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Analysis of the Cytotoxic Effect of Ethanolic Calendula Officinalis Plant Extract on Hepatic Carcinoma

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Abstract. Asteraceae is a family of angiosperms, many members of which exhibit bioactivities against various carcinomas owing to their rich content of terpenes, terpenoids and flavonoids. Common examples are Artemisinin extracted from plants belonging to the genus Artemisia, and Dehydrocostus lactone extracted from the roots of plants belonging to the genus Aucklandia. This essay focuses on the effect of an extract of Calendula officinalis, a widely distributed plant of the Asteraceae family, commonly used as a natural source of chemicals for medical and supplement development, on liver carcinoma cells and normal fibroblasts for comparison. Hepatocellular carcinoma cell line HepG2 and human fibroblast cell line HFF were used. The CCK-8 cell viability assay was applied to determine the inhibitory effect of the extract on cancerous cells and the promotional effect on HFF cells. The result showed that Calendula officinalis extract has a certain concentration of chemicals with a certain degree of anti-proliferative effect and strong specificity.

Keywords: Calendula officinalis, Natural products, Plant extract, Terpenoids, Terpenes, Liver carcinoma

1. Introduction

Liver cancer is among the cancer types with the highest mortality rates worldwide(7.8%), ranked just below lung(18.7%) and colorectal(9.3%) cancers [1]. Since the advent of modern pharmaceutical science, plants have been a major source for drug development, especially for the chemotherapy of cancer. Among angiosperm families, Asteraceae stands out for its exceptional diversity of bioactive terpenoids and flavonoids, many of which exhibit selective cytotoxicity toward cancer cells. Recently, many plants from the family Asteraceae — a widespread angiosperm group commonly used as herbs — have been proven to have antioxidant activity, cytotoxicity and anti-metastasis effects, given by their richness of secondary metabolites such as flavonoids and terpenoids [2]. This article focuses on the anti-proliferation effectiveness on liver cancer cells of the extract from Calendula officinalis, a common species belonging to Asteraceae, using the CCK-8 method for cancer cell viability assay, investigating the potential effect of chemicals in *Calendula officinalis* on cancer, and comparing the effect of sesquiterpene lactones and triterpenoids in Asteraceae for further studies.

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2. Materials and methods

2.1. Cell cultivation

(1) The thawed HepG2 cells were seeded at a density of $1*10^5$ cm^{-2} into a T25 culture flask, supplemented with DMEM culture medium containing 10% FBS to a final volume of 5mL, and then cultured in an incubator at 37°C with 5% of CO_2. After three days of incubation, the medium was discarded, and the cells were collected and cultured in an incubator at 37°C with 5% CO_2 after rinsing with 0.9% sodium chloride solution, supplemented with 1mL of TrypLUS cell dissociation solution for 5 minutes to allow cell detachment.

(2) The thawed HFF cells were seeded at a density of $2*10^4$ cm^{-2} into a T25 culture flask, supplemented with DMEM culture medium containing 15% FBS to a final volume of 5mL, and then cultured in an incubator at 37°C with 5% CO_2. After three days of incubation, the medium was discarded, and the cells were collected and cultured in an incubator at 37°C with 5% CO_2 after rinsing with 0.9% sodium chloride solution, supplemented with 1mL of TrypLUS cell dissociation solution for 5 minutes to allow cell detachment.

2.2. Cell viability assay

(1) A 96-well plate was used. Blank groups, and control and experimental groups were set up respectively, each consisting of 6 replicate wells. The blank groups only received culture medium; the control group received cells and culture medium; and the experimental groups received cells and culture medium supplemented with *Calendula officinalis* extracts at 5 different concentrations (0.25%, 0.5%, 1%, 2%, and 4%). Each group contained 6 replicate wells.

(2) HepG2 and HFF cells were seeded into a 96-well plate to establish the control and 5 experimental groups according to the method described in 3.1, at seeding densities $4.5*10^4$ cm^{-2} and $0.9*10^4$ cm^{-2}, respectively.

(3) After 3-4 days of culture, the optical density values of cells in each group were measured using the CCK-8 assay. 10μL of CCK-8 solution was added to each well, and the plate was then incubated in a 37°C incubator with 5% CO_2 for 3h. The OD value of each well was measured using a microplate reader at a detection wavelength of 450nm and a reference wavelength of 630nm. The cell proliferation promotion/inhibition rates were then calculated.

3. Results

Table 1. Morphological observations of HFF and HepG2 cells treated with different concentrations of *Calendula officinalis* extract

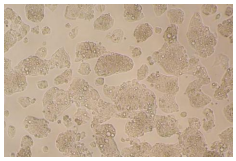
Groups	HFF	HepG2
Control		

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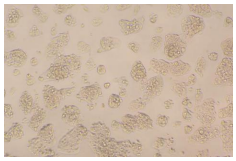
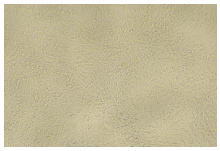
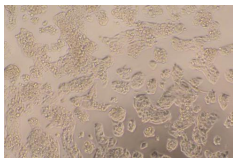
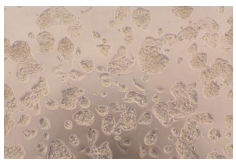
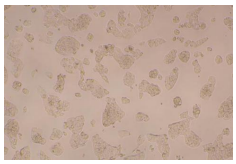
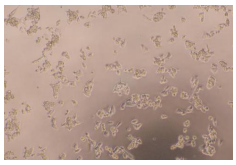
Table 1. (continued)

250μg/ml		
500μg/ml		
1000μg/ml		
2000μg/ml		
4000μg/ml		

To evaluate and predict the inhibitory effect of *Calendula officinalis* extract on HepG2 cells, we collected data from cell viability assays at doses ranging from 250μg/ml to 4000μg/ml (Table 1), which were then fitted with a sigmoid curve. The inhibitory rate was relatively low at doses below

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1000 μg/ml; however, it increased sharply over the range of 2000-4000 μg/ml. As the extract concentration went higher, the inhibitory rate approached 89%. Based on the prediction line, the extract's $IC_{50} \approx 3540$ μg/ml (Figure 1).

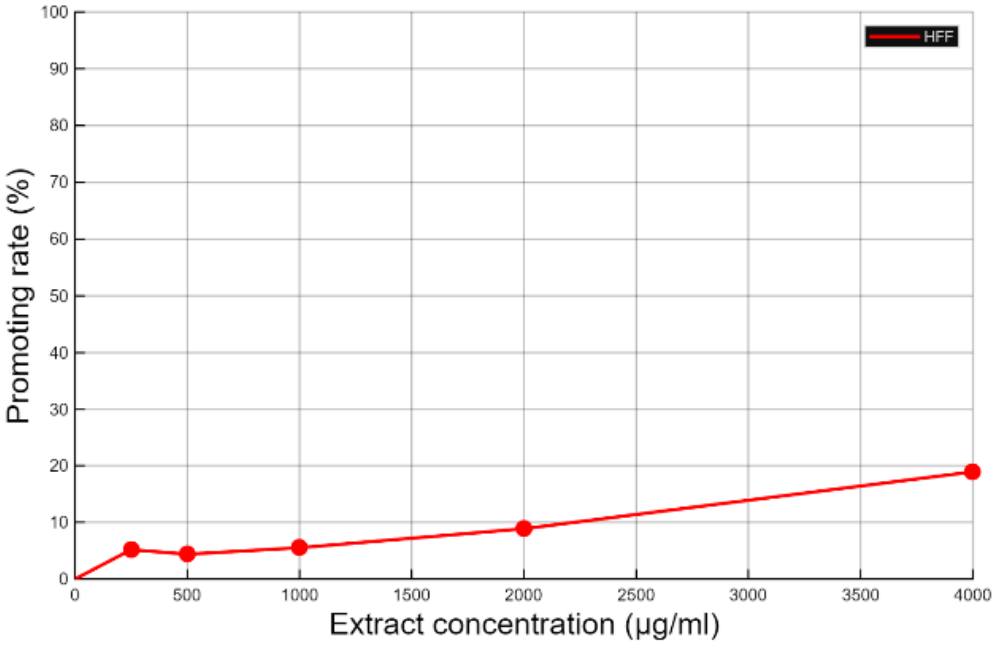

Figure 1. The inhibitory rate of the extract on HepG2 cells over different concentrations, the line was fitted using a sigmoid curve

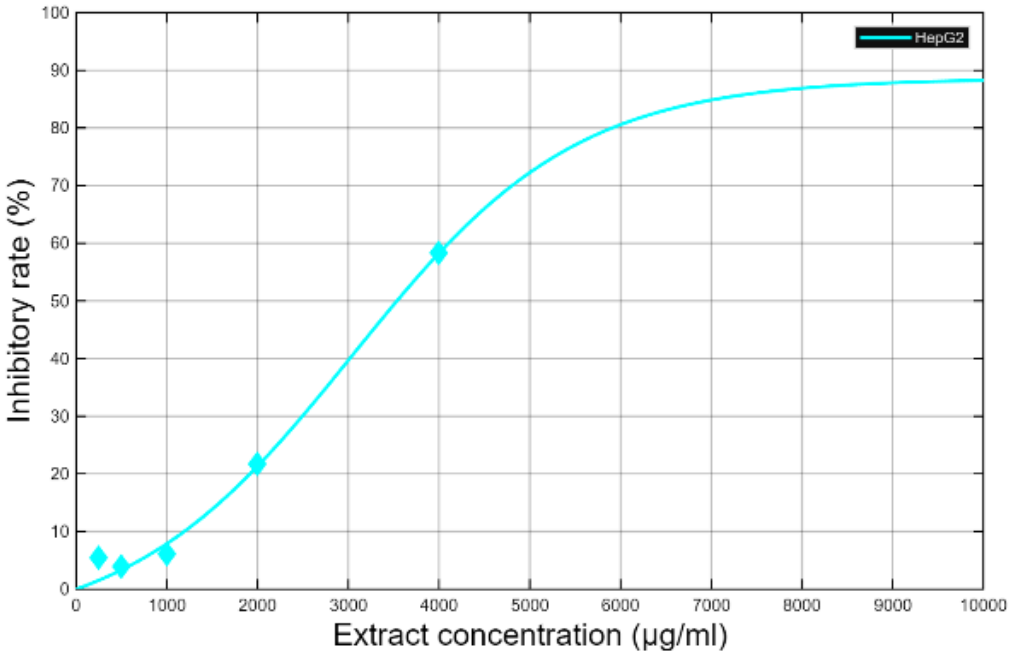

Figure 2. Promoting rate of the extract on HFF cells over different concentrations

The extract also showed a slight promoting effect on HFF cells (Figure 2). At any non-zero concentration, the extract promoted the proliferation of HFF cells, with the effect being most

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pronounced at concentrations of 4000 μg/ml or higher. This suggests that *Calendula officinalis* extract possesses a certain degree of target specificity.

Although the predicted anti-proliferative effect of the extract at high concentrations showed a high maximal activity against HFF cells with specificity, the effective dose was significantly higher than that of mainstream drugs for liver cancer. This suggests the presence of 1 or more low-concentrated highly effective active chemicals, which can be identified for drug production.

4. Discussion

Many sesquiterpenes and sesquiterpenoids are volatile and cannot be obtained by alcoholic extraction; most terpenes and terpenoids obtained are triterpenes and triterpenoids. Various studies have shown that sesquiterpene lactones in Asteraceae have a stronger inhibitory effect than triterpenoids over a short period of time. Marzouk, A. M. et al.'s research has shown that two sesquiterpene lactones (1a-methoxy, 8a-methacryloyloxy-hirsutinolide-13-O-acetate and 8a-methacryloyloxy-hirsutinolide-13-O-acetate) have a higher inhibition effect than lanost-3b,23S-dihydroxy-22(31)-ene which is a triterpene, via cytotoxic assay against HepG2 (Hepatic tumor), HeP2 (Epithelioma), HCT-116 (Colon tumor) and MCF-7 (Breast tumor) [3]. All these compounds were extracted from Vernonia leopoldii (Asteraceae). Toyang NJ et al.'s research has shown that sesquiterpene lactones extracted from *Vernoniaguineensis* exert potent anti-proliferative activity the proliferation of various types of cancer cells, with a low IC50 around 0.4~2.0 μM [4, 5]. Artemisia species (e.g., A. absinthium and A. scoparia), which belong to the tribe Anthemideae and the supertribe Senecionodae (alongside Calenduleae), exhibit very low IC_{50} values (~50-100 μg/mL) [6, 7]. They are well known for their abundant sesquiterpene lactone content, of which artemisinin is a famous example. The listed chemicals above show a large difference in inhibition compared to the results of this article; the reasons could be differences in the mechanisms of inhibition between the two categories of compounds. Sesquiterpene lactones possess specific structural features, such as α-methylene-γ-lactone, which give them the ability to inhibit tumor proliferation through selective alkylation of growth-regulatory biological macromolecules such as key enzymes controlling mitosis, and through the inhibition of nuclear DNA synthesis [8]; for triterpenes and triterpenoids, Yue, S. et al.'s research has suggested that lanostane triterpenes, reduce proliferation by lowering the level of expression of cyclin-dependent kinases to arrest the G2/M phase, and by lowering the level of expression of matrix metalloproteinases, a type of protein that breaks (hydrolyzes) extracellular matrix for metastasis [9]. This suggests that sesquiterpenes have higher cytotoxicity over short timeframes because they directly block the synthesis of DNA, whereas triterpenes & triterpenoids can only show their effect in a longer observation. Arresting the growth phase only delays mitosis, while inhibiting metastasis doesn't reduce proliferation. Therefore, the high IC50 of the Calendula officinalis alcoholic extract observed in this study may reflect the intrinsic characteristics of triterpenoid components in short-term assays, rather than a lack of anti-tumor potential of the plant itself.

5. Conclusion

This study demonstrates that Calendula officinalis extract exerts dose-dependent anti-proliferative effects on HepG2 hepatocellular carcinoma cells while promoting normal fibroblast proliferation, indicating selective bioactivity. However, the effective concentration is substantially higher than that of sesquiterpene lactone-rich extracts from related Asteraceae species, suggesting that alcoholic extraction captures primarily triterpenes with slower-acting mechanisms. The therapeutic potential

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of C. officinalis may therefore depend on alternative extraction methods that preserve volatile sesquiterpenes, or on fractionation to isolate more potent constituents. Future work should compare the chemical profiles of essential oil versus alcoholic extracts, evaluate long-term triterpene effects on cell cycle kinetics, and identify the specific compounds responsible for the observed selectivity.

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