Induction of antioxidant and detoxifying enzymes by oriental bezoar

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Abstract

Oriental bezoar, a gallstone formed in the gall sac of *Bos taurus* Linné var. *domesticus* Gmelin (*Bovidae*), has been used as an antipyretic, sedative, antispasmodic, and detoxifying drug in oriental medicine. It reportedly has an antioxidative effect; however, its underlying mechanism remains unclear. Therefore, we investigated the effects of oriental bezoar and its main components on the nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway, which transcriptionally regulates the gene encoding an antioxidant enzyme, heme oxygenase-1 (*HO-1*), and detoxifying enzymes glutathione-S-transferase alpha 1 (*GSTA1*) and quinone oxidoreductase 1 (*NQO1) in vitro.* Using a dual-luciferase reporter assay and real-time PCR, oriental bezoar and its main constituent bilirubin were shown to induce ARE activity and up-regulate the expression of *HO-1*, *GSTA1*, and *NQO1* in HepG2 cells in a dose-dependent manner. These results suggest that activation of the Nrf2-ARE pathway is partially involved in the antioxidative effect of oriental bezoar, thus providing a scientific basis for oriental bezoar's traditional use for detoxification.

Keywords: Oriental bezoar, Nrf2-ARE, antioxidant, detoxification, bilirubin

Introduction

Oriental bezoar is a gallstone formed in the gall sac of *Bos taurus* Linné var. *domesticus* Gmelin (*Bovidae*) and has been used as an antipyretic, sedative, antispasmodic, and detoxifying drug in oriental medicine[1, 2]. It contains bilirubin, biliverdin, bile acids, and amino acids[1, 2]. Among these ingredients, bilirubin and biliverdin are reported to have direct[3] and indirect[4] antioxidative effect. According to our previous papers[5-7], Reiousan®, a crude drug preparation consisting of oriental bezoar and ginseng, showed an antioxidative activity and facilitated the excretion of foreign substances *in vivo*. Reiousan's effects were attributed to a cooperative effect of oriental bezoar and ginseng; however, the underlying mechanism of oriental bezoar's antioxidative activity remains unclear.

The nuclear factor erythroid 2-related factor 2 (Nrf2)-antioxidant response element (ARE) pathway plays a role in the biological defense against oxidative stress and foreign substances[8]. Upon exposure to oxidative stress and foreign substances, Nrf2 translocates from the cytoplasm to the nucleus and binds to the ARE to transcriptionally activate the genes encoding antioxidant enzymes, such as heme oxygenase-1 (HO-1), γ-glutamyl cysteine ligase, thioredoxin reductase-1, superoxide dismutase, glutathione peroxidase, and catalase, and detoxifying enzymes, such as glutathione-S-transferase alpha 1 (GSTA1), quinone oxidoreductase-1 (NQO1), and UDP-glucuronosyltransferase[8]. Although the Nrf2-ARE pathway is vital for maintaining homeostasis of the body[8-11], Nrf2 levels are reported to decline in aging animals[12, 13]. Therefore, activating the Nrf2-ARE pathway has been considered a strategy to develop drugs for preventing various diseases caused by oxidative stress and foreign substances[8-11].

In this study, we investigated the effects of oriental bezoar and its constituents on the Nrf2-ARE pathway, which is involved in antioxidation and detoxification, using HepG2 cell culture.

Materials

Cells

HepG2 cells (RIKEN BioResource Research Center, Ibaraki, Japan) were stored in liquid nitrogen. Cells with a passage number of 1 to 12 were used.

Test Drugs

Oriental bezoars obtained in Brazil (Kanai Shoten, Tokyo, Japan and Miyachu, Osaka, Japan) were mixed and pulverized in our manufacturing department (Kyushin Pharmaceuticals, Tokyo, Japan). The powdered oriental bezoar was suspended in 10% dimethyl sulfoxide (DMSO, FUJIFILM Wako Pure Chemicals, Osaka, Japan), and extracted using an ultrasonic generator (Model 2510, Branson ultrasonics, CT, USA) for 30 min at 25°C. The extract was sterilized by filtration using a 0.2-μm membrane filter (ADVANTEC, Tokyo, Japan).

Bilirubin, R, S-sulforaphane (FUJIFILM Wako Pure Chemicals), cholic acid (Tokyo Chemical Industry, Tokyo, Japan), deoxycholic acid (Sigma-Aldrich, MO, USA), and hemin (Alfa Aesar, UK) were dissolved in DMSO. The DMSO concentration in the medium was set at 0.1%.

Chemicals

Phosphate-buffered saline (PBS (-)), RPMI 1640 medium (FUJIFILM Wako Pure Chemicals), fetal bovine serum (FBS), 2.5% trypsin (Thermo Fisher Scientific, MA, USA), penicillin-streptomycin (Sigma-Aldrich), and Bambanker® (GC LYMPHOTEC, Tokyo, Japan) were used for cell culture.

For luciferase assays, tris (hydroxymethyl) aminomethane (Tris) (FUJIFILM Wako Pure Chemicals), Opti-MEM® I Reduced Serum Medium (Thermo Fisher Scientific), MultiFectam, plasmid DNA, Nano-Glo® Dual-Luciferase® Reporter Assay (Promega, WI, USA), and hydrochloric acid (HCl) (Yoneyama Yakuhin Kogyo, Osaka, Japan) were used with the target vector pGL4.37 [*luc2P*/ARE/Hygro] and internal control vector pNL1.1.PGK (phosphoglycerate kinase)

[Nluc/PGK].

For RT-PCR, TRIzol® (Invitrogen, MA, USA), chloroform, isopropyl alcohol (Yoneyama Yakuhin Kogyo), ethanol (FUJIFILM Wako Pure Chemicals), Otsuka distilled water (Otsuka Pharmaceuticals, Tokyo, Japan), 5×PrimeScriptTM RT Master Mix, TB Green® Premix Ex Taq™ (Tli RNaseH Plus) (2 ×), and PCR primers for *HO-1* (forward 5′-TTGCCAGTGCCACCAAGTTC-3′ and reverse 5′-TCAGCAGCTCCTGCAACTCC-3′), *NQO1* (5′-GGATTGGACCGAGCTGGAA-3′ and 5′-GAAACACCCAGCCGTCAGCTA-3′) and *GSTA1* (5′-TCTGCCCGTATGTCCACCTG-3′ and 5′-TGCCAACAAGGTAGTCTTGTCCA-3′), and *GAPDH* (Glyceraldehyde 3-phosphate dehydrogenase) (5′-GCACCGTCAAGGCTGAGAAC-3′ and 5′-TGGTGAAGACGCCAGTGGA-3′) (Takara Bio, Shiga, Japan) were used.

Methods

Cell culture

HepG2 cells were cultured in RPMI 1640 medium supplemented with 10% of inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin in a CO_2 incubator (SANYO, Tokyo, Japan) at 37°C and 5% CO_2. The culture medium was replaced once every 3 to 4 days.

Transfection

The cells were cultured in 96-well white plates at a density of 2×10^4 cells in 0.1 mL per well for 24 h. Tris-HCl buffer (20 mM, pH 7.4, 50 µL), 0.5 µg of a target vector pGL4.37 [*luc2P*/ARE/Hygro], and control vector pNL1.1.PGK [Nluc/PGK] were added to a microtube. Then, 25 µL of MultiFectam was added, mixed, and incubated at room temperature for 30 min. Opti-MEM® I Reduced Serum Medium (25 µL) was added, mixed, and incubated at room temperature for 5 min. Lastly, 10 µL of the mixture solution was added to the medium and incubated for 4 h.

Reporter assay[14)]

The transfected cells were treated with a fresh medium containing a test drug for 18 h. After

removing the medium, 80 μL of FBS- and phenol red-free RPMI 1640 medium and 80 μL of One-Glo™ reagent were added to each well. After the microplate was shaken orbitally for 3 min, the luminescence due to activation of firefly luciferase (Fluc) was measured using a microplate reader (Synergy H1, Biotek, VT, USA). Then, 80 μL of Stop & Glo® reagent was added to each well and shaken orbitally for 3 min. After just 7 min, the luminescence due to activation of Nanoluc® luciferase (Nluc), an internal control was measured. The relative light unit (RLU) of Fluc was corrected by the RLU of Nluc (Fluc RLU/Nluc RLU). The relative luciferase activity was calculated as the ratio of the corrected RLU of the treatment groups to the RLU of the control group, and it was shown as the ARE promoter activity.

RNA extraction[15)]

The cells were cultured in a 48-well plate at a density of 8×10^4 cells in 0.4 mL per well for 24 h. The medium was replaced with a fresh medium containing the test drug and incubated for 6 h for *HO-1* RNA extraction or 18 h for *GSTA1* and *NQO1* RNA extraction. After removing the medium, 0.2 mL of TRIzol® was added to the cells. The cells were then scraped with a pipette tip from the plate to disrupt cell membranes, left for 5 min at room temperature, and collected in a microtube. Then, 40 μL of chloroform was added to shake vigorously and stirred. After 5 min, each sample was centrifuged at 12,000 × g at 4°C for 15 min. From the aqueous layer, 50 μL was transferred to a new microtube. Then, 70 μL of isopropyl alcohol was added and stirred. After 10 min, each sample was centrifuged at 12,000 × g at 4°C for 10 min. After removing the supernatant, 200 μL of 75% ethanol was added and stirred. Each sample was centrifuged at 7,500 × g and 4°C for 5 min before the supernatant was removed. The precipitates were air-dried for about 5 min and dissolved in 40 μL of distilled water by stirring.

RNA quantification and reverse transcription

A 20 μL RNA solution and 160 μL of distilled water were added to each well of the UV-permeable

96-well plate. After shaking for 10 s, RNA concentration was measured at the absorbance of 260 nm.

A 20-μL reverse transcription reaction consisting of 2 μL of 5 × PrimeScript RT Master Mix, 0.5 μg of total RNA, and distilled water was conducted at 37°C for 15 min and 85°C for 5 s and then stored at 4°C.

Gene expression analysis[16)]

A 25-μL real-time PCR consisted of 12.5 μL of TB Green® Premix Ex Taq™ II, 0.2 μL of 50 μM forward primer, 0.2 μL of 50 μM reverse primer, less than 100 ng of template DNA in 2.0 μL, and 10.1 μL of distilled water. The PCR was conducted at 95°C for 30 s and 35 cycles of 2-step PCR at 95°C for 5 s and 60°C for 30 s.

The levels of mRNA of *HO-1*, *GSTA1*, *NQO1*, and *GAPDH* were quantified by calculating the ΔCt and ΔΔCt values of each reaction using the following formulas:

$$\Delta Ct = [Ct\ (\text{target gene}) – Ct\ (\text{internal control gene})]$$

$$\Delta\Delta Ct = [\Delta Ct\ (\text{control group}) – \Delta Ct\ (\text{treatment group})]$$

$$\text{mRNA expression} = 2^{\Delta\Delta Ct}$$

The control group was the vehicle-treated group, and the treatment groups were the test drug or positive control-treatment group.

Cytotoxicity assay

HepG2 cells were cultured in a 96-well plate at a density of 1.3×10^4 cells in 0.1 mL per well for 18 h. The medium was replaced with a fresh medium containing test drugs to incubate for 18 h. The medium was then replaced with Cell Titer 96® six-fold diluted in a fresh medium. After 2 h, cell viability was measured at the absorbance of 490 nm.

Statistical analysis

The results were expressed as means ± standard error. The significance of the differences in the data was estimated using the one-way analysis of variance followed by Dunnett's multiple range test.

The differences with $P < 0.05$ were considered statistically significant.

Results

Effects on ARE activity

Oriental bezoar increased ARE activity in a dose-dependent manner, achieving a significant increase at 1000 μg/mL. Bilirubin, a component of oriental bezoar, showed a tendency to increase ARE activity at 171 μM (100 μg/mL). On the other hand, sulforaphane increased ARE activity at 5 μM (Fig. 1).

Effects on mRNA expression of antioxidant enzyme (HO-1) and detoxifying enzymes (GSTA1, NQO1)

Oriental bezoar increased *HO-1* (Fig. 2A), *GSTA1* (Fig. 3A), and *NQO1* (Fig. 3B) expression in a dose-dependent manner, causing significant effects at 1000, 1000, and 3000 μg/mL, respectively. Bilirubin also increased *HO-1* (Fig. 2B), *GSTA1* (Fig. 3A), and *NQO1* (Fig. 3B) expression; bilirubin's effect on *HO-1* expression was significant at 100 μM (100 μg/mL, Fig. 2B). On the other hand, deoxycholic and cholic acid did not affect *HO-1* expression (Fig. 2B). On the other hand, hemin increased *HO-1* expression at 20 μM (Fig. 2A, 2B), and sulforaphane increased *GSTA1* (Fig. 3A) and *NQO1* (Fig. 3B) expression at 5 μM.

Cytotoxicity

Oriental bezoar and bilirubin did not exhibit cytotoxicity at 1000 μg/mL and 171 μM (100 μg/mL), respectively (Fig. 4).

Discussion

Oriental bezoar and bilirubin increased ARE activity and the expression of *HO-1*, *GSTA1*, and *NQO1*. HO-1 is an inducible antioxidant enzyme and is involved in the degradation of heme into

biliverdin, carbon monoxide (CO), and free iron[17]. Biliverdin is reduced to bilirubin by biliverdin reductase[17]. Bilirubin and biliverdin have an antioxidative effect, and CO has an anti-inflammatory effect[18]. Hence, *HO-1* induction may suppress oxidative stress-induced cell injury[18]. GSTA1 and NQO1 are known to act as detoxifying enzymes[19], with GSTA1 catalyzing glutathione conjugation and NQO1 reducing quinones[19]. These enzymes are involved in the metabolism of carcinogens, such as benzo[a]pyrene and aflatoxin, which are metabolically activated by phase I metabolic reactions; therefore, their induction may decrease carcinogens in the body[11, 20]. The activation of the Nrf2-ARE pathway was also reported to induce many other antioxidant enzymes, such as γ-glutamyl cysteine ligase, thioredoxin reductase-1, superoxide dismutase, glutathione peroxidase, and catalase, and detoxifying enzymes such as UDP-glucuronosyltransferase[8, 21, 22]. These findings suggest that oriental bezoar has antioxidative and detoxifying effects by inducing these enzymes by activating the Nrf2-ARE pathway.

Oriental bezoar contains bilirubin, biliverdin, bile acids such as deoxycholic and cholic acid, and amino acids[1, 2]. In this study, bilirubin showed a similar enhancing effect as oriental bezoar on *HO-1*, *GSTA1*, and *NQO1* expression. On the other hand, bile acids did not affect *HO-1* expression. Bilirubin was reported to increase HO-1 protein levels in H9c2 cells derived from a rat heart[4]. These findings suggest that the activating effect of oriental bezoar on the Nrf2-ARE pathway is partially attributed to bilirubin.

On the other hand, oriental bezoar did not exhibit cytotoxicity on HepG2 cells even at the concentration sufficient for activating the Nrf2-ARE pathway. These data suggest that oriental bezoar can enhance the biological defense without causing cell damage.

In summary, we have shown that oriental bezoar and its component bilirubin induce ARE activity and increase the expression of *HO-1*, *GSTA1*, and *NQO1*, the downstream genes of the Nrf2-ARE pathway. Although our results are *in vitro* and require further confirmation, they may help delineate

the antioxidative and detoxifying effects of oriental bezoar.

Conflict of interest

This research was supported by Kyushin Pharmaceuticals. The authors declare no conflicts of interest.

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Figure Legends

Fig. 1 Oriental bezoar and bilirubin activated the ARE promoter activity
Data are expressed as mean ± SEM (N = 5). *: $P < 0.05$, compared to the control group using Dunnett's test.

Fig. 2 Oriental bezoar and bilirubin increased the expression of *HO-1* in HepG2 cells
(A) The effect of oriental bezoar on *HO-1* expression.
(B) The effect of bilirubin, deoxycholic acid, and cholic acid on *HO-1* expression.
Data are expressed as mean ± SEM (N = 3–4). **: $P < 0.01$, *: $P < 0.05$, compared to the control group using Dunnett's test.

Fig. 3 Oriental bezoar and bilirubin increased the expression of *GSTA1* and *NQO1*
(A) The effect of oriental bezoar, bilirubin, and sulforaphane on *GSTA1* expression in HepG2 cells.
(B) The effect of oriental bezoar, bilirubin, and sulforaphane on *NQO1* expression in HepG2 cells.
Data are expressed as mean ± SEM (N = 3–4). **: $P < 0.01$, *: $P < 0.05$, compared to the control group using Dunnett's test.

Fig. 4 Oriental bezoar and bilirubin did not show cytotoxicity in HepG2 cells
Data are expressed as mean ± SEM (N = 3).

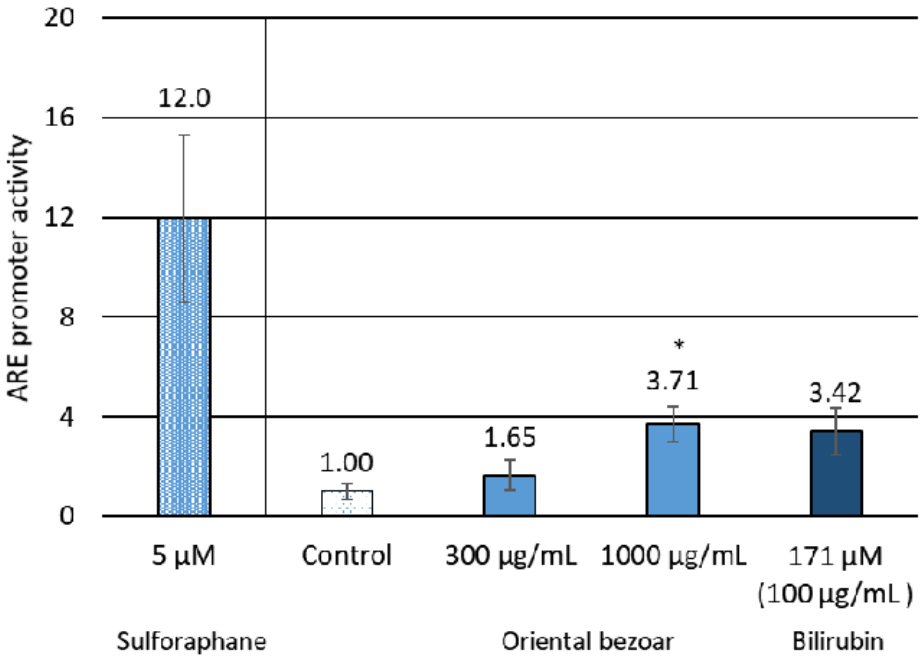
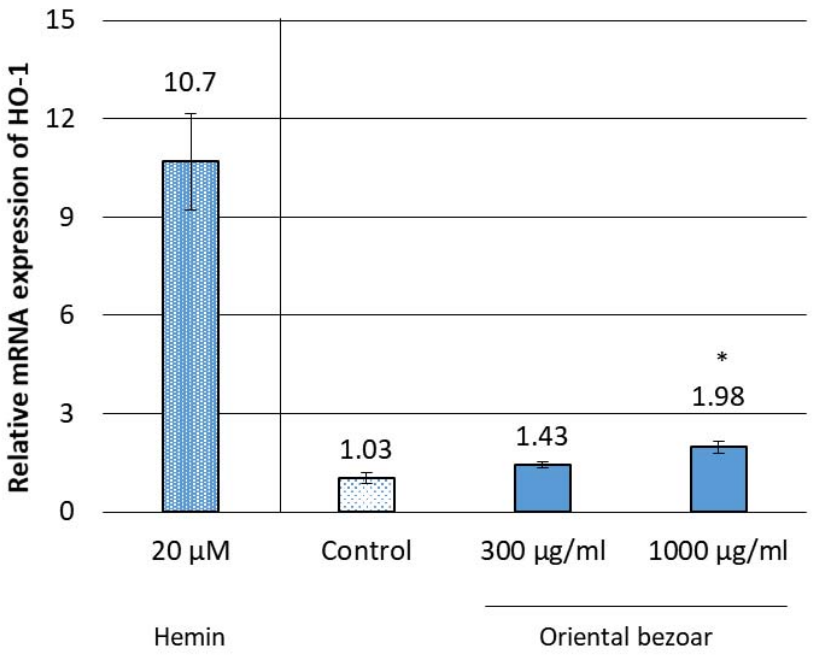

20
16
12
8
4
0
ARE promoter activity
12.0
1.00
1.65
*
3.71
3.42
5 μM
Control
300 μg/mL
1000 μg/mL
171 μM
(100 μg/mL)
Sulforaphane
Oriental bezoar
Bilirubin

Fig. 1


Relative mRNA expression of HO-1
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12
9
6
3
0
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1.03
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*
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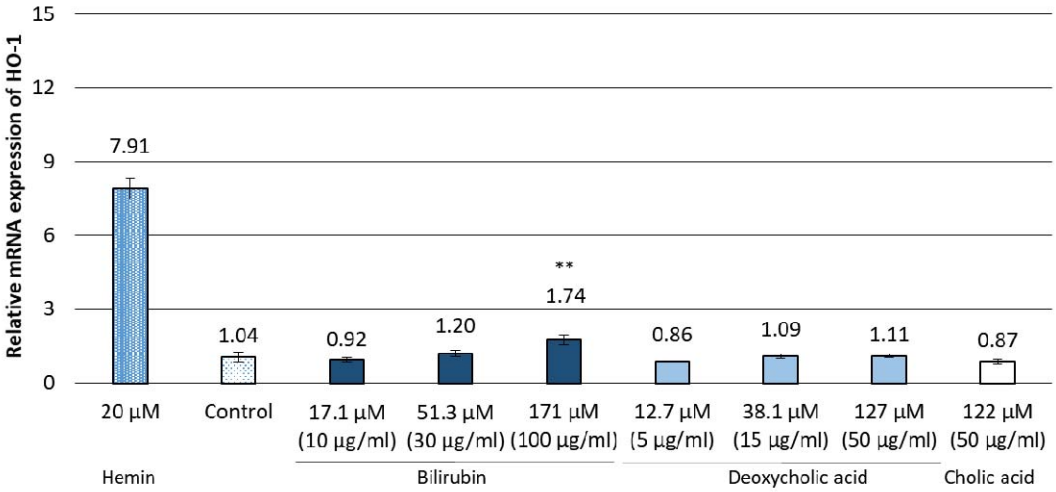
20 µM
Control
300 µg/ml
1000 µg/ml
Hemin
Oriental bezoar

Fig. 2 (A)


Relative mRNA expression of HO-1
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12
9
6
3
0
7.91
1.04
0.92
1.20
**
1.74
0.86
1.09
1.11
0.87
20 μM
Control
17.1 μM (10 μg/ml)
51.3 μM (30 μg/ml)
171 μM (100 μg/ml)
12.7 μM (5 μg/ml)
38.1 μM (15 μg/ml)
127 μM (50 μg/ml)
122 μM (50 μg/ml)
Hemin
Bilirubin
Deoxycholic acid
Cholic acid

Fig. 2 (B)

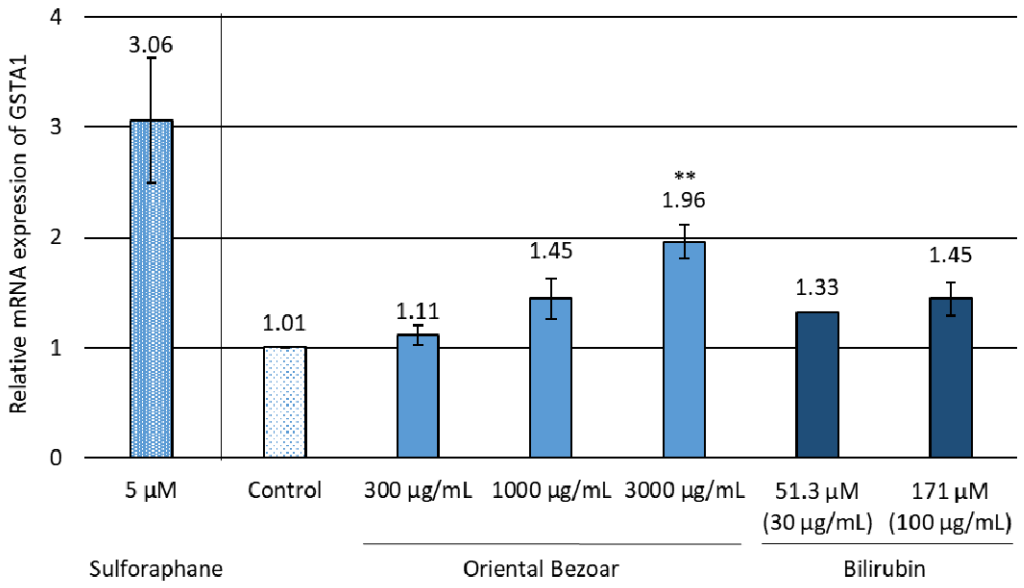
Relative mRNA expression of GSTA1
4
3
2
1
0
3.06
1.01
1.11
1.45
**
1.96
1.33
1.45
5 μM
Control
300 μg/mL
1000 μg/mL
3000 μg/mL
51.3 μM
(30 μg/mL)
171 μM
(100 μg/mL)
Sulforaphane
Oriental Bezoar
Bilirubin

Fig. 3 (A)

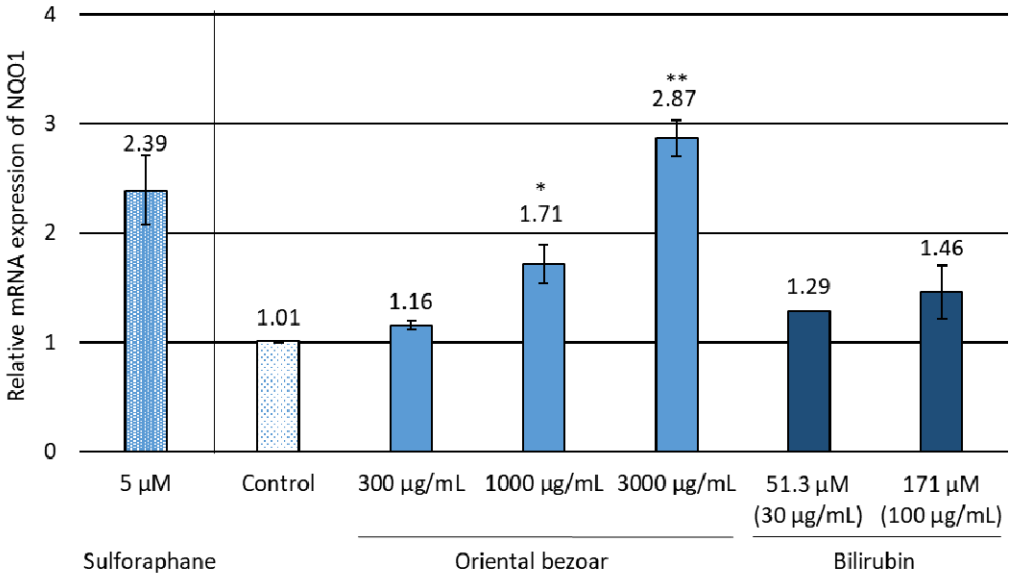
Relative mRNA expression of NQO1
4
3
2
1
0
2.39
1.01
1.16
*
1.71
**
2.87
1.29
1.46
5 μM
Control
300 μg/mL
1000 μg/mL
3000 μg/mL
51.3 μM (30 μg/mL)
171 μM (100 μg/mL)
Sulforaphane
Oriental bezoar
Bilirubin

Fig. 3 (B)

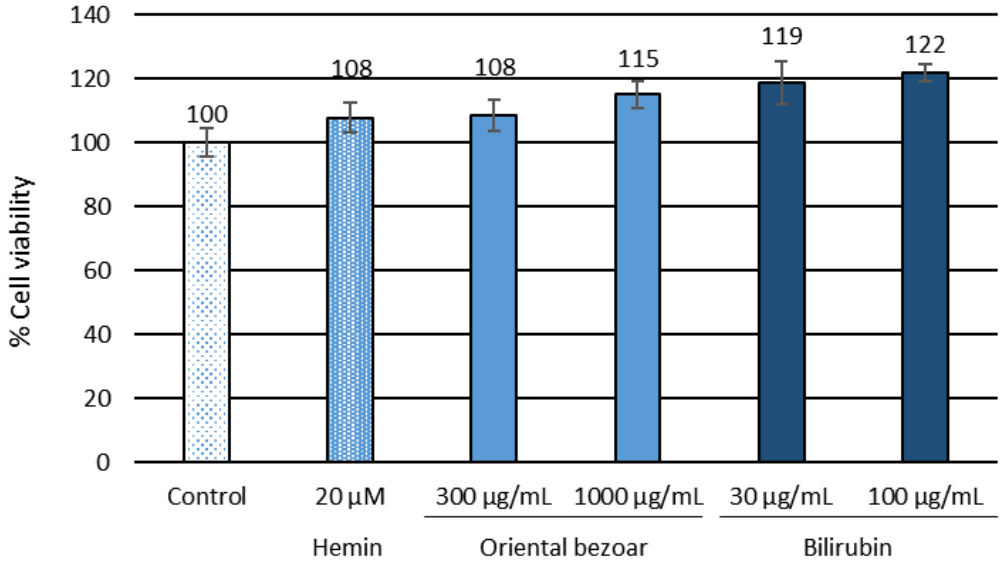
140
120
100
80
60
40
20
0
% Cell viability
100
108
108
115
119
122
Control
20 µM
300 µg/mL
1000 µg/mL
30 µg/mL
100 µg/mL
Hemin
Oriental bezoar
Bilirubin

Fig. 4