applied sciences

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Screening of Antibacterial Activity, Antioxidant Activity, and Anticancer Activity of *Euphorbia hirta* Linn. Extracts

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Received: 1 September 2020; Accepted: 4 October 2020; Published: 26 November 2020

Featured Application: Study of Euphorbia hirta Linn. extracts provide technical insight for functional food and drug development.

Abstract: This study aimed to screen the anticancer and antioxidant potential and antimicrobial activity of methanol, petroleum ether, chloroform, ethyl acetate, butanol of *Euphorbia hirta* Linn. extracts (EH-Me, EH-PE, EH-Ch, EH-EA and EH-Bu, respectively). The results of 2,2-diphenyl-1-pycrylhydrazyl (DPPH) radical scavenging assay and lipid peroxidation inhibition assay showed that EH-EA was the strongest antioxidant (IC_{50} = 10.33 ± 0.01 μg/mL; IC_{50} = 1.48 ± 0.12 μg/mL, respectively) compared to all other extracts. In the antimicrobial activity of the extracts against eight strains of Gram-positive and Gram-negative bacteria using the agar disc diffusion method, we found the EH-EA to be the best antimicrobial agent. Anticancer activities of those extracts were examined by sulforhodamine B (SRB) in vitro cytotoxicity assay on two cancer cell lines, including lung cancer cells NCI-H460 and liver cancer cells Hep G2. EH-EA at concentration of 100 μg/mL has significant inhibitory activity the growth of lung cancer cells NCI-H460 and liver cancer cells Hep G2 compared to all other extracts. Our results suggest that *E. hirta* Linn. extracts possess significant biological activities, including antimicrobial, antioxidant, and moderate anticancer properties. Our results show that this plant could be a good source for natural antioxidants and a possible pharmaceutical supplement. Among five analyzed extracts, EH-EA extract has the strongest activities, and should be used to determine phytochemicals and mechanisms of these activities.

Keywords: *Euphorbia hirta* Linn.; antibacterial activity; antioxidant activity; anticancer activity

1. Introduction

Since ancient times, herbs have been confirmed for their biological ability to relief pain, prevent diseases and cure illnesses. Nowadays, scientists around the world have developed a research system for medicinal plants and their medicinal properties. Each plant species contains hundreds to thousands of different constituent chemicals that interact in complex ways in the bodies of animals and human [1–3]. In developing countries, according to estimates by the World Health Organization, about 80% of the population still depends on folk medicines made from plants for prevention and treatment [4]. Traditional medicine or folk medicine has been proven to be more affordable, clinically effective and has relatively less adverse effects than modern drugs [5–7]. Plant-derived

secondary metabolites that are biosynthesized in plants are not only essential for plant survival, but are also extremely important for development, growth and reproduction and plant protection [8–10]. Secondary metabolites in plants include several groups of molecules, including steroids, alkaloids, phenolic, lignans, carbohydrates and glycosides, etc., which possess several biological activities. For example, phenolic compounds are one big group of secondary metabolites that are popularly distributed in plants, and possess a diversity of biological properties beneficial to humans, such as their antiallergic, anticancer, antimicrobial, anti-inflammatory, antidiabetic and antioxidant activities [11–13].

The antibacterial properties of plants or herbs have been researched popularly. *Ocimum basilicum*, *Momordica charantia*, *Cinnamomum verum*, *Allium sativum*, *Curcuma longa*, for example, exhibit antimicrobial properties against both Gram-positive and Gram-negative bacteria [14–16]. Much research has shown that these plants are rich in a diversity of bioactive compounds found in vitro to inhibit or kill microorganisms effectively [16].

Lipid peroxidation is an ambiguous event in all biological species. Currently, lipid peroxidation is considered as the main molecular mechanism involved in the oxidative damage to cell structures and in the toxicity process that leads to cell death [17]. Lipid peroxidation has been suggested to comprise of three distinct mechanisms: free radical-mediated oxidation, free radical-independent, nonenzymatic oxidation, and enzymatic oxidation [18,19]. Lipid peroxidation is a serious problem for human health. Plant-derived polyphenols have been demonstrated to have a variety of biological effects, due to their antioxidant activities in inhibition of lipid peroxidation in the body [20,21].

Cancer is the second highest cause of mortality globally. In particular, there were 9.6 million cancer-related deaths in 2018 [22,23]. The most common cancers include breast, lung, colorectal, etc. Traditional medicine and phytochemicals extracted from herbs have been increasingly noticed and applied as one of the useful treatments against cancer. A series of clinical studies in recent years have shown the positive effects of herbal drugs on survival, regulation of the immune system and improvement in the quality of life of cancer patients, when combining these herbal medicines with conventional treatments [24]. Diversity of phytochemicals such as phenolic compounds, terpenoids, lignans, tannins, alkaloids, etc., have been investigated from herbal sources that possess strongly effective antioxidant properties to inhibit cell proliferation and stimulate the immune system to enhance prevention or treatment of cancer diseases.

Euphorbia hirta Linn. (*E. hirta*) belongs to the Euphorbiaceae family, a group of small prostrate herbaceous annual weeds in Vietnam. This plant has slender stem of 30–50 cm height, yellow hair, and opposite spear-shaped leaves. It is abundant in open grasslands and is distributed in most Asian countries [25]. In Vietnam, *E. hirta* is commonly distributed in many provinces of the southern area (Figure 1). Many investigations have demonstrated that this plant has been widely used as traditional medicine to treat many diseases, including wound healing, gastrointestinal disorders, tumors, etc. Additionally, this herb was used as a traditional medicine in the treatment of diabetes for a long time [26–29].

Figure 1. *Euphorbia hirta* Linn.

E. hirta contains several secondary metabolites, including flavonoids, terpenoids, phenols, essential oil, etc. Many flavonoids have been identified in *E. hirta* including quercetin, quercitrin, quercitol and derivatives. Some terpenoids in *E. hirta* have been isolated and identified successfully, including triterpenoids, α-amyrin, β-amyrin, friedelin, teraxerol, cycloartenol, 24-methylene-cycloartenol, ingenol triacetate β-sitosterol, campestrol, stigmasterol, etc. [27,28]. The antibacterial activity of *E. hirta* has been discovered and proven by using many different extracts that showed the property against *Shigella species*, *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis* [28]. The aqueous extract of *E. hirta* also showed antioxidant activity and a free radical scavenging capacity [27]. Moreover, *E. hirta* extracts have exhibited anticancer activity [28].

For purpose of evaluation of biological activities of *Euphorbia hirta* Linn., our study was carried out to evaluate the antibacterial activity, lipid peroxidation inhibitory activity and anticancer activity of methanol, petroleum ether, chloroform, ethyl acetate and butanol extracts of this herb.

2. Materials and Methods

2.1. Animals and Materials

2.1.1. Animals

Male Swiss albino mice (25 ± 2 g, aged 5–6 weeks), were supplied by the Institute of Vaccines and Medical Biological Products in Nha Trang City. This research was approved by the University of Science Animal Care and Use Committee (No. 248b/KHTN-ACUCUS). Each extract requires 2 mice, so the total number of mice required for the test is 10 mice. Before any experience, all animals were kept for 1 week under the same laboratory conditions of temperature (22 ± 2 °C), relative humidity (70 ± 4%), and a 12 h light/dark cycle, and received a nutritionally standard diet and tap water.

2.1.2. Chemicals and Reagents

The organic solvents that were used in extraction of *E. hirta* include absolute methanol (Me), petroleum ether (PE), chloroform (Ch), ethyl acetate (EA), and butanol (Bu) purchased from Chemsol Company, Vietnam. Sulforhodamine B, 1-diphenyl-2-picrylhydrazyl (DPPH) and [±]-6-hydroxy-2, 5, 7, 8-tetramethyl-chroman-2-carboxylic acid (Trolox) were purchased from Sigma-Aldrich Co. (USA). Thiobarbituric acid and ascorbic acid were purchased from Merck (Germany). Gallic acid, quercetin and Folin were purchased from Sigma-Aldrich Co. (USA). Dimethyl sulfoxide (DMSO) was purchased from Merck (Germany). All other chemicals were of analytical grade.

2.2. Plant Material Collection and Identification

E. hirta was identified by the Institute of Tropical Biology under the Vietnam Academy of Science and Technology. The fresh *E. hirta* plant was collected in Long Khanh City, Dong Nai Province, Vietnam in February 2015. The collected raw plant was cleaned with tap water to remove any type of contamination and then soaked in 70% ethanol solution to exterminate all enzymes. Next, cleaned material was naturally dried for 3 days under room temperature. Sunlight was strictly prevented from interacting with the preliminarily processed sample. Finally, the dried plant was ground into powder, kept in desiccator to avoid microbial attack before using it for extraction process. The total weight of the raw plant after preliminary preparation was approximately 8 kg.

2.3. Preparation of E. hirta Extracts Using Maceration Method

The powdered plant (8 kg) was macerated with absolute methanol solvent by the ratio of 1:10 (*w/w*) within 7 days [30]. After 7 days, methanol extract solution was obtained and filtered through Whatman No.1 filter paper to remove ground and other impurities. Methanol extract solution from maceration was reclaimed methanol solvent by a rotary evaporator under high pressure to get methanol crude extraction. After yielding total crude extract, EH-Me extract was mixed with distilled water by ratio

1:10 (*w*/*w*) and was processed with PE, Ch, EA, and Bu solvents, respectively. The organic solvents were selected from low polarity to high polarity so as to harvest phytochemicals separately depending on the differences in their polarity. After each fractional extraction, the rotary evaporator was used to evaporate all solvents to form concentrated extracts (Figure 2). Before beginning the experiments about the biological properties of all extracts, the percentage of humidity of all extracts was determined to evaluate the moisture content of the samples. The moisture content was determined by moisture balance, type MOC-120H, no. D207302059. Five extract samples of *E. hirta* (5 mg) were weighed and dried in the oven at 105 °C until its weight was constant [31]. The results are expressed as mean ± standard error of mean (SEM) of three replicates.

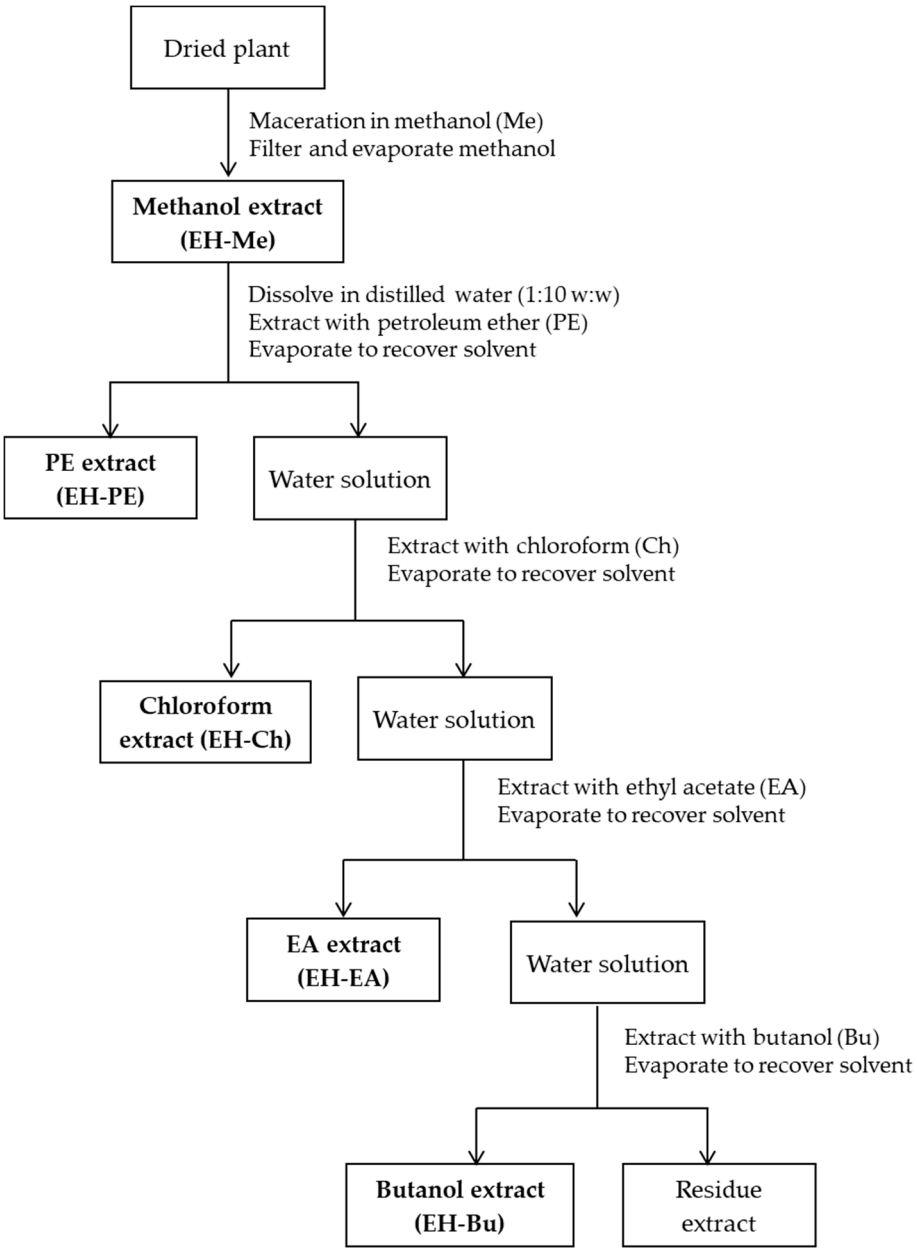

Figure 2. The chart indicates the process of solvent extraction.

The percentage of yield of extract was calculated as:

$$\%\ \text{Yield}\ (\%\text{Y}) = \frac{\text{weight of dried extract (g)}}{\text{weight of sample (g)}} \times 100 \quad (1)$$

2.4. Determination of Total Phenolic and Flavonoid Content of the Extracts from Euphorbia hirta Linn.

2.4.1. Determination of Total Phenolic Content (TPC)

The total phenols content of the extract was determined by the Folin–Ciocalteu method [32,33]. The concentration gradient of gallic acid was prepared as standard solution (0–50 µg/mL), and the calibration curve was established using gallic acid. The samples were diluted with DMSO as sample solutions, after they were made up to 10 mL by methanol. The 6 mL of distilled water and 100 µL of sample or standard solution and 0.5 mL Folin–Ciocalteu reagent were mixed for 5 min, followed by the addition of 1.5 mL of 20% sodium carbonate and 1.9 mL of distilled water. The mixture was placed for 120 min at room temperature. Every experiment was performed in triplicate. The absorbance of the mixture was measured at 758 nm using a UV spectrophotometer. Total phenols content of the samples was expressed as mg of gallic acid equivalents (GAE) per gram dried extract [33,34]. The experimental results are expressed as mean ± standard error of mean (SEM) of three replicates.

2.4.2. Determination of Total Flavonoid Content (TFC)

Total flavonoid content was determined by aluminum chloride colorimetric assay [33]. The concentration gradient of quercetin was prepared as standard solution (0–80 µg/mL), and the calibration curve was established using quercetin. The samples were diluted with DMSO as sample solutions, after they were made up to 50 mL by methanol. The 4 mL of distilled water and 1 mL of sample or standard solution, and 300 µL $NaNO_2$ (5%) were added to a glass cuvette. Three hundred microliters of $AlCl_3$ (10%) were added after 5 min and 2 mL NaOH (1 M) and 2.4 mL distilled water were added after 6 min. The mixture was placed for 10 min at room temperature. Every experiment was performed in triplicate. The absorbance of the mixture was measured at 510 nm using a UV spectrophotometer. Total flavonoid content of the samples was expressed as mg of quercetin equivalents (QE) per gram dry extract. The experimental results are expressed as mean ± standard error of mean (SEM) of three replicates.

2.5. In vitro Antibacterial Activity Using Disc Diffusion Method

The following standard bacterial strains were used in this study belonging to Gram-positive and Gram-negative species: *Bacillus subtilis* (*B. subtilis*), *Escherichia coli* ATCC 25,922 (*E. coli*), *Staphylococcus aureus* ATCC 25,923 (*S. aureus*), *Pseudomonas aeruginosa* ATCC 27,853 (*P. aeruginosa*), *Streptococcus preumoniae* ATCC 49,619 (*S. preumoniae*), *Salmonella typhi* Ty 2 (*S. typhi*), *Vibrio cholearea* AI 4450 (*V. cholearea*), *Shigella flexneri* NCDC 2747-71 (*S. flexneri*) and cultured on specialized media overnight. They were obtained from the laboratory of Ho Chi Minh City Pasteur Institute, Vietnam. Due to their ability to survive in harsh conditions and their multiple environmental habitats, these bacterial organisms including Gram-positive and Gram-negative are the main sources of severe infections in humans.

The antibacterial activity of *E. hirta* extracts was investigated using the disk diffusion method with some modifications [35–37]. The bacterial suspension phase in physiological saline reaches a concentration of McFarland (0.5%) (equivalent to 10^8 CFU/mL). Preparation of Mueller-Hinton (MH) medium plates were prepared containing 5% of sheep blood after being allowed to dry before inoculating bacteria. The bacterial suspension was spread evenly over the surface of MH agar. Thereafter, we conducted holes in the surface of wells, the wells were 20–30 mm apart, and had a diameter of 6 mm. The diluted samples at five different concentrations of 25, 50, 100, 200 and 400 µg/mL for five extracts in DMSO solvent were prepared to add to each well of every disc. All discs were incubated at 37 °C for 18–20 h. Each concentration was repeated 2 times, checking for bacterial growth. The negative control was the same volume of sterilized water. The diameter (in millimeters) of the inhibition zone was measured to estimate the antibacterial activity of *E. hirta*. The inhibition zone was defined as where bacteria cannot grow.

2.6. Study on Antioxidant Activity

2.6.1. DPPH radical scavenging assay

DPPH (1-diphenyl-2-picrylhydrazyl) is a stable free radical that has a maximum absorption of 515–517 nm (purple color). When an antioxidant (as a hydrogen donor) reacts with DPPH, DPPH is reduced to DPPH-H, and as a consequence the solution turns from purple to yellow. Therefore, the antioxidant activity of DPPH samples can be evaluated by the decrease in absorption at 515–517 nm. The potential scavenging abilities of the samples were assessed using ascorbic acid (Merck, Germany) as positive control [38].

Briefly, 0.5 mL of various concentrations of each sample (10, 50, 100, 250, 500, 750 and 1000 µg/mL) or ascorbic acid (0.5, 0.25, 0.1, 0.05, and 0.01 mM) was added into a tube containing 0.5 mL of 0.6 mM DPPH solution dissolved in methanol and the volume was made uniformly to 4 mL using methanol. The solution was mixed and then allowed to stand in the dark at room temperature for 30 min. Absorbance was taken at 515 nm using methanol as blank on UV-visible spectrometer. Then, 0.5 mL of DPPH was added to 3.5 mL of methanol and absorbance was taken for control reading. All analyses were run in triplicate [39].

2.6.2. Lipid Peroxidation Inhibition Assay (Malondialdehyde Assay)

Lipid peroxidation is a well-established mechanism of cellular injury in both plants and animals and is used as an indicator of oxidative stress in cells and tissues. The measurement of malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) has been used as an indicator of lipid peroxidation. MDA is one of the products of lipid peroxidation on cell membranes. The assay is based on the reaction of MDA with thiobarbituric acid (TBA), forming an MDA-TBA2 adduct that absorbs strongly at 532 nm. The potential lipid peroxidation (i.e., MDA formation) inhibition power of extract samples was measured using mouse brain homogenate as lipid rich substrate. Trolox, a water-soluble analog of vitamin E, was used as positive control [40].

After the sampling, the mouse brain tissue was immediately placed in ice-cold 5 mM phosphate buffer (pH 7.4) with the brain and phosphate buffer ratio of 1:10 (w/v). The tissue was then homogenized at 13,000 rpm in an ice-cold condition. Then, 0.1 mL of various concentrations of each sample or Trolox (10, 5, 1, 0.5, and 0.1 mM) was added to 0.5 mL of brain homogenate and the volume was made to reach 2 mL using 50 mM phosphate buffer. The mixture was then incubated at 37 °C for 15 min to allow lipid peroxidation and thus MDA production. The reaction was stopped with 1 mL of 10% trichloroacetic acid. The tube was then centrifuged at 10,000 rpm at 5 °C for 5 min. The supernatant (2 mL) was transferred into a different tube and then allowed to react with 0.8% TBA solution (1 mL) at 95 °C for 15 min. The control was prepared as above without the test sample. Absorbance of the mixture was measured at 532 nm against a blank solution without sample and TBA after the tubes reached room temperature.

2.6.3. Data Analysis

The percentage of antioxidant activity was calculated as $[(\Delta A_0 - \Delta A_s)/\Delta A_0] \times 100$, where ΔA_0 was the absorbance of the control ($\Delta A_0 = A_{control} - A_{blank}$), and ΔA_s was the absorbance of the extract/standard ($\Delta A_s = A_{sample/standard} - A_{blank}$).

The extract concentration providing 50% inhibition (IC_{50}—the half maximal inhibitory concentration) was obtained by interpolation from linear regression analysis.

2.7. Anticancer Activity Using Sulforhodamine B Assay

2.7.1. Cell Lines and Cell Culture

The NCI-H460 cell line was derived by A.F. Gazdar and associates in 1982 from the pleural fluid of a patient with large cell cancer of the lung. HepG2 hepatocellular carcinoma cell line was

purchased from the American Tissue Culture Collection (ATCC; catalog no. HB-8065; Manassas, VA). NCI-H460 (HTB-177) and Hep G2 cells (HB-8065) were purchased from the American Type Culture Collection (Manassas, Rockville). Cells were cultured at 37 °C and 5% CO_2 in Eagle's minimal essential medium (EMEM) supplemented with 10% (*v/v*) FBS (Sigma), 2 mM L-glutamine (Sigma), 20 mM HEPES (Sigma), 0.025 μg/mL amphotericin B (Sigma), 100 IU/mL penicillin G (Sigma), and 100 μg/mL streptomycin (Sigma).

2.7.2. The Protocol of Toxicological Activity Experiment by Sulforhodamine B (SRB) Method

The assay was performed as previously described with some modifications [41]. Cells, seeded at a density of 10,000 cells/well (Hep G2) and 7,500 cells/well (NCI-H460) in 96-well plates, were cultured for 24 h before being incubated with samples at different concentrations for 48 h. Treated cells were fixed with cold 50% (w/v) trichloroacetic acid (Merck) solution for 1–3 h, washed, and stained with 0.2% (w/v) SRB (Sigma) for 20 min. After five washes with 1% acetic acid (Merck), protein-bound dye was solubilized in 10 mM Tris base solution (Promega). Optical density values were determined with a 96-well micro-titer plate reader (Synergy HT, Biotek Instruments) at the wavelengths of 492 nm and 620 nm.

After 24 h of culture, the cell population was incubated with the analyte at concentrations for 48 h. The total protein from the test cell was then fixed with cold 50% (w/v) trichloroacetic acid (Sigma) and stained with 0.2% sulforhodamine B solution (Sigma) for 20 min. After five washes with 1% acetic acid (Merck), protein-bound dye was solubilized in 10 mM Tris base solution (Promega). Results are read by ELISA reader at 492 nm and 620 nm. The experiments were repeated three times and the results are presented as mean ± standard deviations.

The percentage of growth inhibition (I%) was calculated according to the formula:

I% = (1 − [ODt/ODc] × 100) %, in which ODt and ODc are the optical density value of the test sample and the control sample, respectively. Camptothecin (Calbiochem) was used as a positive control.

2.8. Statistical Analysis

SPSS version 20 (statistical package for the social sciences) software (Schrödinger, LLC New York, NY, USA) was used for data analysis and all comparisons were made by using one-way ANOVA followed by Dunnett's test. Values were expressed as means ± SEM (standard errors of means), for which the P-values less than 0.05, 0.01, and 0.001 were considered statistically significant.

3. Results

3.1. Plant Extraction

Five fractional extracts of *E. hirta* were obtained after the extraction process. They are EH-Me, EH-PE, EH-Ch, EH-EA, EH-Bu extracts. Detailed information about these extracts are mentioned in Table 1.

Table 1. Weight and yield of extract after liquid–liquid extract.

Name of Extract	Weight (g)	Yield (%)	Humidity (%)
Dried plant	8000	-	8.01 ± 0.38
EH-Me	900	11.25	15.05 ± 0.05
EH-PE	300	3.75	6.01 ± 0.29
EH-Ch	9	0.11	3.03 ± 0.12
EH-EA	92	1.15	5.31 ± 0.21
EH-Bu	117	1.46	17.44 ± 0.33
Total	1418	17.725	-

3.2. Determination of Total Phenolic and Flavonoid Contents

According to previous investigations, *E. hirta* extracts contain a large amount of phenolic and flavonoid components [10]. The total phenolic content of the extracts was quantified. The regression equation of the standard curve of gallic acid was y = 0.0113x + 0.02261 with R^2 = 0.9958. The results show that the linear relationship was good in the detection ranges. In the present study, the total phenolic and flavonoid contents are shown in Table 2. The value of the phenolic content was 109.86 ± 1.38, 90.89 ± 1.45, 55.86 ± 0.66, 254.96 ± 10.05 and 70.90 ± 0.65 mg of GAE/g extract for EH-Me extract, EH-PE extract, EH-Ch extract, EH-EA extract, and EH-Bu extract, respectively. EH-EA had the highest total phenol content (254.96 ± 10.05 mg GAE/g extract), followed by EH-Me, EH-PE, EH-Bu, EH-Ch.

Table 2. Total phenolic and flavonoid contents in *E. hirta* extracts. Each value is the average of three analyses ± standard error of mean (n = 3). Values with different superscripts in the columns are significantly different based on one-way ANOVA followed by Dunnett's test.

Samples	Total Phenolic Content (mg GAE/g Dried Extract)	Total Flavonoid Content (mg QE/g Dried Extract)
EH-Me	109.86 ± 1.38 ^{a}	18.92 ± 1.33 ^{f}
EH-PE	90.89 ± 1.45 ^{b}	8.48 ± 1.16 ^{g}
EH-Ch	55.86 ± 0.66 ^{c}	16.41 ± 1.44 ^{h}
EH-EA	254.96 ± 10.05 ^{d}	27.66 ± 0.73 ^{i}
EH-Bu	70.90 ± 0.65 ^{e}	12.43 ± 1.66 ^{j}

The total flavonoid content of the extracts was quantified. The regression equation of the standard curve of quercetin was y = 0.008x − 0.0447 with R^2 = 0.9927. The results show that the linear relationship was good in the detection ranges (Table 2). The value of the total flavonoid content was 18.92 ± 1.33, 8.48 ± 1.16, 16.41 ± 1.44, 27.66 ± 0.73 and 12.43 ± 1.66 mg of quercetin/g extract for EH-Me extract, EH-PE extract, EH-Ch extract, EH-EA extract, and EH-Bu extract, respectively (Table 2). EH-EA had the highest total flavonoid content (27.66 ± 0.73 mg quercetin/g extract), followed by EH-Me, EH-Ch, EH-Bu, EH-PE.

3.3. In Vitro Antibacterial Activity Using Disc Diffusion Method

Five *E. hirta* extracts were investigated to evaluate their antibacterial activity against pathogenic bacteria including two strains of Gram-positive bacteria (*S. pneumoniae, B. subtilis*, and *S. aureus*) and three strains of Gram-negative bacteria (*E. coli*, *S. typhi*, *S. flexneri, P. aeruginosa,* and *V. cholearea*) using the agar disc diffusion method. The results for the antibacterial activity of the five *E. hirta* extracts are displayed in Table 3. The antibacterial activity of extracts stopped the growth of most of the colonies of bacteria by forming significant inhibition zones (inhibition zone 10 mm).

The results reveal that three extracts (EH-PE, EH-Ch, EH-EA) are potentially effective in suppressing bacterial growth with variable potency. EH-PE extract was effective against the growth of *S. typhi* and *B. subtilis* at concentrations of 25 mg/mL, 50 mg/mL, 100 mg/mL, 200 mg/mL, 400 mg/mL. With EH-Ch extract, at concentration of 400 mg/mL, there are inhibition zones on six pathogenic strains, including *B. subtilis*, *S. aureus*, *S. preumoniae*, *S. typhi*, *V. cholearea*, *S. flexneri*. In addition, EH-Ch extract exhibited an inhibitory effect against three pathogenic strains at concentration of 200 mg/mL including *V. cholearea*, *B. subtilis*, *S. preumoniae*. Moreover, at a concentration of 100 mg/mL, this extract was effective against *Bacillus subtilis.* With EH-EA extract, at concentration of 100 mg/mL, 200 mg/mL and 400 mg/mL, there are inhibition zones on four pathogenic strains, including *S. typhi*, *B. subtilis*, *S. flexneri, V. cholearea.* At a concentration of 400 mg/mL, EH-EA extract was able to inhibit *S. aureus*, *S. preumoniae* and at a concentration of 200 mg/mL, this extract exhibited an inhibitory effect against *S. preumoniae*.

For the remaining extracts, they have weak effectiveness in the inhibition of investigated bacteria. Both EH-Me extract and EH-Bu extract just exhibited significant antibacterial activities against two strains (*V. cholerae* and *B. subtilis*) at a high concentration of 400 mg/mL.

Table 3. Antibacterial activity of *E. hirta* Linn extracts by disc diffusion method. - No inhibition zone observed.

Tested Bacterium	EH-Me (mg/mL)		EH-PE (mg/mL)		EH-Ch (mg/mL)		EH-EA (mg/mL)		EH-Bu (mg/mL)	
	Concentration	DIZ	Concentration	DIZ	Concentration	DIZ	Concentration	DIZ	Concentration	DIZ
B. subtilis	**-**	**-**	**200**	**10**	**400**	**11**	**-**	**-**	**-**	**-**
E. coli	-	-	-	-	-	-	-	-	-	
S. aureus	-	-	-	-	-	-	-	-	-	-
P. aeruginosa	-	-	-	-	-	-	-	-	-	-
S. preumoniae	-	-	-	-	-	-	400	12	-	-
S. typhi	-	-	-	-	-	-	200	11	-	-
V. cholearea	400	11	-	-	200	11	200	13	400	10
S. flexneri	-	-	-	-	-	-	400	13	-	-

3.4. Study on Antioxidant Activity

3.4.1. DPPH Radical Scavenging Assay of *E. hirta* Extracts

As shown in Table 4, IC_{50} values of EH-Me, EH-PE, EH-Ch, EH-EA, EH-Bu were 17.26 ± 0.12 µg/mL, 122.86 ± 2.65 µg/mL, 92.48 ± 1.47 µg/mL, 10.33 ± 0.01 µg/mL, 55.54 ± 0.09 µg/mL, respectively in DPPH radical scavenging activity. According to IC_{50} values, it can be speculated that EH-EA showed the highest scavenging activity. However, DPPH scavenging activity of EH-EA was weaker than the positive control ascorbic acid (IC_{50} of 5.11 ± 0.02 µg/mL).

Table 4. 2,2-diphenyl-1-pycrylhydrazyl (DPPH) radical scavenging activity of extracts.

Samples	IC_{50} (µg/mL)
EH-Me	17.26 ± 0.12
EH-PE	122.86 ± 2.65
EH-Ch	92.48 ± 1.47
EH-EA	10.33 ± 0.01
EH-Bu	55.54 ± 0.09
Ascorbic acid	5.11 ± 0.02

3.4.2. Lipid Peroxidation Inhibitory Activity of *E. hirta* Extracts

For lipid peroxidation inhibitory activity, IC_{50} values of EH-Me, EH-PE, EH-Ch, EH-EA, EH-Bu were 4.69 ± 0.13 µg/mL, 25.99 ± 0.09 µg/mL, 56.19 ± 0.98 µg/mL, 1.48 ± 0.12 µg/mL, 5.19 ± 0.02 µg/mL, respectively (Table 5). EH-EA extract appeared to be 18.74-fold as potent as Trolox, whereas EH-Me, EH-Bu, EH-PE were about 5.93, 5.36, 1.07-fold, respectively, as potent. EH-Ch extract was weaker than the positive control Trolox (IC_{50} of 27.85 ± 1.22 µg/mL).

Table 5. Lipid peroxidation inhibitory activity of extracts.

Samples	IC_{50} (µg/mL)
EH-Me	4.69 ± 0.13
EH-PE	25.99 ± 0.09
EH-Ch	56.19 ± 0.98
EH-EA	1.48 ± 0.12
EH-Bu	5.19 ± 0.02
Trolox	27.85 ± 1.22

3.5. In vitro Screening for Cytotoxic Activity of E. hirta Linn.

The SRB assay (sulforhodamine B) is a simple and sensitive colorimetric method for determining the cytotoxicity of a substance. SRB is a negatively charged dye containing two sulfonic groups that bind static electricity to positively charged parts of protein under mild acidic conditions and dissociate under basic conditions. The amount of bound dye will reflect the total protein amount of the cell.

In this experiment, cells were fixed, washed and stained with SRB. The SRB then binds to the dissolved cell protein to form a pink, transparent solution. The measured optical density of the solution correlates with the total protein amount or the number of cells. The change in cell count compared to controls reflects the cytotoxicity of the study substance. In the present study, at a constant concentration of 100 µg/mL, the cancer inhibitory activity on the growth of lung cancer cells NCI-H460 and liver cancer cells Hep G2 of five extracts were measured (Figure 3).

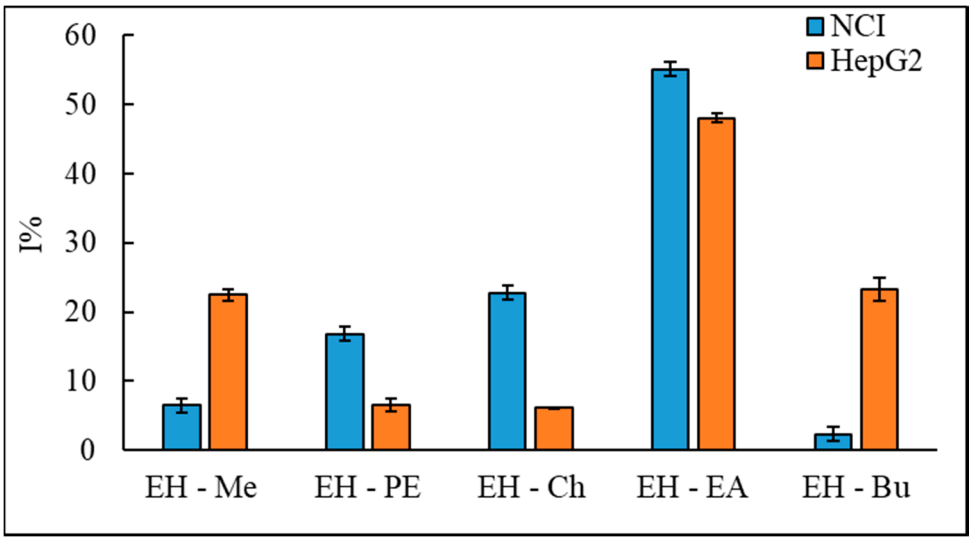

Figure 3. Cytotoxicity of *E. hirta* L. on NCI H460 and Hep G2 cells.

4. Discussion

The tendency to use traditional medicines in the treatment of many diseases has been increasing. Numerous studies have proven that plant-based medicines have not only demonstrated the ability to prevent and treat diseases effectively, but also limited side effects and adverse effects of modern medicines. Plants are rich in natural bioactive compounds, such as phenolics, flavonoids, and their derivatives. These compounds have obtained attention because of their bioactivities and physiological functions, including antioxidant, anti-allergic, anti-inflammatory, antimicrobial, and antidiabetic activities [42].

From methanol crude extract of *E. hirta* (EH-Me extract) containing multiple different groups of phytoconstituents, liquid–liquid extraction technique can be employed to separate many different fractions. As a result, five extracts of *E. hirta* were obtained based on their polarity by specific solvents including petroleum ether, chloroform, ethyl acetate and butanol extracts. Qualitative phytochemical analysis of five extracts of *E. hirta* was carried out to evaluate the bioactive compounds in each extract. The concentration of bioactive compounds in the plant extracts depends on the solvent polarity that was used in the extraction preparation [33,43]. The extraction method is the first step to separate phytochemicals from raw materials. According to the principle of extraction, solvent extraction is the most popularly used method. In this method, solvent polarity significantly affects the efficiency of extraction and constituents of phytochemicals. Depending on chemical nature, a variety of secondary metabolites are extracted in solvents of different polarity as no single solvent may reliably extract all the phytochemical and antioxidant compounds present in the plant material. The serial exhaustive extraction method involves the successive extraction with solvents of increasing polarity from non-polar (petroleum ether) to more polar solvent (water) to ensure the extraction of a wide range of compounds with different polarity. The ascending order of the polarization of solvents is petroleum ether < chloroform < ethyl acetate < butanol < methanol. Therefore, methanol was used to extract raw material. In relation to the solvent used, the highest concentrations of total phenolic and flavonoid contents were found in EH-EA extracts. EH-EA had the highest total phenol content

(254.96 ± 10.05 mg GAE/g extract) and the highest total flavonoid contents (27.66 ± 0.73 mg quercetin/g extract). The results of the determination of total phenolic content and total flavonoid content of these extracts were almost compatible with previous studies [44]. From the above results, it can be predicted that EH-EA extract has better activity in biological activity trials. For the purpose of screening biological activities of these extracts, we investigated antibacterial, antioxidant, lipid peroxidation inhibitory and anticancer properties.

Five *E. hirta* extracts were investigated to evaluate their antibacterial activity against pathogenic bacteria including two strains of Gram-positive bacteria and three strains of Gram-negative bacteria using the agar disc diffusion method. The strains of bacteria selected for antibatesting cause diarrhea, acute diarrhea and pneumonia, etc., which are diseases with a high mortality rate. *E. coli* are a large and diverse group of aerobic bacteria in the environment and food, and either live in the digestive tract of humans or animals. *E. coli* have contributed to many important functions in human life such as stimulating immunity, improving the metabolization of sugars, and preventing the attack of other bacteria from entering the digestive system. However, they can cause diseases such as urinary tract infection, meningitis, etc. *S. aureus* is a major bacterial human pathogen that causes a wide variety of clinical manifestations. The results show that EH-Ch extract and EH-EA exhibited antibacterial inhibitory effect better than other extracts. Among them, EH-EA has the strongest antibacterial activity. However, all extracts almost have no inhibitory activity against *E. coli, S. aureus* and *P. aeruginosa* at most concentrations. The result of antimicrobial property of *E. hirta* extracts is almost compatible with previous studies [45–50].

DPPH free radical scavenging is one of the important mechanisms for the evaluation of the antioxidant activity of a sample. The effect of antioxidants on DPPH were predicted through their hydrogen donating ability of the sample [51]. Literature suggested that total polyphenols content and radical scavenging antioxidant activity are highly correlated. Radical scavenging activities are very important to prevent the deleterious role of free radicals in different diseases, including cancer [52–54]. To investigate the antioxidant activity of *E. hirta* extracts, DPPH radical scavenging assay of *E. hirta* extracts was carried out. The IC_{50} values of EH-Me, EH-PE, EH-Ch, EH-EA, EH-Bu were measured to estimate the potential scavenging abilities of the samples. Our results reveal that EH-Me and EH-EA extracts had stronger free radical scavenging activity than other extracts. This result could be explained by EH-Me and EH-EA extracts containing many phenolic compounds and flavonoids. According to IC_{50} values, it can be speculated that EH-EA showed the highest scavenging activity. This result shows that there is similarity to previous research results [55].

For lipid peroxidation inhibitory activity, IC_{50} values of EH-Me, EH-PE, EH-Ch, EH-EA, EH-Bu were also measured to evaluate antioxidant properties of *E. hirta* extracts. According to IC_{50} values given, EH-Me, EH-PE, EH-EA and EH-Bu extracts possess strong inhibitory properties against lipid oxidation. In particular, EH-EA extract appeared to be 18.74-fold as potent as Trolox, whereas EH-Me, EH-Bu, EH-PE were about 5.93, 5.36, 1.07-fold, respectively (Table 4). These experimental results can be explained by the high concentration of bioactive ingredients extracted from extracts using highly polar solvents. Taken together, these results indicate that *E. hirta* extracts may have therapeutic effects such as inhibiting lipid peroxidation and preventing radical damage. Moreover, previous studies have also reported that *E. hirta* extract had efficacy against selective cancer cell lines such as Hep-2, malignant melanoma, and squamous cell carcinoma [56,57]. In 2014, Neelesh Sharma et al. evaluated anticancer activities of *E. hirta* ethanolic extract [55]. The ethanolic extract showed selective anticancer activity at a concentration of 100 µg/mL ($p < 0.05$). Anticancer activity and cytotoxicity of silver nanoparticles tested against neuroblastoma cell (SH-SY5Y cells) and breast cancer (MCF-7 cell) was reported. That study proved that the ethanolic extract of *E. hirta* showed significant anticancer activity against neuroblastoma cell (SH-SY5Y cells) and breast cancer (MCF-7 cell) [58]. In the present investigation, in the in vitro screening for cytotoxic activity of *E. hirta* extracts, at a concentration of 100 µg/mL, EH-EA extract has significant inhibitory activity against the growth of lung cancer cells NCI-H460 and live cancer cells Hep G2, about 55% and 48%, respectively.

Similar to the biological activities described above, EH-EA extract was also shown to be much more effective in inhibiting two line of cancer cells than the crude extract EH-Me and three remaining fractions. At the same concentration of 100 µg/mL, EH-Me, EH-PE, EH-Ch and EH-Bu did not show significant high cytotoxicity on NCI H460 and Hep G2. For the mentioned results, EH-EA extract may be rich in bioactive compounds, and these compounds exhibited significant antibacterial, antioxidant and anticancer activities.

5. Conclusions

Recent investigations have proven that secondary metabolites from natural resources containing bioactive components have a wide variety of biological properties. This study provides significant evidence about the biological activity of different extracts of *E. hirta*. These results indicate that *E. hirta* extract can be a good source for antioxidant, antimicrobial, and anticancer herbal-related drugs. From the above-mentioned results about biological activities, the EH-EA extract outperformed the EH-Me extract and the remaining extracts. Therefore, further studies to isolate and identify of bioactive compounds from EH-EA extract for in vitro and in vivo investigations of observed activities are highly recommended. In addition, it is necessary to elucidate the mechanisms of action of these extracts and bioactive compounds isolated from this plant at the cellular and molecular level to evaluate biological capacity of substances on specific therapeutic properties.

Author Contributions: Conceptualization, N.T., N.N. and L.L.; methodology, N.T., M.N. and K.P.L.; software, K.P.L. and M.N.; validation, N.T., M.N. and N.N.; formal analysis, N.T. and M.N.; investigation, N.T., N.N. and M.N.; resources, L.L. and Q.T.; data curation, N.N. and L.L.; writing—original draft preparation, N.T.; writing—review and editing, N.T., M.N. and L.L.; visualization, N.N.; supervision, L.L.; project administration, Q.T. and L.L. All authors have read and agreed to the published version of the manuscript.

Funding: This work was funded by Vietnam National University at Ho Chi Minh City under grant number GEN2019-28-01/HD-KHCN together with the ethical approval for the research content.

Conflicts of Interest: The authors declare no conflict of interest.

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