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Research Article

Cytotoxic effect of *Eleutherococcus trifoliatus* (L.) S.Y. Hu stem bark extracts on Gastric, Lung, and Hepatocellular cancer cells

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Abstract: *Eleutherococcus trifoliatus* is a popular medicinal species used in Asian folk medicine with many uses in treating human diseases. Scientific research on this plant has been limited, but some scientific publications have described this herb's anti-inflammatory, antibacterial, antioxidant, and anti-cancer activities. Although it is a frequently used part of traditional medicine, current scientific evidence on the biological activities of *E. trifoliatus* stem bark is still lacking. By performing chemical reactions, free radical scavenging experiments, toxicity experiments on brine shrimp, and cytotoxicity tests using MTT dye, the study has shown the diversity in the metabolic composition of *E. trifoliatus* stem bark as well as the antioxidant capacity and safety of the total extract. The anticancer effect of the total extract was investigated and indicated the dramatical ability to inhibit the cell growth of liver cancer HCC-J5 cells (IC50 = 19.35 ± 4.89 µg/mL), lung cancer cells A549 (IC50 = 5.34 ± 1.62 µg/mL) and gastric cancer cells AGS (IC50 = 0.22 ± 0.20 µg/mL); the selectivity in effects was also observed. The chloroform fraction had the most potential to be further exploited in the direction of inhibiting cancer cells.

1. INTRODUCTION

Eleutherococcus trifoliatus (L.) S.Y. Hu (synonym:*Acanthopanax trifoliatus* (L.) Voss.) is a small tree with medium-high (below 7 meters), robust branching, and thorning (Loi, 2004). Leaves are triangular in shape, like duck feet, with staggered distribution, and leaf veins with spines (Loi, 2004). Flowers develop at the tip of the branch with more than 3 crowns. Berried fruits are in a flat sphere shape, in black, harboring 2 seeds. Essential oils can be extracted from the whole plant with fragrance (Loi, 2004). This plant is mainly distributed in areas of Asia such as Korea, China, Far East Russia, Vietnam, and Japan. This plant has been discovered in Vietnam in Cao Bang, Lao Cai, Lang Son, Lai Chau, Son La, Hoa Binh, Thanh Hoa, Nghe An, Quang Nam, Quang Ngai, and Kom Tum. This plant is exploited in folk medicine as a medicinal herb to treat human diseases (Huyen *et al.*, 2006; Loi, 2004). Traditional herbalists believe that the root bark and stem bark are the most pharmacologically practical parts of *E. trifoliatus* and

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should be harvested in the summer (Huyen *et al.*, 2006; Loi, 2004). The medicinal powder has a bitter taste and is used in treating bone and joint diseases, inflammation, and erectile dysfunction (Loi, 2004; Thu & Tuan, 2013). In modern medicine, this medicinal herb is used for sedation, anti-inflammation, and dilation of blood vessel walls, thereby lowering blood pressure and stopping coughing and expectoration (Loi, 2004; Thu & Tuan, 2013).

Despite its widespread application, scientific research on *E. trifoliatus* has still been quite vague, preventing the exploitation of this plant in modern medicine. Thus far, scientific evidence has shown several bio-activities of *E. trifoliatus*, such as the antioxidant effect (Sithisarn & Jarikasem, 2009); anti-inflammatory on RAW264.7 cells and BALB/c mice (Chen *et al.*, 2021; Chien *et al.*, 2015; Thu & Tuan, 2013); antibacterial impact tested on *Salmonella, Escherichia coli*, *Staphylococcus aureus*, *Staphylococcus albicans*, and *Micrococcus luteus* (Chen *et al.*, 2021); analgesic effects were observed on rats (Chen *et al.*, 2021); hemostatic effects evaluated on New Zealand white rabbits (Chen *et al.*, 2021); type 2 diabetic treatment investigated on C57BL/6 mice (Lin *et al.*, 2023). *E. trifoliatus*'s antitumor studies have also been conducted on PC-3, SF-268, MCF-7, HepG2, and NCI-H460 cells; several toxicity pathways have been described (Li *et al.*, 2016; H.-C. Wang *et al.*, 2014; H. Q. Wang *et al.*, 2014). However, research on the herb's stem bark has been still lacking. This work aimed to demystify the anti-proliferation of the *E. trifoliatus* stem bark total and fractional extracts on lung carcinoma, gastric adenocarcinoma, and hepatocellular carcinoma cells, and describe the ingredient background and antioxidant capacity of *E. trifoliatus* stem bark.

2. MATERIAL and METHODS

2.1. Extract Preparation

2.1.1. *Plant sample and extraction*

Eleutherococcus trifoliatus stem bark was derived from An Giang Province, Vietnam, with voucher 2021NCBG-AG01. The sample was dried after double washing with distilled water. The dried sample was ground into a fine powder and macerated with absolute methanol at 1:3 (weight/volume). After five days of maceration with the filtrate collection every 24 hours, the total stem bark extract (abbreviated as ET) was obtained by solvent removal. For the fractional extraction, the crude extract was dissolved with chloroform to obtain the chloroform fraction. The insoluble extract was further dissolved with ethyl acetate to obtain ethyl acetate fraction. The procedure continued with methanol and water. The results were 4 different fractions dried using the Soxhlet system, including chloroform (extract abbreviated as ETC), ethyl acetate (extract abbreviated as ETE), methanol (extract abbreviated as ETM), and water (extract abbreviated as ETW) extracts. The droughty total and fractional extracts were weighted and dissolved with DMSO (Dimethyl sulfoxide, Sigma-Aldrich, USA) to achieve the stock solution of 400 µg/mL for crude extract and 100 µg/mL for fractional extracts.

2.1.2. *Phytochemical screening*

In order to optimize the detection of plant compounds, medicinal powders were soxhleted with three solvents of different polarities as described by Ciulei (Ciulei, 1993). A mass of 30 grams of the herbal was wrapped with filter paper and extracted using the Soxhlet system with diethyl ether, ethanol, and water, respectively. The chemical reactions were performed for phytochemicals detection.

2.2. Antioxidant Effect of Total Extract

The free radical scavenging capacity of the ET was determined by performing the DPPH (2 2-diphenyl-1-picrylhydrazyl, Sigma-Aldrich, USA) and ABTS (2,2′-Azinobis-(3-Ethylbenzthiazolin-6-Sulfonic Acid), Sigma-Aldrich, USA) assays. Active ABTS solution was formed by the reaction of 2.6 mM ABTS with 7.4 mM potassium peroxydisulfate [$K_2S_2O_8$] (Sigma-Aldrich, USA) for 16 hours in the dark. The active ABTS solution was adjusted with methanol to achieve the final solution with an absorbance of 1.00 ± 0.02 at 734 nm. The extract

at different concentrations was reacted to DPPH (with a ratio of 1:1) and active ABTS (with a ratio of 1:5). After a 30-minute incubation; the mixtures measured the absorbance at 517 nm and 734 nm for DPPH assay and at 734 nm for ABTS. Nonlinear regressions computed the EC50 (half maximal effective concentration) values of the ET on DPPH and ABTS radicals.

2.3. Toxicity on Brine Shrimp

Brine shrimp cysts (*Artemia nauplii*) were hatched in an incubator with continuous light and aeration. A volume of 2 mL saline containing thirty individual shrimps was divided into separate cups. The ET extract at different concentrations ranging from 0 to 100 µg/mL was supplemented into larvae cups. The DMSO equivalent to the amount added in the most concentrated treatment was set as a negative control. After 24 hours of treatment, the dead shrimps, which showed no movement for at least 10 seconds, were counted and computed for the survival proportion (Santos Filipe *et al.*, 2022).

2.4. Cytotoxicity Evaluation

2.4.1. *Cell lines and cell culturing*

Lung carcinoma cells A549 (ATCC, USA), gastric adenocarcinoma AGS (ATCC, USA), and hepatocellular carcinoma HCC-J5 (Cell Culture Center of the National Taiwan University, Taipei, Taiwan) were used to investigate the anticancer impact of *Eleutherococcus trifoliatus* stem bark extracts. The non-cancerous cells, fibroblast BJ (ATCC, USA), were used as control. Cells were cultured in Roswell Park Memorial Institute (RPMI, Thermo Scientific, USA) 1640 Medium, added 10% Fetal Bovine Serum (FBS, Sigma-Aldrich, USA) and 1% Penicillin-streptomycin (Sigma-Aldrich, USA). As 80% of the culture surface was occupied by cells, the cells were sub-cultured with the initial density of 10^5 cells/mL.

2.4.2. *Cytotoxicity assay*

Cells were seeded at 10^5 cells/mL density into 96-well plates. After 24 hours, the medium harboring the extract at different concentrations with homologous volumes was added to each well. The treatment was conducted for 72 hours. The cell viability was calculated by MTT ((3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide), Sigma-Aldrich, USA) reagent; the crystal dissolution solution was measured the absorbance at 490 nm. Nonlinear regression indicated the half-maximal inhibitory concentration (IC_{50}) values of the extracts on tested cells.

2.4.3. *Selective index*

The selective index of the ET was indicated by dividing the IC_{50} value of the ET on BJ cells by the one on cancer cells.

2.5. Data Computation

Data collection occurred at least three independent times. Documents were analyzed using Excel 365 (Microsoft, USA) and GraphPad Prism version 9.0.0 (GraphPad Software, USA). Nonlinear regression was performed according to the model Y=100/(1+10^((LogIC50-X)*HillSlope))) to deduce the IC50 and EC50 values. Comparative statements were determined from the results of ANOVA one-way analysis combined with the Tukey post-hoc test and Student T-test with an Alpha significance level of 5%. The symbol notes (*), (**), (***), and (****) stood for the *p*-value below 0.033, 0.0021, 0.0002, and 0.0001, respectively.

3. FINDINGS

3.1. Phytochemical Description

The chemical reactions exhibited the presence of polyphenol, flavonoid, alkaloid, saponin, anthocyanin, reducing sugar, cardiac glycoside, and steroid compounds (Table 1).

Table 1. Screening for phytochemicals in *E. trifoliatus* stem bark.

Compounds	Reactions	Extracts		
		Diethyl ether	Ethanol	Water
Alkaloid (Jha *et al.*, 2012)	Mayer	+	+	+
	Wagner	+	+	+
Flavonoid (Ly *et al.*, 2019)	Alkalize with 10% NaOH	+	+	-
	Acidified with concentrated H2SO4	+	+	-
Steroid (Nath *et al.*, 1946)	Liebermann – Burchard		+	+
Polyphenol (MacWillliam & Wenn, 1972)	Reducing $FeCl_3$		+	+
Anthocyanin (Ly *et al.*, 2019)	Reddening induced by HCl 10% Cyanotion induced bt NaOH 10%		-	-
Saponin (Edeoga, 2005)	Foam formation			+
Cardiac glycoside (Mujeeb *et al.*, 2014)	Keller-Kiliani		+	-
Reducing sugar (Ayoola *et al.*, 2008)	Fehling			+
Organic acid (Thi *et al.*, 2023)	Na_2CO_3		-	-
Polyuronid (Thi *et al.*, 2023)	Flocculation		-	-

Presence (+); absence (-); empty cells stand for no need for detection.

3.2. Antioxidant Effect of The Total Extract

As shown in Figure 1, the radical scavenging ability of the ET was described. The EC50 (µg/mL) values were demystified at 339.50 ± 48.60 and 138.10 ± 24.70 for DPPH and ABTS. The *t*-test one-tail result showed the effect of the ET on ABTS was greater than on DPPH, P-value < 0.0379. The vitamin C at the concentration of 50 µg/mL illustrated to scavenge 87.14 ± 0.24 % DPPH and 99.66 ± 0.60 ABTS; the effect of 100 µg/mL one was 89.94 ± 0.24 % and 100 ± 0.02 % for DPPH and ABTS.

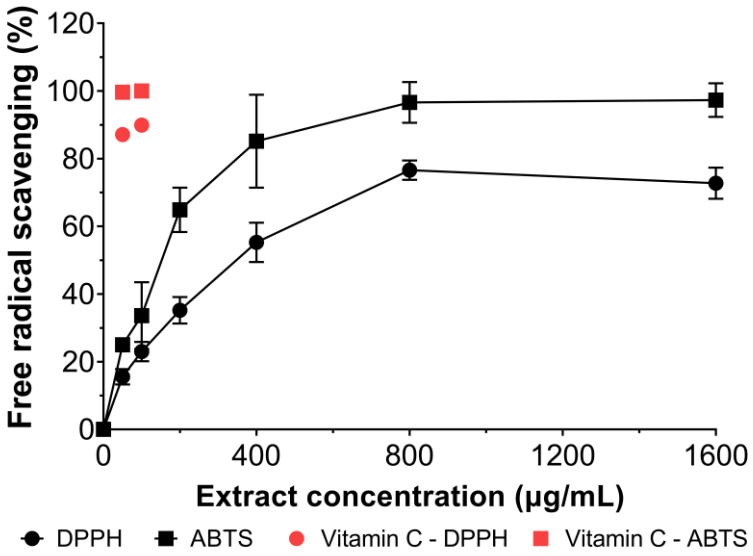

Figure 1. The free radical scavenging of the *E. trifoliatus* stem bark total extract evaluated by DPPH and ABTS assays.

3.2. Toxicity on Brine Shrimp

The ET extract showed no negative impact on the vital of *Artemia nauplii* after 24 hours of treatment, *p*-value = 0.2127 (Figure 2). There were no statistical differences between the experiment with DMSO and negative control, *p*-value = 0.1854.

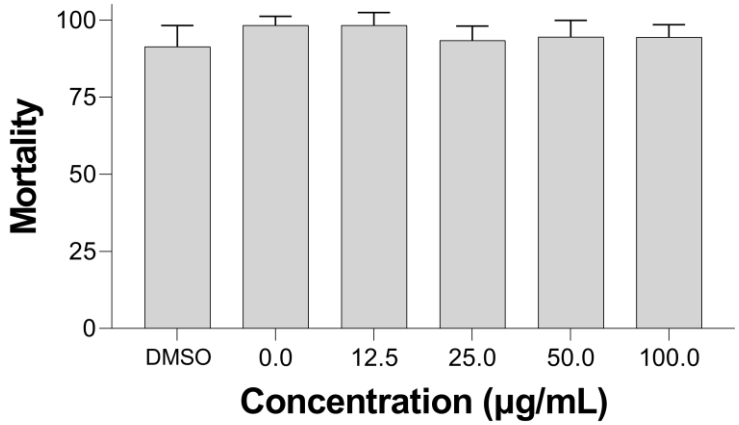

Figure 2. The toxic impact of *E. trifoliatus* stem bark total extract on brine shrimp.

3.4. Anticancer Effect of the ET on Selective Cancer Cells

The anticancer effect of ET results were shown in Figure 3. The influence was illustrated in a dose-dependent manner (Figure 3A). Non-linear regression analysis determined the IC_{50} values of ET on hepatocellular carcinoma (HCC-J5), lung carcinoma (A549), and gastric adenocarcinoma (AGS) at 19.35 ± 4.89 µg/mL, 5.34 ± 1.62 µg/mL, and 0.22 ± 0.20 µg/mL, respectively. The impact of ET on the three cell lines was statistically significant, *p*-value < 0.0001. The lethality of ET was recorded strongest on AGS and weakest on HCC-J5 (Figure 3B).

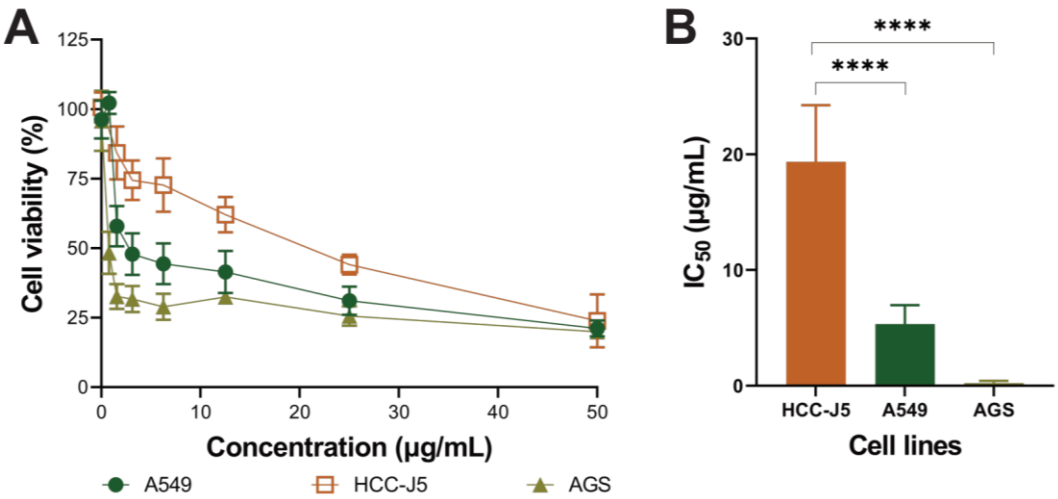

Figure 3. The cytotoxic effect of *E. trifoliatus* stem bark total extract on evaluated cancer cells (A), and the IC values of the impact (B).

3.5. ET Retards the Growth of Fibroblast

The impact of ET on BJ cells was depicted in Figure 4A. The IC_{50} of the extract on the fibroblast was calculated at 21.33 ± 6.97 µg/mL. Based on the IC_{50} values, the SI values were computed at 1.10 ± 0.28, 3.99 ± 1.21, and 95.39 ± 31.17 for HCC-J5, A549, and AGS. There is a statistically significant difference in the selectivity of ET's effects on AGS cells compared to HCC-J5 (*p*-value = 0.0017) and A549 (*p*-value = 0.0019).

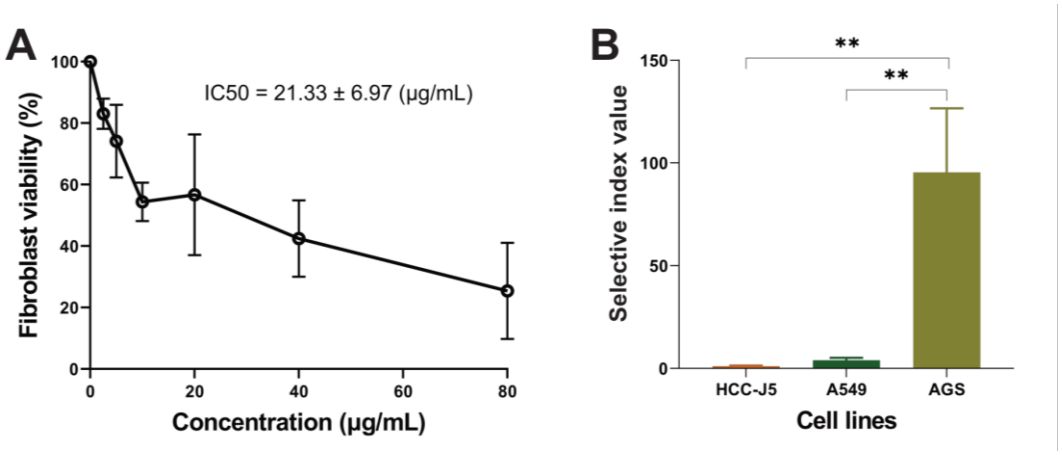

Figure 4. The inhibitory of *E. trifoliatus* stem bark total extract on fibroblast (A), and the selective index in anticancer capacity of the extract (B).

3.6. The Anticancer Effect of *E. trifoliatus* Fractions

The inhibitory of four fractional extracts of *E. trifoliatus* stem bark was illustrated in Figure 5. The impact was observed to be in a dose-dependent manner. The IC_{50} values of the fraction on tested cells are shown in Figure 6.

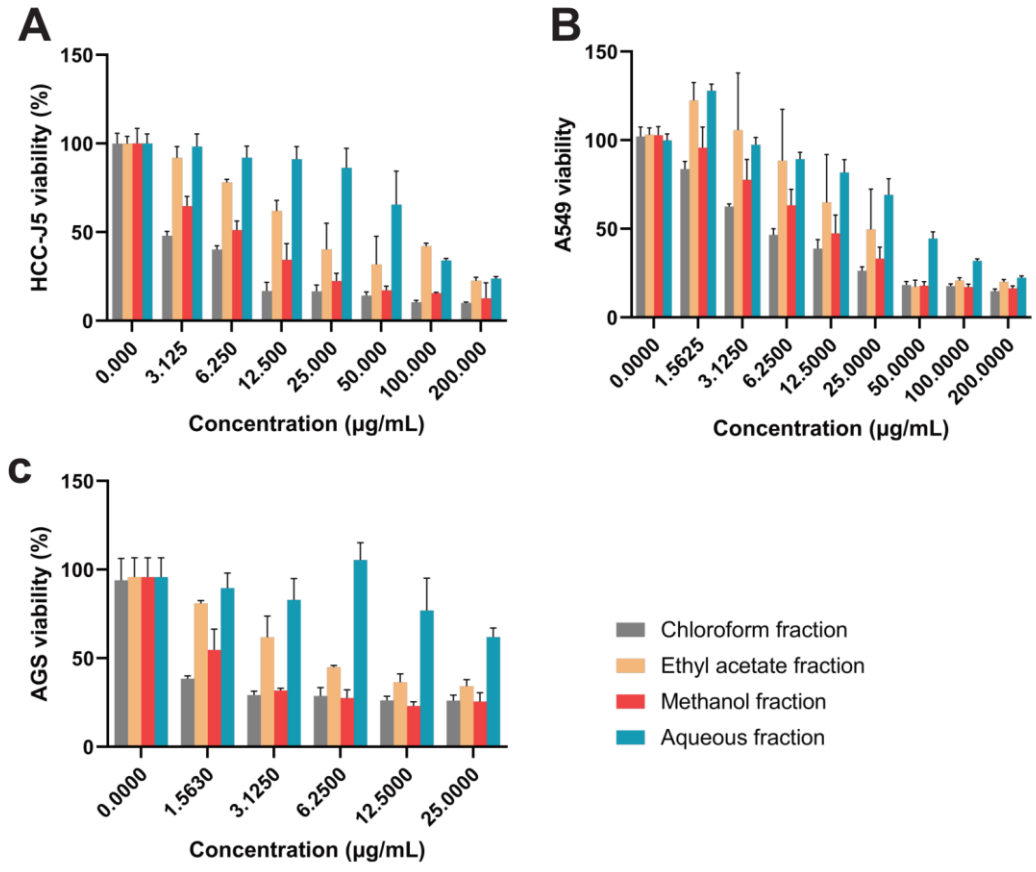

Figure 5. The cytotoxic impact of the fractional extract of *E. trifoliatus* stem bark on HCC-J5 (A), A549 (B), and AGS (C).

The expressed the IC_{50} values on HCC-J5, A549, and AGS were 2.22 ± 0.84 µg/mL, 7.10 ± 1.13 µg/mL, and 0.08 ± 0.50 µg/mL for chloroform fraction; 23.92 ± 5.56 µg/mL, 22.84 ± 5.94 µg/mL, and 6.71 ± 1.65 µg/mL for ethyl acetate fraction; 5.84 ± 1.35 µg/mL, 12.21 ± 2.03 µg/mL, and 1.28 ± 1.00 µg/mL for methanol fraction; 75.62 ± 10.61 µg/mL, 48.01 ± 7.07 µg/mL, and 34.69 ± 11.88 µg/mL for aqueous fraction. The effects of chloroform and methanol

fractions were the most effective on the three cell lines tested when compared with water and ethyl acetate fractions, *p*-value < 0.0001.

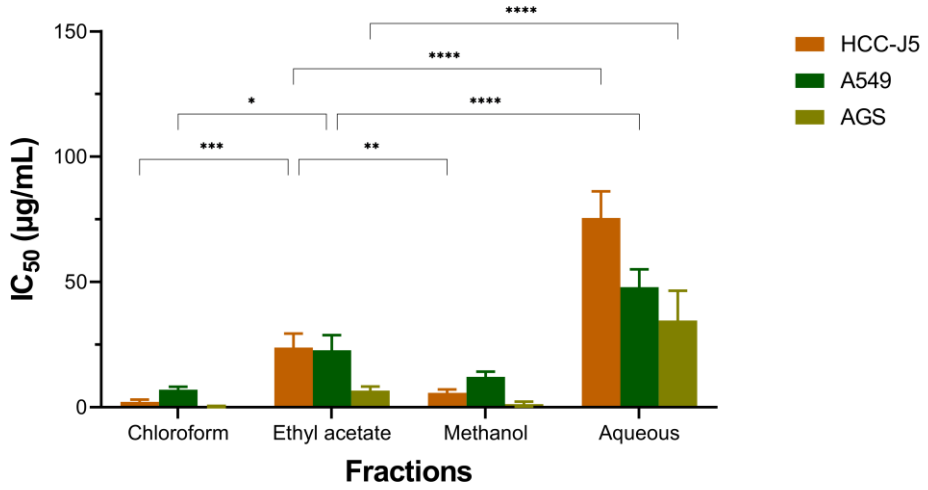

Figure 6. The IC_{50} values of the fractional extracts on evaluated cancer cells.

4. DISCUSSION and CONCLUSION

E. trifoliatus stem bark has been widely used in folk remedies in Asia; however, scientific information about this medicinal herb has not been fully described (Wiart, 2007). The presence of secondary metabolites is an important sign in predicting the bio-activity of plant extracts (Baumli *et al.*, 2023). Investigations on the phytochemical composition of *E. trifoliatus* stem bark have been still limited. The diversity in ingredients of the stem bark was described as suitable with previous reports. Some studies showed an abundance of phenolic compounds (0.65 ± 0.06 mg/g), flavonoids (0.60 ± 0.06 mg/g), and saponin (23-30 mg/g) in this plant (Chen *et al.*, 2021; Dedvisitsakul & Watla-Iad, 2022). Due to its aromatic scent, many previous studies have focused on analyzing its terpene-derived compounds, such as α-pinene, sabinene, terpinen-4-ol, β-pinene, and *p*-cymene (Muselli *et al.*, 1999; Phuong *et al.*, 2006). In the leaves, various vitamins were found, including vitamin A (428.47 ± 3.00 µg/100g), vitamin B1 (0.41 ± 0.01 µg/100g), vitamin B2 (0.17±0.00 µg/100g), vitamin C (11.95±0.86 µg/100g) (Ganogpichayagrai & Suksaard, 2020). The antioxidant bioactive of the ET could be predicted due to the presence of relevant secondary compounds.

In this study, the capacity to trap free radicals reflected the antioxidant ability of the ET extract. As the ET concentration increased, the rate of free radicals collected increased. The antioxidant impact of the *E. trifoliatus* leaf extract was recorded by using oxygen radical absorbance capacity (9057.29 ± 43.08 µmol) and ferric reducing antioxidant power (1230.88 ± 19.51 µmol), and the whole plant effect on DPPH free radical scavenging activity was 0.93 ± 0.09 mg L-ascorbic acid equivalent per gram extract (Dedvisitsakul & Watla-Iad, 2022; Ganogpichayagrai & Suksaard, 2020). The ET extract was considered to have a weak antioxidant effect, because the EC50 values were greater than 100 µg/mL (Blois, 1958). Radical neutralization has been reported to come from electron donation by natural compounds such as phenolic compounds (Lobo *et al.*, 2010). Various scientific evidence has proven the relationship between antioxidants and their bio-effect (Kancheva & Kasaikina, 2013). The presence of secondary metabolites and antioxidant capacity gave predictions about the activities of this plant.

The safety of plant extracts has an impact on their application (Ferraz *et al.*, 2022). For the purpose of toxicity determination, the brine shrimp lethality assay, which is widely used for the preliminary cytotoxicity assay of plant extract, was conducted (Harwig & Scott, 1971). The lethality of the ET was unremarkable on *A. nauplii* viability; the DMSO was also ineffective on larvae. The survival rate of shrimp in experiments always remained above 95%, and there was

no statistically significant difference in the impact of ET concentrations on larval vitality. The median lethal dose (LD50) value was considered to be greater than the value of 100 µg/mL which was classified as non-active for the crude extract, for it was not demystified during the evaluation (Muhammed *et al.*, 2021; Rieser *et al.*, 1996). In the report elsewhere, the protective ability of *E. trifoliatus* extract investigated on the liver of rats with liquor overdose showed that the extract could induce liver cell degeneration (Li *et al.*, 2021). The low toxicity facilitates ET extract in medical applications.

Notably, the ability of *E. trifoliatus* to inhibit cancer cell growth has been investigated. The results illustrated that the inhibition of cell proliferation by ET was in a dose-dependent manner. In the concentration of 50 µg/mL, the ET retarded around 75% of cell proliferation in all cell lines. The non-linear regression indicated the IC50 values of ET on HCC-J5, A549, and AGS were lower than the value of 20 µg/mL creating a boundary to classify the strong effective plant extract in cytotoxicity, in which, the effect on AGS was weighed in as excellent cytotoxic for a total extract (Indrayanto *et al.*, 2021). The impact of ET on AGS was more effective than the other two cell lines. Thus far, several studies have investigated the effects of this plant extract on cancer. PC-3 cells were observed to be inhibited under the leaf extract via interruption of the NF-kB, Erk1/2, Akt pathway (H. Q. Wang *et al.*, 2014). *E. trifoliatus*-derived terpenoids was determined as SF-268, MCF-7, HepG2 and NCI-H460 anti-growth elements (Li *et al.*, 2016). Taiwanin E isolated from *E. trifoliatus* was demonstrated as a pRB inhibitor in MCF-7 treatment (H.-C. Wang *et al.*, 2014). Moreover, the lethality impact of the ET was also investigated on fibroblast BJ with the IC50 of 21.33 ± 6.97 µg/mL leading to the classification of the effect as moderate cytotoxic. By dividing the IC50 value of ET on BJ cells by the test cancer cells, the SI values were indicated. The selectivity of an extract with an SI value less than one should be considered against its use as an herbal drug (Indrayanto *et al.*, 2021; Nogueira & Rosário, 2010). Therefore, the selectivity in the effect of ET on HCC-J5 (SI = 1.10 ± 0.28) was considered unexploitable. On the contrary, the SI values of ET on A549 and AGS exhibited the potential to exploit this medicinal plant as a source of raw materials for future treatment research. The effect of ET on A549 (SI = 3.99 ± 1.21) was classified as a prospective anti-cancer, while the impact on ASG (SI = 95.39 ± 31.17) was elected as a highly potential herb (Indrayanto *et al.*, 2021; Peña-Morán *et al.*, 2016; Weerapreeyakul *et al.*, 2012). The cytotoxic influence of *E. trifoliatus* stem bark extract on liver cancer, lung cancer, and stomach cancer was discussed for the first time in this study.

Chloroform and methanol fractions were recorded to be more cytotoxic than ethyl acetate and aqueous fractions. The inhibition induced by ETC was astounding with the IC50 on three cell lines under 10 µg/mL; the effect on AGS was most substantial with IC50 = 0.0782 + 0.50 µg/mL. The impact of ETM was also classified as a potential extract with strong cytotoxic; the ETM's IC50 was below 10 µg/mL for the one on HCC-J5 and below 2 µg/mL for AGS. The effects of the fractionated extracts were observed to be divided into two groups when compared with the effects of the total extract, including those with better inhibitory activity and those with poorer inhibitory activity. Compared to the ET, the anti-proliferated effect of ETM and ETC increased on HCC-J5; the effect of ETC augmented on AGS. The chloroform extract fraction was also shown to be effective in cytotoxicity observed in some previous studies compared to other fractions (Einafshar *et al.*, 2024; Li *et al.*, 2015; Zhang *et al.*, 2020). It could be concluded that chloroform was the effective solvent for extracting the compounds inducing cytotoxicity, and more in-depth studies are needed to be carried out to clarify it.

In conclusion, *E. trifoliatus* stem bark extract expressed a weak antioxidant effect, but the cytotoxic impact was remarkable. The inhibited impact was first recorded on lung carcinoma cells A549, gastric adenocarcinoma cells AGS, and hepatocellular carcinoma cells HCC-J5. Additionally, the tremendously selective impact of the extract was on AGS that needed to be further researched. The safety of the total extract was proven via toxicity assay. The effect was more powerful in chloroform fractionalization.

Declaration of Conflicting Interests and Ethics

The authors declare no conflict of interest. This research study complies with research and publishing ethics. The scientific and legal responsibility for manuscripts published in IJSM belongs to the authors.

Authorship Contribution Statement

Nguyen Trung Quan and **Hoang Thanh Chi** designed and performed the experiments. **Nguyen Trung Quan** analyzed the data. **Bui Thi Kim Ly** wrote the original draft. All authors read, edited, and approved the final manuscript.

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