Effect of the Geijibokryunghwan on human hepatocarcinoma cells

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We invesgated the GBH water extracts can be used as a potential cancer chemopreventive agent in humans, especially in hepatological cancer cell lines. The GBH was found to act as an potent inhibitor of COX-I only, but not as COX-2 inhibitor. Furthermore, the extract mediated anti-inflammatory effects and inhibited COX-associated hydroperoxidase functions(antipromotion activity). Inhibitory effect of the GBH water extracts on the growth of cancer cell lines such as HepG2 cell and Hep3B cell was shown.

Key words : Geijibokryunghwan(桂枝茯苓丸), hepatocarcinoma chemoprevention cyclooxygenase (COX)

Introduction

The prevention of cancer by ingestion of chemical agents that reduce the risk of carcinogenesis[1], is one of the most direct ways to reduce morbidity and mortality. Cancer chemopreventive agents include nonsteroidal anti-inflammatory drugs (NSAIDS) such as indomethacin, aspirin, piroxicam and sulindac, all of which inhibit cyclooxygenase (COX)[2)3)4)5)]. This inhibitory activity is relevant to cancer chemoprevention because COX catalyzes the conversion of arachidonic acid to pro-inflammatory substances such as prostaglandins, which can stimulate tumor cell growth and supress immune surveillance[6)7)]. In addition, COX can activate carcinogens to forms that damage genetic materials[8)9)].

Many oriental medicines have been shown to be growth-inhibitory against mouse-implanted, allogenic tumors[10)11)]. The extracts of several yeasts have also been shown to manifest similar antitumor activity[12)]. In a previous paper, it was confirmed that the Geijibokryunghwan (GBH) water extracts showed anti-thrombosis activity[13)14)15)16)17)].

In this report, author invesgated the GBH water extracts can be used as a potential cancer chemopreventive agent in humans, especially in hepatological cancer cell lines. For further study, the antioxidant and antimutagenic activities of the GBH are needed. Also, the effects of the GBH on induction of phase II drug-metabolizing enzymes (anti-initiation activity) and on induction of human promyelocytic leukemia cell differentiation (antiprogression activity) should be carried out.

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Materials and Methods

1. Cells and Materials

HepG2 cell and Hep3B cell lines were from Cell bank, Korea Research Insitute of Bioscience and Biotechnology, KIST (Taejon, Korea). Sheep red blood cells (sRBCs) were obtained from Korea Media Co., Ltd. (Seoul, Korea). RPMI 1640 were purchased from Gibco BRL (Grand Island, NY, USA). Lipopolysaccharide (LPS) and carrageenan were purchased from Sigma. GBH was obtained from Dongguk oriental hospital, Kyungju, Korea (Table 1).

Table 1. Composition of GBH[13)14)15)16)17)]

Cinnamomi Ramulus (桂枝)	1.33 g
Hoelen (茯苓)	1.33 g
Moutan Cortex Radicis (牧丹皮)	1.33 g
Paeoniae Radix (芍藥)	1.33 g
Persicae Semen (桃仁)	1.33 g
總 量	6.65 g

2. General analytical methods

Protein was determined by the procedure of Lowry et al.[18)] using bovine serum albumin (BSA) as a standard.

3. Extraction, fractionation and purification

The dried samples were homogenized using a mechanical disintegrator with Tekmar Tissue homogenizer (Tekmar Co., Cincinati, OH, USA) in distilled water, and the crude fraction was collected by centrifugation (15,000 ×g, for 20 min) at 4℃. The supernatant solution was concentrated to about 120 ml, and used for the experiments(Fig. 1.)

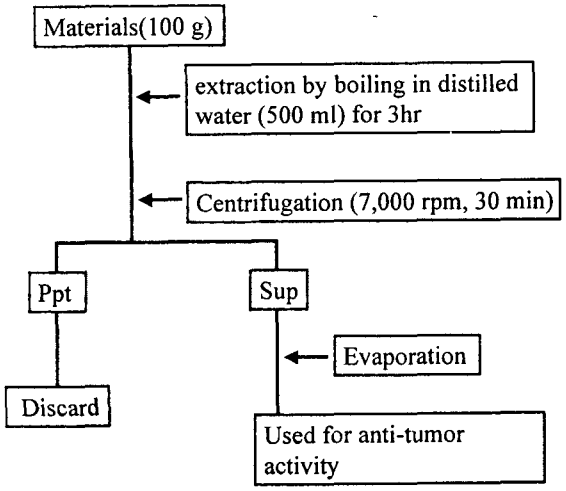

Fig. 1. Preparation of the water-extracts

4. Measurement of COX activity

COX activity was measured by assessing oxygen consumption at 37℃[19)20)]. Reactions were started by adding 0.6 mM arachidonic acid to a mixture containing 0.1 M sodium phosphate buffer (pH 7.5), 1.0 mM phenol, and 0.01 mM hemin; microsomes (0.2 mg of protein) derived from sheep seminal vesicles as a crude source of COX-1 or recombinant human COX-2 (0.1 mg of protein); and the test compound.

5. Measurement of hydroperoxidase activity

Hydroperoxidase activity was determined by spectrophotometry. Reaction mixtures contained 0.1 M Tris-HCl (pH 8.5), 1.2 μM hemin, 0.24 mM N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD), COX-1 (40 μg of protein) or COX-2 (50 μg of protein), and test compounds. H_2O_2 (250 μM) was used to initiate the reaction, and changes in absorbance at 595 nm were measured. Inhibitory activity was calculated by comparing the initial rate of changes in absorbance in the presence of test compounds with that observed with solvent (DMSO) only. Inhibition test of DMBA-induced preneoplastic lesions in mouse cultured by treatment with the extracts.

Mammary glands were incubated with the extracts for 10 days and DMBA for 24 hrs on 3 days[21)]. Percent incidence of mammary lesions were determined after an additional 14 days of incubation. The data from the extract-treated groups were compared with control groups and the results expressed as a percentage. Test of tumorigenesis in the two-stage mouse skin cancer model of initiation and promotion

Six groups of 20 female CD-1 mice (4 to 6 weeks old) were treated topically with 300 μmol of DMBA in 0.3 ml of acetone on the shaved dorsal region. One week later, the mice were treated with 5 μmol of TPA (12-O-tetradecanoylphorbol-13-acetate) in 0.3 ml of acetone alone or together with 5, 10, 40, 100 or 200 mg of BGHextracts, twice a week for 16 weeks. Animals were weighed weekly and observed for tumor development once every week. Then, observable skin tumors were numberd.

6. Statistical analysis

The statistical significance of difference among groups were evaluated by Student's t-test or Duncan's new multiple range test; $p<0.05$ was considered significant.

Results

1. Antitumor properties of the extracts

The results of antitumor assay indicate that the extracts show growth-inhibitory activity against HepG2 and Hep3B cell lines. It is suggested that the extracts coul be further assyaed for a potent antitumor activity against against HepG2 and Hep3B cell lines. When survival rate of HepG2 and Hep3B cells treated with the extracts were examined, the rate was decreased by dose-dependent manner(Fig. 2 and Table 2).

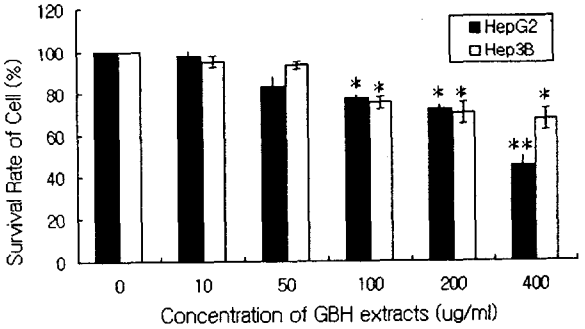

Fig. 2. Inhibitory effect of the extratcs on HepG2 and Hep3B. When survival rate of HepG2 and Hep3B cells treated with the extracts were examined, the rate was decreased by dose-dependent manner. Data represent means±S.E., n=3. * : p<0.05, ** : p<0.01.

Table 2. Inhibitory effect of the extratcs on HepG2 and Hep3B

Dose (μg/ml)	Survival rate (%)	
	HepG2	Hep3B
0	100	100
10	98 ± 3	95 ± 3
50	83 ± 5	93 ± 2
100	77 ± 2*	75 ± 3*
200	72 ± 2*	70 ± 5*
400	45 ± 5**	67 ± 5*

When survival rate of HepG2 and Hep3B cells treated with the extracts were examined, the rate was decreased by dose-dependent manner. Data represent means±S.E., n=3. * : p<0.05, ** : p<0.01.

However, it was more effective to the HepG2 cells: when HepG2 cells were treated with 50, 100, 200 and 400 μg/ml of

the extracts, the survival rates were 83%, 77%, 72% and 45%, respectively. Seemingly, in case of Hep3B cells, it showed 93%, 75%, 70% and 67% of survival rates, respectively.

On the other hand, the morphological change was observed, showing that the round form of HepG2 cells and flat form of Hep3B cells were being necrosised when 60 μg/ml concentration of the extracts was treated.

2. Effects on tumor initiation inhibition through inhibition COX-1 activity of the extracts in cancer cells

As noted above, the extracts was identified on the basis of its ability to inhibit the COX activity of COX-1 (median effective dose ED50 = 32 mg/ml), as shown in Fig. 3 and Table 3, and this activity correlates with antitumor promotion. There is no any effect on COX-2 activity (Fig. 4 and Table 4).

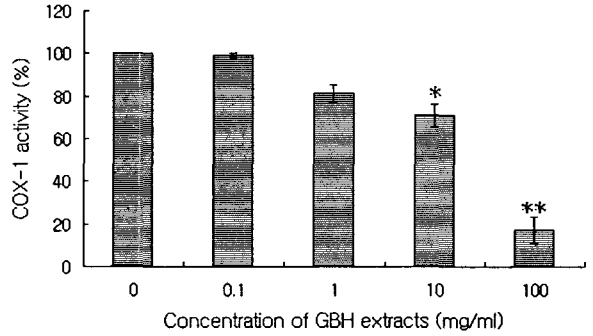

Fig. 3. Effects of the extracts on COX-1 activity. Each point represents the mean ± S.E. of two determinations. * : P<0.05, ** : P<0.01.

Table 3. Effects of the extracts on COX-1 activity

Concentration (mg/ml)	Percent activity (%)
Control	100
0.1	99±1
1.0	81±4
10	71±5*
100	17±6**

Each point represents the mean ± S.E. of two determinations., * : P<0.05, ** : P<0.01.

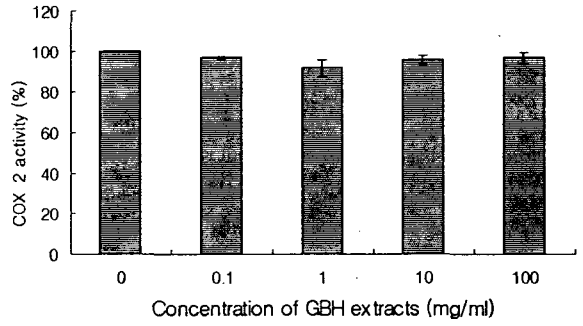

Fig. 4. Effects of the extracts on COX-2 activity. Each point represents the mean ± S.E. of two determinations. * : P<0.05, ** : P<0.01.

Table 4. Effects of the extracts on COX-2 activity.

Concentration (mg/ml)	Percent activity (%)
Control	100
0.1	96.6±0.5
1.0	91.6±4.1
10	95.6±2.0
100	96.6±2.5

Each point represents the mean ± S.E. of two determinations.

Thus, the extracts-mediated inhibition was specific for the COX activity of COX-1. Although its inhibitory activity was less than that of certain NSAIDs such as indomethacin (ED50 = 6.3 μM) (Fig. 5 and Table 5), it was much greater than that mediated by compounds such as aspirin (ED50 = 1121 μM).

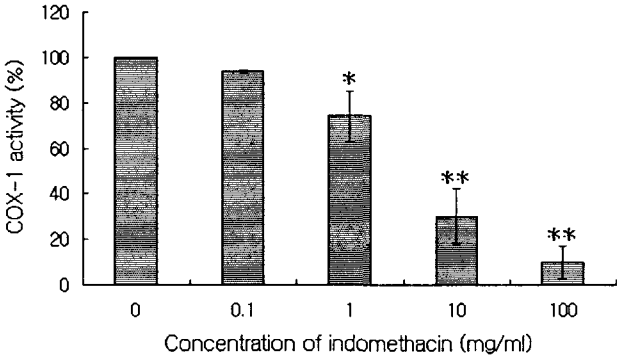

Fig. 5. Effects of indomethacin on COX-1 activity. Each point represents the mean ± S.E. of two determinations. * : P<0.05, ** : P<0.01.

Table 5. Effects of indomethacin on COX-1 activity

Concentration (mg/ml)	Percent activity (%)
Control	100
0.1	93.6±0.5
1.0	74±11*
10	30±12.1**
100	9.6±7.3**

Each point represents the mean ± S.E. of two determinations. * : P<0.05, ** : P<0.01.

Iindomethacine and most other NSAIDs did not inhibit any acticity of COX-1 (Fig. 6 and Table 6).

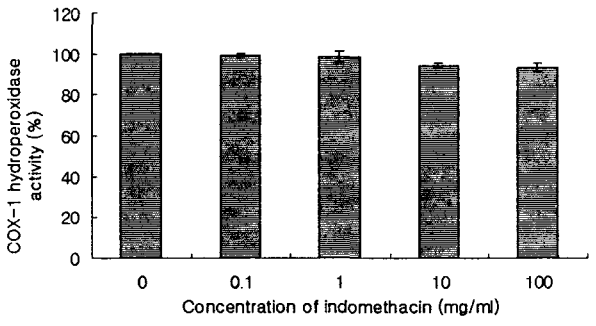

Fig. 6. Effect of indomethacin on COX-1 hydroperoxidase activity.

Table 6. Effect of indomethacin on COX-1 hydroperoxidase activity.

Concentration (mg/ml)	Percent activity (%)
Control	100
0.1	99±1
1.0	98±2.6
10	94±1
100	93±2

Inhibitory activity was calculated by comparing the initial rate of changes in absorbance in the presence of test counponds with that observed with solvent (DMSO) only. Each point represents the mean ± S.E. of two determinations.

In contrast, the extracts inhibited the hydroperoxidase activity of COX-1 (ED50 = 24 mg) (Fig. 7 and Table 7). Although, the extracts-mediated inhibition was specific for the COX activity of COX-1 because there was no discernable activity when oxygen uptake was assessed with COX-2 (Fig. 8 and Table 8), inhibition of the hydroperoxidase activity of COX-2 (ED50 = 123 mg) was greatly reduced relative to the activity observed with COX-1 (Fig. 8 and Table 8).

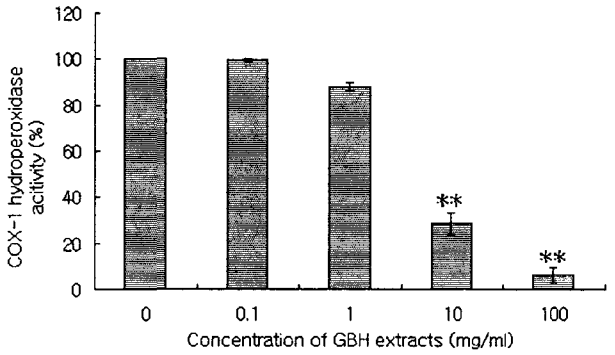

Fig. 7. Effect of the extracts on COX-1 hydroperoxidase acitivity. * : P<0.05, ** : P<0.01.

Table 7. Effect of the extracts on COX-1 hydroperoxidase acitivity.

Concentration (mg/ml)	Percent activity (%)
Control	100
0.1	99.6±0.5
1.0	88±1.7
10	28.6±4.7**
100	6.3±3.2**

Inhibitory activity was calculated by comparing the initial rate of changes in absorbance in the presence of test counponds with that observed with solvent (DMSO) only. Each point represents the mean ± S.E. of two determinations. * : P<0.05, ** : P<0.01.

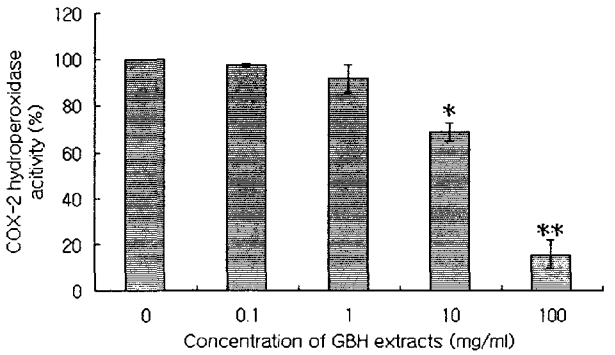

Fig. 8. Effect of the extracts on COX-2 hydroperoxidase acitivity. * : P<0.05, ** : P<0.01.

Table 8. Effect of the extracts on COX-2 hydroperoxidase acitivity.

Concentration (mg/ml)	Percent activity (%)
Control	100
0.1	97.6±0.5
1.0	91.6±6.1
10	68.6±4.0*
100	15.6±6.3**

Inhibitory activity was calculated by comparing the initial rate of changes in absorbance in the presence of test counponds with that observed with solvent (DMSO) only. Each point represents the mean ± S.E. of two determinations. * : P<0.05, ** : P<0.01.

Discussion

GBH formula has as formula recorded in the <Synopsis of the golden chamber>, action of 'eleminating the evil and not impairment of healthy energy' and 'promoting the flow of QI and cold and heat, so used for the expel blood stasis herbs from the ancient[14)16)17)]. Clincal application of GBH is for cases of irregular menstruation, amenorrhea, retention of the dead fetus, lochiostasis, etc., which ara attributive to the accumulation of blood stasis and phlegm in the lower jiao. And GBH is also applicable to cases of hysteromyoma, metropolypus, ovarian cyst, salpingitis, pelvic cellulitis, etc., marked by pain or masses over the liwer abdomen, which are attributive to the accumulation of blood stasis and phlegm[22)].

In oriental medicine, carcinoma is belong to category of 'ZingHa and JukChui'. This Zing and Juk are clinically characterized by a palpable and immovable mass of a definite shape in the abdomen, with pain at a definit site. In this case, the Parenchymatous Viscera are affectd at the Xue(blood)Phase. This Ha and Chui are characterized by the intermittent feeling of an indefinite mass in the abdomen, with pain at no definite site. In this case, the Hollow Viscera are affected at the Qi(vital energe) Phase[23)].

In this Study, author invesgated the GBH water extracts can be used as a potential cancer chemopreventive agent in humans, especially in hepatological cancer cell lines. For further study, the antioxidant and antimutagenic activities of the GBH are needed.

The main feature of models derived from experimental system is of a discrete, ordered series of changes to which terms such as initiation, promotion, progression and immortality can be applied. Progression in invasion and metastasis occur infrequently in primary animal tumors. The process of chemical carcinogenesis can be divided into three general stages and chemopreventive agents have been categorized according to the stage that they inhibit[24)]. Our extract inhibits cellular events associated with tumor initiation, promotion, and progression.

The extracts inhibited COX-1 and this correlates with antitumor promotion, while it did not inhibit COX-2 activity.

Thus, the extracts-mediated inhibition was specific for the COX activity of COX-1 because there was no discernable activity when oxygen uptake was assessed with COX-1, an inducuble form of the enzyme associated with responses such as inflammation[25], Although its inhibitory activity was less than that of certain NSAIDs such as indomethacin.

Also, unlike indomethacine and most other NSAIDs, the extracts inhibited the hydroperoxidase activity of COX-1. Thus, the extracts-mediated inhibition was specific for the COX activity of COX-1 because there was no discernable activity when oxygen uptake was assessed with COX-2.

Conclusion

Some oriental herbal prescriptions used for a syndrome expressed in oriental medical concept as chest paralysis and heartache are thought to be effective for angina pectoris. We investigated the effects of an oriental medicinal prescriptions, GBH consisting of herbes of Paeoniae Radix, Pachymae Fungus, Mountan Cortex Radicis and Cassiae Cortex on growth-inhibitory activity and cancer chemopreventive activity in assays representing three major stages of carcinogenesis. The extracts was found to act as an potent inhibitor of COX-I only, but not as COX-2 inhibitor. Furthrmore, the extract mediated anti-inflammatory effects and inhibited COX-associated hydroperoxidase functions (antipromotion activity). Inhibitory effect of the GBH water extracts on the growth of cancer cell lines such as HepG2 cell and Hep3B cell was shown. These data suggest that GBH extracts merits investigation as a potential cancer chemopreventive agent in humans, especially in hepatological cancers.

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