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Chemoprevention Against Hepatocellular Carcinoma of *Cornus officinalis in vitro*

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Abstract: The water extracts of *Cornus officinalis* Sieb. et Zuce against hepatocellular carcinoma (HCC) was studied for its chemopreventive potential. Three HCC cell lines (HepG2, SK-Hep1 and PLC/PRF/5) and three leukemic cell lines (U937, K562 and Raji) were tested with XTT assay. Extracts of *C. officinalis* inhibited all these HCC cells and leukemic cells at a concentration of 100 μg/ml ($P < 0.05$) and was dose-dependent ($P < 0.0001$). P53 ($P < 0.0001$) and Ras ($P = 0.001$) significantly affected its activity against HCC. Extracts of *C. officinalis* also possessed the anti-oxidant activity through free radicals scavenging activity at a concentration of 50 μg/ml ($P < 0.05$). In summary, our experiment implied that *C. officinalis* might be a candidate for chemopreventive agent against HCC through the antioxidant and anti-neoplastic effects.

Keywords: *Cornus officinalis*; Free Radical; Hepatocellular Carcinoma; p53; Ras.

Introduction

Primary hepatocellular carcinoma (HCC) is frequently found in Asia. It is usually fatal without treatment (Dienstag and Isselbacher, 2001). To date, there are limited effective therapeutic modalities. The prognosis is further worsened by the frequent recurrence. Poor prognosis makes the prevention more important. Suppression of hepatic necroinflammation by drugs may prevent hepatocarcinogenesis (Okuno *et al.*, 2001). Free radicals are involved in inflammation (Wulf, 2002). They can also react with biomolecules in cells, including DNA.

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The resulting genotoxic damage to DNA, particularly double-strand breaks, or hindrance to DNA repair is implicated in mutagenesis and carcinogenesis (Dizdaroglu *et al.*, 2002; Lala and Chakraborty, 2001; Kagan *et al.*, 1999; Toyokuni and Sagripanti, 1993; Hochstein and Atallah, 1988). Furthermore, free radicals, such as reactive oxygen species (ROS), have growth-promoting effects related to redox-responsive signaling cascades. Even normal cells show increased expression of growth-related genes and cellular proliferation if exposed to ROS (Wulf, 2002). It was also postulated that the free radical scavenger system is impaired in HCC (Bellisola *et al.*, 1987). Free radicals might be involved in carcinogenesis of HCC during chronic inflammation.

Several natural products have been found to possess the ability of ameliorating free radicals induced DNA damage (Anderson *et al.*, 2001) and inducing apoptosis of malignant cells (Thatte *et al.*, 2000). Natural products have also been proven to prevent the development of HCC in patients with cirrhosis, particularly in patients without hepatitis B virus infection (Oka *et al.*, 1995). It was supposed that natural product might possess the activity against HCC. *Cornus officinalis* Sieb. et Zuce (Cornaceae) has been used in traditional Chinese medicine for many centuries. Several pharmacologic effects of its crude extracts were recently found. It possesses other effects including anti-neoplasm, anti-inflammation, inhibition of lipid peroxidation, hepatoprotection and other activities (Zheng *et al.*, 1998). Nevertheless, the anti-neoplastic effect of *C. officinalis* was reported against Erlish ascitic tumor cell only (Zheng *et al.*, 1998). Its effect on HCC has not yet been explored. To determine whether *C. officinalis* possesses the anti-HCC activity and free radical scavenging, we tested the effect of water extracts of *C. officinalis* on the HCC and leukemic cell lines by XTT assay in order to find natural crude drugs for chemoprevention against HCC.

Materials and Methods

Preparation of Plant Extracts and Chemicals

Fifty grams of the whole plant of *C. officinalis* was shade-dried and decocted for 1 hour with 1 liter of boiling reverse-osmotic water 3 times. The decoctions were combined, filtered, concentrated and lyophilized. The hot water extracts of *C. officinalis* (HWCO) was dissolved with 50 mM KH_2PO_4 (pH 7.8) (Ferak) solution or with re-distilled water for the evaluation of antioxidant activity and anti-neoplastic activity, (Lin *et al.*, 2000; Chang and Yeung, 1988). 5-Flurouracil (5-FU, Sigma) was suspended in dimethyl sulfoxide (DMSO, Sigma) as the positive control in cytotoxicity test. The final concentration of DMSO was less than 0.1% in experiments. Xanthine, xanthine oxidase (XO), cytochrome C, thiobarbituric acid (TBA), L(+)-ascorbic acid, sodium dodecyl sulphate (SDS) and 1,1,3,3-tetraethoxypropane (TEP) were all purchased from Sigma Chemical Co. Ferrous chloride ($FeCl_2$) was bought from Wako Pure Chemical Industries Ltd (Japan). XTT was obtained from Roche Diagnostics GmbH (Germany). *n*-butanol (Merck) and trolox (Aldrich) were all purchased.

Cell Lines and Culture

Three HCC cell lines, namely HepG2 [American Type Culture Collection (ATCC HB 8065), p53-positive; Ras-mutated; HBV-negative], SK-Hep1 (ATCC HTB 52, p53-positive; Ras-normal; HBV-negative) and PLC/PRF/5(ATCC CRL 8024, p53-negative; Ras-normal; HBV-positive) (Hsu *et al.*, 1993; Puisieux *et al.*, 1993) were used for the evaluation of anti-HCC activity. Three leukemic cell lines, namely U937 (ATCC-CRL-1593), K562 (ATCC-CCL-243) and Raji (ATCC-CCL-86) were used as the reference control of malignant cell. Leukemic cells were cultured with RPMI 1640 and HCC cells were cultured with Dulbeco's Modified Eagle Medium (DMEM). They were all supplemented with antibiotics (100 U/ml penicillin G sodium, 100 µg/ml streptomycin and 0.25 µg/ml amphotericin sulfate; Sigma) and 10% fetal bovine serum. Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2.

Cell Proliferation Assay

Inhibition of cell proliferation by HWCO was measured by XTT (2,3-bis [2-methoxy-4-nitro-5- sulfophenyl]-5-[(phenylamino)carbonyl-2H-tetrazolium hydroxide] assay. Briefly, cells were plated in triplicate in 96-well culture plates (1×10^4 cells/well). After 24 hours incubation, the cells were treated with HWCO (0, 100, 250 and 500 µg/ml) or 5-FU (0, 0.5, 1 and 5 µg/ml) for 3 days. DMSO 0.1% (w/w) was used as the solvent control of 5-FU. Fifty µl of XTT test solution, which was prepared by mixing 5 ml of XTT-labeling reagent with 100 µl of electron coupling reagent, was then added to each well. After incubation for 2 hours, the absorbance was measured on an ELISA reader (Multiskan EX, Labsystems) at a test wavelength of 492 nm and a reference wavelength of 690 nm.

Data was calculated as a percentage of inhibition by the formula: inhibitive rate (%) = $[1 - (OD_T)/(OD_C)] \times 100\%$, where OD_T and OD_C indicated the absorbance of the test compounds and the solvent control, respectively. The concentration of 50% inhibition (IC_{50}) was calculated as the concentration that achieved 50% cytotoxicity against cancer cells. The concentration of 50% cytotoxicity (CC_{50}) was estimated using normal human lymphocy (4×10^6 cells/ml) instead of malignant cell by the above methods. The selectivity index (SI) was determined by the ratio of the CC_{50} to the IC_{50}.

Animals and Preparation of Liver Homogenate

Male Wistar albino rats (4–6 weeks, 120–150 g) were purchased through the animal center, College of Medicine, National Cheng Kung University. The care and use of animals were in accordance with the "*Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*, 6th edition 1997, NHMRC." They were housed in an air-conditioned room at 23 ± 1°C, 55 ± 5% humidity, 12-hour light, and fed with a standard laboratory diet and tap water. Liver tissues were removed after animals were sacrificed. Liver was cut into

about 2 g pieces and stored individually at −70°C in plastic tubes. The frozen liver samples were homogenized in Tris-HCl buffer to give a 20% (w/v) homogenate before experiments.

Free Radicals Scavenging Activity

HWCO was diluted with working solution (xanthine/cytochrome c) and 20 µl of 1 unit/ml of xanthine oxidase was added. The final concentrations of HWCO were 0.05, 0.1, 0.5, 1 and 5 mg/ml. They were screened at a test wavelength of 550 nm for 2 minutes (Lin *et al.*, 2000; Chang *et al.*, 1995; McCord and Fridovich, 1969). Solvent was used as the negative control. The inhibitive rate and IC_{50} were calculated according to the method described above.

Inhibition of Xanthine Oxidase (XO)

HWCO was diluted with working solution (xanthine/cytochrome c) and 20 µl of 1 unit/ml of xanthine oxidase was added. The final concentrations of HWCO were 0.05, 0.1, 0.5, 1 and 5 mg/ml. They were screened at a test wavelength of 295 nm for 2 minutes (Lin *et al.*, 2000; Chang *et al.*, 1995; McCord and Fridovich, 1969). Solvent was used as the negative control. Allopurinol was used as the positive control. The inhibitive rate and IC_{50} were calculated according to the method described above.

Anti-Lipid Peroxidation in Rat Liver Homogenate

Liver homogenate of 250 µl was mixed with 100 µl Tris-HCl (pH 7.2), 50 µl of 0.21 mM ascorbic acid, 50 µl of 4 mM $FeCl_2$ and 50 µl tested samples. Trolox, at concentration of 1 mg/ml, was used as a positive control. Solvent was used as the negative control. After incubation at 37°C for 1 hour, 500 µl of 0.1N HCl, 200 µl of 9.8% SDS, 900 µl re-distilled water and 2 ml of 0.6% TBA were added sequentially. They were shaken vigorously and placed in a boiling water bath for 30 minutes. After cooling, 5 ml *n*-butanol was added and shaken vigorously. The *n*-butanol layer was separated by centrifugation at 1000 *g* for 25 minutes. The absorbance of the *n*-butanol layer was then determined by a spectrophotometer (HITACHI U-2000) at a test wavelength of 532 nm (Lin *et al.*, 2000; Kimuya *et al.*, 1981). The inhibitive rate and IC_{50} were calculated by the method described above.

Statistical Analysis

Analysis of variance (ANOVA) and Mann-Whitney U test were used to determine the significance of results of triplicate by statistical software of JMP5. The least square means Tukey HSD test was used for comparisons of pairs of groups in ANOVA. Multivariate linear regression was used for factorial analysis thereafter. Difference between pairs of groups with $P < 0.05$ was considered statistically significant.

Results

Cell Proliferation Assay

Hot water extracts of *C. officinalis* (HWCO) significantly inhibited the proliferation of HCC cells and leukemic cells compared to the solvent control. The effects were dose-dependent ($P < 0.0001$). HWCO inhibited HCC cells and leukemic cells at a concentration as low as 100 µg/ml ($P < 0.05$, Table 1). Among HCC cells, SK-Hep1 (71.4%) was more responsive to HWCO than HepG2 (55.4%) and PLC/PRF/5 (47.7%) ($P < 0.05$). HWCO was more effective against K562 (61.1%) than U937 (48.7%) and Raji (43.9%) ($P < 0.05$). HWCO inhibited HCC cells with IC_{50} between 110 µg/ml and 337 µg/ml, compared to those of leukemic cells between 186 µg/ml and 282 µg/ml. Most of the selective indexes of anti-neoplastic activity of HWCO were more than 5 (Table 2).

When we compared the anti-neoplastic effect of HWCO between HCC and leukemic cells, the overall anti-neoplastic effect of HWCO was greater against HCC cells (58.1%) than leukemic cells (51.2%) ($P = 0.0275$). Nevertheless, the effect was significant only at a concentration of 100 µg/ml (HCC cell versus leukemic cell: 35% versus 17%, $P < 0.05$). In contrast, the effect of 5-FU was greater against leukemic cells (53%) than HCC cells (44.5%) ($P = 0.0012$), but the significance was only found at the concentration of 5 µg/ml (leukemic cells versus HCC cells: 81.5% versus 68.8%, $P < 0.05$).

When we compared the inhibitory rates of HWCO between HCC cells with Ras and p53, we found that Ras (positive versus negative: 59.5% versus 43.5%; $P = 0.001$) and p53 (positive versus negative: 63.4% versus 39.7%; $P < 0.0001$) significantly affected the effect of HWCO against HCC cells. The effect of 5-FU was only significantly related to Ras (positive versus negative: 50.9% versus 26.6%; $P = 0.0003$).

Antioxidant Activity

HWCO showed free radicals scavenging activity at a concentration as low as 50 µg/ml ($P < 0.05$, Table 3) with an IC_{50} of 100 µg/ml. The higher concentration of HWCO was needed to inhibit xanthine oxidase (XO) and lipid peroxidation. HWCO inhibited XO and lipid peroxidation at concentrations above 500 µg/ml ($P < 0.05$) with IC_{50s} of 2620 µg/ml and 892 µg/ml (Table 3), respectively. That concentration is much higher than the dosage usually used in traditional medicine. These antioxidant activities were also dose-dependent ($P < 0.0001$).

Discussion

C. officinalis has been used as food and as Chinese traditional medicine for centuries. Its anti-neoplastic effect was only reported on Erlish ascitic tumor in culture (Zheng *et al.*, 1998). Its effect against HCC and leukemic cells has never been reported. We demonstrated that the hot water extracts of *C. officinalis* (HWCO) had the anti-neoplastic activity against

Table 1. The Inhibitive Rate of *C. officinalis* and 5-FU Against HCC Cells and Leukemic Cells

	Concentration (μg/ml)	Hepatocellular Carcinoma Cell lines			Leukemic Cell Lines		
		HepG2 (%)	SK-Hep1 (%)	PLC/ PRF/5 (%)	K562 (%)	U937 (%)	Raji (%)
Control		0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0.1 ± 0.1	0 ± 0.1	0.1 ± 0.1
C. officinalis	500	74.1 ± 1.5*	88 ± 3.1*	75 ± 3.8*	91 ± 1.7*	79 ± 3.2*	71.1 ± 1.6*
	250	67 ± 3.6*	77 ± 0.7*	37 ± 3.5*	71.1 ± 2.5*	51.1 ± 3*	46.8 ± 6.9*
	100	25 ± 1.2*	49 ± 3.3*	31 ± 1.7*	21.1 ± 2.6*	16 ± 4.5*	14 ± 1.9*
5-FU	5	57.9 ± 3.8*	72.8 ± 5.2*	75.6 ± 4.2*	84.3 ± 3.9*	80.3 ± 5.8*	80 ± 1.9*
	1	28.7 ± 2.7*	62.8 ± 4*	48.3 ± 3.4*	49.6 ± 4.4*	46.5 ± 3.8*	46.5 ± 3.3*
	0.5	8.9 ± 1.9*	32.8 ± 2.7*	12.8 ± 2.5*	35.7 ± 3.1*	27.2 ± 4*	27.2 ± 3.1*

HCC: Hepatocellular carcinoma.

*P < 0.05 comparing between test drugs and solvent control by ANOVA.

Table 2. The 50% Inhibitive Concentration (IC_{50}) of *C. officinalis* and 5-FU Against Leukemic Cells and Hepatocellular Carcinoma Cells

		Hepatocellular Carcinoma Cell Lines				Leukemic Cell Lines			
		HepG2	Hep3B	SK-Hep1	PLC/PRF/5	K562	U937	P3HR1	Raji
C. officinalis	IC_{50} (μg/ml)	208	> 500	110	337	186	245	208	282
	SI†	7.8	ND	14.6	4.7	8.6	6.6	7.7	5.7
5-FU	IC_{50} (μg/ml)	3.1	2.6	0.8	1.2	1.05	1.41	3.67	1.64
	SI†	> 13	> 19	> 50	> 33	> 38	> 28	> 11	> 24

*The 50% cytotoxic concentration (CC_{50}) of *C. officinalis* was 1608 μg/ml. The CC_{50} of 5-FU was > 40 μg/ml.

†The selectivity index (SI) = $^*CC_{50}/IC_{50}$.

Table 3. The Antioxidant Activity of *C. officinalis*

Concentration (μg/ml)	50	100	500	1000	5000	IC50 (μg/ml)
Free radicals scavenging activity (%)	30.8 ± 2.7*	50 ± 1.9*	80.8 ± 4.8*	ND	ND	100
XO inhibitory activity (%)	ND	ND	24.3 ± 2.0*	30.9 ± 2.4*	79 ± 4.8*	2620
Inhibitory effect on lipid peroxidation (%)	ND	ND	13.7 ± 2.3*	57.2 ± 3.4*	68.8 ± 3.6*	891.6

XO: xanthine oxidase, ND: not done, IC_{50}: The concentration of 50% inhibition.

The IC_{50} of allopurinol (positive control) against xanthine oxidase was 47 μg/ml.

The inhibitory effect of positive control (trolox 1 mg/ml) for $FeCl_2$-ascorbic acid induced lipid peroxidation was 95%.

*P < 0.05 comparing between the inhibitory effects of test drug and solvent control by Mann-Whitney U test.

HCC and leukemic cells. This activity of HWCO might help to explore the pharmacologic study of HWCO against other neoplasms. Traditionally, HCC has been thought of as a relatively chemoresistant cancer and leukemia as a chemosensitive cancer. Our experiment showed that HWCO inhibited HCC more significantly than leukemic cells. Clarification of mechanisms of action and purification of active components might make *C. officinalis* a useful chemopreventive and/or adjuvant therapeutic agent in the future.

There are several factors contributing to the carcinogenesis of hepatocyte and proliferation of HCC. Any agent or factor that contributes to chronic, low-grade liver cell damage and mitosis makes hepatocyte DNA more susceptible to genetic alterations. Thus, chronic liver inflammation of any type is a risk factor and predisposes the liver to the development of HCC (Dienstag and Isselbacher, 2001). Free radicals are generated by several reactions and can react with biological molecules such as DNA, proteins and phospholipids. They are also involved in inflammation, mutagenesis and carcinogenesis (Dizdaroglu *et al.*, 2002; Wulf, 2002; Lala *et al.*, 2001; Kagan *et al.*, 1999; Toyokuni and Sagripanti, 1993; Hochstein and Atallah, 1988). Free radicals attack DNA and generate a multiplicity of DNA damage. ROS are potential carcinogens because they facilitate mutagenesis, tumor promotion and progression. Signaling pathways of JNK, p38 MAPK and the transcription factors AP-1 and NF-kB involving cellular proliferation are particularly responsive to redox regulation (Wulf, 2002). Oxidants might act at several stages in the malignant transformation of cells (Wulf, 2002). Long-term supplementation of agents inhibiting hepatic cytosolic lipid peroxidation can protect the cell from free-radical mediated damage and can be useful in the inhibition of hepatic carcinogenesis (Basak *et al.*, 2001). Thus, antioxidant agents might be important in preventing formation of malignancy. Xanthine oxidase is involved in the generation of free radicals. The serum level of xanthine oxidase is found to increase in hepatic inflammation (Shamma *et al.*, 1965). It was expected that xanthine oxidase inhibitors could be useful for the treatment of liver disease caused by generation of the superoxide anion radical. Our results showed that HWCO could scavenge the free radicals at a concentration that simultaneously inhibited HCC cells. It could also inhibit activity of xanthine oxidase and lipid peroxidation at higher concentrations. These effects of HWCO might prohibit the carcinogenesis of HCC induced by free radicals and increase its chemopreventive potential. Tumor growth and metastasis may also be triggered by activation of cyclooxygenase (COX)-2. Thus, Anti-inflammation might be an effective tool for the chemoprevention and treatment of hepatocellular carcinomas (Cheng *et al.*, 2002; Rahman *et al.*, 2000). HWCO has also been reported to possess anti-inflammatory activity (Zheng *et al.*, 1998).

Signal transduction proteins, such as p53 and Ras, are involved in cellular growth of neoplasm. P53 mutation is common in HCC, but mutation of Ras is rare (Hsu *et al.*, 1993; Puisieux *et al.*, 1993). The anti-HCC activity of HWCO was significantly affected by p53 and Ras. The result might imply that HWCO could inhibit HCC by p53-mediated apoptosis and/or by prohibiting Ras-mediated proliferation. Thus, p53 mutation in HCC might not be a limiting factor of HWCO against HCC. HWCO could still be useful in p53-mutated HCC.

5-FU inhibits thymidylate synthesis to achieve anti-proliferation of cells. It has several acute and delayed toxicities (Lacy *et al.*, 2002 and 2003). The application of 5-FU against HCC was limited clinically. 5-FU was more effective to leukemic cells than to HCC cells in

our experiments. HBV, Fas and p53 did not significantly affect the anti-neoplastic activity of 5-FU. However, Ras has the significant effect. It is possible that 5-FU might achieve anti-neoplastic effect by pathways other than inhibiting thymidylate synthesis. HWCO and 5-FU might have different mechanisms to inhibit HCC growth. Combination of HWCO and 5-FU might be promising in the chemoprevention of HCC. High CC_{50} (1608 μg/ml) of HWCO indicated good tolerability by human cells. This might make it useful in treating human leukemia and HCC in the future.

A good chemopreventive agent against HCC should possess antioxidant, anti-inflammatory activities to decrease new carcinogensis; the anti-neoplastic activities to clear the newly formed malignant cell, and hepatoprotective activity to decrease the hepatic damage. *C. officinalis* seemed to possess these activities as supported by our experiment and previous literatures. *C. officinalis* could be a qualified candidate of chemopreventive agent against HCC. In spite of frequently mutated p53 protein in HCC, HWCO still could exert its anti-HCC activity by inhibiting Ras-mediated pathway. Combination of HWCO with other anti-HCC agents and purification of its active component might make *C. officinalis* more useful against HCC in the future.

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