inside a drying chamber at 50˚C until there was no difference in mass and all water had been drained out of the sample. Next, 100 grams of dry specimen were grinded to a smooth powder to increase diffusion in the solution. Samples were soaked in pure methanol with a concentration of 1:10 (g/mL) for 48 hours. The mixture after the immersion was decanted to obtain leachate.[13]

Preparation of the extract

To begin, we poured 500 mL of leachate into a 1 L pear-shaped container to avoid overflow. Next, we utilized a rotary evaporator at 50˚C, 250 mbar tension in 40-minute duration. The extract was be left to dry naturally and preserved in a cool tray in the refrigerator at 4˚C for later examination.[13]

Determination of chemical composition through GC/MS method

Gas chromatography-mass spectroscopy (GC/MS) is a material analysis method that employs gas chromatographs (GC) fitted with mass selective detectors called mass spectrometers (MS). GC/MS analysis is an ideal tool for identifying unknown substances or contaminants that are present in extremely low quantities.

The sample was injected into a gas chromatograph port which was heated to up to 300˚C where the material was then volatilized. Gaseous components were separated as they flowed through the column; the column was wound within a special oven which modulates temperatures between -20˚ to 320˚C. Its surface is coated with a material that separates the various chemical compounds in the sample based on size and polarity. The separated components flow directly out of the column and into the MS which has three internal steps:

1. Ionization source – components are blasted with electrons, causing them to break up and turn into positively charged ions.
2. Filter – the ions pass through an electromagnetic field and are filtered based on mass. Analysts set a predetermined range of masses to be allowed to pass through from the ionization source.
3. Detector – counting the number of filtered ions, the information is sent to a computer, and a mass spectrum and distribution of ions of different sizes are generated.

The mass spectrum is used to identify the components by comparing each to extensive reference libraries. To quantify compounds within the analyzed sample, analysts establish a standardized curve of known concentrations of each material.[13]

DPPH free radical scavenging method

The a-diphenyl-B-picrylhydrazyl (DPPH) method gives the oxidative activity of a substance or other biological basis by developing free radicals as EtOH saturates. The outcome shows high precision. The method is simple, manageable, and suitable for multiple oxidative activity resistant compounds.

Oxidative resistant compounds neutralize DPPH radicals by allowing hydrogen to absorb wavelengths as the solution pigment fades, resulting in the ability of oxidative activity when the solution switches from purple pink to pale yellow. The lower the OD value the higher the DPPH free radical scavenging activity.[13]

To begin, we poured 5 mL of DPPH (0.8mM, diffused in methanol) into tubes containing the extract at various concentrations, ranging between 0 to 1000 μg/ml. Under no-light condition, we annealed for 30 minutes then analyzed the OD values in which the active resistance depends on the light abortion percentage with a 517 nm wavelength. The positive control sample stands as acid ascorbic (15 μ/mL) and a negative control sample was twice-distilled water. The formula for the percentage of activity is calculated by:

Percentage DPPH free radical scavenging= $\frac{(ODc - ODm)}{ODc} \times 100$

ODm: Optical density (OD) value

ODc: Optical density (OD) of the control sample (-)

For the percentages we obtained, note the IC_{50} value (Concentration of reactant which is able with a 50% chance of analyzing a free radical) as the premise for comparison. The lower IC_{50} values measured, the greater the oxidative activity.[13]

Monks tested in vitro cell toxicity method

Monks tested in vitro cell toxicity method provides a high-quality outcome at a low expense. This technique has been authenticated by The National Cancer Institute - NCI which can identify potential compounds that reduce the development of cancer cells.

According to the toxicity status of cells, in order to define compounds that have the possibility to reduce the development of cancer cells by identifying the protein amount based on optical density as the cells are dyed in Sulforhodamine B (SRB). OD values are recorded by the machine. In addition, it contains the SRB quantity that yields a propitious ratio with the proportion attached to protein molecules. As the OD value gain gives a greater number of cells.

Reductant compound (10 μl) was dissolved in DMSO 10% and into wells of 10 μl/ml. Trypsin separated cells, at the same time adjust the suitable density. We poured more cells with a reasonable amount in 190 μl environment to develop for 3 to 5 days. The tray containing cancer cells (180 μl) was used as a comparison for day 0. After 1 hour, we situated the cells in Trichloroacetic acid-TCA.

The cells were soaked in a warm container of CO2 in order to be fixed into the bottom of the culture well TCA. After 30 minutes, we dyed them in SRB for 1 hour at 37 Celsius. Next, we poured out SRB and then washed it 3 times with acetic acid 5%. We then let it dry at room temperature. We used tris (hydroxymethyl) aminomethane 10 mM and dissolved the leftover SRB. We also dyed the protein molecules and then shook it slightly for 10 minutes.

The ELISA Plate Reader (Bio-Rad) machine was used to examine the outcome of the color content SRB dye under the spectrum observed at 515 nm wavelength. The formula is shown below:

$$\%Alive\ cells = \frac{OD(Reductant) - OD(Day0)}{OD(Negative\ confront) - OD(Day0)} \times 100$$

$$\%Dead\ cells = 100\% - \%\ Alive\ cells$$

This was repeated several times to increase reliability. Ellipticine (Sigma) as a positive control sample and DMSO 10% as the negative control sample were used. We evaluated IC50 values by TableCurve software.[14]

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