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ANTICANCER PROPERTIES OF *Cyperus amuricus*: *IN VITRO* AND *IN SILICO* STUDIES TARGETING HEPG2 LIVER CANCER CELLS VIA THE AKT/SURVIVIN/BCL-2 SIGNALING PATHWAY

Khoi Thanh Tu[1], Kien Cuong Tran[1,2], Hoa Ninh Luong[1,2], Huu Thuan Anh Nguyen[1,2], Thanh Luan Nguyen[3], Minh Quan Pham[4,5], Quoc Long Pham[4,5] and Thi Hai Ha Pham[1,2,✉]

[1]*Center for Hi-Tech Development, Nguyen Tat Thanh University, Saigon Hi-Tech Park, D2 street, Long Thanh My, Ho Chi Minh city 700000, Vietnam*

[2]*NTT Hi-Tech Institute, Nguyen Tat Thanh University, 300A Nguyen Tat Thanh street, Xom Chieu, Ho Chi Minh city 700000, Vietnam*

[3]*HUTECH Institute of Applied Science, HUTECH University, 475A Dien Bien Phu, Thanh My Tay, Ho Chi Minh city 700000, Vietnam*

[4]*Institute of Natural Products Chemistry, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Hanoi, Vietnam*

[5]*Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet, Nghia Do, Hanoi, Vietnam*

✉To whom correspondence should be addressed. Email: pthha@ntt.edu.vn

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ABSTRACT

Cyperus amuricus has been used in folk medicine to treat urolithiasis and prevent cancer. To confirm the biological activities of *C. amuricus*, the aim of this study is to determine and investigate the potential of anticancer activities against the HepG2 liver cancer cell line. For this purpose, the binding inhibiting activities of phenolic and flavonoid compounds docked to proteins of the Akt/survivin/Bcl-2 signaling pathway were investigated *in silico* and *in vitro*. Before analyzing the bioactivities, the crude methanol extract of *C. amuricus* was fractionated with various solvents. The total phenolic content (TPC) was quantified using the Folin-Ciocalteu reagent assay, whereas the total flavonoid content (TFC) was determined using the aluminum chloride assay. The MTT assay and Western blotting study were performed to determine the potential cytotoxicity of the ethyl acetate fraction (E fraction) of *C. amuricus*. At 221.86 ± 2.17 µg GAE/mg and 386.67 ± 4.83 µg QE/mg, the E fraction had significant levels of phenolic and flavonoid compounds. The E fraction surpassed the crude methanol extract and other fractions in terms of radical scavenger capacity with an IC_{50} value of 21.07 ± 0.30 µg/ml. In particular, the E fraction had high cytotoxic activity against the HepG2 cell line of hepatocellular carcinoma (IC_{50} = 166.50 µg/ml) but not against the standard fibroblast cell line. Western blot analysis demonstrated that the E fraction effectively induced caspase-3 activation while inhibiting the expression of Akt, survivin, and Bcl-2 in a concentration-dependent manner. Mechanistically, the molecular docking analysis

results revealed that the phenolic and flavonoid compounds found in the Cyperaceae family could serve as potential phytochemicals with antioxidant and anticancer effects by targeting proteins associated with cancer proliferation. These initial findings suggested that *C. amuricus* could inhibit the growth of HepG2 cancer cells *in vitro* via the Akt/survivin/Bcl-2 signaling pathway.

Keywords: Akt/survivin/Bcl-2 signaling pathway, anticancer activity, *Cyperus amuricus*, caspase-3, molecular docking study.

INTRODUCTION

Hepatocellular carcinoma (HCC), commonly known as liver cancer, ranks third in the world in terms of cancer-related death, primarily due to the association with cirrhosis, especially in regions such as Southeast Asia with high hepatitis prevalence (Ferenci *et al.*, 2010). Although progress has been made in understanding HCC, there is still an urgent need for creative and effective pharmaceutical therapies to treat the disease. While current treatment options involve chemotherapy, radiation, and chemically derived drugs, these therapies can create barriers such as drug resistance, adverse effects on patients' health, and elevated treatment costs (Rahbari *et al.*, 2011). Research into alternative therapeutics is, therefore, of crucial importance.

The PI3K/Akt signaling pathway is essential for the development and progression of cancer. The pathway plays a role in various cellular processes, including cell survival, proliferation, growth, and metabolism (Paglino *et al.*, 2014). This signaling is closely related to the regulation of apoptosis through interactions with survivin, Bcl-2, and caspase-3 (Paglino *et al.*, 2014, Tungsukruthai *et al.*, 2021). Caspase-3 is critical in the early stages of apoptosis, playing a crucial role in inhibiting ROS production, cleaving essential proteins, and contributing significantly to chromatin condensation and DNA fragmentation (Reed, 2000). Survivin, an inhibitor of apoptosis protein (IAP), is more overexpressed in the G2/M phases of cancer cell cycles than in typical phases. Survivin is upregulated by the PI3K/Akt signaling pathway, which promotes cell survival and proliferation. Therefore, survivin has been implicated as an adverse prognostic factor and has been observed to confer resistance to apoptosis induced by anticancer agents (Altieri, 2008). Similar to survivin, overexpression of Bcl-2 in cancer cells prevents the release of apoptosis-promoting factors from mitochondria and promotes cell survival by indirectly inhibiting caspase-3 activation, modulation of cell division, and suppression of apoptosis (Youle *et al.*, 2008). This coordinated inhibition of apoptotic processes enables cancer cells to evade programmed cell death and resistance to treatment (Bose *et al.*, 2017).

Phenolics and flavonoids have emerged as promising candidates as anticancer and chemotherapy agents (Mirza-Aghazadeh-Attari *et al.*, 2020). Various research studies have indicated that these compounds possess powerful antioxidant, anti-inflammatory, and anti-carcinogenic properties (Li *et al.*, 2018). Their ability to intercept ROS and modulate oxidative stress is particularly relevant in liver cancer, where oxidative damage plays a crucial role in disease progression (Kopustinskiene *et al.*, 2020). In addition to their antioxidant activities,

phenolics and flavonoids have shown that they can interfere with other signaling pathways, such as the PI3K/Akt/mTOR pathway, which is critical for regulating cell growth, metabolism, and apoptosis, thus contributing to their anti-carcinogenic effects (Li *et al*., 2018).

Cyperus amuricus, a member of the Cyperaceae family, possesses anti-urolithiasis and chemopreventive properties in traditional medicine. Known for the sweet and aromatic flavor, rhizomes have a long history in folk medicine, where their astringent, diuretic, diaphoretic, drying, and savory properties have been highly valued (Pham *et al.*, 2017). Additionally, the tubers are extensively applied to alleviate urinary retention, hydrocephalus, bloating, poor digestion, spleen enlargement, menstrual problems, postpartum symptoms, nausea, and vomiting (Taheri *et al.*, 2021). Previous studies on *C. amuricus* identified three antioxidant phenolic compounds, such as 3,4-dimethoxybenzoic acid, 4-hydroxybenzoic acid, and piceatannol, demonstrating potent free radical scavenging abilities, especially against DPPH and superoxide anions (Im Lee *et al.*, 2008). In addition, steam distillation of *C. amuricus in vitro* exhibited an inhibitory effect of 57.3% on pancreatic lipase (Sharma *et al.*, 2005). The findings suggest that *C. amuricus* has considerable therapeutic potential, prompting further investigation. However, there is minimal understanding regarding critical *C. amuricus* compounds that target the Akt/survivin/Bcl-2 pathway, particularly in Vietnam. This study aims to identify the anticancer properties of *C. amuricus* extract against liver cancer cell line HepG2 through molecular docking studies on pathway-related proteins such as caspase-3, Akt, survivin, and Bcl-2. This area has barely been published so far.

MATERIALS AND METHODS

Materials and chemicals

Methanol (Analytical grade), Folin-Ciocalteu's phenol reagent, aluminum chloride, quercetin (HPLC ≥ 98%), gallic acid, α, α-diphenyl-β-picrylhydrazyl reagent, ascorbic acid (HPLC ≥ 99%), Eagle's Minimal Essential Medium, and fetal bovine serum were purchased from Sigma-Aldrich® Co., USA. The membranes were probed with rabbit monoclonal antibodies specific to anti-cleaved Caspase-3 (E83-77), and Bcl-2 (#3498), as well as rabbit polyclonal antibodies against pan-AKT (ab8805), AKT1 (phospho S473) (ab8932), anti-Survivin (ab469), and β-Actin (ab8227) (Abcam, Cambridge, UK and Cell Signaling Technology, Danvers, MA, USA).

Preparation of crude *C. amuricus* extract

The entire *C. amuricus* plant was collected between February and June in Tra Vinh province (9° 59′ 41″ N 106° 12′ 53″ S), Vietnam. After collection, the plant was recognized and authenticated by the Department of Ecology and Evolutionary Biology at the University of Science in Vietnam (No. 06-2023/GXN). The dried plant was then crushed into a powder using an electric blender.

Plant materials were extracted and fractionated using maceration and liquid-liquid extraction methods (Tiwari *et al.*, 2011). The plant extracts of *C. amuricus* were prepared by soaking plant powder and methanol in a ratio of 1:7 (g powder: ml solvent) in a conical flask and storing them for three days. The extraction was repeated

three times. The mixtures were then filtered and evaporated until dry using a rotary evaporator to obtain a crude extract, yielding 17.55 ± 1.81% with a moisture content of 14.67%, meeting the requirements stated in the Vietnamese Pharmacopoeia V. The crude extract was dissolved in water and consecutively fractioned with increasing polarity solvents to obtain the hexane fraction (H fraction), the chloroform fraction (C fraction), and the ethyl acetate fraction (E fraction), respectively. In each experiment, all portions were dissolved in a suitable solvent to get a stock solution.

Determination of total phenolic content (TPC) and flavonoid content (TFC)

The total phenolic content (TPC) of the tested samples was assessed using the Folin-Ciocalteu method with some minor alterations (Mwamatope *et al.*, 2020). Briefly, a 100 µl sample solution with a concentration of 100 µg/ml was mixed with 100 µl of distilled water and 150 µl of 10% Folin-Ciocalteu reagent solution. After an 8-minute incubation, 450 µl of 30% Na_2CO_3 was added and incubated at room temperature for 30 minutes. The absorbance was measured at 765 nm. The TPC was determined as micrograms of gallic acid equivalents per milligram extract weight (µg GAE/mg).

The total flavonoid content (TFC) of the crude extract and fractions were determined using a colorimetric method based on the $NaNO_2$-$AlCl_3$-NaOH complex, as described above, with a slight modification (Martono *et al.*, 2019). The samples were prepared for a stock solution in a final concentration of 500 µg/ml with methanol solvent. The stock solutions were adjusted with distilled water and combined with 0.3 ml of 5% $NaNO_2$. After 6 minutes, 0.3 ml of 10% $AlCl_3$ was added and allowed to stand for 1 minute before 2 ml of 1M NaOH was added. The distilled water was then added to the mixture until a final volume of 10 ml was reached. The solutions were spectrophotometrically recorded at 510 nm. Quercetin was used to generate the TFC standard curve, and the outcome data was expressed in µg quercetin equivalents (QE) per mg of extract.

Determination of antioxidant activity

The antioxidant activity of the sample was measured using the α, α-diphenyl-β-picrylhydrazyl (DPPH) method with some minor alterations (Mensor *et al.*, 2001). The reaction mixture consisted of 150 µl DPPH (0.2 µM) and 150 µl sample or a standard solution (ascorbic acid) or Milli-Q water (control group). This mixture was kept stable at room temperature for 30 minutes in the dark, after which the absorbance of the DPPH was measured with a spectrophotometer. The purple color of the sample solution faded into yellowish shades due to the presence of antioxidants, causing a reduction in absorbance at 517 nm by α, α-diphenyl-β-picrylhydrazine (DPPH-H) formation. The DPPH radical scavenging activity was calculated using the following formula:

$$I\,\% = \frac{A_{control} - A_{sample}}{A_{control}} \times 100\%$$

***In vitro* cytotoxic activity**

In this study, the HepG2 cancer cell line (ATCC HB-8065™) and the fibroblast cell line (ATCC PCS-201-012™) were used to investigate the cytotoxic effect of *C. amuricus* through an MTT assay (Cragg *et al.*, 2016). HepG2 and fibroblast cells were cultured in a 96-well plate with high glucose

medium (DMEM) supplemented with 10% FBS and penicillin/streptomycin and then maintained at 37ºC with 5% CO_2 for 24 hours. Ly294002, a known PI3K inhibitor (Imai *et al.*, 2012), and DMSO were used as positive and negative controls for comparison, respectively. Various concentrations of the E fraction, negative control (DMSO 0.05%), and positive control (LY294002 0.07 µg/ml) were introduced into the cells. After treatment, an MTT solution (5 mg/ml) was added to each well and incubated for 4 hours for formazan crystal formation. The medium was then removed from each well, and the crystals were dissolved with DMSO solution before the optical absorbance was measured at 595 nm using an ELISA reader. The percentage of cytotoxicity was calculated using the following formula:

$$\text{Cell death (\%)} = 100 \times \frac{A_{sample} - A_{blank}}{A_{DMSO} - A_{blank}}$$

The IC_{50} value, which represents concentration and results in a viability rate of approximately 50%, was determined by plotting the percentage of viable cells against different concentrations of the *C. amuricus* fraction.

Western blotting study

The HepG2 cells treated with the E fraction were lysed using the Litovchick method (Litovchick, 2020). The supernatant of the extracted protein was collected and measured using a Nanodrop 1000 system (Thermo Scientific, USA). An equal amount of protein was then electrophoresed in a 10% SDS-PAGE gel and transferred to nitrocellulose membranes. The membrane was blocked with 5% skim milk. After blocking unspecific targets, the blots were probed with the desired primary antibodies, anti-cleaved caspase-3 and anti-survivin (Santa Cruz, USA), and then washed in PBST thrice before permeabilizing them for one hour with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG as secondary antibodies (Cell Signaling Technology). The blot was washed three times with PBST and analyzed with an enhanced chemiluminescence (ECL) detection solution in accordance with the recommended procedure (Pierce, Rockford, IL, USA). Relative protein expression levels were normalized by rejecting the same membrane when anti-β-actin was detected.

Molecular docking study

Ligand preparation

The structures of the phenolics and flavonoids of the Cyperaceae family were collected from the PubMed databases, which showed antioxidant and inhibitory effects against cancer. The three-dimensional structures were optimized using Avogadro software and converted to PDBQT format for further use in docking studies. Ly294002, a commercial cancer treatment drug, was also docked as a positive group.

Protein preparation

The crystal structures of molecular targets were downloaded from the Protein Data Bank (PDB) and listed in Table 1. All nonessential water molecules and heteroatoms were removed, leaving only the co-crystallized target ligands to identify a grid box using Discovery Studio 2024 software. The receptor proteins were then supplied with polar hydrogen and Kollman charges using AutoDockTools. The prepared protein files were saved in PDBQT format for subsequent docking analyses.

Table 1. The selected crystal protein structures were used for molecule docking.

No.	Protein Data Bank	Description	Resolution
1	5KCV	Crystal structure of allosteric inhibitor, ARQ 092, in complex with an autoinhibited form of Akt1	2.70 Å
2	2W3L	Crystal Structure of Chimaeric Bcl2-xL and Phenyl Tetrahydroisoquinoline Amide Complex	2.10 Å
3	2XYG	Caspase-3: CAS329306	1.54 Å
4	3UIH	Crystal structure of human Survivin in complex with Smac/DIABLO (1-15) peptide	2.10 Å

Molecular docking method

The molecular docking procedure was performed using AutoDock Vina software to explore the synergistic interactions between ligands and target proteins (Fan *et al.*, 2019). The interactions between ligands and target proteins were then investigated using PyMOL software. To validate the docking protocol, co-crystallized inhibitors (native ligands, NL) of these proteins were re-docked to the active site of the selected protein structure. It was found that the root-mean-square deviation (RMSD) was less than 2 Å (Trott *et al.*, 2010).

Statistical analysis

All experiments were performed three times; the results were presented as mean ± standard deviation. The data analysis was carried out using version 16 of Minitab software, employing the Student's *t*-test and one-way ANOVA. Statistical significance was defined as a *P*-value of less than 0.05.

RESULTS AND DISCUSSION

Total phenolic and flavonoid contents

Figure 1 showed the TPC and TFC analysis results for the crude extract and *C. amuricus* fractions. The E fraction possessed the highest total phenolic and flavonoid contents at 221.86 ± 2.17 µg GAE/mg and 386.67 ± 4.83 µg QE/mg, respectively. The TPC was lowest in the H fraction (11.86 ± 0.92 µg GAE/mg), while the TFC was lowest in the crude extract and the C fraction at 130.04 ± 2.55 and 130.06 ± 3.85 µg QE/mg, respectively.

Phenolic compounds, known as secondary metabolites in plants, have multiple functions, including promoting health, protecting against infections and predators, preventing UV damage, and attracting pollinators (Li *et al.*, 2018). Flavonoids are the most common phenolic substances with a chemical structure consisting of two aromatic rings connected by a three-carbon bridge, typically a heterocyclic ring C (Pietta, 2000). The proportions of the TPC and TFC contents increased as the polarity of the solvent increased. The elevated polarity of the extraction solvents can increase the solubility of the phenolic and flavonoid compounds in the extraction solution, except for flavonoid aglycones, which do not dissolve well in polar solvents. Furthermore, the ability of an extract to dissolve phenolic and flavonoid constituents depends on their polarity (Chaudhari *et al.*, 2015, Kim *et al.*, 2011). In this study, the high levels of phenolics and flavonoids in the E fraction

could be attributed to purification and concentration during the fractionation process. The E fraction is likely to scavenge significant radicals, highlighting potential applications in cancer treatment as it neutralizes free radicals and inhibits cancer cell proliferation mechanisms. These findings are promising for developing therapeutic strategies based on natural compounds that offer the benefits of reduced side effects and improved treatment effectiveness.

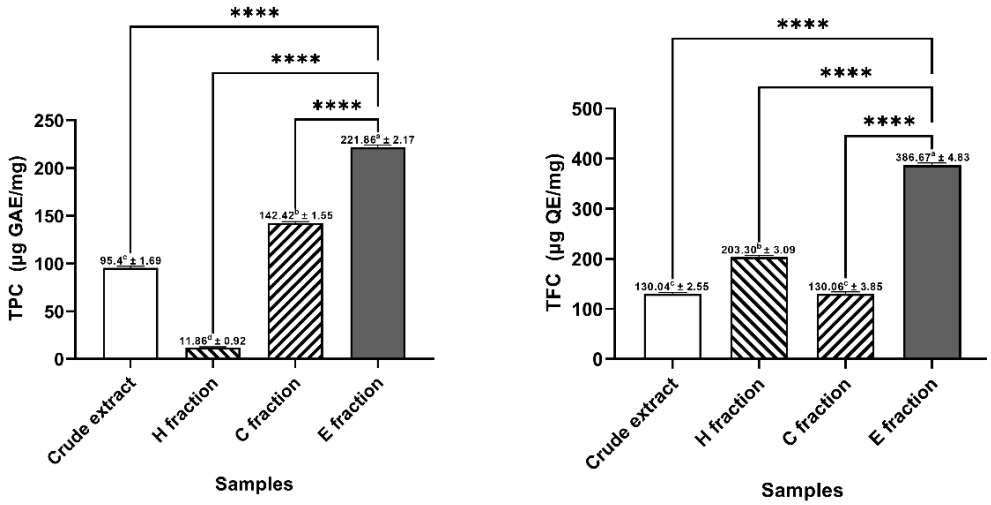

Figure 1. The TPC and TFC of crude and fractions of *C. amuricus*. The values followed by different letters were significantly different at $p < 0.05$.

Antioxidant activity

The incidence of diseases caused by radiation-induced cell damage is declining, affecting the aging process and contributing to a wide range of diseases, including Alzheimer's, Parkinson's, and cancer (Maynard *et al.*, 2015). As a result, antioxidants have attracted much attention due to their tendency to reduce the negative effects of free radicals. Figure 2 depicted the radical scavenger potential of the *C. amuricus* crude extract and different fractions as examined using the DPPH method, one of the most commonly used techniques to measure antioxidant activity. All extracts exhibited dose-dependent DPPH scavenging activity. Following the C fraction, crude extract, and H fraction, which had IC_{50} values of 63.14 ± 2.13, 82.59 ± 3.47, and 169.87 ± 8.95 µg/ml, respectively, the E fraction exhibited the highest DPPH radical scavenging ability (IC_{50} = 21.07 ± 0.30 µg/ml). In comparison, ascorbic acid, used as a standard, exhibited an IC_{50} of 4.02 ± 0.70 µg/ml. More antioxidant activity was indicated by a lower IC_{50}. The outcome aligned with Lee *et al.*'s discovery that three phenolic substances extracted from *C. amuricus* exhibited powerful antioxidant properties, particularly in neutralizing DPPH free radicals and superoxide anions (Im Lee *et al.*, 2008).

The E fraction displayed crucial antioxidant activity, as was predicted, given the high concentration of total phenols and flavonoids (TPC and TFC in the fraction were higher than in the others). The critical role of phenolics and their subclass compounds as free radical scavengers has been emphasized in some previously

published research papers. According to one report, the hydroxyl groups in phenolic compounds destroy free radicals by attaching to metal ions and promoting the generation of reactive oxygen species, which causes lipids to oxidize. The redox potential of these substances governed their ability to function as antioxidants (Kumbhare *et al.*, 2012). These substances either depressed peroxides, extinguished singlet and triplet oxygen, or absorbed and deactivated free radicals. Variants of flavonoids interfered with cyclin-dependent, oxidative, and hydrolytic kinase enzymes, causing cancer cells to undergo apoptosis and reduce inflammation (Rao *et al.*, 2005).

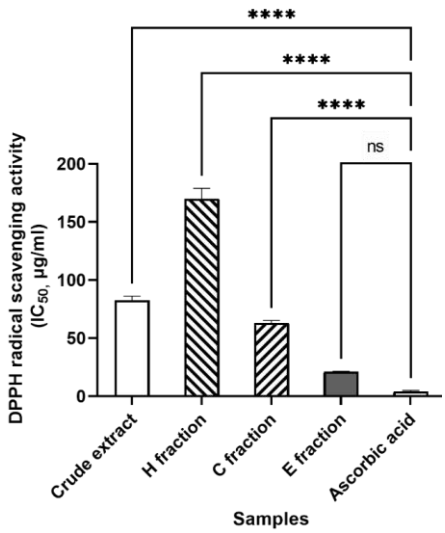

Figure 2. The antioxidant activity of *C. amuricus* crude extract and extract fractions. ns $p > 0.05$ and **** $p < 0.05$ compared with the ascorbic acid group.

Cytotoxic activity

Based on the *in vitro* results above, the E fraction with the highest TPC and TFC values was selected to be tested *in vitro* against the human HepG2 cell line using an MTT assay. According to Figure 3, the *C. amuricus* E fraction significantly reduced the viability of HepG2 cells in a dose-dependent manner. At the highest concentration of 200 µg/ml, the viability of HepG2 cells dropped significantly to 40.68 ± 2.71%, which demonstrated strong cytotoxic effects. On the other hand, fibroblast cells maintained a viability of approximately 100.42 ± 0.13%, suggesting minimal cytotoxicity at this level. At 150 µg/ml, the viability of HepG2 cells was significantly lower (56.55 ± 0.55%) than fibroblast cells, which remained at 101.2 ± 6.98% (Table 2), highlighting the selective effect of the E fraction. (Table 2). Remarkably, the E fraction in HepG2 cells reached an IC_{50} of 166.50 µg/ml in HepG2 cells, which increased effectiveness in reducing cancer cell survival. Additionally, the study confirmed that DMSO had minimal toxicity with an inhibition of less than 5% at a concentration of 0.05%, and no adverse effects were observed due to the presence, suggesting that the observed cytotoxicity was primarily due to the active constituents of the E fraction and not to the solvent. At a concentration of 10 µM, LY294002 exhibited cytotoxicity of 52.55 ± 2.15% against HepG2 cells and

51.79 ± 1.75% against normal fibroblast cells. As LY294002 acts by inhibiting the activation of the PI3K/AKT/mTOR signaling pathway, the compound effects are observed in both cancerous and normal cells (Zhang *et al.*, 2019). The inclusion of this positive control serves as a preliminary assessment for subsequent investigations into cell signaling mechanisms. As a result, the cytotoxicity of the E fraction was more effective against HepG2 cells, while the effect on fibroblast cell survival was lower than that of cancer cells, possibly due to bioactive compounds such as flavonoids, phenolics, and alkaloids that trigger apoptosis. Flavonoids have accumulated plenty of evidence of their dual action by modulating enzyme activities that scavenge ROS. Under normal conditions, the compounds act as antioxidants, while in cancer cells, these compounds act as powerful pro-oxidants. This dual action triggers cell cycle arrest, which leads to apoptosis and the autophagy pathway and thus suppresses the proliferation and invasiveness of cancer cells (Kopustinskiene *et al.,* 2020).

This result was consistent with a study from 2017, which found that *C. amuricus* had considerably stronger cytotoxicity activities on Hep3B cells than on HEK293 cells (Pham *et al.*, 2017). The inhibitory effect on the HepG2 cell line was determined on another representative of the same genus, *Cyperus articulates*, with the effective concentration of essential oil (IC_{50} = 28.5 µg/ml) (Nogueira *et al.*, 2020).

Table 2. *In vitro* cytotoxicity of the *C. amuricus* E fraction.

Samples	Cell viability (%)	
	HepG2	**Fibroblast**
E fraction (150 µg/ml)	56.55 ± 0.55	101.2 ± 6.98
DMSO 0.05%	95.84 ± 1.76	97.05 ± 5.69
LY294002 0.07 µg/ml	52.55 ± 2.15	51.79 ± 1.75

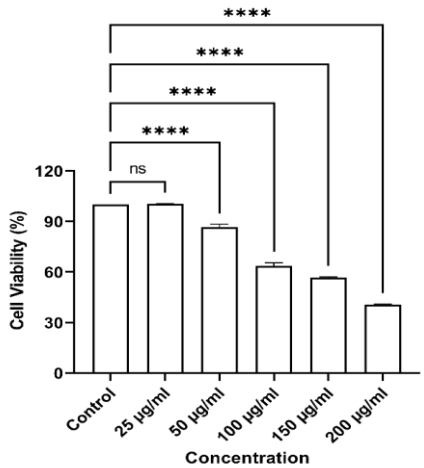

Figure 3. Effect of *C. amuricus* E fraction on the cytotoxicity of HepG2 cells *in vitro*. ns $p > 0.05$ and **** $p < 0.05$ compared with the control group.

Moreover, notable variations in nuclear size and morphology were also observed in HepG2 cells treated with the E fraction for 24 hours (Figure 4). In the control group, the cell nucleus maintained a uniform and normal appearance. Conversely, at 150 µg/ml, distinct apoptotic properties were evident, including bright DAPI staining, chromatin condensation, apoptotic body formation, loss of membrane integrity, and cell fragments in the medium. This suggests that the E fraction effectively induces apoptosis in HepG2 cells (Clarke *et al.*, 1996).

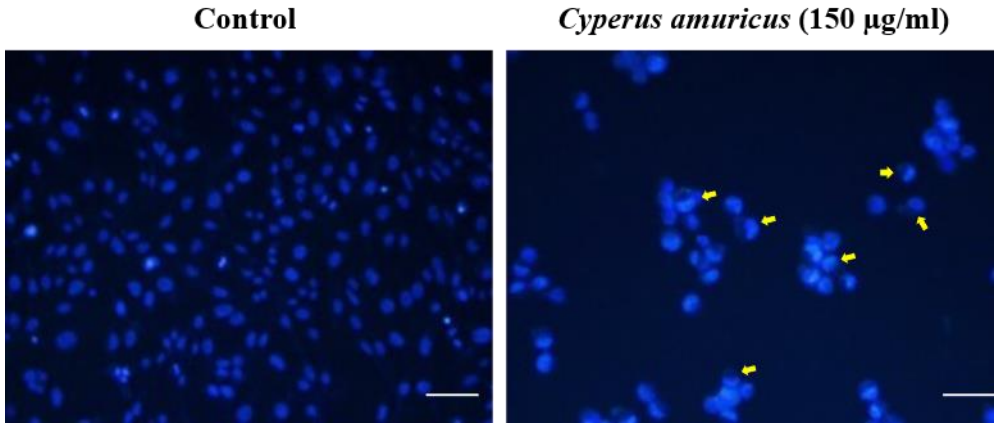

Figure 4. Effects of E fraction on HepG2 cells. Yellow arrows: bright DAPI staining; white bar: scale bar: 100 µm.

Western blotting study

To determine the underlying effect of the E fraction on HepG2 cells, the expression of apoptosis-related proteins, including cleaved caspase-3 and survivin, extracted from HepG2 cells treated with different concentrations of the E fraction, was detected by Western blot analysis, with β-actin serving as the loading control. The experimental results identified that the E fraction could inhibit the expression level of survivin in a concentration-dependent manner compared to the control group (Figure 5). It was remarkable that the bands of cleaved caspase-3 were observed in the HepG2 cells treated with the E fraction compared to the control group. The inhibition of Akt phosphorylation (Ser473), survivin expression, and Bcl-2 levels were confirmed by immunoblotting analyses of HepG2 cell lysates in response to the E fraction. Similarly, β-actin expression remained constant in all HepG2 cell samples.

The PI3K/Akt pathway is important for cellular signaling for cell survival and apoptosis suppression (Reed, 2000). PI3K, which is activated by stimulating growth factor, activates Akt serine/threonine kinase, which in turn inhibits apoptosis and promotes cell survival, proliferation, migration, and angiogenesis by modifying their downstream effectors (Sun *et al.*, 2021). The Akt pathway has been linked to survivin overexpression and, consequently, cell survival in several malignancies, including liver cancer (Chen *et al.*, 2016). Survivin, a member of the IAP, is critical for cell cycle control and apoptosis (Altieri, 2008). It is constantly expressed during embryonic and fetal development but is invisible in most normal adult tissues (Chen *et al.*, 2016). Survivin amplification frequently confers resistance to therapy and decreases apoptotic

agent-induced cell death (Cheng *et al.*, 2013). Here, the inhibition of Akt phosphorylation (Ser473), survivin expression, and Bcl-2 levels were confirmed by immunoblotting analysis of HepG2 cell lysates in response to the E fraction of *C. amuricus* with specific antibodies. The E fraction mediated an effective suppression of the phosphorylation levels of Akt (Ser473) and survivin, as the expression of these key markers of the metabolic pathway in HepG2 cells is detectable in a concentration-dependent manner. In addition, Bcl-2 levels were reduced in cells treated with the E fraction compared to those in control cells. The grade of cleaved caspase-3 was constantly enhanced in the E fraction-treated HepG2 cells relative to the control.

Caspase-3 typically exists as an inactive precursor of 32 kDa (procaspase-3) and, upon stimulation, is converted into the active form, known as cleaved caspase-3. This process results in the cleavage of DNA and protein fragments in cancer cells and induces cell death by activating the apoptosis pathway (Brentnall *et al.*, 2013). Survivin is abundantly expressed in cancer cells, where this protein binds to and inhibits caspase, preventing cell death triggered by various stimuli. The disruption of survivin leads to enhanced caspase-3-dependent apoptosis and demonstrates significant antitumor activity in several cancer cell types (Carrasco *et al.*, 2011). Akt enhances survivin expression by using transcription factors such as NF-κB, which suppresses caspase-3 activation and stabilizes mitochondrial integrity. Additionally, Akt inhibits pro-apoptotic proteins such as Bad and Bax, shifting the balance further toward cell survival (Paglino *et al.*, 2014). Bcl-2, which is located in the outer membrane of mitochondria, prevents cytochrome c release and caspase activation and thus contributes to apoptosis resistance (Youle *et al.*, 2008). This suggested that the E fraction could induce the apoptosis process in HepG2 cells by upregulating the expression of cleaved caspase-3 and inhibiting survivin. Overall, the E fraction of *C. amuricus* improved inhibition and apoptosis in HepG2 cells by reducing the PI3K/Akt/survivin signaling pathway.

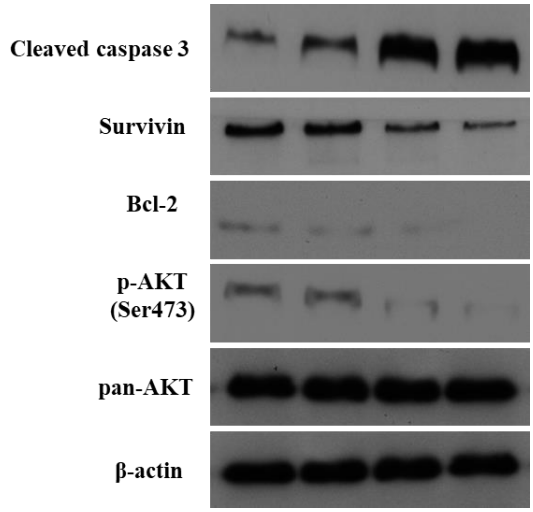

Figure 5. Effects of E fraction on the expression of cleaved caspase-3 and Akt/survivin/Bcl-2 proteins in HepG2 cells. Images show chemiluminescent detection of the blots, which are representative of three independent experiments.

Molecular docking study

Computer-based docking experiments were carried out to predict and simulate the interactions between the compounds and their targets, serving as an effective method to search for active constituents and predict their mechanisms of action (Ul Bari *et al.*, 2019). Because of the limited studies on the isolated phytochemicals in *C. amuricus*, the phenolic and flavonoid compounds with strong antioxidant and anticancer properties within the Cyperaceae family were examined for their binding interactions with various amino acids in the active site of proteins associated with the Akt/survivin/Bcl-2 signaling pathway. Thirty compounds were docked to molecular docking studies, and the results showed that 10 compounds exhibited the lowest docking scores for the target protein, as presented in Table 3.

Table 3. Docking results of 10 phenolics and flavonoids from the Cyperaceae family, NL, and positive control with target proteins.

No.	Compounds	Docking scores (kcal/mol)			
		5KCV	2W3L	2XYG	3UIH
1	Luteolin 7-O-β-D-glucuronopyranoside	-10.7	-6.6	-5.1	-6.5
2	Myricetin 3-O-β-D-galactopyranoside	-10.7	-6.7	-5.9	-5.7
3	Epi-orientin	-10.6	-7.0	-6.2	-5.9
4	7,3'-dihydroxy-5,5'-dimethoxy-8-prenylflavan	-10.6	-7.9	-6.0	-5.3
5	Rutin	-10.5	-7.4	-5.0	-6.2
6	5,7,3'-trihydroxy-5'-methoxy-8-prenylflavan	-10.2	-7.4	-6.0	-5.5
7	Luteolin 4'-O-β-D-glucuronopyranoside	-10.0	-6.1	-5.2	-6.8
8	Orientin	-10.0	-7.1	-5.9	-5.7
9	Ellagic acid	-9.9	-7.0	-6.2	-5.3
10	Quercetin	-9.8	-6.4	-6.1	-5.7
11	NL binding to 5KCV	-13.9	-	-	-
12	NL binding to 2W3L	-	-10.3	-	-
13	NL binding to 2XYG	-	-	-5.7	-
14	NL binding to 3UIH	-	-	-	-4.5
15	Ly294002	-10.7	-7.4	-6.0	-5.3

Most of these compounds showed good binding affinity for targeted proteins. The interactions between these compounds and targets, such as hydrophobic, hydrogen, Pi-Sigma, and Pi-Pi bonds, were displayed in Figures 6, 7, 8, and 9. When analyzing the docking interaction, it was found that luteolin 7-O-β-D-glucuronopyranoside and myricetin 3-O-β-D-galactopyranoside had the lowest docking energy scores of -10.7 kcal/mol with Akt compared to other compounds. However, these scores were comparable to Ly294002, which was used to inhibit cancer metastasis and was inhibited

as an inhibitor of the PI3K/Akt pathway. 7,3'-dihydroxy-5,5'-dimethoxy-8-prenylflavan exhibited the strongest binding affinity against Bcl-2 with a score of -7.9 kcal/mol and thus surpassed both positive control drugs. Epi-orientin and ellagic acid displayed strong binding energies against caspase-3 and exceeded NL with docking scores of -6.2 kcal/mol. Furthermore, luteolin 4'-O-β-D-glucuronopyranoside exhibited a binding affinity of -6.8 kcal/mol compared to a binding affinity of -4.5 kcal/mol of the native ligand.

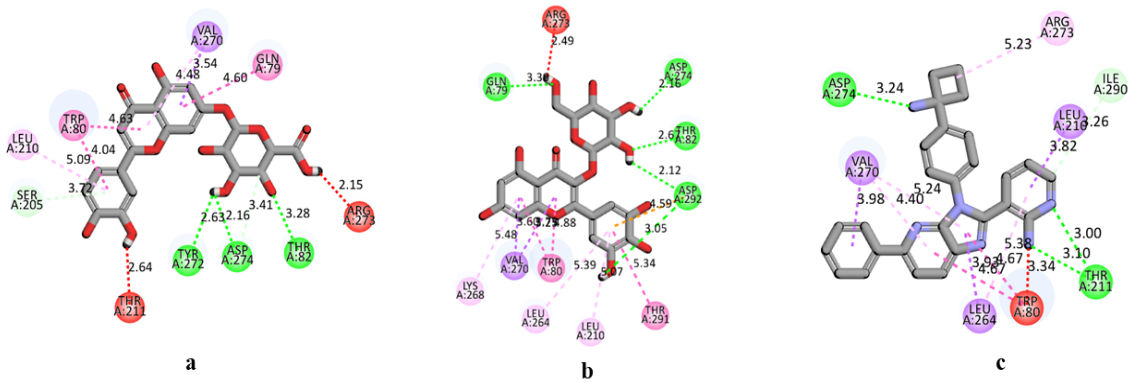

Figure 6. Luteolin 7-O-β-D-glucuronopyranoside (a), Myricetin 3-O-β-D-galactopyranoside (b), and NL (c) with Akt.

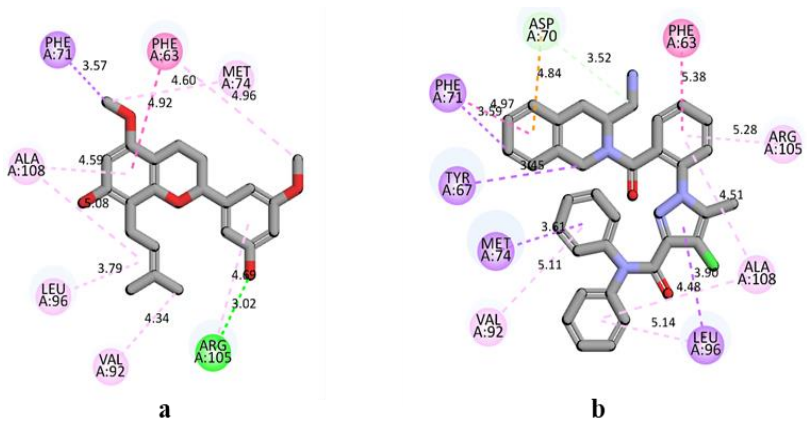

Figure 7. 7,3'-dihydroxy-5,5'-dimethoxy-8-prenylflavan (a) and NL (b) with Bcl-2.

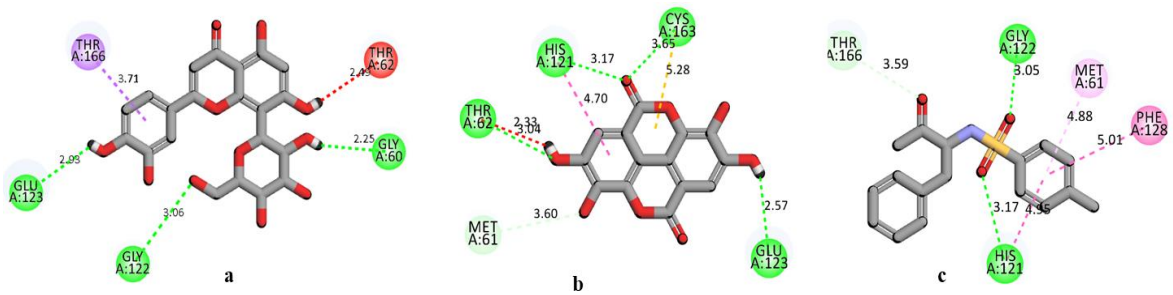

Figure 8. Epi-orientin (a), Ellagic acid (b), and NL (c) with caspase-3.

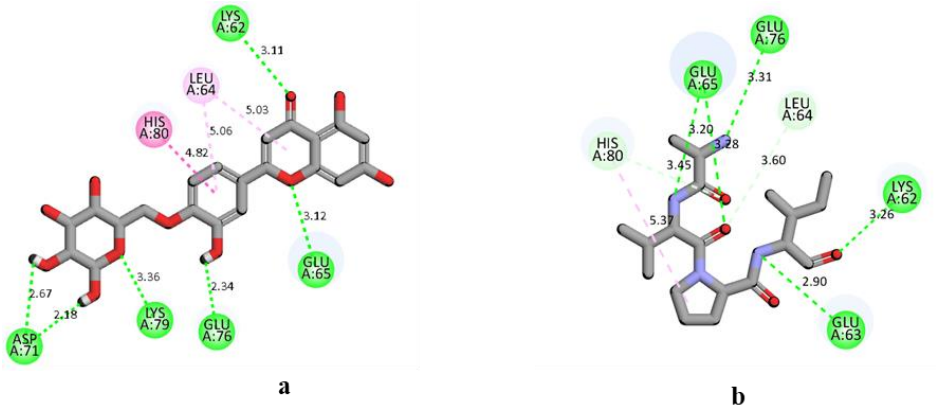

Figure 9. Luteolin 4'-O-β-D-glucuronopyranoside (a) and NL (b) with survivin.

The molecular docking process was carried out extensively to elucidate the interaction between the compounds and proteins, offering consideration of the molecular mechanisms by which ligands bind to the amino acid residues of the target proteins. Luteolin 7-O-β-D-glucuronopyranoside and myricetin 3-O-β-D-galactopyranoside were found to target Akt activation due to a binding pattern similar to that of miransertib, an oral allosteric Akt-1 inhibitor. This inhibition is due to the fact that it occurs as a result of hydrophobic interactions with Trp80 (Figure 6), an important residue, as previously reported (Lapierre *et al.*, 2016). During apoptosis, members of Bcl-2 and survivin play essential functions. It was observed that the selected phenolics and flavonoids suppress the anti-apoptotic proteins Bcl-2 and survivin. Therefore, this result concludes that these compounds induce apoptosis, at least in part, via the mitochondrial metabolic pathway. A connection between apoptosis induced by phenolic and flavonoid compounds and Akt inhibition is also suspected. It is generally accepted that Akt plays a critical role in inducing apoptosis by regulating members of the Bcl-2 and IAP families. Additionally, phenolic and flavonoid compounds were demonstrated to inhibit Akt activity, and a combination with the Akt inhibitor LY294002 exhibited an additive effect (Tungsukruthai *et al*., 2021).

Consequently, these docking scores reveal that most phenolic and flavonoid compounds within the Cyperaceae family have low docking scores and a high binding affinity for key proteins involved in inhibitory signaling pathways. This supports the potential of *C. amuricus* to inhibit cancer cells and provides insights into the inhibitory mechanism (Figure 10).

Only limited research has been conducted in Vietnam to screen and identify the cytotoxic effects of *C. amuricus* on liver cancer cells. Furthermore, a widely used cancer treatment strategy combines chemotherapy with natural chemicals that can suppress cell growth and trigger cancer cell death, reducing side effects. This study was initiated to obtain preliminary positive results and explore new ways to identify and develop potential biological agents for *C. amuricus*. Such efforts not only improve the effectiveness of current medications but also help reduce the time and cost of clinical trials and accelerate the use of technology in drug manufacturing and market distribution in Vietnam.

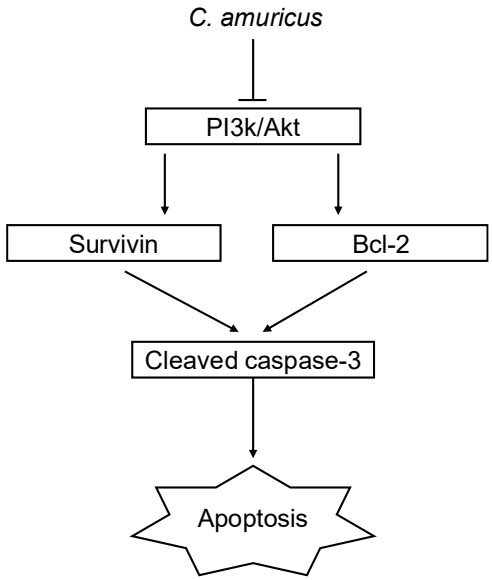

Figure 10. Predicted mechanism of apoptotic cell death induced by *C. amuricus* in HepG2 cancer cells by specifically influencing the PI3K/Akt pathway: *C. amuricus* suppresses Akt activity, which then downregulates survivin and Bcl-2 and simultaneously activates caspase-3, which leads to apoptotic cell death.

CONCLUSION

C. amuricus possessed high inhibitory activity against the apoptosis route, as it showed a potential binding affinity of related proteins through the molecular *in silico* docking process. Based on the results, this study proposes combined therapies using the E fraction of *C. amuricus*, which targets the PI3K/Akt/survivin signaling pathway, as a viable strategy to achieve therapeutic effectiveness that cannot be achieved when the agents are used individually to treat hepatocellular carcinomas. Three important conclusions were drawn from the results of this investigation. First, the E fraction of *C. amuricus* showed a strong correlation between biological activities (antioxidant and anticancer properties) and phenolics/flavonoid levels. Second, apoptotic cell death triggered by the E fraction was amplified in HepG2 cancer cells *in vitro* and *in silico* in the inhibitor of the PI3K/Akt/survivin signaling pathway. Third, PI3K/Akt/survivin could serve as a valuable biomarker to assess the response of *C. amuricus* in future preclinical and clinical studies. The findings suggested that the integration of traditional knowledge with modern scientific tools offers great potential for discovering lead compounds for effective pharmaceuticals. However, further studies are needed to elucidate the molecular mechanisms of *C. amuricus*, which are involved in its anti-proliferative action *in vitro* and its effectiveness *in vivo*.

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CONFLICT OF INTEREST

The authors declared no conflict of interest with respect to the research, authorship, and/or publication of this article.

AUTHOR CONTRIBUTIONS

Conceptualization, Q.L.P., M.Q.P. and T.H.H.P.; methodology, K.C.T., H.N.L. and H.T.A.N.; software, K.T.T., H.N.L. and M.Q.P.; investigation, T.L.N. and T.H.H.P.; writing-original draft preparation, M.Q.P., K.C.T., and K.T.T.; writing-review and editing, M.Q.P., T.L.N and T.H.H.P.; supervision, H.T.A.N., Q.L.P. and T.H.H.P. All authors have read and agreed to the published version of the manuscript.

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