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Ancient forest plants possess cytotoxic properties causing liver cancer HepG2 cell apoptosis

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Here, we collected 154 plant species in China ancient forests looking for novel efficient bioactive compounds for cancer treatments. We found 600 bioactive phytochemicals that induce apoptosis of liver cancer cell in vitro. First, we screen the plant extract’s \textit{in vitro} cytotoxicity inhibition of cancer cell growth using \textit{in vitro} HepG2 cell lines and MTT cytotoxicity. The results from these initial MTT in vitro cytotoxicity tests show that the most efficient plants towards hepatoma cytotoxicity is \textit{Cephalotaxus sinensis}, mint bush (\textit{Elsholtzia stauntonii}) and winged spindle tree (\textit{Euonymus alatus}). We then used in cell-counting kit-8 (CCK-8) to further understand in vivo tumor growth using nude mice and GC-MS and LC-QTOF-MS to analyze the composition of compounds in the extracts. Extracted chemically active molecules analyzed by network pharmacology showed inhibition on the growth of liver cancer cells by acting on multiple gene targets, which is different from the currently used traditional drugs acting on only one target of liver cancer cells. Extracts from \textit{Cephalotaxus sinensis}, mint bush (\textit{Elsholtzia stauntonii}) and winged spindle tree (\textit{Euonymus alatus}) induce apoptosis in hepatoma cancer cell line HepG2 with a killing rate of more than 83% and a tumor size decrease by 62–67% and a killing rate of only 6% of normal hepatocyte LO2. This study highlight efficient candidate species for cancer treatment providing a basis for future development of novel plant-based drugs to help meeting several of the UN SDGs and planetary health.

1. Introduction

Due to growth and aging of the world’s population, the burden of malignant cancerous tumors continue to increase being a major cause of disease and mortality (Zheng et al., 2018). According to the WHO International Agency for Research on Cancer (IARC), the number of new cancer cases worldwide is 19.3 million, with men slightly overrepresented by ca. 10% (Fig. 1) (Sung et al., 2021). Of these, primary liver cancer is the sixth...
most common cancer, and is responsible for a third of all cancer mortalities estimated to be around 906,000 annual cases and 830,000 deaths (Sung et al., 2021). Other prevalent cancers include breast, lung, colorectal, prostate and stomach cancers and altogether with liver cancer these account for about 50% of all cancer diagnosis being overrepresented in low to middle-income countries (LMICs). The LMICs house 82% of the global population (Zheng et al., 2018), and cancer is causing the majority of mortalities, while in developing countries it is the second largest mortality factor (Bundhamcharoen et al., 2016; Organization, 2019). These malignant tumors bring large economic burdens and in developing countries often unfordable for a high number of families (Huang et al., 2016; Oliveira et al., 2021). In China for example, self-paying medical expenses for treatment accounts on average for 58% of the total household income and in some circumstances more than 100% (Huang et al., 2016; Oliveira et al., 2021).

Targeted therapy and immunotherapy constitute efficient means in terms of treatment of patients with advanced liver cancer (El-Khoueiry et al., 2021; Galle et al., 2021; Weidanz, 2021), warranting the development of new anti-cancer drugs (Fu et al., 2020; Kleijnen et al., 2017; Schnekenburger et al., 2014). Of these, natural phytochemicals may

Fig. 1. Cancer incidence statistics in 24 regions of different genders in the world in 2020. (a) Global new male cancer cases. (b) Global new female cancer cases. Figures in brackets represent the number in thousands of new cancers in 2020 (Sung et al., 2021). (c) Top ten types of cancer incidence in 2020. Numbers in in brackets represents the proportion of new cancers in 2020.

Fig. 2. The location map of samples collection places. Collected from Henan Province, China.
possess advantages, and many of anti-cancer preparations derive from natural products (Kato et al., 2016; Ke et al., 2021; Koh et al., 2017; Waiyaput et al., 2012) (Table S1). The development of new drugs is therefore continuously improving and plant medicines are shown to reduce liver cancer cell proliferation and cell apoptosis, reversing cancer cell resistance and immunity (Luo et al., 2020; Wang et al., 2020). These plant medicines are a mixture of phytochemicals that inhibits enzyme activities (Bik-San et al., 2015; Zhu et al., 2015). Among the commonly used plant medicines in China is ginseng used among other to treat liver cancer by inhibiting pathological relations to tumorogenesis, including cell survival, proliferation, invasion and angiogenesis (Hu et al., 2019; Taigo et al., 2018; Zhu et al., 2021). Sinisan is another example of Chinese plant-based medicine formulate from bupleurum, paenodia, fructus and licorice, used to test in vivo inhibition of liver cancer cell HepG2 in nude mice (Wei et al., 2014).

To investigate the biological activity of phytochemicals further, we collected 154 plant species in China virgin-forest looking for novel bioactive compounds with cancer killing effects. We studied the biologically active compounds in vitro cytotoxicity and inhibition on in vivo tumor growth. In addition, GC-MS and LC-QTOF-MS were used to analyze the composition of compounds in the extracts after which network pharmacology was used to study biochemical inhibition of multiple gene targets, providing a basis for future development of novel forest-based drugs.

2. Materials and methods

2.1. Sampling and field work

Since December 29, 2017, the Henan Agricultural University, Henan Xiaojinling National Nature Reserve Administration Bureau, the Luanchuan Laojunshan Forest Farm, the Biyang MaDao Forest and Boshan Forest collected 154 plant species of four seasons in the remote forests of Funiu Mountains, Wangwu Mountains, and Laojun Mountains (Fig. 2). See extended materials and methods in supplementary information for further details.

2.2. Chemical extraction

The collected material was divided into six sub-samples: leaves, branches, bark, wood, roots, and root bark, resulting in more than 600 samples in total. These were then pulverized, and homogenized and extracted with ethanol, benzene/ethanol (1:1), distilled water/ethanol (1:1) and distilled water (the ratio of sample to solvent being 1:30). Using different solvents to extract phytochemicals enables the comparison of extraction rates and inhibitory efficiency of different extracts on cancer cells. Finally, the extracts were concentrated to 10 mL. The ethanol and benzene were obtained from Tianjin Fuyu Fine Chemical Co., Ltd. and YanTai ShuangHuang Chemical Co., Ltd., and distilled water produced from the water plant of Henan Agricultural University. We then performed GC-MS and LC-QTOF-MS to analyze the composition of the extract compounds in bark, branch, root and root bark from the three most potent species Cephalotaxus sinensis, Elsholtzia stauntonii and Euonymus alatus.

2.3. GC-MS and LC-QTOF-MS analyses

We used GC-MS and LC-QTOF-MS to analyze the composition of the extract compounds in bark, branch, root and root bark from the three most potent species Cephalotaxus sinensis, Elsholtzia stauntonii and Euonymus alatus. First, we implemented an Agilent 7890B-5977A GC-MS equipped with a HP-5 MS (60 m × 0.25 μm × 0.25 μm) electroquadrupole capillary column. The carrier gas used was high purity helium, with flow rate of 1.5 mL/min, and no divergence. The initial temperature program of the GC was 50 °C, increased to 250 °C at a rate of 8 °C/min. Then the temperature increased to 280 °C at a rate of 5 °C/min and maintained for 5 min. The mass range scanned by the MS program was 30–600 amu, with an ionization voltage of 70 eV and an ionization current of 150 μA using electron ionization (EI). The ion source and the quadrupole temperature were set to 230 °C and 150 °C, respectively. Then, we used Liquid Chromatography-Quadroplpe Time of Flight-Mass Spectrometry (LC-QTOF-MS) analysis. LC: the chromatographic column was ACQUITY UPLC I-Class-Xevo G2-XS (2.1 × 50 mm, 1.7 μm). Mobile phase: Positive ion mode: 0.10% (v/v) formic acid (A), acetonitrile with 0.10% (v/v) formic acid (B). Flow rate: 0.30 mL/min. Column temperature: 30 °C. Post time: 5 min. Gradient elution: [Time (min), B (%)] was [0, 5], [2, 5], [20, 100], [25, 100] in turn. MS: Ion source: AJS ESI (Agilent Jet Stream electrospray ionization). Detection mode: Positive ion mode. Capillary voltage: 3.0 kV. Sample cone: 40 V. Source temperature: 100 °C. Dissolution temperature: 400 °C. Cone gas: 50 L/h. Dissolution gas: 800L/h. Database Screening: Traditional Chinese Medicine Database in Waters UNIFI software. Scan mass range program: 50-1200 m/z. Reference ion:121.0509 (64.0158), 922.0098 (Positive ion mode).

2.4. MTT cytotoxicity assay

A 20% trypsin digest was added to prepare cell suspension after which the cell density was controlled at 5 × 10^4 cells/mL. The cell suspension was modulated into a 96-well plate at 200 μL/well in DMEM medium (10% fetal calf serum, 100 μg/mL penicillin and 100 μg/mL streptomycin) and placed in a CO₂ incubator. Subsequently, the medium was cultured at 37 °C for 24 h, after which the sample to be tested was added at a preset mass concentration gradient of 50 μl per well, and a blank control experiment was performed with an equal amount of DMSO; the cells were cultured in a CO₂ incubator at 37 °C for 48 h. After centrifugation, the culture solution was discarded, carefully washed 3 times with PBS (phosphate-buffered saline) buffer. Then the culture medium containing MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) 5 mg/mL was added, and incubation was continued for 4 h in a CO₂ incubator. The supernatant was aspirated, 150 μl of DMSO was added to each well, and shaken at low speed for 10 min to dissolve the crystals sufficiently. Then we used an enzyme-linked immunosorbent to measure well absorbance (OD value) at a wavelength of 493 nm. For the survival rate the following formula was used; survival rate (100%) = (experimental group/control group OD) * 100%. For the statistics and image production Graph Pad Prism 9.0 software was used (Mbemi, 2018; Sauri et al., 2016).

2.5. Cytotoxic cell counting Kit-8 (CCK-8) assay

Viable HepG2 cells with logarithmic growth were centrifuged and counted after which 1.2 × 10^6 cells per well were added to 100 μl DMEM medium containing 10% FBS (fetal bovine serum) 96-well plates. After 24 h, plant extract concentration ratios of 1:9 or 1:4, respectively, were added and following 7 h, 10 μl CCK-8 reagent was added to each well and incubated for 2 h. Finally, we measured the OD value of each sample at 450 nm.

2.6. Flow cytometry for cell apoptosis

HepG-2 cells with logarithmic growth were centrifuged and counted. A total of 1.0 × 10^6 cells were added into 2 mL DMEM medium containing 10% FBS in each well. Seven wells were used in the experiment and after 24 h, PBS diluted plant extracts of 1:9 and 1:4 were added, respectively, to observe inhibitory differences Table S2. The culture medium was discarded, the cells were washed twice with PBS, digested with trypsin and centrifuged at 1000 r for 3 min. The supernatant was discarded, PBS was added, centrifuged (1000 r, 3 min), and the procedure was repeated. Then, we prepared a propidium iodide (PI) solution from Annexin V binding buffer diluted 1:10 using sterile water, after which 5 mL Annexin V Binding Buffer was added. Then, Eppendorf tubes were labelled, and cell suspension containing 500 μL 1X Annexin V
Binding was added 5 μL PI solution, 5 μL PI solution, cultured in the dark for 15 min, and tested using the Annexin V-FITC Apoptosis Staining / Detection Kit (Fan et al., 2020; Li et al., 2015).

2.7. Cell line derived xenografts and in vivo treatment

5 × 10⁶ HepG2 cells were subcutaneously injected into the right medial hind leg of nude mice to establish the xenograft model of cell lines. The tumor allowed growth for seven days before treatment. Mice were randomly divided into 7 groups (Control, CSL1, CSL2, CSL3, CSL4, ESB4 and EAS6) according to tumor volume and body weight, with 3 mice in each group. The mice were treated by gavage for 2 weeks, respectively. The plant extracts were diluted 10 times with PBS and each nude mouse was given 200 μL of PBS (control group) and 200 μL PBS/extract mixture (treatment group). The body weight and tumor volume of the mice was measured every three days, and the tumor weighed after the treatment cycle (Ginestier et al., 2010; Shahrzad et al., 2008). The present study was approved by the Ethics Committee of Xinxiang Medical University (Xinxiang, China). The authors would like to thank Yuan Ze University and Saveetha Institute of Medical and Technical Sciences for the facilities and support provided.

2.8. Screening of target genes associated with liver cancer

We compared targets genes related to the chemical composition in the extracts using the Swiss Target Prediction, STITCH, STRING and NCBI-gene database. In order to analyze the complex relationship between these active components and their targets, an interaction network was constructed through network pharmacology. All network maps visualized and analyzed by Cytoscape 3.8.2 (http://www.cytoscape.org/).

2.9. Statistical analyses

Graph Pad Prism 9.0 software was used for the graphical presentations, while a paired student’s t-test was used to test for group comparison (significant level set to p < 0.05).

3. Results and discussion

3.1. Cell viability

MTT cytotoxicity test showed that out of 600 extracts from 154 plants, 53 of these killed up to 83.2% of the HepG2 cancer cells. The killing rates of extracts from the three plants Cephalotaxus sinensis (CSL), Elsholtzia stauntonii (ESB) and Euonymus alatus (EAS) were the highest, up to 80.8%, and their toxicity to LO2 human hepatocyte cell line was as low as 6.1%. For ESB, the effects were most pronounced for the spring and winter samples. The trunk of ESB in spring has the highest killing rate of liver cancer cells, reaching 82.3% (Fig. 3). For EAS, autumn root bark possessed the highest killing rate of liver cancer cells reaching 82.1% (Fig. 3). To confirm these extracts with toxicity towards liver cancer cells, we carried out multi season collection and extraction of plants with a variety of solvents. In this round of screening, tree species with a killing rate greater than 60% in the first round of test were selected to obtain their cytotoxicity data (see Supplementary Information). The killing rate of plant CSL on HepG2 cancer cells was more than 50% with 23 extracts providing a maximum killing rate of 72.1%, with CSL summer root bark extracts having a cytotoxicity to human normal hepatocyte LO2 of 12.5%. The killing rate of ESB spring bark on HepG2 cancer cells was more than 50%, with a maximum killing rate of 80.8% and a cytotoxicity to human hepatocyte LO2 cell line of only 6.1%. The killing rate of EAS summer extract on HepG2 cancer cells was more than 50% for 11 extracts, of which the
3.2. Chemical analyses and modelling of target genes

We analyzed the extracts of the 3 plants by GC-MS and LC-QTOF-MS to determine the composition of the extracts and their main compounds. GC-MS was used for the analysis and identification of volatile thermally stable compounds, while LC-QTOF-MS was used for non-volatile and ionized metabolites. The GC-MS analysis of three plant extracts showed 40–69 compounds (Fig. 3b and c), while the more sensitive and accurate LC-QTOF-MS showed 100–283 compounds in same three extracts (Fig. 3d and e). The 280 types of compounds detected in CSL branch extracts form R–OH (<5.58%), R–O–H/R–O–R’ (<11.99%), R–COOH (<1.17%), R–N (<12.54%) and others (<63.63%) (Table S3). The 100 active compounds detected in ESB wood were R–OH (<33.82%), R=O–H/R=O–R’ (<9.13%), R–COOH (<1.42%), R–N (<2.71%) and others (<30.16%) (Table S4), while the 230 compounds in EAS root–bark extracts were R–OH (<27.45%), R=O–H/R=O–R’ (<4.29%), R–COOH (<2.20%), R–N (<0.96%) and others (<43.15%) (Table S5). These compounds include alcohols, esters, phenols, ketones, ethers and acids (Tables S3–5).

Table S6 lists several anti-cancer active components in the extracts of the three plants (CSL, EAS and ESB). Lupeol has a growth inhibitory and induce apoptosis in prostate cancer cell line LNCaP and CWR22Rv1 (Liu et al., 2013). Procyanidin A2 is a flavonoid compound present in cranberry and cowberry. It is known for its anti-cancer, antioxidant, anti-bacterial, and anti-inflammatory activities (Wen, 2014). Amentoflavone is a natural flavonoid compound with diverse biological activities, including anti-inflammatory, antioxidant, blood sugar-lowering, and anti-tumor properties (Fei et al., 2012). The acid substances include roburic acid, linolenic acid, caenothac acid, lithospermic acid, and studies have shown that they have anti-tumor effects under certain conditions (Attar-Bashi et al., 2004; Chan and Ho, 2015; Chen et al., 2017). Jatrorrhizine is a tetrahydroprotoberine alkaloid, which induces tumor cell growth by increasing the expression of cell-cycle inhibitors.
P21 and P27, inhibiting cell cycle stagnation of melanoma cells which results in restricted tumor cell growth (Wang et al., 2019). When emodin concentration is 10 μg/mL, the maximum growth density of human lung cancer A-549 cells can be reduced, and the split index is lowered, thereby inhibiting lung cancer cell growth (Zhang et al., 2022).

The three plants CSL, EAS and ESB inhibit liver cancer through multiple drug targets (Fig. 5). Our modelling of target genes showed that apigenol from plant CSL inhibits PMA mediated tumor promotion through inhibition of protein kinase C and oncogene expression involving 18 target genes such as AKT1 and ALOX5 (Fig. 5-a). Also, lupeol from plant EAS inhibit liver cancer and leukemia cells growth and apoptosis through estrogen receptor (ER) and target hepatoma cells through seven target genes including NR1H4 and CDC25A (Fig. 5-b). In addition, the phytochemical panaxydol from plant ESB that inhibits the proliferation of hepatoma cells including NTRK1 and MAPK1 is among the 14 involved genes (Fig. 5-c).

### 3.3. Active substances

Using FCM combined with LC-QTOF-MS offers a high-speed screening technology for qualitative and quantitative analysis in apoptosis studies providing high throughput, precision and reliability (Bendall et al., 2011; Robinson and Roederer, 2015; Wang et al., 2015; Zeng et al., 2010). Laboratory studies show that among these active substances, the natural alkaloid piperlongumine increase the level of reactive oxygen species (ROS) and selectively kill cancer cells (Mohler et al., 2014). In addition, piperlongumine can also promote autophagy and mediate tumor cell death by inhibiting Akt/mTOR signaling pathway. Esculentoside A alleviate LPS (lipopolysaccharide) induced acute lung injury by inhibiting the production of inflammatory factors, and reduce the release of LPS, while the plant flavonoid apigenol inhibits cell proliferation through blocking of cell cycle in G2/M phase (Lee et al., 2007; Ujiki et al., 2006). Morin has photosensitive killing effect on ascitic hepatoma cells by inhibiting DNA synthesis of ascitic hepatoma cells. The active ingredient crocin-A inhibits the proliferation of tumor cells by affecting the transcription of RNA and the synthesis of related proteins (Amin et al., 2016; Zhang et al., 2020).

Laboratory studies show that umbelliferone in combination with anticoagulant, antioxidant and antitumor treatments reduced cell proliferation and induced dose-dependent apoptotic events in human renal cell carcinoma cells, while crocin A project against liver injury and inhibit a broad number of cancer cell lines (Ali et al., 2021; Wang et al., 2019). Other studies show how panaxydol has a time and concentration dependent inhibitory effect on liver cancer cells causing cancer cell death (Guo et al., 2009; Prakash et al., 2009), while lupeol down-regulate the expression level of marker CD133 of TIC cells, and thereby inhibit liver cancer cells (Liu et al., 2013). Aucubin is a iridoid glycoside compound existing in natural plant species, promoting liver protection through anti-inflammatory and anti-tumor effects (Kim et al., 2014). Moreover, flavonoid extract from Zingiber officinale rhizome also induce cytotoxic effects on cancer cell lines through DNA damage (Ajith, 2010). Both water and ethanol extracts from roots of Tinospora cordifolia exhibit effective growth inhibition in MCF-7, MDA-MB-231 and HeLa cancer cells (Maliyakkal et al., 2013). The contents of active substances obtained from different plant extracts are listed in Tables S8 and S9. Several of these active substances that inhibit the growth of cancer cells need further isolation, extraction, and multi-step testing for future research purposes.

Fig. 5. Gene target network model of the chemical components of samples from CSL, EAS and ESB acting on liver cancer HepG-2 cells. Purple represents gene targets consistent with liver cancer while others are not consistent.
3.4. Targeted therapy

Targeted therapy is one of the effective means of transformation therapy in cancer treatment. At present, the kinase inhibitors such as sorafenib are used for targeted therapy of liver cancer cells reducing hepatocellular carcinoma volume of intrahepatic and extrahepatic metastases in combination with bioactive plant extracts such as corosolic acid found it in Actinidia chinensis (Johnson and Billingham, 2009; Wu et al., 2018; Yoshimoto et al., 2018; Zhang et al., 2022). The development of plant-based medicine opens up new windows for targeted therapy in cancer treatment. At present, the kinase inhibitors such as -lipoic acid (α-LA) prevent oxidative stress while inducing apoptosis in liver cancer cells (Pibiri et al., 2020) while berberine alkloid protect plasmid DNA from H2O2 induced lesions in human promyelocytic cancer cells (HL-60) (Khan et al., 2010). Resveratrol in soybean, peanut and pomegranate lead to apoptosis and anti-proliferation effects on human cervical cancer cells through upregulation of Bcl-2 related X protein and inducing p53 expression (Talib et al., 2020). Apoptosis causes a series of changes in morphological, biochemical and molecular biological properties, including cell shrinkage, nuclear chromatin condensation, and changes in cell membrane permeability, induced protease regulation to molecular activation. Therefore, these specific phytochemicals may be potential one drug one-target candidates for future development and Phase-1 III testing.

In conclusion, the plant extracts in our study are mixtures with many anticancer and antitumor chemicals having multiple target endpoints. Malignant tumors bring large economic burden to the society. The extracts of the three plants we screened had significant cytotoxicity to liver cancer cells. Extracts of Cephalotaxus sinensis, Elsholtzia stauntonii and Euonymus alatus induced apoptosis and death of HepG-2 cells in vivo and in vitro, which was the proportion of apoptosis rate in normal cells over 80%, which had a small side effects on LO2 of normal liver cells. The maximum reduction of tumor volume and tumor weight in animals treated with plant extract reached 67.6% and 62.5% within 2 weeks. In conclusion, this paper presents forest plant extracts that kills liver cancer cells that may provide a foundation for the development of forest plant anticancer drugs.

Author contributions


Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2023.117474.

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