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Effects of Hepaclin-4 on Hepatocellular Carcinoma (Hep G2)

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Objective: Liver cancer is the fifth most frequent cancer worldwide and is usually diagnosed at an advanced stage. Natural products play an important role in anticancer drug therapy because they are effective in treating specific characteristics of cancer while also effective at reducing side effects. However, the cytotoxicity and anti-proliferative effects of *Scutellaria baicalensis Georgi, Saussurea amara L, Chiazospermum erectum (L) Bernh, Carthamus tinctorius L*., remain unclear. Our research aims to investigate the anti-cancer properties of Hepaclin-4 (Hep-4) extracts from the above mentioned plants. **Methods:** To evaluate the in vitro cytotoxicity of Hep-4, an ethanol extract of Hep-4 (500, 250, 125, 100, 50, and 10 µg/mL)L, doxorubicin, and 5-fluorouracil (5-FU) were assessed against the Hep G2 hepatocarcinoma cell line by monitoring proliferation using the MTT assay method. **Results:** The results demonstrated growth inhibition of cells in both dose-and time-dependent manners. Hep-4 inhibited Hep G2 transformation in 45% and the comparative group, 5-FU, effects on 40% of it. **Conclusion:** Our findings suggest that Hep-4 extracts have the potential to be a potent cytotoxic agent against Hep G2 cells, as it has commendable anti-proliferative effects against hepatocarcinoma cell lines and can be considered an effective adjuvant therapeutic agent.

Keywords: Medicinal Plants, Biological Products, Hepatocellular Cancer, Cell Line

Introduction

Cancer, a complex disease that results from genetic and epigenetic modifications of tumour suppressor genes or oncogenes, can be developed because of alterations of apoptosis-signalling. The breakdown of the apoptosis process is observed in many human tumours, which may transform a normal cell to a tumour cell [1]. Apoptosis is one of the primary targets for a majority of conventional anti-cancer drugs. These drugs are able to induce fatal intracellular damage, which often activates a downstream cascade of molecular events [2].

Hepatocellular carcinoma (HCC), a type of liver cancer, is a

high incidence and fatal disease that is usually diagnosed at an advanced stage; it is the fifth most frequent cancer worldwide, and it is the third most fatal cancer [3-5]. In 2008, 748,000 liver cancer cases and 696,000 mortalities were estimated worldwide [6]. HCC is the second leading cause of cancer-related deaths in many Asian regions, with peak incidences in East Asia [7-8]. Despite recent scientific advancement in hepatology, liver problems have continued to increase [9]. According to 2014 epidemiological statistics from International Agency for Research on Cancer, 97.8 males and 61 females per 100,000 in Mongolia suffered from liver cancer; liver cancer occupied 78.8% of all the morbidity; and the mortality rate was registered as 7.4 times higher than the global average [10].

Natural products, derivatives of natural products, and synthetic pharmaceuticals based on natural products play a principal role in anti-cancer drug therapy [11, 12]. Natural products as medicines are effective in treating specific characteristics while also effective at reducing side effects [13]. Thus, research of natural products to help humans overcome many newly emerging and known diseases, particularly cancers, is in high demand. In recent years, ethnomedicine has developed new pharmaceutical products based on the traditional medicine. Hepaclin-4 (Hep-4) is contained in plants used for treating different kinds of fever, as well as the liver diseases: *Scutellaria baicalensis Georgi, Saussurrea amara L, Chiazospermum erectum (L) Bernh, and Carthamus tinctorius L. Baicalin* is found in several species in the genus *Scutellaria*, including in *Scutellaria baicalensis* and *Scutellaria lateriflora. Baicalin* is a known prolyl endopeptidase inhibitor. It induces apoptosis in cancer cells [14]. Furthermore, *Saussurea amara L* (sesquiterpene) has been shown to inhibit the mRNA expression of iNOS by lipopolysaccharide stimulated macrophages, thus reducing nitric oxide production [15]. Thus, we conducted a in vitro study on the anti-cancer effects) of Hep-4 on Hep G2.

Methods

Plant Material

The plant sample was collected from Khentii, Tuw, Umnugobi, and Bulgan provinces in Mongolia in June through August, 2016. The plant sample was identified by Dr. Ganbold. E (Assistant Professor of Plant Systems, Institute of Botany, Mongolia). Fresh herbs of Hep-4 were collected, washed, and air dried at room temperature. The dried material was homogenized to obtain a coarse powder and stored in airtight bottles. Approximately 5g of the powdered material was subjected to soxhlet extraction (Electrothermal Eng. Ltd., Rochford, UK) using 150 mL 70% ethanol. The dry residue of ethanol extract was dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) to obtain the stock solution (1000 µg/mL).

Cell Culture

Human hepatoma cells, Hep G2, were obtained from the American Type Culture Collection (Manassas, VA, USA). Hep G2 cells were grown in RPMI-1640 supplemented with 10% FBS and 100 IU/mL penicillin streptomycin. The cultures were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

MTT Cytotoxicity assay

Hep G2 cells were seeded at a density of 1×10 6/well into 96-well culture plates and incubated overnight before being exposed to various concentrations of Hep-4 extract (500, 250, 125, 100, 50, and 10 µg/mL). Doxorubicin was used as the positive control, and untreated media was used as the negative control. After 24, 48, and 72 hours, 20 µg/mL of MTT solution was added to each well and incubated for 4 hours. The procedure was repeated at least three times. After adding 100 µL of DMSO, the absorbance was measured using an ELISA reader (BMG Labtech, Ortenberg, Germany) at a test wavelength of 450 nm. The absorbance of the treated and control Hep G2 cells were used to determine the cytotoxicity of extract according to the following formula: cytotoxicity (%) = ((absorbance of treated cells)/(absorbance of negative control)) × 100 [16].

Migration Assay

Migration is a key property of live cells and critical for normal development, immune response, and disease processes such as cancer metastasis and inflammation. Methods to examine cell migration are very useful and important for a wide range of biomedical research, including cancer biology, immunology, vascular biology, cell biology, and developmental biology. In our study, we used a cell culture wound closure assay in which a scratch was generated on a confluent cell monolayer to assess the speed of wound closure.

Statistical Analysis

The data were expressed as mean ± SD and difference among treated (ethanol extract and water extract) and untreated cells were analyzed using two-way ANOVA followed by the Bonferroni's multiple comparisons test. Statistical significance was set at p-value <0.05 or p-value <0.01. GraphPad Prism Software 7.0 was used to conduct all statistical tests.

Results

The anti-proliferative effects Hep-4 extracts were illustrated by the inhibition effect on the cell proliferation in a time and dose-dependent manner. Water extract of Hep-4 did not show the statistical significance, when the compared the DMSO. The ethanol extract of Hep-4 (100 µg/mL), significantly increased anti-proliferative effects on the Hep G2 cell line, when the compared to the DMSO. The ethanol extract of Hep-4 (100 µg/mL and 250 µg/mL) significantly inhibited and reduced the growth of Hep G2, whereas water extract only prevented the growth Hep G2 (Table 1). The anti-proliferative effects of Doxorubicin versus 5-Fluorouracil (5-FU) can be seen in their significant inhibition of Hep G2 growth in 24 hours, compared to the DMSO. However, anti-proliferative effects of Doxorubicin are significantly low compared to 5-FU (Table 2). Hep G2 cells were treated with water extract and 70% ethanol extract of Hep-4 for 72 hours. 70% ethanol extract of Hep-4

Table 1. Anti-proliferative activity of Hepaclin-4 extracts on Hep G2 over 24 hours

Hepaclin-4			Bonferroni's Multiple Comparisons Test	
Dose	Water Extract	Ethanol Extract (70%)	95.00% CI of diff.	Adjusted p-value
500µg/mL	101.32±5.8	102.18±8.9	-15.88 to 14.16	>0.9999
250µg/mL	105.97±10.8	88.41±2.8**	2.538 to 32.58	0.0054
125µg/mL	100.95±4.7	88.16±4.7	-2.232 to 27.81	0.3002
100µg/mL	99.13±9.0	83.36±6.5#*a	0.7484 to 30.79	0.0264
75µg/mL	97.86±9.2	85.39±9.2#a	-2.552 to 27.49	0.3826
50µg/mL	93.01±3.1	87.68±7.9	-9.692 to 20.35	>0.9999
25µg/mL	95.53±2.1	86.17±5.7	-5.662 to 24.38	>0.9999
10µg/mL	95.29±2.4	86.09±3.2	-5.822 to 24.22	>0.9999
DMSO	100±1.5	99.08±3.4	-14.1 to 15.94	>0.9999

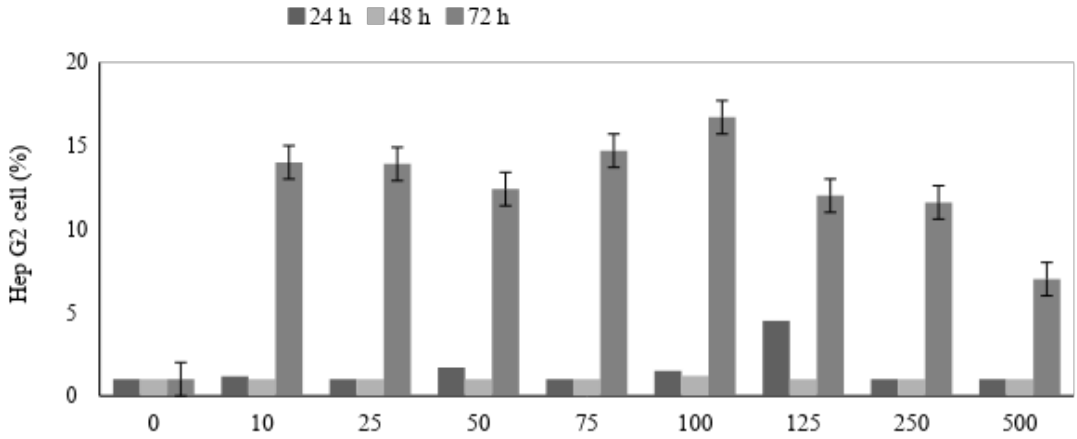
#p<0.05 Water Extract and Ethanol Extract vs DMSO; ap<0.05 Ethanol Extract 100 µg/mL and 75µg/mL vs Ethanol Extract 500µg/mL;*p<0.05, **p<0.01 Doxorubicin vs 5-Fluorouracil. Two-way ANOVA post hoc Bonferroni's Multiple Comparisons Test (Row factor F (8,72)=4.691, p=0.0001; Column Factor F (1, 72) = 47.87, p<0.0001; Interaction F (8, 72) = 2.54, p=0.0170)

Table 2. Anti-proliferative activity of Doxorubicin and 5-Fluorouracil on Hep G2 over 24 hours

Percentage of Viability (%)			Bonferroni's Multiple Comparisons Test	
Dose	Doxorubicin	5-Fluorouracil	95.00% CI of diff.	Adjusted p-value
150µg/mL	60.50±3.2#**	75.44±2.3#	-21.93 to -7.945	<0.0001
100µg/mL	68.03±3.4#**	78.34±3.3#	-17.3 to -3.315	0.0002
75µg/mL	71.93±1.9#**	82.43±2.5#	-17.49 to -3.505	0.0001
50µg/mL	76.04±3.3#**	84.45±1.8#	-15.4 to -1.415	0.0051
25µg/mL	80.47±2.2#*	88.21±1.5#	-14.73 to -0.745	0.0153
DMSO	100±4.4	101±5.0	-7.995 to 5.995	>0.9999

#p<0.05 Doxorubicin and 5-Fluorouracil vs DMSO; *p<0.05, **p<0.01 Doxorubicin vs 5-Fluorouracil.

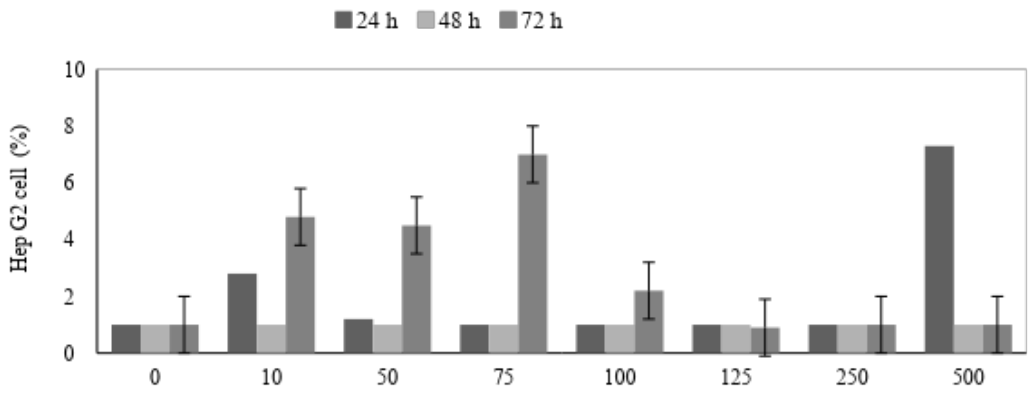
Two-way ANOVA post hoc Bonferroni's Multiple Comparisons Test (Row factor F (5,48)=134.6, p<0.0001; Column Factor F (1, 48) = 123.4, p<0.0001; Interaction F (5, 48) = 5.554, p=0.0004)



Vertical Axis: Cytotoxic Effect of Hepaclin-4 (EtOH) on Hep G2 (%)

Horizontal Axis: The Concentrations of Hepaclin-4 (EtOH), doses 10 µg/mL-500 µg/mL, in hours

Figure 1. The Effect of 70% Ethanol Extract of Hepaclin-4 on Hep G2 (%).



Vertical Axis: Cytotoxic Effect of the Hepaclin-4 (OWE) on Hep G2 (%)

Horizontal Axis: The Concentration of Hepaclin-4 (OWE), doses 10 µg/mL-500 µg/mL, in hours.

Figure 2. The Effect of Water Extract of Hepaclin-4 on Hep G2 (%).

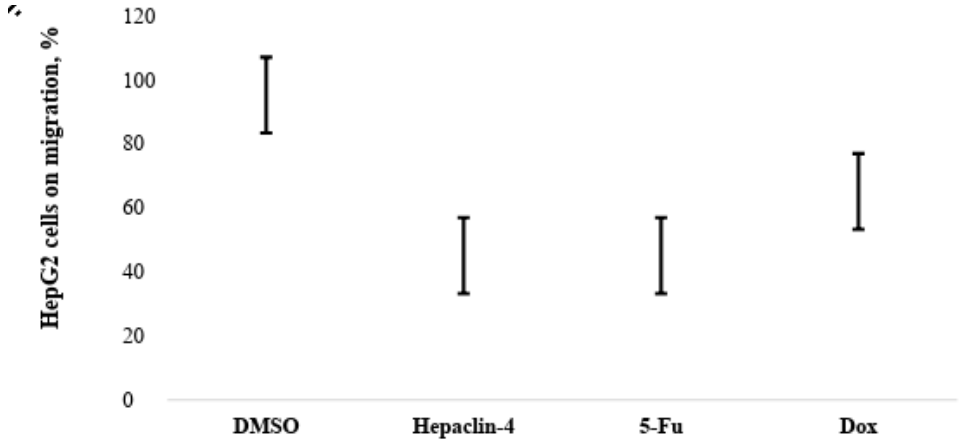
had significantly better effects, though it was dose-dependent ($p<0.05$). Hep-4 ethanol extract 500 µg/mL had similar results to the control group ($p>0.05$) (Figure1).

After 72 hours, the 100 µg/mL ethanol extract and 50 µg/mL water extract held back Hep G2 by 16.7% and 7.5%, respectively. The Hep-4 extracts showed inhibitory effect on replication, growth, invasion, and adhesion of cancer cells (Figure 2).

Hep-4 inhibited 45% of Hep G2 transformation in 45%, compared to 40% inhibition by 5-FU (Figure 3).

Discussion

Natural products and pharmaceutical compounds have been studied as cancer chemopreventive agents, in vitro and in vivo [17]. There is limited information on the medicinal value of *Scutellaria baicalensis* Georgi, particularly regarding its cytotoxicity against cancer cells. Our study demonstrated that Hep-4 was an effective inhibitor against Hep G2 proliferation, suggesting the presence of bioactive compounds in Hep-4 [18]. However, the positive control, doxorubicin, showed



Vertical Axis: Hep G2 Cell Migration (%)

Horizontal Axis: Hepaclin-4 & comparative groups

Figure 3. The Effect of 70% Ethanol Extract of Hepaclin-4 and the comparative groups on the cell migration of Hep G2 (%).

a considerably stronger effect on Hep G2 in comparison to Hep-4. Furthermore, Hep-4 only exhibited stronger cytotoxic activity after 72 hours of exposure at higher concentrations, whereas the cytotoxic effects of lower concentrations were not significantly different between 24 and 48 hours. This indicates that, under low concentrations, some Hep G2 cells had been killed initially, but over time, surviving cells were stimulated for proliferation or had adapted to the treatment and recovered. However, high Hep-4 doses inhibit proliferation over time.

According to a previous study, *Scutellaria baicalensis Georgi* extract was able to inhibit the Madin-Darby Canine Kidney (MDCK) cell line by 36%, compared to the control, but the skullcap preparation at in100 µg/mL was able to inhibit MDCK cell division by 77%. This study showed that the skullcap preparation has a greater ability to inhibit MDCK (and pheochromocytoma) cell division than S*cutellaria baicalensis Georgi* extract [19].

In reality, cancer cells that exhibit resistance at one concentration may indicate growth inhibition at higher doses of the same preparation [20]. In addition, the applicability of a substance as a pharmacological drug depends on the balance between its therapeutic and toxicological effects [21]. Our results showed that Hep-4 extracts did not produce a cytotoxic effect towards normal cells, suggesting that the anticancer activity of Hep-4 might be specific to Hep G2 cells, in contrast to doxorubicin which was toxic against normal cells. The suppression of cancer cell growth may occur through interference with fundamental cellular functions, including through apoptosis [22]. The difference between cytotoxicity and apoptosis is demonstrated by a series of specific morphological features [23].

In a previous study, it was shown that some plants used in the traditional Mongolian medicine to treat hepatic disorders (i.e., *Saussurea amara L*) have a considerable choleretic effect, as studied in an isolated rat liver perfusion system, and have a low toxic potential [24]. To evaluate the cytotoxic effect of plant extract that are used in Mongolian traditional medicine, another study performed a sulforhodamine B colorimetric assay with Hep G2. The "original water extract" (OWE) from *Chiazospermum erectum (L)* Bernh., exhibited a pronounced cytotoxic effect on Hep G2. At concentrations of 4000 µg/mL, 400 µg/mL, 40 µg/mL, 4 µg/mL, and 0,4 µg/mL, cellular viability decreased by 25-50% [25].

These results have important clinical implications, as they show that traditional Mongolian medicinal plants have commendable anti-proliferative effects against human HCC without harming the normal cells. Further study of gene expression over time and study of more genes related to apoptosis will improve our findings and give us useful results to find a novel drug. Because of limited laboratory capacity, our study could not explore the mechanisms whereby Hep-4 extracts inhibit Hep G2. Future research should focus on the detailed studies of the cancer pathways and apoptosis mechanisms of Hep-4. Our findings suggest that Hep-4 extracts

have the potential to be a potent cytotoxic agent against Hep G2 cells, as it has commendable anti-proliferative effects against HCC cell lines and can be considered an effective adjuvant therapeutic agent.

Conflict of interest

Authors declare no conflict of interests.

Acknowledgements

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