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Research Article
Anticancer and Antioxidant of Chloroform Extracts from Medical Plants in the Mekong Delta, Vietnam

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Abstract

Background and Objective: Natural compounds derived from plants are one of the promising therapies for various diseases, especially cancer. This study aimed to evaluate and compare the proficiency against oxidative stress and liver cancer cells of ten medicinal plants; to define the correlation between alkaloids, flavonoids and polyphenols, as well as to examine their cell protection ability. **Materials and Methods:** The samples were extracted with chloroform before conducting antioxidant activity determination by 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay, then evaluated anticancer activity via 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay using HepG2 cell lines. **Result:** The results indicated that *E. sonchilofia* had the highest amount of three bioactive compounds. *M. pigra* in ABTS assay gave the most efficient IC_{50} value compared to the second large value of *E. indica*. Further investigation with MTT assay showed a significant difference in the anticancer capability of *W. trilobata* among others. The ethyl acetate fractioned extract showed a higher anticancer cell activity. The thin layer chromatography indicated the presence of terpenoids with the specific purple spot. **Conclusion:** Base on our results, the medicinal plant is a rich source of valuable bioactive compounds that can be potentially toxic to cancer cells.

Key words: herbs, HepG2, liver cancer, bioactive compounds, *E. sonchilofia*

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Free radicals are now a top concern among scientists around the world. In addition to accelerating the aging process in the human body, they are also potential causes of abnormalities in the organism (e.g., macromolecule damage, membrane destruction and irregular cell division), leading to many dangerous diseases, including cancer. Free radicals can be inactivated by antioxidants, which are substances that neutralize, slow down or reverse the oxidation of compounds in the body[1]. According to Yadav *et al.*[2], antioxidants can be artificially synthesized or naturally available in food and are commonly added into dietary supplements to prevent free-radical related diseases.

Since natural products are considered more biologically friendly than chemically synthesized ones, the demand for plant-derived antioxidants in anticancer therapies is increasing day by day, urging scientists to find more ways to make full use of products both from wild plants and traditional medicinal plants. These plants contain a lot of secondary metabolites that could be researched and used for medicinal purposes. According to Cragg *et al.*[3], 41% of the anticancer drugs approved by the U.S FDA are derived from plants.

Along with being added to medications curing diabetes, cardiovascular diseases and several cancers, natural antioxidants are also essential for the treatment of hepatocellular carcinoma. This disease is often called liver cancer and is considered the third most fatal disease in humans[4]. Usually, liver cancer is attributed to the existence of hepatitis B, hepatitis C and the overuse of alcohol. To investigate this study is due to the cytotoxicity of phytochemicals on Hep-G2 cancer cells – a cell line that secretes many plasma proteins and has a high rate of proliferation.

Several studies have been conducted to exploit useful extracts from plants and apply them to liver cancer treatment. The chloroform extract of *Strobilanthes crispa* was proved to possess cytotoxicity against Hep-G2 cells[5]. *Abelmoschus esculentus* L. was demonstrated to have anticancer ability against Huh7it liver cancer cells[6]. The study conducted on *Viscum album* also pointed out that extracts of this plant may inhibit the proliferation of Hep-3B liver cancer cells[7].

Chloroform extracts of ten traditionally medicinal plants and wild grass in the Mekong Delta were evaluated for their antioxidant and anticancer activities, alkaloid, flavonoid and polyphenol contents.

MATERIALS AND METHODS

Study area: The experiments were conducted from May 2019 to January 2020.

Plant samples: Plants were collected from different areas of Can Tho City. The collected samples were identified and deposited at the Plant Laboratory, Department of Biological Pedagogy, Pedagogy Faculty, Can Tho University. The samples were separated, washed, dried at 60°C in an oven and finely ground into powder.

Extraction: A sample of 100 grams of dried plant powder was soaked in hexane (1 L) for a day each to remove lipids and chloroplasts. The crushed plants were impregnated with 50 mL of NH_4OH and macerated in 1 L of chloroform for 24 hrs. The mixture was filtered and concentrated under reduced pressure in a rotary evaporator at 50°C. The plant samples were extracted in triplicate. The extract with the highest activity was fractioned to identify the main bioactive compound as shown in Fig. 1.

Quantitative analysis of phytochemicals: Total Alkaloid content: The total alkaloid was determined based on the reaction with Bromocresol Green (BCG) by UV-Spectrophotometer[8]. A concentration (1 mg mL^{-1}) of each sample extract in methanol was prepared. One milliliter of the extracts was mixed with 1 mL of 2 N HCl in 5 min. The mixture was added 5 mL of BCG 1×10^{-4} M and 5 mL of McIlvaine buffer (pH 4.7) in the extraction flask. After 2 min reaction, the mixture was shaken with 10 mL of chloroform. The absorbance of the reaction mixture was measured at 470 nm using a spectrophotometer. The total alkaloids content was identified by using a standard curve with atropine (0-250 µg mL^{-1}). The mean of triplicate was used and the total alkaloids of plant extracts were showed as µg of atropine equivalents (AE)/g of the dried sample.

Total flavonoid content: The total flavonoid was estimated due to the Aluminum chloride colorimetric method by Bag *et al.*[9]. A 400 µL of sample extract (100 µg mL^{-1}) in methanol was prepared. A 40 µL $NaNO_2$ 5% was added to the sample. The solutions were mixed thoroughly and incubated at room temperature for 5 minutes. The mixture was added 40 µL $AlCl_3$ 10% and incubated at room temperature for 6 min after thorough shaking. A volume of 400 µL NaOH 1 M and 120 µL H_2O were poured into the mixture. The absorbance of

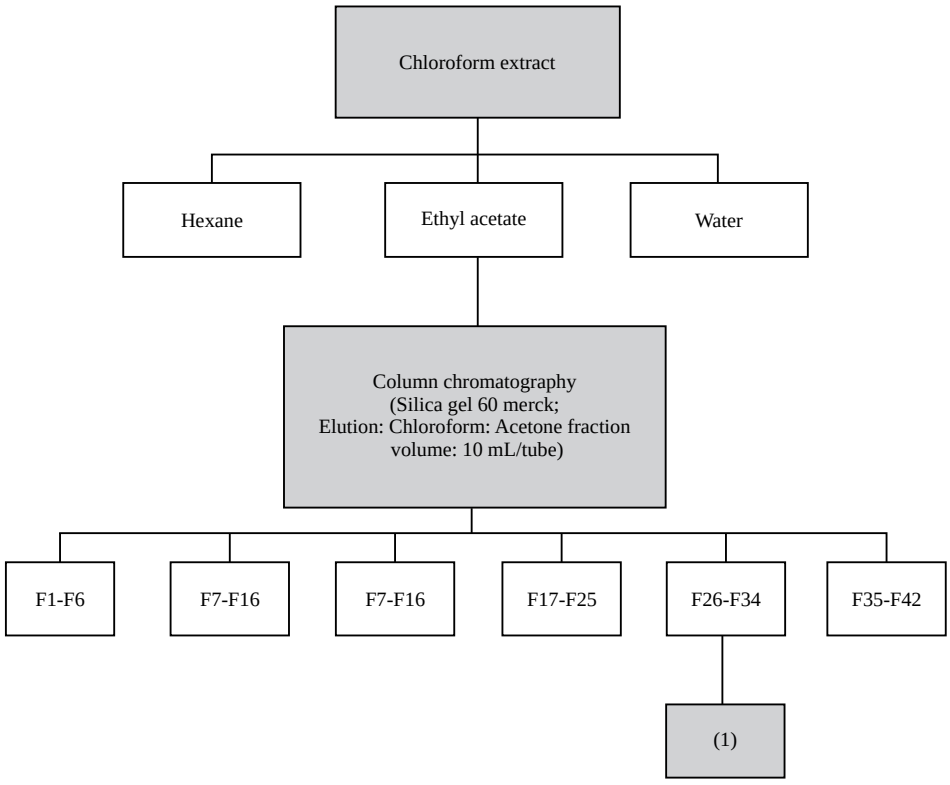

Fig. 1: The fractioning to identify the main bioactive compound

the reaction mixture was measured at 510 nm using a spectrophotometer. The total flavonoid content was identified by using a standard curve with quercetin (20-100 μg mL^{-1}). The mean of triplicate was used and the total flavonoids of plant extracts were showed as μg of quercetin equivalents (QE)/g of the dried sample.

Total polyphenols content: The total phenol content is determined by adjusting the description of Singleton *et al.*[10]. A concentration (100 μg mL^{-1}) of each extract in methanol was prepared. A 500 μL of extracts were mixed with 250 μL Folin-Ciocalteu reagent 20% then shaken and incubated at room temperature for 5 minutes. After incubation, 250 μL of Na_2CO_3 10% was poured into the mixture then mixed and incubated at 37°C for 30 min in the dark. The final mixture was centrifuged at 4°C. The absorbance of the reaction mixture was measured at 765 nm using a spectrophotometer. The total polyphenols content was identified by using a standard curve with gallic acid (2-10 μg mL^{-1}). The mean of triplicate was used and the total polyphenols of plant extracts were shown as μg of gallic acid equivalents (GA)/ g of the dried sample.

Antioxidant assay: The antioxidant capacity of plant extracts was estimated based on the scavenging $ABTS^{\bullet+}$ (2,2 -azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) of antioxidants as depicted by Re *et al.*[11] with edited. To form $ABTS^{\bullet+}$ solution, 7 mM ABTS solution and 2.45 mM $K_2S_2O_8$ solution was mixed at the same volume. The mixture stands in the dark at room temperature for 14-16 hrs. After incubation, the solution was diluted with ethanol and adjusted to an absorbance of 0.70±0.05 at a wavelength of 734 nm. A 100 μL of $ABTS^{\bullet+}$ solution was added into 100 μL plant extract at discrepancy concentration (5, 25, 50 μg mL^{-1}). The mixture was incubated at room temperature for 30 min. The absorbance of the reaction mixture was measured at 734 nm using a spectrophotometer. The standard curve of gallic acid (0.17-0.68 μg mL^{-1}) was used in this assay. The assay was done in triplicates.

Cell culture: A human hepatocellular carcinoma (HepG2) was cultured in MEME (Minimum Essential Medium with Eagle salt) with 7-10% FBS (Fetal Bovine Serum) and supplemented other essential elements. Cell lines were incubated at 37°C at 5% CO_2 and 98% humidity with absolute aseptic. Cells were used to evaluate anticancer activity at the log phase.

Anticancer assay: HepG2 cells were separated by trypsin and diluted to 1-3×10^4 cell/mL with the medium. Thereafter, 190 µL HepG2 cells were treated with 10 µL different concentrations of plant extract (256, 64, 16, 4 µg mL^{-1} in DMSO) for 72 hrs. The anticancer activity of plant extract was evaluated in hepatocellular carcinoma HepG2 through MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) assay by Mosmann[12]. Then, 10 µL of 5 mg mL^{-1} MTT was added to the cells treated with plant extracts for 4 hrs. After removing medium, living cells were reduced MTT from yellow to purple indissoluble formazan, which was diluted by 100 µL DMSO. The absorbance of the solution was measured at 734 nm using a spectrophotometer. Ellipticine 0.01 mM in DMSO was used as a control sample in this assay. The assay was done in triplicates.

Statistical analysis: All the data in this study were analyzed separately and expressed as Mean±standard deviation. Correlation analysis (with 95% confidence interval) of biological activity (anticancer HepG2 and antioxidant) versus the total alkaloids, phenolics and flavonoids contents were calculated by one-way analysis of variance (ANOVA) with the Tukey test with Minitab 16.0 and Microsoft Excel 2013.

RESULTS

Total phytochemicals content: Total alkaloids and flavonoids content (TAC and TFC): The results of alkaloids content determined by spectrophotometry are described in the left column in Fig. 2. The highest content of alkaloids was found in *T. procumbens* (7.2±0.18 µg g^{-1}) and *E. sonchifolia* (7.01±1.5 µg g^{-1}). The disparity between the highest, *T. procumbens* and the lowest, *C. rotundus*, was approximately 5.2 µg total alkaloids per gram of dried powder. Besides, the total flavonoids content quantified by the Aluminium chloride colorimetric method revealed a slight similarity in most of the samples. Eight of the ten extracts achieved the highest statistical value. However, *A. conyzoides* (4.97±0.47 µg g^{-1}) is the highest measured content, whereas *C. rotundus* (1.91±0.43 µg g^{-1}) and *M. pudica* (1.28±0.13 µg g^{-1}) present the lowest in flavonoids content. The disparity of the highest and the lowest flavonoids content is about 3.7 µg total flavonoids for every gram of dried powder.

It can be seen that the concentration in both alkaloids and flavonoids appear to be considered in most plant, excepting *C. rotundus, M. pudica* and *E. Indica* (as indicated in Fig. 2). Additionally, *T. procumbens* and *E. sonchifolia* are superior to the others due to the highest content in both statistical values.

Total polyphenols content (TPC): That the Folin–Ciocalteu assay quantified the polyphenol contents of ten extracts are shown in Fig. 3. The highest amount of polyphenols measured belongs to three plants: *E. sonchifolia, A. Conyzoides* and *E. indica,* but *E. sonchifolia* outweighed the other two plants, with 0.91±0.54 µg g^{-1}. Regardless of low in the polyphenols content are relatively similar in the rest of the ten.

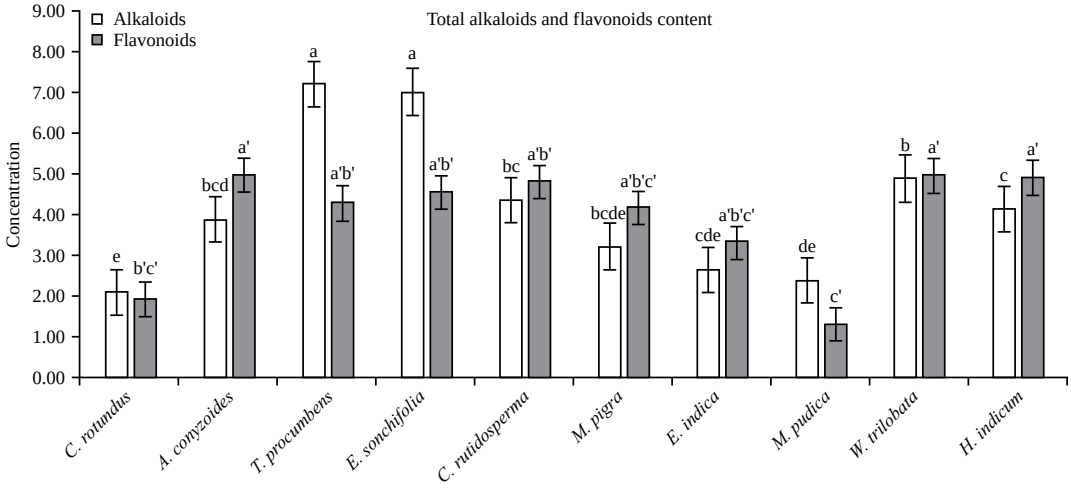

Fig. 2: Total alkaloids and flavonoids content (TAC and TFC)
Statistically significant differences ($p<0.05$) of both alkaloids and flavonoids content are in column with the different letters. Error bars represent standard errors of the means

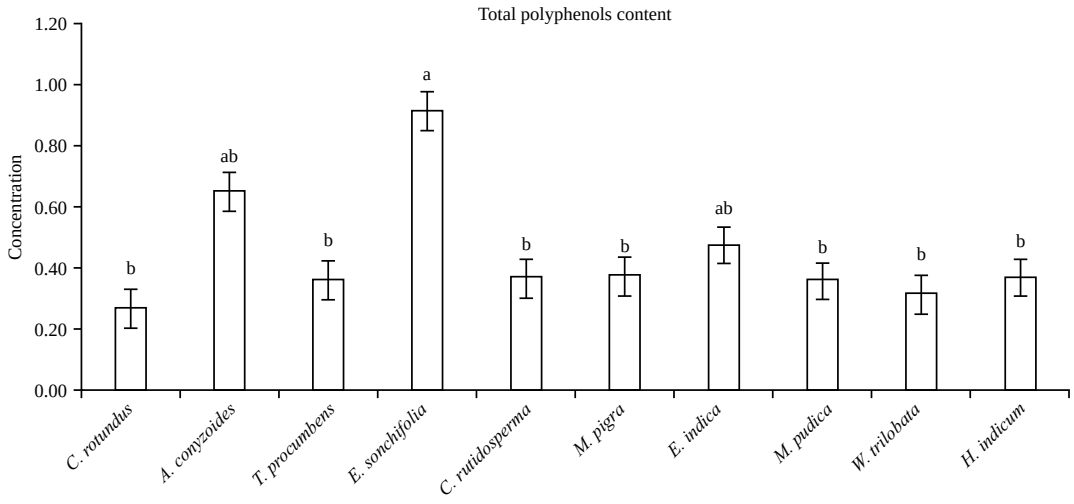

Fig. 3: Polyphenols content determined by Folin-Ciocalteu assay

Statistically significant differences ($p<0.05$) of polyphenols content are in column with the different letters. Error bars represent standard errors of the means

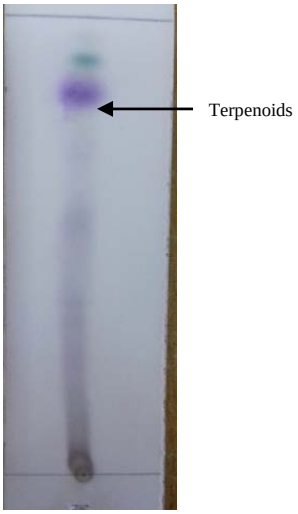

Fig. 4: TLC of the ethyl acetate fraction (Hexane: Ethyl acetate-50:50)

Table 1: The half-maximal inhibitory concentration (IC_{50}) of ABTS assay and MTT assay

Samples	ABTS assay (μg mL^{-1})	MTT assay (μg mL^{-1})
C. rotundus	31.033±3.249	64.0±2.5
A. conyzoides	25.907±1.088	83.2±3.0
T. procumbens	30.110±2.155	117.0±4.5
E. sonchifolia	35.980±3.057	>256.0
C. rutidosperma	33.340±1.051	>256.0
M. pigra	20.497±0.346	153.3±3.5
E. indica	24.500±1.586	>256.0
M. pudica	31.050±3.986	256.0
W. trilobata	27.047±0.928	38.0±1.5
H. indicum	24.643±1.485	224.0±6.5
Standard	Gallic acid = 0.327±0.006	Ellipticine = 0.32±0.03

Biological activities

Antioxidant activity: In this research, the ABTS assay was used to evaluate the antioxidant activity of plant extracts. The ABTS assay is based on the ability of extraction to scavenge the $ABTS^{+\bullet}$ cation produced by the 2,2 -azinobis-3-ethylbenzothiazoline-6-sulphonate oxidation reaction. Table 1 shows the IC_{50} values of 10 herb chloroform extracts. The highest is *M. pigra* (IC_{50} = 20.497±0.346 μg mL^{-1}) while *E. sonchifolia* (IC_{50} = 35.980±3.057 μg mL^{-1}) has the lowest free radical scavenging ability.

Anticancer activity: All plant extracts were tested against the human liver cancer cell line by MTT method. As presented in Table 1, IC_{50} values were detected anticancer efficiency contents in samples ranging from 38.0 μg mL^{-1} to over 256.0 μg mL^{-1}. Test with *W. trilobata* gave a significant effect on cancer cells at the lowest concentration (IC_{50} = 38.0±1.5 μg mL^{-1}). The *C. rotundus* is the following plant with a lower effect (IC_{50} = 64.0±2.5 μg mL^{-1}).

Further investigation was conducted with the *W. trilobata* extract because of its high anticancer activity. Three fractions including hexane, ethyl acetate and water were examined for the toxicity to HepG2 cancer cells. The result showed that the ethyl acetate fraction had the highest activity with IC_{50} = 22.81±0.20 (μg mL^{-1}). Consequently, the main compound in the ethyl acetate fraction was fundamentally identified as terpenoids (Fig. 4).

Correlation: As can be seen from Table 2, no correlation was observed between scavenging activity, antitumor HepG2 and

Table 2: The correlation was calculated between phytochemicals screening and biological activity

Samples	Antioxidant activity	Anticancer activity	Total alkaloid	Total flavonoids
Anticancer activity	0.245			
Total alkaloid	0.408	-0.014		
Total flavonoid	-0.174	-0.127	0.611	
Total polyphenol	0.329	0.310	0.463	0.324

phytochemical contents because the r^2 values were lower 0.5. Only total alkaloids and total flavonoids had the correlation, but it was not high.

DISCUSSION

In this research, it focuses on the preliminary assay of crude extracts. Plant extract includes alkaloids, flavonoids, polyphenols, phytoestrogens, terpenoids, carotenoids, limonoids, phytosterols, glucosinolates and anthocyanidins[13]. In leaves of *A. conyzoides* also contains alkaloids, quinones, cardiac glycoside and tannin[14]. Previous studies showed that *E. sonchifolia* has various phytochemicals like tannin anthraquinones, triterpenoids, saponins, flavonoids and cardiac glycosides. Among them, tannin is highly present[15]. Steroids, saponins, coumarins, alkaloids, amino acids, diterpenes, phenol and flavonoids are phytochemical groups that were found in the chloroform extract of *T. procumbens*[16]. There is a large number of phytochemical groups in the crude extracts, so the correlation between these compounds and their biological activity could not be observed. This means that the biological activities of the extracts are thanked to other compounds.

The total alkaloids content of chloroform extracts was determined by utilizing atropine as a standard while total flavonoids using quercetin and gallic acid was used for quantification of total polyphenols. As shown in Fig. 1, two plants that have potential in alkaloids and flavonoids among the ten are *T. procumbens* and *E. sonchifolia*. The latter also contains the highest amount of polyphenols. Edagha *et al.*[17] confirmed about the richness of alkaloids and flavonoids in the extract of *E. sonchifolia* and concluded that the promotion of this on erythropoiesis and hepatoprotective activity in *Plasmodium berghei* infected mice. Furthermore, Areekul *et al.*[18] reported the polyphenols in total of fresh and dried *E. sonchifolia* in 80% ethanol extracts are 7.02±0.56 and 26.56±2.04 mg GAE/g db, respectively. Additionally, Savithramma *et al.*[19] reported the absence of alkaloids in the chloroform extract of *T. procumbens,* which contradicts to the considerable amount(7.2±0.18 μg g^{-1}) in our findings. *Ageratum conyzoides* also possesses a noticeable concentration in terms of alkaloids, flavonoids and polyphenols (0.65±0.04 μg g^{-1}). Bidie *et al.*[20] also declared the presence of polyphenols and flavonoids in this plant.

Antioxidant capacity was assessed by IC_{50} value. The lowest IC_{50} values illustrated the strongest free radical scavenging ability. In the present study, antioxidant value of *M. pudica* (IC_{50} = 31.050±3.986 μg mL^{-1}) is lower than previous study of Guha *et al.*[21] (IC_{50} = 65.4±0.73 μg mL^{-1}). Chloroform extract of *E. sonchifolia* (IC_{50} = 35.980 ± 3.057 μg mL^{-1}) in this study showed a higher antioxidant effect compared with methanol and hexane extract, IC_{50} = 276.7±15.1 μg mL^{-1}, >500 μg mL^{-1}, respectively by Peisino *et al.*[22].

Based on our findings, the chloroform extract of *H. indicum* inhibited fifty percentages of HepG2 cell line at 224.0±6.5 μg mL^{-1}. Additionally, the methanolic extract of *H. indicum* showed a good cytotoxic effect on the HeLa cell line with IC_{50} = 200 μg mL^{-1} for both stem and leaf extracts by Sivajothi *et al.*[23]. A previous study with *M. pudica* revealed that Daudi cells treated with hydroalcoholic extract showed significant inhibition of cell proliferation at 201.65 μg mL^{-1} by Parmar *et al.*[24]. In human liver cancer, *M. pudica* showed anticancer efficiency with IC_{50} = 256.0 μg mL^{-1}.

The bioactive effects of elements in the plant extracts depend on the extraction procedure and the characteristics of the used solvent by Hayouni *et al.*[25]. The quality of plant extracts depends on the properties of raw materials, the origin of plants, degree of processing, sample moisture and crushed size of sample by Pandhi and Poonia[26].

Regarding the bioactive compound in the ethyl acetate fraction of *W. trilobata*, many publications reported that the plant contains tannin, saponins, flavonoids, phenol and terpenoids[27]. Flavonoid and phenol classes were not correlated to the bioactivities of this plant, therefore, the phytoconstituents caused the toxicity to liver cancer cells could be tannin, saponins and terpenoids. With the evidence found in Thin Layer Chromatography (TLC), it could be concluded that terpenoids had the anticancer activity of *W. trilobata*.

CONCLUSIONS

E. sonchifolia, M. pigra and *W. trilobata* were potential plants that have the highest result of the total content of three compounds, antioxidant and anticancer activity, respectively. Further researches on these three plants should be conducted to clarify specific substances that are responsible for protecting the human body against diseases.

SIGNIFICANCE STATEMENT

This study discovered the antioxidant and anticancer of some wild plants in Vietnam that can be beneficial for producing functional foods or drugs. This study will help the researchers to uncover the critical areas of anticancer medicines from wild plants that many researchers were not able to explore. Thus a novel natural compound may be arrived at.

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