


ORIGINAL CONTRIBUTION

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Phytochemical screening and anticancer activity of the aerial parts extract of *Xanthium strumarium* L. on HepG2 cancer cell line

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Abstract

Background: Cancer is one of the most considerable concerns because of increasing the death rate all over the world. Recent studies have disclosed that plant extracts exhibit anticancer activity through various mechanisms. *Xanthium strumarium* has been used by Vietnamese in herbal medicines to support the medication of infirmities. This study is to consider the secondary metabolites, antioxidant and anticancer capacities of extract from the aerial parts (stems and leaves) of *X. strumarium* (AP-XS).

Methods: AP-XS was analyzed for the presence of phytochemicals via qualitative chemical tests and determined total polyphenol and flavonoid contents. DPPH (1,1-diphenyl-2-picrylhydrazyl) quenching assay and sulforhodamine B (SRB) assay were selected to investigate antioxidant capacity and anti-proliferative activity, respectively. Besides, acridine orange-ethidium bromide (AO-EB) dual staining was applied to evaluate the ability to induce apoptosis on HepG2 cancer cells.

Results: Results of present study indicated that AP-XS contains the main phytochemicals such as flavonoids, tannins, saponins, alkaloids, and triterpenes. Ethanol extract had highest content of polyphenol (84.86 mg gallic acid equivalent/g dry mass), and exhibited the great total antioxidant property ($IC_{50} = 184.13 \mu\text{g/mL}$) and anti-proliferative activity on HepG2 cancer cells ($IC_{50} = 81.69 \mu\text{g/mL}$). Furthermore, the characteristics of apoptosis including shrinkage of the cell and apoptotic bodies were found following 60 h of AP-XS extract treatment through AO-EB dual staining.

Conclusion: The data suggest that AP-XS extract had antioxidant potential and anti-proliferative effect. The anti-proliferative property was considered to have an association with a rising of apoptosis. These results were reliable for further research on *X. strumarium* as a source of phytochemicals with anticancer activity potential for cancer therapeutics.

Keywords: *Xanthium strumarium* L., Phytochemicals, Antioxidant, Anticancer, Apoptosis, HepG2 cells

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Background

An imbalance of systems oxidation and anti-oxidation caused by free radicals can destroy biological macromolecules and cause cellular oxidative stress. These can cause some degenerative and chronic pathologies, for example, cancer, diabetes mellitus, Alzheimer's, and Parkinson's [1]. Hepatocellular carcinoma or liver cancer is the most popular widespread cancer, accounting for approximately 90% [2]. It causes high annual mortality rates, especially in Southeast Asia, this is an area with a high rate of hepatitis [3]. Some current treatment therapies include chemotherapy, radiotherapy and chemically derived drugs. However, treatment like using chemotherapy can cause many side effects and adversely affects the health of patients [4]. Simultaneously, the major issue of chemotherapy therapies to cure hepatocellular carcinoma is the resistance mechanism of cancer cells because of increase in multidrug-resistant protein and apoptotic protein reduction [5]. Therefore, more effective methods for cancer control and apoptosis induction are needed, contributing to cancer treatment.

According to statistics, over 60% of the the population in the world and around 80% in developing countries use traditional and medicine plants for their treatment purposes [6]. Many previous published have indicated that medicinal herbs have a very important role in cancer treatment, helping to control cancer and induce apoptosis in cancer cell [4, 7]. *Xanthium strumarium* L. (Asteraceae) is a traditional herb that has been applied to cure many different diseases. This plant native to China and is widely distributed all over the world, including Vietnam. It often grows in plains, hills, mountains and wilderness roadsides. The flowering time ranges from July to August, and fruiting stage lasts from September to October. Studies of pharmacology have demonstrated that *X. strumarium* have a variety of biological activities, for instance, anticancer, antibacterial, antifungal, antioxidant, antitumor, anti-inflammatory, antinociceptive, antilipidemic, hypoglycaemic, and other activities. *X. strumarium* has been proven to contain sesquiterpenoids, steroids, polyphenols, flavonoids, triterpenoids, alkaloids, anthroquinones, coumarins, and glycosides [8]. Polyphenols are one of the most vital phytochemicals distributing in medicinal plants, known as critical anticancer agents. Flavonoids are polyphenol compounds found in a variety of plants as bioactive secondary metabolites. Polyphenols and flavonoids have been shown to possess a wide variety of anticancer effects such as preventing oxidative stress caused by free radicals, downregulating pro-inflammatory signaling pathways, participating in arresting the cell cycle, inducing apoptosis, autophagy, and suppressing cancer cell proliferation and invasiveness. Thus, polyphenols as well as flavonoids are of great interest in research as potential

anticancer agents [9, 10]. Some reports have also demonstrated that compounds and extracts of *X. strumarium* presented anticancer activity [8, 11, 12]. In particular, there were many studies on the chemical composition and biological effects of fruits related to anticancer ability. However, there have been no reports regarding the evaluation of anti-proliferation activity and activating apoptosis of aerial parts extract of *X. strumarium* on HepG2 cancer cell line. Hence, the research objectives was to consider antioxidant and anticancer activities on HepG2 cancer cell line of the aerial parts extract of *X. strumarium* extract (AP-XS). At the same time, this study determines the phytochemical characteristics of AP-XS by qualitative chemical tests and total polyphenol and flavonoid contents.

Materials and methods

Chemicals and reagents

Ethanol (98% v/v) was purchased from OPC Pharmaceutical Company. Camptothecin (HPLC $\geq 99\%$), methanol (HPLC $\geq 99.9\%$), acetic acid (HPLC 100%), trichloroacetic acid (HPLC $\geq 99\%$), Folin-Ciocalteu's phenol reagent (Quality level 200), aluminum chloride (99.999% trace metals basis), quercetin (HPLC $\geq 98\%$), gallic acid (HPLC $\geq 98\%$), 1,1-diphenyl-2-picrylhydrazyl reagent (Quality level 200), vitamin C (HPLC $\geq 99\%$), sulforhodamine B (Quality level 100), Eagle's Minimal Essential Medium (Quality level 300), fetal bovine serum, L-glutamine (Quality level 300), HEPES (Titration $\geq 99.5\%$, quality level 300), amphotericin B (Quality level 200), penicillin G (Quality level 200), and streptomycin (Quality level 100) were purchased from Sigma-Aldrich® Co. Ltd. (USA).

Preparation of medicinal plant and extract

The aerial parts (stems and leaves) of *X. strumarium* were picked on May 2018 from Ho Chi Minh City. The plant sample were identified and authenticated by MSc. Le Duc Thanh (Natural Resources – Medicinal Materials Department, Research Center of Ginseng and Medicinal Materials, Ho Chi Minh City), this medicinal material has a scientific name of *X. strumarium* L.. The herbal samples were rinsed with tap water before having them cleaned with distilled water to remove the dirt on their surfaces. They were then air dried to the standard of losing weight due to drying in accordance with the Vietnam Pharmacopoeia 5th Edition (the loss on drying of raw materials is less than 13%). Afterwards, these dried samples were ground into powder and kept in sealed bag (Sample code: TTS-AGP-XS-001) at the Research Center Ginseng and Medicinal Materials in Ho Chi Minh City.

Raw material powder with a moisture content of 10.14% was extracted with the ratio of 1: 10 (g powder/

mL solvent) by ethanol using percolator apparatus at room temperature. After collecting the extract (a rate of 2 mL/min), it was concentrated at 60 °C in reduced pressure condition by a Buchi rotary evaporator to gain ethanol extract, which corresponds to a yield of 11.73% (w/w). The extract was preserved in sterilized vials at 4 °C. In each assay, the extract was dissolved in a suitable solvent to yield a stock solution.

Phytochemical screening

The identification of secondary metabolic groups in AP-XS was done through phytochemical analysis by chemical reactions for lipids, volatile oils, carotenoids, triterpenoids, alkaloids, flavonoids, anthraquinones, anthocyanosides, proanthocyanidins, coumarins, tannins, saponins, reducing agents, and organic acids. This screening was performed based on the Ciulei's process [13] with slight alterations.

Estimation of total polyphenols content (TPC)

The TPC of AP-XS was estimated using Folin-Ciocalteu's reagent as previously described with minor alterations [14]. Briefly, 200 µL test sample was combined with 500 µL of Folin-Ciocalteu's reagent in 6 mL of double distilled water. The mixture was kept at 25 °C for 5 min. After that, 1.5 mL of Na₂CO₃ (20% *weight/volume*) solution was poured into this mixture and the volume was reached up to 10 mL by double distilled water. The mixtures were reacted for 2 h at 25 °C in a light-free environment. The optical density was recorded at 758 nm and all estimates were repeated three times. The TPC was calculated from the calibration plot ($Y = 0.0097x - 0.0278$, $R^2 = 0.997$) and expressed as mg of gallic acid equivalent (GAE)/g of dry mass.

Estimation of total flavonoids content (TFC)

The TFC of AP-XS was estimated based on the described method using AlCl₃ reagent with minor adjustments [15]. Briefly, an amount of 1 mL diluted extract or quercetin solutions was incorporated with 1 mL of AlCl₃ (2% *weight/volume*) separately and with methanol, the mixture was reached up to 10 mL in quantity. Then, the solution was vortexed and kept at 25 °C for 15 min. The optical density of the reaction mixtures was acquired at 454 nm. The measurements were performed in repetition three times. The TPC was determined from the calibration plot ($Y = 0.0219x - 0.0554$, $R^2 = 0.998$) and expressed as mg of quercetin equivalent (QE)/g of dry mass.

In vitro antioxidant activity assay

DPPH free radical quenching assay was used in this research in order to assess the antioxidant activity of AP-XS extract based on a previously described method [14].

To briefly illustrate, the mixture (The total volume is 4 mL) in methanol consisting of different concentrations (62.5, 125, 185.5, 250, 312.5 µg/mL) of test extract or vitamin C (positive control) and DPPH reagent (0.6 mM) with equal volume of 0.5 mL, was reacted for 30 min at 25 °C in a light-free condition. Subsequently, the absorbance was acquired at 515 nm. All reactions were made in triplicate. The antioxidant ability was evaluated via IC₅₀ value that was determined from the proportion of the radical scavenging activity using the expression:

$$(\% \text{scavenging effect}) = \frac{A_c - A_t}{A_c} \times 100$$

In which, A_c and A_t are the absorbance of the control sample (without test extract) and the test sample (with test extract), respectively.

Cell line and cell culture

HepG2 cells (HB-8065) were bought from the ATCC®, cells were cultured at 37 °C and 5% CO₂ in EMEM medium with several other ingredients including FBS, L-glutamine, HEPES, amphotericin B, penicillin G, and streptomycin with a concentration of 10% *v/v*, 2 mM, 20 mM, 25 ng/mL, 100 IU/mL, 0.1 mg/mL, respectively..

Sulforhodamine B (SRB) assay

Cytotoxicity of the extract on HepG2 cancer cells was assessed by SRB assay using described method with camptothecin as a positive control [16]. On 96-well plates, cells were loaded at a density of 10⁴ cells/well and grown for 24 h before being treated with AP-XS extract at different concentrations for 48 h. Then, cells were fixed in cold-TCA (50% *weight/volume*) for 2 h, they were continuously washed and stained with SRB (0.2% *weight/volume*) for 20 min. After that, the cells were continuously washed with acetic acid (1% *volume/volume*) 5 times, Tris base (10 mM) was used to dissolve protein-bound dye. Subsequently, absorbances were recorded at 492 nm and 620 nm using a Synergy HT plate reader. The rate of the cytotoxicity (CT%) was estimated by the expression:

$$CT\% = \frac{A_c - A_t}{A_c} \times 100$$

In which, A_c and A_t are the absorbance of the control sample and the test sample, respectively.

Acridine orange - Ethidium bromide (AO-EB) double staining

On 6-well plates, cells were loaded at a density of 2 × 10⁵ cells/well and kept at 37 °C and 5% CO₂ for 24 h to grow. Subsequently, cells were exposed to AP-XS extract at concentration of 60, 80 and 100 µg/mL. After cells

were continuously incubated for 60 h under the same conditions, which were washed with phosphate buffered saline and stained with AO (100 µg/mL)-EB (100 µg/mL) reagent. Cell morphology was examined using fluorescence microscopy.

Data analysis

The results were displayed as mean ± SEM (Standard error of the mean), data were analyzed by Graphpad Prism software (Inc., La Jolla, CA, USA) using t-test.

Results

Phytochemical screening

The extract was subjected to qualitative chemical tests for the identification of various secondary metabolites present in AP-XS. The results showed that the AP-XS the possible presence of flavonoids, alkaloids, tannins, triterpenes, saponins, volatile oils, and organoic acids (Table 1). In which, the major components found are flavonoids and triterpenoids.

Total polyphenol and flavonoid contents (TPC and TFC)

Total polyphenol and flavonoid contents of dry powder and AP-XS extract of *X. strumarium* were expressed as mg quercetin equivalents/g and mg gallic acid equivalents/g of dry mass, sequentially. Table 2 represents the analytical data for polyphenol and flavonoid contents of the dry powder and AP-XS extract.

In vitro antioxidant activity

There is conglomerating evidence that reactive free radicals cause cell damage, which is one of the causes of aging and leading to a lot of diseases such as Alzheimer's, Parkinson's and cancer. Thus, antioxidants have get significant consideration due to the potential of reducing adverse effects of free radicals. In this study, DPPH assay was used to confirm the free radical scavenging proficiency of AP-XS extract, which are ubiquitously applied to evaluate the antioxidant activity of plant extracts. Result illustrated that the quenching ability of AP-XS extract was concentration-dependent, the

Table 2 TPC and TFC in the aerial parts of *X. strumarium*

Sample	TPC (mg GAE/g d.w.)	TFC (mg QE/g d.w.)
Raw powder	4.68 ± 0.19	1.23 ± 0.02
Ethanol extract	84.86 ± 5.13 ^a	3.66 ± 0.08 ^a

^a*p* < 0.001 significantly different (t-test), mg GAE/g d. w.: mg of gallic acid equivalents/1 g of dry weight, mg QE/g d. w.: mg of quercetin equivalents/1 g of dry weight. All values are reported as means ± SEM (n = 3)

AP-XS extract presented over 50% inhibition against DPPH free radical by 50.3% at 187.5 µg/mL and the *IC*₅₀ value reached 184.13 µg/mL (Fig. 1).

Cytotoxicity of ethanol extract of AP-XS on HepG2 cancer cell line

In order to investigate the anticancer property of AP-XS extract, HepG2 cancer cell line was treated throughout 48 h with divergent concentrations of AP-XS extract (40, 60, 80, 100 µg/mL). The result of SRB assay revealed that the AP-XS extract gradually decreased the survival percent of HepG2 cancer cells as the concentration of the extract was increased (Table 3). AP-XS extract exhibited *IC*₅₀ value of 81.69 µg/mL compared to the *IC*₅₀ value of Camptothecin was 0.079 µg/mL. These results divulged changes in cell morphology and contraction of cells generating cell death induced by AP-XS extract in the HepG2 cancer cell line compared to control cells (Figure S1 in the supplementary data).

Ethanol extract of AP-XS induced apoptosis on HepG2 cells

The ability to induce apoptosis of AP-XS extract was detected using AO-EB dual staining on HepG2 treated with extract. Results indicated that HepG2 cells showed some characteristics of apoptosis after 60 h treatment with AP-XS extract. AP-XS extract or CPT treated cells exhibited nuclear and chromatin condensation, and late apoptotic cells with condensed or fragmented chromatids (Fig. 3). Simultaneously, there was a deduction in the number of cells and changes in cell morphology at concentrations of AP-XS extract and CPT compared to control cells after 60 h treatment (Figs. 2 and 3).

Discussion

In recent years, the use of medicinal materials in cancer control and treatment has been increasingly concerned because of the diverse presence of phytochemicals with multiple biological properties [4, 7]. Preliminary phytochemical analysis illustrated that flavonoids, alkaloids, tannins, triterpenes, saponins, volatile oils, and organoic acids was present in the AP-XS. This result is similar to previous study, both leaves and stems of *X. strumarium* contain flavonoids, alkaloids, tannins, triterpenes, saponins, and cardiac glycosides [17]. These phytochemicals were announced to have a lot of biological effects [18].

Table 1 Preliminary phytochemical screening results of AP-XS via chemical reactions

Metabolites	AP-XS	Metabolites	AP-XS
Alkaloids	+	Proanthocyanidins	–
Flavonoids	+	Anthocyanosids	–
Tannins	+	Lipids	–
Triterpenoids	+	Volatile oils	+
Saponins	+	Carotenoids	–
Coumarins	–	Organic acids	+
Anthraquinones	–	Reducing agents	–

(+): the presence; (–): the absence

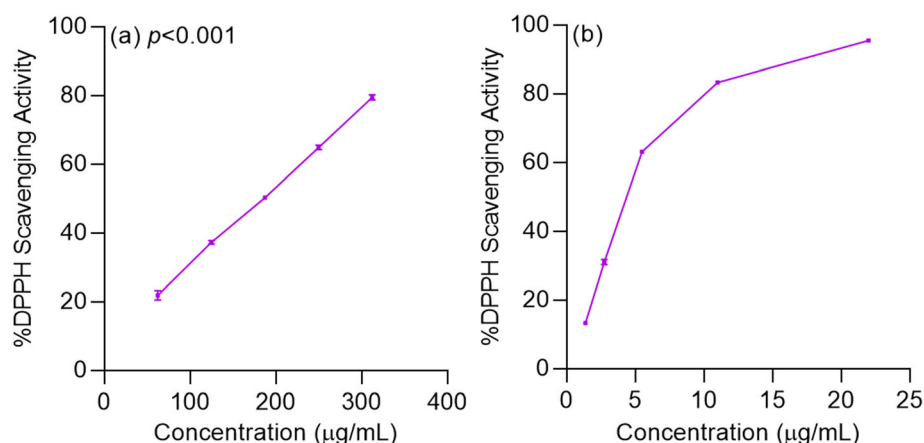


Fig. 1 DPPH scavenging activity of AP-XS extract (a) and vitamin C (b). $p < 0.001$, a significant difference one by one in each test (t-test). All values are reported as means \pm SEM ($n = 3$)

Among the variety of phytochemicals, polyphenols compounds have attracted the consideration of scientists as well as its application in different fields such as pharmaceutical, nutraceutical, health, and cosmetic industries. These compounds are common in the plants that can be considered as the part of the daily diet and are attracted as natural antioxidants [19]. In this study, dry powder and ethanol extract of AP-XS was determined total polyphenol and flavonoid contents using colorimetric methods based on the gallic acid and quercetin standard, respectively. Result indicated that AP-XS extract presented the TPC and TFC with 84.86 mg GAE/g dry mass and 3.66 mg QE/g dry mass, sequentially.

Free radicals have been demonstrated to make a substantial contribution in many pathologies like cancer, Alzheimer's, Parkinson's, arthritis, and diabetes mellitus. Overloading free radicals causes many negative impacts on biological systems. It can combine and oxidize biological molecules such as proteins, lipids, and carbohydrates. This causes damage to cells, tissues and organs, leading to cancer progression [1]. In this study, antioxidant capacity of AP-XS extract was evaluated using DPPH free radical scavenging assay and compared with vitamin C as positive control. Result demonstrated that the AP-XS extract presented DPPH free radical scavenging with IC_{50} value of 184.13 µg/mL. Previous studies have shown that polyphenols, flavonoids, and tannin are considered as sources of antioxidants and scavenging activity. Polyphenols or flavonoids has been shown to have antioxidant activity and inhibit the initiation or spread

of oxidative reaction chains via donating a hydrogen molecule of hydroxyl groups, resulting in a more stable, less-reactive radical [19].

The anticancer activity of AP-XS extract was also investigated through SRB assay and AO-EB dual staining on HepG2 cancer cell line. The SRB assay is used in order in screening to evaluate the toxicity of the ethanol extract of AP-XS. The SRB assay has been ubiquitously applied for determining the cytotoxicity of toxic substances and plant extracts against cancer cell lines. The anionic dyes SRB will bind electrostatically with the positively charged part of the protein. The amount of binding dye will reflect the total protein of the cells. In the assay, SRB will bind to the cell's protein, which is dissolved to form a pink solution. The optical density of the solution correlates with the total protein or number of cells. The change in number of cells compared with the control (Camptothecin) reflects the cytotoxicity of the test samples [20]. The result revealed that AP-XS extract showed cytotoxic activity on HepG2 with IC_{50} value of 81.69 µg/mL. In addition, results also indicated that AP-XS extract induced apoptosis on HepG2 cells through changes in cell morphology and apoptotic bodies formation after 60 h treatment. HepG2 cells showed some characteristics of apoptosis after 60 h treatment with AP-XS extract such as a decrease in the number of cells and changes in cell morphology, nuclear and chromatin condensation and late apoptotic cells with condensed or fragmented chromatids. The presence of alkaloids, polyphenols, sesquiterpenoids and terpenoids

Table 3 Cytotoxicity of AP-XS extract on HepG2 cancer cell line

AGP-XS	Concentration (µg/mL)				IC_{50} (µg/mL)
	40	60	80	100	
% cytotoxicity	12.44 \pm 2.22	30.22 \pm 1.14	45.57 \pm 3.72	73.27 \pm 3.35	81.69 \pm 1.55

All values are reported as means \pm SEM ($n = 3$)

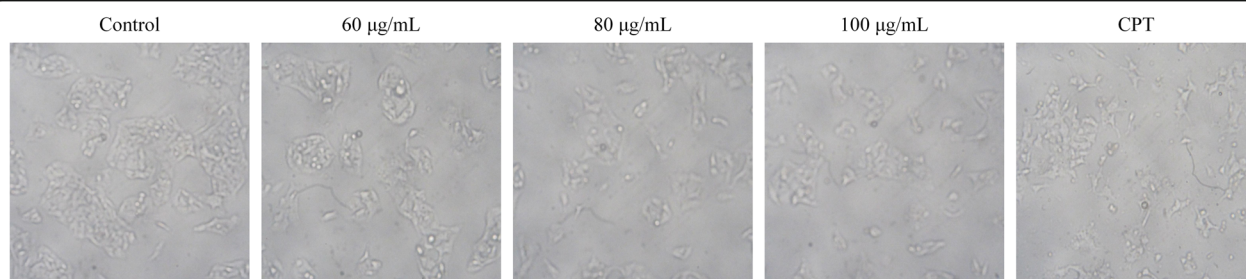


Fig. 2 Morphological transforms were observed in HepG2 cancer cells. Cells were treated without (Control) or with different concentrations of AP-XS extract and Camptothecin (CPT) for 60 h

in AP-XS may be related to anticancer activity. The alkaloids from many medicinal herbs have been shown anticancer activity with different mechanisms of action [21]. Anthraquinones, coumarin and flavonoid belonging to the polyphenols group have been warranted to have anticancer, antioxidant, and anti-inflammatory capacities [19, 22–24]. Terpenoids were shown as antitumor effect and anti-inflammatory proficiency [25]. Sesquiterpenoids is a phytochemical of many crucial biological properties, which are plentiful in *X. strumarium*, exhibiting strong activities with antitumor, antibacterial, antiviral, and anti-inflammation [26]. Xanthatin and xanthinosin, sesquiterpenoids isolated from *X. strumarium* was found to confer anticancer activity [27, 28]. Therefore, these individual chemical components may have contributed to the anticancer activity of the AP-XS extract. The anticancer ability can be considered the main pharmacological effect of *X. strumarium* and has been extensively

studied on several cancer types as liver, breast, cervical, and lung cancers. Similar to present study, many previous studies also demonstrated that leaves, stems and roots extracts as well as isolated compounds such as 8-epi-xanthatin-1 α ,5 α -epoxide, xanthatin, and xanthinosin from *X. strumarium* exhibited anti-proliferation and induced apoptosis activity on the variety of cancer cell lines, for instance, HepG2, A549, HeLa, MDA-MB-231, MCF-7, and HTC-15 via different signal pathways [8].

In summary, this study demonstrated that the aerial parts extract of *X. strumarium* contains a wide variety of secondary metabolites that expressed antioxidant activity and anticancer ability on HepG2 cancer cell line based on the experiments performed. However, more scientific evidence is needed to comprehensively evaluate the biological effects of the aerial parts extract of *X. strumarium*.

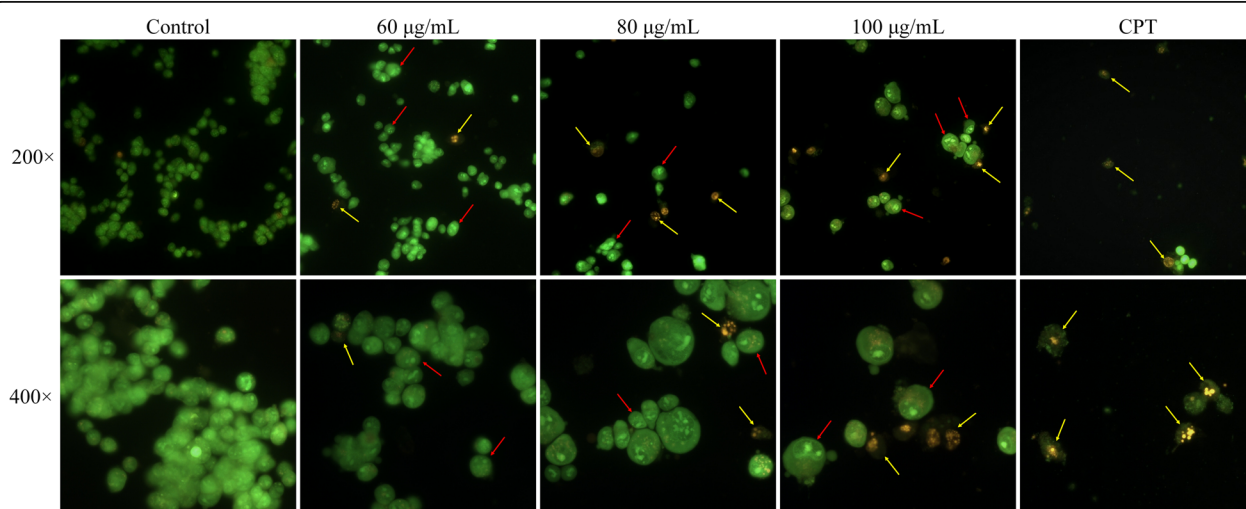


Fig. 3 Apoptotic characteristics of HepG2 cells were observed via AO-EB dual staining. Cells were incubated with AP-XS extract at variety of concentrations and Camptothecin (CPT) for 60 h. Some characteristics are expressed as condensation and fragmentation of chromatins (red arrows) and late apoptotic cells (yellow arrows)

Conclusion

This study demonstrated that the antioxidant potential and anticancer capacity of the aerial parts extract of *X. strumarium*. These results collectively indicated that the aerial parts (stems and leaves) of *X. strumarium* have the potential to exert anticancer effect by inducing apoptosis on HepG2 cells. However, AP-XS extract exhibited very low potency compared to Camptothecin. This extract is not the candidate for anticancer effect. This results study also showed that the benefits of using medicinal herbs in the topical cure and administration of cancer.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s40816-021-00252-w>.

Additional file 1: Figure S1. Morphological transforms were observed in HepG2 cancer cells treated without (Control) or with different concentrations of AP-XS extract (60, 80 and 100 µg/mL) and Camptothecin (CPT) for 24, 48 and 60 h

Abbreviations

AP-XS: Aerial parts of *X. strumarium*; DPPH: 1,1-diphenyl-2-picrylhydrazyl; SRB: Sulforhodamine B; IC₅₀: 50% inhibitory concentration i.e. the concentration of extract that can achieve 50% of intended activity; AO-EB: Acridine orange-Ethidium bromide; HPLC: High performance liquid chromatography; FBS: Fetal bovine serum; EMEM: Eagle's minimal essential medium; TPC: Total polyphenols content; TFC: Total flavonoids content; SEM: Standard error of the mean

Acknowledgments

The authors would like to thank the National Institute of Medicinal Materials and the Ministry of Science and Technology, Vietnam for providing funding for this research under grant number of 09/2019/HD-DTCS-TTS and NVQG-2017/23, respectively.

Authors' contributions

LHT has performed the literature search, designed the experiments, carried out some experiments, collected data, and written manuscripts. LVM conceived the original idea and supervised the project and worked on the manuscript. TTM has participated in a number of experiments. NTH has involved in the experimental design and reviewed the manuscript. NHD was involved in some concepts. YXZ was involved in some concepts and reviewed the manuscript. All authors have read and approved the manuscript.

Funding

The authors are thankful to the National Institute of Medicinal Materials and the Ministry of Science and Technology, Vietnam for providing funding for this research.

Availability of data and materials

All the data generated or analysed during this study are included in this published article.

Ethics approval and consent to participate

Not applicable.

Consent for publication

The authors approved the manuscript for this publication.

Competing interests

The authors declare that they have no competing interest.

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Received: 4 August 2020 Accepted: 20 January 2021

Published online: 23 January 2021

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