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Research Article
Liver Protective and Reactive Oxygen Species Scavenging Effects of Emodin in Lipopolysaccharide/Bacillus Calmette Guerin-injured Mice by Optical Molecular Imaging

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Abstract

Background: Hepatocytes damage is sometimes closely related to oxidative stress and reactive oxygen species which are the major contributors to lipopolysaccharide-induced liver injury. Emodin, the active natural product in rhubarb of hydroxyanthraquinone skeleton, has been reported of protective activity to liver tissue, whose mechanism is generally thought of antioxidation based on chemical reaction or indirect evidence. There is no visualized evidence proved the reactive oxygen species scavenging effect of emodin *in vivo*. **Materials and Methods:** The dynamic reactive oxygen species luminescent signal in mice injured by bacillus calmette guerin and lipopolysaccharide was monitored by using the optical molecular imaging approach. **Results:** The elevations of serum alanine aminotransferase and aspartate transaminase activities in bacillus calmette guerin/lipopolysaccharide-injured mice were reversed by emodin, indicating the protection of emodin to hepatocytes. And emodin significantly and dose-dependently attenuated the reactive oxygen species luminescent signal elicited by bacillus calmette guerin/lipopolysaccharide, indicating visually the *in vivo* reactive oxygen species scavenging effect of emodin. In addition, emodin significantly and dose-dependently elevated the activity of superoxide dismutase, content of reduced glutathione and total antioxidant capacity and meanwhile decreased the contents of hydrogen peroxide, lipid peroxides and malondialdehyde in livers of bacillus calmette guerin/lipopolysaccharide-injured mice. It could be attributed to the anti-oxidative effect of emodin which helps to maintain the reactive oxygen species balance *in vivo*. **Conclusion:** Emodin can protect liver against bacillus calmette guerin/lipopolysaccharide-induced injury and the mechanism includes reactive oxygen species scavenging effect and anti-lipid peroxidation at least.

Key words: Emodin, hepatoprotection, reactive oxygen species, molecular imaging, lipopolysaccharide, nuclear factor kappa B, anti-oxidation, alanine aminotransferase, aspartate transaminase

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Recently, the application of herbal medicines in treatment of liver diseases are greatly expending in not only China but also Western countries. However, the wide uses of traditional Chinese herbal medicines are still restricted due to their under-clarified mechanisms. Rhubarb, a worldwide used Chinese herbal medicine has been usually prescribed as a liver protecting remedy in China to treat acute or chronic hepatitis caused by variant pathogenesis, such as viral hepatitis, non-alcoholic or alcoholic fatty liver disease[1], autoimmune hepatitis[2-4]. Emodin, one of the main components isolated from rhubarb, was primarily elucidated of hepatoprotective activity[5] in experimental animal models and liver cell line culture[6,7]. However, the liver protective mechanisms of emodin are not fully illustrated.

It was well documented the Reactive Oxygen Species (ROS) are generally involved in the pathological mechanisms of liver injury caused by virus, inflammation and toxins[8-11]. The ROS can directly react with lipids, proteins and nucleic acids causing structural changes of such biomacromolecules and consequently leading to abnormality of biological functions[12-14]. The ROS can also activate cell apoptosis and death progress inducing up-regulation of nuclear factor kappa B (NF-κB) and then cause over generation of inflammatory mediators[10,15]. On the other hand, emodin has an anthraquinone skeleton substituted by phenolic hydroxyls which are potential of anti-oxidation[16]. Some literatures demonstrated the anti-oxidative effect of emodin *in vitro* and *in vivo*, proposing mechanisms through scavenging ROS like oxygen radical, superoxide anion and lipid peroxide, which are generated or induced by light[17], chemical inducers[18] and inflammation[19]. However, the previous *in vivo* studies have been limited in indirect assessment of scavenging ROS activity of emodin by detecting the lipid peroxidation metabolites like malondialdehyde (MDA) and the anti-oxidants like glutathione in tissues or cells, rather than the direct monitoring of scavenging ROS process. So we are wondering whether emodin protects liver through scavenging ROS *in vivo*.

In this study, an optical molecular imaging approach[20] was utilized to monitor the dynamic reaction between emodin and ROS in mice injured by Bacillus Calmette Guerin (BCG) and lipopolysaccharide (LPS) to elucidate the anti-oxidative mechanism of emodin in liver protection. The LPS is a generally studied bacterial toxin causing liver injury through massively generating of ROS[21,22]. Such ROS in tissues can be monitored by reactive fluorogenic probes[23], such as lucigenin[24], luminol[25], dihydroethidium and the other luciferin analogues[26]. In this study, a sensitive fluorogenic probe[27,28], L-012 was used. The serum transaminase activity and oxidation metabolites concentration in liver were also tested. The purpose of this study is to protect the livers via scavenging the reactive oxygen species of emodin visually *in vivo*.

MATERIALS AND METHODS

Phytochemicals, reagents and instruments: Emodin (HPLC purity of 99%, Lot., 050504) was isolated from rhubarb extract and supplied by Jiangsu Jiutai Biological Chemistry Company, China. Emodin was freshly dispersed in the 0.5% carboxymethylated cellulose aqueous solution for drug administration. The BCG (Lot., 20100905) was purchased from the National Institutes for Food and Drug Control, Beijing, China. The LPS (Lot., L2880) was purchased from Sigma-Aldrich Co., LLC., St., Louis, USA. The L-012 (8-Amino-5-chloro-7-phenylpyrido[4,6-8] pyridazine-1,4-(2 H, 3 H) dione sodium salt, Lot., 120-04891) was purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Cell mitochondria isolation kit was purchased from Beijing Solarbio Science and Technology Co., Ltd. Assay kits for alanine aminotransferase (ALT), aspartate transaminase (AST), Reactive Oxygen Species (ROS), hydrogen peroxide (H_2O_2), lipid peroxides (LPO), MDA, superoxide dismutase (SOD), reduced glutathione (GSH) and the total antioxidant capacity (T-AOC) were purchased from Nanjing Jiancheng Biological Engineering Institute.

The T10 basic homogenizer (IKA®-Werke GmbH and Co., KG, Germany). Multiskan MK3 microplate reader (Thermo Fisher Scientific, United States). The optical molecular imaging system was supplied by the Institute of Automation, Chinese Academy of Sciences.

Experimental animals and modeling: Male and female BALB/c mice, weighing 20±1 g were obtained from the Laboratory Animal Center of Academy of Military Medical Sciences (License No. SYXK 2007-004). The animals were raised in an environmentally controlled breeding room (temperature 22±2°C, humidity 60-80%). The breeding room was illuminated by an artificial light cycle with 12 h of light and 12 h of darkness every day and was regularly disinfected. The animals had unlimited access to food and water. The standard rat feed was supplied by the Academy of Military Medical Sciences.

Twenty mice were used in the methodological study of the molecular imaging approach. Another 60 mice were divided randomly into five groups: Namely the normal control group (N), the BCG/LPS model group (M), the low dosage

(30 mg kg^{-1}) of emodin treated group (E_{30}), the middle dosage (60 mg kg^{-1}) of emodin treated group (E_{60}) and the high dosage (120 mg kg^{-1}) of emodin treated group (E_{120}). Except for group N, all the mice were injected intravenously through caudal vein with BCG saline solution (10 mg mL^{-1}, 0.2 mL $capita^{-1}$) at the beginning of the experiment. Then different dosages of emodin in the form of suspension using 0.5% carboxymethylated cellulose (CMC) in distilled water were administered intragastrically to the mice in group E_{30}, E_{60} and E_{120}, once per day, lasting for 14 days. Groups M and N were administered with vehicle solution. Twenty-four hours after the last administration of emodin, all mice except for the normal control ones were injected intravenously through caudal vein with LPS saline solution (7.5 μg $capita^{-1}$)[9]. Meanwhile, mice in group N were administered with saline of equivalent volume. Twelve hours after LPS injury, four mice were selected randomly from each group to perform molecular imaging test and the other eight mice in each group were sampling blood and liver tissue.

This study was conducted in strict accordance with the recommendations of the Guidelines for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of China. The animal protocol was approved by the Committee on the Ethics of Animal Experiments of the 302 Military Hospital (Approval ID: 11-022).

Molecular imaging test: The molecular imaging system was cooled by liquid nitrogen and the CCD was maintained at -110°C. The rats were pre-anesthetized by intraperitoneally (i.p.) administered with 20% ethyl carbamate solution (0.1 mL kg^{-1}). The luminescent probe L-012 was dissolved in ultrapure water. The L-012 was i.p., administered at four different doses (1, 5, 25 and 75 mg kg^{-1}) and then the luminescent signal was recorded from the 8-11 min after injection of L-012. The difference between subcutaneous (s.c.) and i.p. administration of L-012 was tested at dose of 25 mg kg^{-1}.

Sampling and biochemical test: The blood samples were centrifuged at 4000 rpm to separate serum. The activities of serum ALT and AST were determined by the assay kits, respectively. Accurately weighed liver tissue of 0.5 g was cut into pieces and homogenized with 9 times of cold normal saline (w/v) in ice-bath. The homogenate was centrifuged at 3000 rpm under 4°C for 15 min. The supernatant was determined for the contents of H_2O_2, LPO, MDA, GSH and the activities of SOD, GSH, T-AOC in liver homogenate and were determined, respectively. The protein in liver homogenate was also determined with biuret reagent.

Data processing and statistical analysis: The data were processed by WinMI software, calculating automatically the segmentation mode of luminescent region and photon number, adding pseudocolor and merging with white light images. All data were expressed as Mean±Standard Deviation (SD). Comparison among groups was analyzed by ANOVA and the significant level of probability was set at 0.05.

RESULTS

Activities of ALT and AST: The serum ALT and AST activities were summarized in Table 1. The results showed that the ALT and AST activities were both significantly elevated by the irritation of BCG/LPS in the model mice, compared to the normal control group (N). These two indices decreased significantly after the treatment of emodin in a dose-dependent manner, compared to group M.

***In vivo* ROS signals in mice:** There was no significant difference of luminescent signal between i.p. and s.c. administration routes of L-012 (Fig. 1a). The luminescent signal intensity was positively correlated to the dose of L-012 and the dose of 25 mg kg^{-1} produce enough luminescent signal (Fig. 1b). So we used 25 mg kg^{-1} of L-012 (i.p.) in the rest of the experiments. It was significant elevation of ROS signal in the mice of BCG/LPS-injured group (M) (Fig. 2a), compared to the normal control group (N) ($p<0.01$, Fig. 2b). And there were significant and dose-dependent declines of ROS signals in the emodin-treated mice of groups E_{30}, E_{60} and E_{120} ($p<0.01$, Fig. 2b), among which the luminescent signal in the large dose group of emodin could not be observed (Fig. 2a).

MDA, LPO and H_2O_2 in liver tissues: Compared to the normal control group (N), the values of liver MDA, LPO and H_2O_2 were all significantly elevated in the BCG/LPS-injured mice (group M) (Table 2). And such indices were significantly decreased by the treatment of emodin with dose-related trends. The value of MDA was restored to normal level at the

Table 1: Effects of emodin on serum ALT and AST activities in BCG/LPS- injured mice

Groups	Dosage (mg kg^{-1})	sALT (U L^{-1})	sAST (U L^{-1})
N		32.8±5.9	35.1±4.2
M		201.7±18.7**	197.5±10.4**
E_{30}	30	184.1±16.1**▲	167.2±19.2**▲▲
E_{60}	60	125.6±11.9**▲▲	102.3±9.6**▲▲
E_{120}	120	83.5±7.6**▲▲	61.6±6.9**▲▲

Data are presented as Mean±SD, n = 8 per group, *$p<0.05$, ** $p<0.01$ compared to the control group (N), ▲$p<0.05$, ▲▲$p<0.01$ compared to the BCG/LPS-injured model group (M)

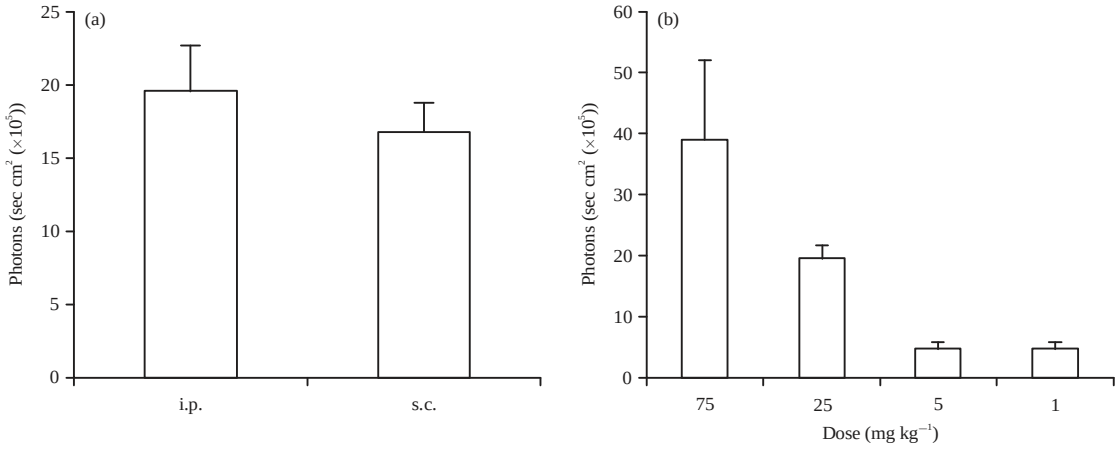

Fig. 1(a-b): Administration route and dose of L-012 influence the luminescent signal, (a) Difference between intraperitoneal (i.p.) and subcutaneous (s.c.) administration of L-012 at dose of 25 mg kg^{-1} and (b) Signals of different dose (mg kg^{-1}) of L-012 administered by i.p., route. Error bars indicate Standard Deviation (SD)

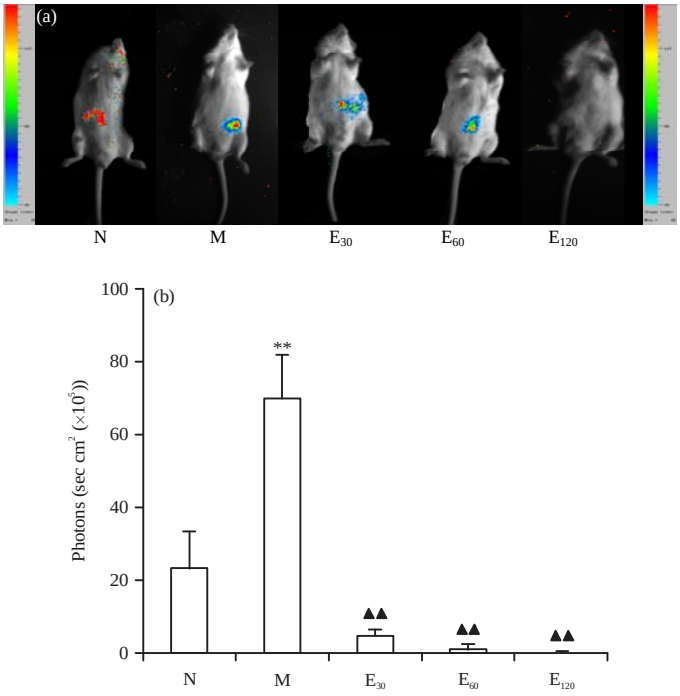

Fig. 2(a-b): Emodin suppresses ROS luminescent signal in BCG/LPS-injured mice, (a) Pictures of the *in vivo* imaging of different groups (n = 4) and (b) Average intensities of luminescent signal in different groups (n = 4), N: Normal control group, M: BCG/LPS model group, E_{30}: Low dosage (30 mg kg^{-1}) of emodin treated group, E_{60}: Middle dosage (60 mg kg^{-1}) of emodin treated group and E_{120}: High dosage (120 mg kg^{-1}) of emodin treated group. The ROS luminescent signal declined as the increment of the emodin dosage. There was significant elevation of ROS signal in the mice of BCG/LPS-injured group (M) compared to the normal control group (N). And the ROS signals of the emodin-treated mice of groups E_{30}, E_{60} and E_{120} declined significantly in a dose-dependent manner, among which the luminescent signal in the large dose group of emodin could not be observed. Error bars indicate Standard Deviation (SD), **p<0.01 as compared with the normal control group (N), ▲▲p<0.01 as compared with the BCG/LPS model group (M)

large dosage of emodin (120 mg kg^{-1}). The values of LPO and H_2O_2 were restored to normal level at the low dosage of emodin (30 mg kg^{-1}) and continually decreased along with the dosage increased.

Table 2: Effects of emodin on liver MDA, LPO and H_2O_2 in BCG/LPS-injured mice

Groups	MDA (nmol mg^{-1} prot)	LPO (μmol L^{-1})	H_2O_2 (mmol g^{-1} prot)
N	0.603±0.385	1.508±0.096	7.100±1.604
M	3.119±1.325**	1.704±0.231*	9.811±1.896**
E_{30}	0.735±0.606▲▲	1.509±0.078▲	7.985±1.492▲
E_{60}	0.702±0.251▲▲	1.474±0.111▲	7.159±2.363▲
E_{120}	0.691±0.399▲▲	1.399±0.293▲	5.184±0.959▲▲

Data are presented as Mean±SD, n = 8 per group, *p<0.05, **p<0.01 compared to the control group (N), ▲p<0.05, ▲▲p<0.01 compared to the BCG/LPS-injured model group (M)

Table 3: Effects of emodin on liver SOD, GSH and T-AOC in BCG/LPS-injured mice

Groups	SOD (U mL^{-1})	GSH (mg g^{-1} prot)	T-AOC (U mg^{-1} prot)
N	66.091±25.151	1.068±0.101	1.079±0.874
M	39.861±19.393*	0.867±0.114**	0.499±0.277
E_{30}	65.849±20.975▲▲	1.278 ±0.276▲▲	0.596±0.365
E_{60}	89.475±8.947▲▲	1.360±0.518▲▲	1.120±0.626▲
E_{120}	98.319±15.578▲	1.490±0.319▲▲	1.632±1.048▲

Data are presented as Mean±SD, n = 8 per group, *p<0.05, **p<0.01 compared to the normal control group (N), ▲p<0.05, ▲▲p<0.01 compared to the BCG/LPS-injured model group (M)

GSH, SOD and T-AOC in liver tissues: Compared to the normal control group (N), the values of liver SOD, GSH and T-AOC were all significantly declined in the BCG/LPS-injured mice (group M) (Table 3). While treated with emodin, such indices were all restored with dose-dependent trends. The SOD could be restored to normal level and the GSH could be restored up to the normal level by emodin treatment even at the low dosage (30 mg kg^{-1}). In the large dose group, the values of SOD, GSH and T-AOC were all larger than the normal group.

DISCUSSION

Mice primed with BCG are highly sensitive to endotoxin such as LPS-mediated hepatotoxicity[29], resulting in a massive secretion of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF-α)[30]. The LPS induced liver injury associated with up-regulation of toll-like receptor 4 (TLR4)[31], which is the central signaling receptor for LPS in mammals[32]. The TLR4 activation also activates ROS, including nitric oxide (NO·), superoxide ($O_2{\cdot}^-$), H_2O_2 and hydroxyl radical (·OH) etc., which are toxic to cells because they can react with most cellular macromolecules, including proteins, lipids and DNA[8,12-14,33]. There is now substantial evidence that ROS contribute significantly to the pathogenesis of acute hepatocyte injury in the liver[34,35]. Thus, anti-oxidants are considered of protective potential in treating ROS induced liver injury[9,10,36,37]. Emodin has been proved of exact anti-oxidative effects *in vitro* and *in vivo*[17-19,38,39] and of hepatoprotective effects in different experimental animal models and liver cell lines culture[7]. However, there is no proof that the antioxidative activity of emodin is contributed to scavenging ROS *in vivo*. However, ROS are evanescent species and consequently, their measurement within integrated systems, such as animal models and humans is a complex challenge[27]. *In vivo* monitoring ROS has drawn great attention to researchers[26,40]. Recently, the molecular imaging technology has been used to detect LPS-induced ROS in rat tissues *in vivo*[28]. In this study, we found the emodin showed dose-dependent effect in scavenging ROS signal *in vivo* (Fig. 2). Moreover, the contents of H_2O_2 in the livers of emodin-treated groups (E_{30}, E_{60} and E_{120}) showed significant decreases with a dose-related trend, compared to the group M (Table 2), which indicated the peroxidation induced by LPS was blocked or inhibited in tissue. In lipid peroxidation, ROS, especially hydroxyl radicals (·OH), initiate the chain reaction through "Stealing" the hydrogen atoms from the lipids in cell membranes and at last result in cell damage. One of the end-product of lipid peroxidation is malondialdehyde (MDA), which reacts with deoxyadenosine and deoxyguanosine in DNA and results DNA damages[41]. Thus, the contents of lipid peroxides (LPO) and MDA can reflect the peroxidation status in tissue. Our results showed that emodin-treated groups had significantly lower contents of LPO and MDA in livers, compared to the group M, which illustrated the inhibitory or blocking effect of emodin to LPS-induced peroxidation status in rats.

In normal conditions, there is a redox-optimized ROS balance in body[42]. Some intracellular anti-oxidant regulators, such as superoxide dismutase (SOD) and glutathione can block the ROS overflow and their chain reactions. However, the overproduction of ROS induced by LPS will exhaust anti-oxidants and then the cell antioxidant defenses can be compromised and eventually overwhelmed[42]. When the balance is destroyed, several enzymatic (SOD) and non enzymatic (GSH) markers of oxidative stress can be found of significant decreases[21,43]. Our results showed that the contents of SOD and GSH, along with the total anti-oxidative capability (T-AOC) in emodin-treated rat livers had significant increase with dose-related trend compared to the group M. It could be attributed to the anti-oxidative effect of emodin which helps to maintain the ROS balance *in vivo*. In summary of *in vitro* and *in vivo* evidences, emodin can scavenge ROS and block the overproduction of ROS induced by LPS, then consequently protect against the ROS-induced tissue injury.

Except for blocking the ROS-mediated direct injury, emodin might involved in the regulation of ROS-mediated signal transductions resulting in cell injury. Generally,

increased ROS production in a cell leads to the activation of extracellular signal-related kinases (ERKs), mitogen-activated protein kinase (MAPK) and MAPK-mediated tumor necrosis factor-α (TNF-α) biosynthesis[44,45]. The TNF-α plays an important role in BCG/LPS-induced acute liver injury[46]. It was reported that emodin significantly alleviated the increases of TNF-α and its mRNA expression levels induced by LPS *in vivo*[22]. The NF-κB can also be activated by ROS[15] and NF-κB activation is a common pathway that mediates LPS-induced up-regulation of gene encoding for proinflammatory cytokines[11]. It was also reported that emodin can inhibit extracellular regulated protein kinase (ERK) 1/2 and suppress the transcriptional activity of nuclear factor-kappaB (NF-κB)[47]. These evidences support the regulating effect of emodin on ROS-mediated signal transductions. Considering the direct reaction of emodin to ROS, whether emodin regulates these signal transduction pathways via ROS is still unclear.

The light emission was observed from the abdominal area (Fig. 2). It was reported that substantial amounts of ROS are produced in the intestinal epithelial cells[48] and the distal part of the small intestine emits the strongest luminescence signal of ROS[28]. The liver, lungs, skin, spleen and brain also emit luminescence signals of ROS probed by L-012 after LPS administration[28]. Since emodin could be distributed in liver, kidneys, lungs, spleen, heart and the other organs[49,50], there might be interests in whether emodin has protective effect to these organs and whether its therapeutic effect is related to ROS scavenging.

There are some structural analogues of emodin of anti-oxidative activity. A study showed 2-hydroxyemodin has better inhibitory activities in reactive oxygen- and nitogen-mediated reactions as well as anti-lipid peroxidation than emodin does[18]. The recent studies showed emodin can also protect livers against chemical toxins (carbon tetrachloride, α-naphthylisothiocyanate and acetaminophen) induced injury[6,51-53]. Emodin can also prevent hepatosteatosis and fibrogenesis in rats[7]. Whether such liver protection of emodin is related to the inhibition to ROS needs further illustration.

CONCLUSION

Emodin can protect liver against bacillus calmette guerin/lipopolysaccharide-induced injury and the potential mechanisms underlying hepatoprotection functions of emodin include two aspects in this study: The first one is that emodin scavenges reactive oxygen species, the second one is that emodin posseses anti-lipid peroxidation effect. The present study illustrated emodin might become a potential agent for the treatment of bacillus calmette guerin/lipopolysaccharide-induced liver injury.

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