The effect of emodin on cytotoxicity, apoptosis and antioxidant capacity in the hepatic cells of grass carp (*Ctenopharyngodon idellus*)

**Abstract**

We determined the effect of emodin on the lactate dehydrogenase (LDH) release, superoxide dismutase (SOD), glutathione (GSH), total antioxidant capacity (T-AOC), reactive oxygen species (ROS), mitochondrial membrane potential (ΔΨm), and apoptosis in the hepatic cells of grass carp (*Ctenopharyngodon idellus*). Cultured cells were treated with different concentrations of emodin (0.04–25 μg/ml) for 24 h. We found that the cytotoxic effect of emodin was mediated by apoptosis, and that this apoptosis occurred in a dose-dependent manner. Emodin (1–25 μg/ml) significantly induced apoptosis accompanying ΔΨm disruption and ROS generation and significantly reduced the SOD activities and T-AOC compared to the control. Thus, the oxidative effect of emodin may be attributed to the loss of the cell's ability to maintain the activity of its radical-scavenging enzymes. GSH was also significantly higher after 0.2–1 μg/ml emodin exposure, indicating that cells failed to maintain their redox balance when compensating for the increased oxidative stress. Our results suggest that emodin (1–25 μg/ml) exerts its cytotoxic effects via apoptosis by directly affecting the mitochondria.

**1. Introduction**

Antimicrobial compounds are commonly used in fish and shellfish to control diseases [12]; however, these antimicrobials can suppress animal growth. There is a growing interest for using medicinal herbs in aquaculture because they are rich sources of immune-enhancing substances. These medicinal herbs are used in many countries to promote health, increase the body’s natural resistance to infection, and prevent and treat various diseases [3,4].

Emodin (1, 3, 8-trihydroxy-6-methylandraquinone) is a medicinal extract present in rhubarb (*Rheum officinale* B.). It has been widely used as a traditional medicine in many areas, especially Eastern Asia. Emodin has numerous pharmacological effects, such as laxative, anti-allergic [5,6], anti-inflammatory, anti-cancer, and anti-diabetic activities [7–9]. However, potential emodin side effects have also been reported, including genotoxic [10], nephrotoxic [11], and hepatotoxic effects [12]. Interestingly, emodin also exerts cytotoxic and protective effects on rat C6 glioma cells [13].

Emodin induces oxidative stress and causes damage to lipids, DNA, and proteins [14]. Organisms have evolved many defense mechanisms to protect against oxidative stress, which include enzymatic and non-enzymatic antioxidants [15]. Antioxidative enzymes are the first line of defense against free radicals [16]. Superoxide dismutase (SOD) is a well-known antioxidative enzyme that converts superoxide to hydrogen peroxide [17,18]. Glutathione (GSH) is another endogenous antioxidant scavenger that acts as a major cellular redox buffer [19].

Grass carp (*C. idellus*) is an herbivorous freshwater fish that is native to China and has a long cultivation history, high yield, and low cost [20–22]. It plays an important role in aquaculture with 4.22 million tons produced in 2010, the highest in fish production worldwide [23]. However, cultured grass carp have suffered serious mortality problems due to disease outbreaks. Previous studies have mainly focused on promoting grass carp growth, enhancing their nonspecific immunity, or increasing their natural resistance to infection [24–26]; few have studied toxicity.
The liver is the main defense against oxidative stress caused by excessive ROS [27,28]. It constantly metabolizes various materials containing different nutrient and toxin levels. Thus, it is important to examine the effects of emodin on oxidative damage in the grass carp liver. The aim of this study was to determine the effects of emodin on lactate dehydrogenase (LDH) release, SOD, total antioxidant capacity (T-AOC), GSH, reactive oxygen species (ROS), and Annexin V/FITC.

2. Materials and methods

2.1. Materials

The hepatic grass carp cell line (L8824). Emodin (purity >99%), Kits measuring LDH release, SOD activity, T-AOC, total GSH (GSH + GSSG) and oxidative GSH (GSSG), and Annexin V/FITC. Other drugs and reagents, such as Rhodamine 123, MEM medium, fetal calf serum, penicillin, streptomycin, Tripure reagent, and PBS were of biochemical quality.

2.2. Cell culture

The L8824 cells were maintained in MEM medium, supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 U/ml streptomycin, and in a humid atmosphere of 5% CO₂ at 27 °C. Following treatment, cells were harvested using 0.25% trypsin (0.2% EDTA), centrifuged at 1000 × g for 5 min, and washed twice with PBS. Three replicates were performed for each treatment, including controls, and each assay described below was performed using samples from each replicate flask.

L8824 cells were plated in 96- or 6-well plates. Cells were treated with various emodin concentrations (0.04, 0.2, 1, 5, 25 μg/ml) for 24 h. Emodin was dissolved in 8 mg/ml of DMSO. The stock emodin solution was diluted to the desired concentration immediately before use. Control cells were cultured in the same medium but without emodin. Vehicle cells were added to the medium in 0.3% DMSO.

2.3. LDH release assay

LDH is a soluble cytosolic enzyme present in most eukaryotic cells that is released into the culture medium upon cell death due to plasma membrane damage. The increase in the LDH activity in culture supernatant is proportional to the number of lysed cells [29]. L8824 cells were seeded in 96-well (5 × 10³ cells/well) culture plates at a final volume of 200 μl/well of culture medium, supplemented with 10% fetal calf serum, for 24 h. After cells were exposed to emodin for 24 h, LDH release was measured according to manufacturer’s instructions.

2.4. ROS measurements

Changes in intracellular ROS levels were determined by measuring the oxidative conversion of cell permeable 2', 7'dichlorofluorescein diacetate (DCFH-DA) to fluorescent dichlorofluorescein (DCF) in a fluorescence microplate (Fluoroskan Ascent FL). Cells in 6-well (1 × 10⁶ cells/well) culture plates were incubated with emodin for 24 h. The cells were then incubated with DCFH-DA at for 20 min at 37 °C and washed twice with serum-free medium. The distribution of DCF fluorescence in 20,000 cells was detected by fluorospectrophotometry at an excitation wavelength of 488 nm and an emission wavelength of 535 nm [30].

2.5. SOD activity and T-AOC assay

Cells were seeded in 6-well (1 × 10⁶ cells/well) culture plates after emodin exposure and were evaluated for SOD activity and T-AOC according to manufacturer’s instructions. SOD activity was determined using xanthine oxidase-derived superoxide, which reacts with WST-1 result to form formazan; formazan is effectively inhibited by SOD [31]. SOD activity was then monitored at 450 nm. One unit of enzyme activity was defined as the quantity of SOD required for 50% inhibition [32]. SOD activity was then normalized to milligram protein.

T-AOC was measured with 2,2'-azino-bis (3 ethylbenzthiazoline)-6-sulfonic acid (ABTS) using a microplate reader (BIO-RAD Model 3550, CA, USA) at 414 nm. The relative T-AOC values of the samples were normalized to protein concentration.

2.6. GSH and GSSG measurement

GSH and GSSG were measured by colorimetric microplate assay kits. Briefly, 40 μl meta-phosphoric acid was added to 10 ul sub-brain homogenates and then centrifuged at 10,000 × g for 10 min at 4 °C. The supernatant was subsequently used for the total GSH (GSH + GSSG) and GSSG assays. Total GSH was measured by the DTNB-GSSG recycling assay [33]. GSSG level was quantified by the same method, except the supernatant was pretreated with 1% 1 M 2-vinylpyridine solution to remove GSH. GSH was calculated using the following equation: GSH = (total GSH activity) – 2*GSSG.

2.7. Mitochondrial membrane potential

ΔΨm was monitored using fluorescent Rhodamine 123 dye, which preferentially localizes to active mitochondria based on highly negative ΔΨm [30]. Rhodamine 123 (final concentration of 10 mM) was added to cells after emodin treatment for 24 h. After 30 min at 37 °C, the cells were collected by pipetting, washed twice with PBS, and then analyzed by fluorospectrophotometry at an excitation wavelength of 488 nm and at emission wavelength of 535 nm.

2.8. Flow cytometric detection of apoptotic cells

Apoptotic cells were evaluated using Annexin V/FITC. In brief, Annexin V+/PI– cells were considered apoptotic, while Annexin V–/PI+ cells were considered necrotic [34]. Cells were stained according to manufacturer’s instructions and analyzed by flow cytometry (Cytomixics FC 500). The cells were collected by centrifugation after emodin exposure and washed with PBS. The pellets were resuspended in the Annexin V/FITC staining reagent and fixed at 20–25 °C for 10 min. The cells were then washed and resuspended in the PI staining reagent. Staining was stable at 4 °C for 30 min [35]. Samples were then analyzed by flow cytometry.
2.9. Statistical analyses

All results are shown as the mean ± standard error (X ± SEM) and were analyzed using SPSS statistics software, version 16.0. Data were subjected to one-way analysis of variance and Duncan’s multiple range tests. A P < 0.05 was considered statistically significant.

3. Results

3.1. The effect of emodin on cytotoxicity

L8824 cells were exposed to different concentrations of emodin for 24 h, and cytotoxicity was measured by LDH release (Fig. 1). Compared to the control cells, the amount of LDH release significantly increased in a dose-dependent manner with exposure to 0.2, 1, 5, and 25 μg/ml emodin (P < 0.05). LDH release of cells treated with 25 μg/ml emodin was significantly higher than that of other groups (P < 0.05).

3.2. The effect of emodin on ROS

We measured ROS production using the fluorescent dye DCFH-DA (Fig. 2). Compared to control cells, the treatment of 1, 5, and 25 μg/ml emodin significantly increased ROS (P < 0.05). ROS treated with 1, 5, and 25 μg/ml emodin was significantly higher than that of 0.04 and 0.2 μg/ml groups (P < 0.05).

3.3. The effect of emodin on antioxidant capacity

SOD activity in cells treated with 0.2, 1, 5, and 25 μg/ml emodin was significantly lower than that of the control group (Fig. 3(A); P < 0.05). T-AOC in the emodin-treated cells was also significantly lower (Fig. 3(B); P < 0.05). However, GSH in cells treated with 0.2 and 1 μg/ml emodin significantly increased (Fig. 3(C); P < 0.05).

3.4. The effect of emodin on apoptosis

We also examined the effect of emodin on apoptosis in the hepatic cells of grass carp. We found that the percentage of apoptotic cells in cells treated with 1, 5, and 25 μg/ml emodin was significantly higher (Fig. 4(A) and (B); P < 0.05). We further investigated whether emodin had any effect on ΔΨm. Compared to control cells, ΔΨm in L8824 cells was much lower after 24 h of exposure to 1, 5, and 25 μg/ml emodin (Fig. 4(C); P < 0.05).

4. Discussion

In the present study, the grass carp cell line L8824 was used to study the cytotoxic effects of emodin in vitro. Exposure to 1, 5, and 25 μg/ml emodin for 24 h significantly increased LDH release in a dose-dependent manner. Ko et al. [36] also reported that cell viability was inhibited by exposure to varying concentrations of emodin for 24 h. Differences in drug concentrations in an in vitro toxicity system may depend on the biology of the cultured cells (e.g., species, strain sex, age, tissue) as well as handling during cell
isolation and cultivation [37]. The cellular damage caused by emodin was further confirmed by apoptosis assays.

In a quantification study, we observed that emodin-induced apoptosis occurred in a dose-dependent manner. This was consistent with findings that emodin can trigger apoptosis in vitro. Wang et al. [38] showed that emodin inhibited cell proliferation by locking cells in the G1 phase and inducing caspase 3-dependent apoptosis in HK-2 cells, a human proximal tubular epithelial cell line, in both a dose- and time-dependent manner. Other reports found that canine tendon cells and chondrocytes treated for 4 days with 200 µg/ml enrofloxacin exhibited apoptotic features and fragmented DNA [39]. These data emphasize that emodin leads to unavoidable apoptotic cell death [40].

The signaling mechanisms that regulate apoptosis are very complicated. There are several conditions (e.g., oxidative stress), molecules (e.g., Ca²⁺, proteases, or nucleases), and organelles (e.g., mitochondria) involved in apoptosis. At present, it is thought that diverse apoptotic stimuli converge on a common apoptotic pathway mediated by the mitochondria [41,42]. Mitochondria are crucial for cell function and viability. They play a central role in cell biology not only in ATP production, but also in Ca²⁺ sequestration and ROS generation [43]. To gain further insight into emodin-induced apoptosis in this study, we focused on the mitochondria and analyzed ΔΨm and ROS. ΔΨm decreased in a dose-dependent manner in emodin-treated cells. Within 24 h of treatment, the ΔΨm declined to

![Fig. 4. The effects of 24 h of emodin on apoptosis (A) and mitochondrial membrane potential (C) in L8824 cells. Quantitative analyses of apoptosis (B) are also shown.](www.aladdin-e.com)
approximately 75% of control levels, and this was apparently enhanced at emodin concentrations of 1, 5 and 25 μg/ml.

ROS are also important for apoptosis. The mechanisms driving apoptosis in cells under oxidative stress may involve high ROS levels, which directly inhibit caspase activity, disrupt intracellular Ca^{2+} homeostasis, and lead to ATP depletion [44]. ROS in emodin-treated cells was significantly higher compared to controls. This was consistent with findings that apoptosis is accompanied by elevated cellular levels of ROS, such as superoxide [45]. Thus, oxidative stress likely plays a role in emodin-induced apoptosis in hepatic grass carp cells. Previous studies have also shown that emodin generates ROS [14,46] and triggers apoptosis [47]. Feeding aqueous animals with an extract-incorporated diet has been shown to enhance ROS after one week [48]. Similarly, *Labeo rohita* on a diet containing 0.5% *Achyranthes asperaseed* also experienced significantly enhanced superoxide anion production [49], while trout that were fed nettle and mistletoe extracts increased extracellular superoxide anion production [50]. Therefore, our results further suggest that emodin produces ROS, affects ∆Ψm, and subsequently triggers apoptosis in the hepatic cells of grass carp.

ROS can rapidly be removed by enzymatic and non-enzymatic antioxidants, thereby maintaining a healthy pro-oxidant/antioxidant balance [15]. Changes in antioxidant enzyme activity are often considered a biomarker of the antioxidative response. The activation of endogenous antioxidant enzymes and non-enzymatic antioxidants, such as SOD and GSH, respectively, has a protective role against oxidative damage [51]. In the present study, we examined SOD, GSH, and T-AOC in cells treated with emodin. We found that emodin significantly decreased SOD and T-AOC, but not GSH, activities. This may indicate a deficiency in some enzymatic antioxidants or severe oxidative injury [52]. Therefore, the oxidative effect of emodin may be attributed to the loss of the affected cell’s ability to maintain the activity of its radical-scavenging enzymes. In addition, the current study demonstrated that GSH significantly increased in response to emodin, which could cellular attempts to maintain its redox balance. However, these mechanisms may not be able to compensate for the increased emodin-induced oxidative stress since ROS levels remained high. This could be due to upregulated GSH synthesis by gamma-glutamylcysteine synthetase or de novo GSH synthesis resulting from the elevated transport of amino acids, such as cysteine and glutamate. Elevated GSH levels have been observed in lung cells 24 h after oxidative stress [53]. In Wuchang breams, dietary emodin supplements can contribute to antioxidative abilities that help mitigate oxidative organ damage [26].

The effects of herbs on aquaculture depend on several factors, such as time, dosage, method of administration, and the physiological condition of the fish. However, since these effects are dose-dependent, there is a chance that overdosing will occur [54]. Thus, the dosages of these herb extracts must be taken into consideration.

In conclusion, emodin (1–25 μg/ml) can produce ROS, which alter the subcellular redox equilibrium. Emodin-induced apoptosis is mediated by these ROS as well as ∆Ψm in the hepatic cells of grass carp. Due to the cytotoxic effects it causes, high concentrations of emodin is not suitable for its use in fish.

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