Luteolin Alleviates AflatoxinB₁-Induced Apoptosis and Oxidative Stress in the Liver of Mice through Activation of Nrf2 Signaling Pathway

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Abstract: Aflatoxin B₁ (AFB₁), a threatening mycotoxin, usually provokes oxidative stress and causes hepatotoxicity in animals and humans. Luteolin (LUTN), well-known as a strong antioxidant, acts as a strong antioxidant. This research was designed to investigate whether LUTN exerts protective effects against AFB₁-induced hepatotoxicity and explore the possible molecular mechanism in mice. A total of forty-eight mice were randomly allocated following four treatment groups (n = 12): Group 1, physiological saline (CON). Group 2, treated with 0.75 mg/kg BW aflatoxin B₁ (AFB₁). Group 3, treated with 50 mg/kg BW luteolin (LUTN), and Group 4, treated with 0.75 mg/kg BW aflatoxin B₁ + 50 mg/kg BW luteolin (AFB₁ + LUTN). Our findings revealed that LUTN treatment significantly alleviated growth retardation and rescued liver injury by relieving the pathological and serum biochemical alterations (ALT, AST, ALP, and GGT) under AFB₁ exposure. LUTN ameliorated AFB₁-induced oxidative stress by scavenging ROS and MDA accumulation and boosting the capacity of the antioxidant enzyme (CAT, T-SOD, GSH-Px and T-AOC). Moreover, LUTN treatment considerably attenuates the AFB₁-induced apoptosis in mouse liver, as demonstrated by declined apoptotic cells percentage, decreased Bax, Cyt-c, caspase-3 and caspase-9 transcription and protein with increased Bcl-2 expression. Notably, administration of LUTN up-regulated the Nrf2 and its associated downstream molecules (HO-1, NQO1, GCLC, SOD1) at mRNA and protein levels under AFB₁ exposure. Our results indicated that LUTN effectively alleviated AFB₁-induced liver injury, and the underlying mechanisms were associated with the activation of the Nrf2 signaling pathway. Taken together, LUTN may serve as a potential mitigator against AFB₁-induced liver injury and...
could be helpful for the development of novel treatment to combat liver diseases in humans and/or animals.

**Keywords:** aflatoxin B$_1$; luteolin; oxidative stress; apoptosis; liver injury; Nrf2 signaling pathway

1. **Introduction**

Aflatoxins are one of the most dangerous mycotoxins, produced mainly by *Aspergillus flavus* and *Aspergillus parasiticus* and usually found in agricultural environments and food commodities [1]. According to studies, even low levels of aflatoxins in the diet can be harmful to human health [2,3]. Currently, approximately 4.5 billion people are in danger of being exposed to aflatoxins, which cause 4.6–28.2% of all occurrences of hepatocellular carcinoma worldwide [4,5]. Aflatoxin B$_1$ (AFB$_1$) is the most potent liver carcinogen among the aflatoxins, and it has been categorized as a Class I carcinogen by the International Agency for Research on Cancer (IARC) [6]. Additionally, AFB$_1$ is reported to cause severe health issues, including growth retardation, hepatotoxicity, neurotoxicity, teratogenicity, mutagenicity and immunotoxicity in humans and animals [7–9]. The liver is believed to be the primary target organ for AFB$_1$ toxicity, a major metabolizing and detoxifying organ in the body [10]. Previous research has shown that reactive oxygen species (ROS) formation appears to be a significant contributor to the toxicity caused by AFB$_1$ in the liver [11]. ROS overproduction could lead to mitochondrial oxidative stress, resulting in lipid peroxidation and decreased antioxidant enzyme activity, causing cellular and organismal hepatic damage [5,12]. Therefore, it is imperative to find an effective antioxidant to protect the liver and alleviate the AFB$_1$ toxicity. Natural active biological compounds derived from plants have recently received much attention due to their low toxicity. Herbal metabolites are effective alternatives for tackling the hazardous effects of AFB$_1$ [13,14].

Flavonoids are bioactive compounds primarily present in plants with a wide range of pharmacological and health-promoting properties [15]. Luteolin (3,4,5,7-tetrahydroxyflavone) is a type of natural flavonoid found in various plants worldwide, such as fruits, vegetables, and some herbal medicines [16]. Luteolin possesses a diverse range of biological properties, including antioxidant [17], antimicrobial [18], anti-inflammatory [19], anticancer [20], and neuroprotective capabilities [21]. The nuclear factor erythroid 2-related factor (Nrf2) plays a central role in the activation of cytoprotective genes in response to xenobiotics and oxidative stress by binding to the antioxidant response element (ARE) [22,23]. Moreover, the Nrf2 gene is typically expressed in metabolically active tissues such as the liver [10]. A recent study revealed that AFB$_1$-induced liver damage in broiler chicks has been associated with dysregulating the Nrf2 signaling pathway [13]. Therefore, Nrf2 signaling is regarded as the most significant therapeutic target for preventing and treating oxidative stress-induced liver damage [24,25]. Although, a study reported that luteolin has a therapeutic impact on ochratoxin A-induced oxidative injury in NRK-52E kidney cells [17]. Moreover, LUTN has been reported to exert antifibrogenic effects against carbon tetrachloride-(CCL4) on hepatic satellite cells and liver fibrosis via multiple mechanisms [26,27]. However, the preventative actions of LUTN against AFB$_1$-induced liver damage and underlying mechanisms have still not been explored. Therefore, the current research was designed to investigate whether LUTN exerts protective effects against AFB$_1$-induced hepatotoxicity and explore the possible molecular mechanism in mice. Presumably, this is the first study to highlight the protective role of luteolin against AFB$_1$-induced hepatic damage in mice.

2. **Materials and Methods**

2.1. **Chemicals and Antibodies**

The luteolin (LUTN, #41753–43-9, purity ≥ 98%) was bought from (Shanghai Yuanye Bio-Technology Co., Ltd.). Aflatoxin B$_1$ (AFB$_1$, #1162-65-8) used in the present study was supplied by Sigma-Aldrich (St. Louis, MO, USA). The ELISA assay kits for malondi-
aldehyde (MDA, #A003-1), reactive oxygen species (ROS, #E004), glutathione peroxidase 
(GSH-Px, #A005), total antioxidant capacity (T-AOC, #A015), catalase (CAT, #A007-1) and 
total superoxide dismutase (T-SOD, #A001) were from (Jiancheng Bioengineering Institute, 
Nanjing China). Apoptosis detection kit annexin V-FITC/PI (#KGA-108) was supplied by 
(Jiangsu KGI, Biotech, CO., Ltd. China). A test kit for the bicinchoninic acid assay was 
provided by Thermo Fisher Scientific (Waltham, MA, USA). Chemiluminescence Western-
Bright ECL substrate kit (# ab65623) was supplied by (Abcam, Shanghai, Trading, Co., Ltd. 
China). The primary antibodies, heme oxygenase-1 (HO-1, #A1346), glutamate-cysteine 
ligase catalytic subunit (GCLC, #A1038), quinone oxidoreductase 1 (NQO1, #A1518), nu-
clear factor erythroid 2-related factor 2 (Nrf2, #A0674), Bcl-2-associated X protein (Bax, 
#A19684), caspase-3 (#A2156) and B-cell lymphoma 2 (Bcl-2, A0208) were procured from 
(Abclonal Tech, Woburn, MA, USA). Secondary antibodies, anti-mouse IgG (#4410), anti-
rabbit IgG (#4414), and β-actin (#3700), were obtained from (Cell Signaling Technology, 
Boston, MA, USA).

2.2. Animals

Four weeks old male C57BL/6 mice were bought from Wuhan University (Wuhan, 
China). As an adoption period, mice were housed in separate cages and given an appro-
priate environment one week before the start of the experiment. Standard feed pellets 
and freshwater were accessible to the animal’s ad libitum. All mice were housed under 
laboratory conditions, light-dark period (12 h light/12 h dark), relative humidity of 45–60%, 
and the temperature of 22 ± 2 °C. Huazhong Agricultural University permitted the ani-
mal experiments under the Laboratory Animals Care and Ethics Committee (Permit No. 
HZAUMO-2018-07). Besides this, the health of experimental mice was closely monitored, 
and necessary measures were taken to assure the maximum welfare of the animals.

2.3. Experimental Design and Treatment

The 48 mice were randomly assigned into four groups as follows: (n = 12):

- Group 1, (CON) received physiological saline.
- Group 2, (AFB₁), treated with 0.75 mg/kg BW aflatoxin B₁.
- Group 3, (LUTN), treated with 50 mg/kg BW luteolin.
- Group 4, (AFB₁ + Luteolin), treated with 0.75 mg/kg BW aflatoxin B₁ + 50 mg/kg BW 
luteolin.

LUTN and AFB₁ were dissolved in phosphate buffer saline. Following our preliminary 
experiment and the results of previous researchers, we chose a dose of 0.75 mg/kg BW for 
AFB₁ as reported this dose could induce hepatotoxicity [28], and oral gavage of 50 mg kg 
BW LUTN could ameliorate liver damage in mice [29]. The experiment lasted for 15 days, 
and all groups received oral administration once a day at 9.00 a.m. During the whole 
experiment, feed intake and body gain were recorded.

2.4. Sample Collection

The mice were individually weighed and euthanized at the end of the experiment 
to collect the blood and liver samples. The serum was separated from blood samples 
following the centrifugation and was kept at −20 °C for serum biochemical assays. The 
Liver coefficient (%) = \frac{Liver weight (g)}{Mice weight (g)} \times 100

(1)
2.5. Determination of Serum Biochemical Indicators

An automatic biochemistry analyzer (Beckman Synchron CX4 PRO, Fullerton, CA, USA) was used to quantify the amounts of globulin and albumin, as well as GGT, ALP, AST, and ALT in serum samples following the manufacturer’s suggested protocol.

2.6. Hematoxylin and Eosin (H&E) Staining

The H&E staining was conducted as reported in our prior study [30]. Liver specimens were fixed for 24 h in 4% fresh paraformaldehyde solution, dried with alcohol solvent, and then embedded. The 5 µm fragments were sectioned and processed for H&E staining to assess the pathological observation in the liver of mice under a microscope (Nikon, Tokyo, Japan).

2.7. Determination of Oxidative Stress Indices

The 10% tissue homogenates were prepared from collected liver samples following our previously reported procedure [6]. The bicinchoninic acid determination kit was used to measure the protein concentration of tissue homogenate. ROS, MDA, T-SOD, GSH-Px, CAT and T-AOC were detected using commercially available ELISA kits. The measurements were carried out following the kit’s protocols.

2.8. Apoptosis Assay by Flow Cytometry

Single-cell suspensions were prepared to detect the apoptosis rate in the liver of mice following our previously described procedure [31,32]. Briefly, an annexin V-FITC/PI apoptosis detection kit was used to determine the apoptosis rate in the liver following the manufacturer’s recommended instructions. The cells were stained with annexin V-FITC (5 µL) and PI (5 µL) in the dark for 30 min at room temperature. Finally, apoptosis rates were determined using flow cytometry (Beckman-CytoFLEX Coulter, CA, USA). The data were examined by using FlowJo (BD Biosciences, NJ, USA).

2.9. Quantitative Real-Time PCR (qRT-PCR) and Western Blotting Analysis

The transcription levels of pertaining genes used in the present study were determined by qRT-PCR following the method previously mentioned in our study [1]. The primers tested in the current research are presented in Supplementary Table S1. Relative mRNA expression was normalized to the CON group. The $2^{-\Delta\Delta C_t}$ formula was used to quantify with the β-actin as a reference gene [33]. The protein expression of Nrf2 signaling and mitochondrial apoptotic pathways in mouse liver was evaluated by Western blot according to the previously described procedure [34,35]. The bicinchoninic acid assay kit was used to quantify the protein contents of samples. Chemiluminescence WesternBright™ ECL substrate kit was used to identify the blots, and then FluroChem FC2 Imaging System was used to visualize and quantify the results.

2.10. Statistical Analysis

The experimental data were analyzed for significance by using SPSS (version 22., IBM Corporation, Armonk, NY, USA) software. A one-way analysis of variance (ANOVA) was used for statistical analysis, followed by the least significant difference (LSD) test. The results are presented as mean ± SD. The significance level of data was set at $p$-value < 0.05.

3. Results

3.1. Luteolin Alleviates Growth Retardation of Mice Induced by AFB$_1$

The protective effects of luteolin (LUTN) on the growth of mice exposed to aflatoxin B$_1$ (AFB$_1$) are depicted in Figure 1. During the entire experimental period, the group exposed to AFB$_1$ recorded the ($p < 0.05$) lowest ADG and ADFI in the comparison of CON, LUTN and AFB$_1$ + LUTN groups, respectively. Contrastingly, LUTN therapy considerably improved growth performance in mice, as evidenced by increased ADG and ADFI ($p < 0.05$) compared to the AFB$_1$ group. Furthermore, LUTN treatment significantly reduced liver
coefficient \((p < 0.05)\) increased by AFB\(_1\) (Figure 1C). These findings indicated that LUTN could eliminate the harmful effect of AFB\(_1\) on the growth of mice.

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![Figure 1](image)

**Figure 1.** Luteolin treatment alleviates growth retardation of mice induced by aflatoxin B\(_1\). (A) Average daily gain (ADG), (B) average daily feed intake (ADFI) and (C) liver coefficients (%). The results are presented as mean ± SD \((n = 12)\). \(a–c\) Columns with different lowercase letters indicated significant differences between the compared groups \((p < 0.05)\).

3.2. Luteolin Protects AFB\(_1\)-Induced Liver Damages in Mice

The effects of LUTN treatment on the biochemical profile of mice exposed to AFB\(_1\) are summarized in Figure 2A–F. Compared with the CON group, AFB\(_1\) exposure considerably \((p < 0.05)\) increased serum liver enzymes activities such as ALP, ALT, AST, and GGT, while decreased globulin and albumin content. On the other hand, LUTN treatment considerably reversed AFB\(_1\)-induced alterations on the biochemical profile of mice. Moreover, histological analysis revealed that AFB\(_1\) exposure damaged the liver structure of mice, as evident by microvesicular appearance of the lipid droplets with small and large area of blood infiltration were observed in the AFB\(_1\) treated group (Figure 3B). Strikingly, LUTN treatment evidently \((p < 0.05)\) ameliorated and restored liver injury induced by AFB\(_1\), indicating that LUTN could protect the liver from AFB\(_1\)-induced hepatotoxicity.

3.3. Luteolin Ameliorates Oxidative Damage in the Liver of Mice Induced by AFB\(_1\)

To detect the redox status in the liver of experimental mice, ROS, MDA, CAT, T-SOD, GSH-Px, and T-AOC were detected. As shown in Figure 4A,B, mice exposed to AFB\(_1\) revealed a significant \((p < 0.05)\) increase in ROS and MDA levels. At the same time, the activities of T-AOC, CAT, GSH-Px and T-SOD were significantly reduced as compared to the CON, LUTN and AFB\(_1\) + LUTN groups, respectively. Contrastingly, LUTN treatment significantly \((p < 0.05)\) reversed AFB\(_1\)-induced alterations in the oxidative stress markers and antioxidant variables in the liver of mice, as evident by decreased ROS and MDA levels by 38% and 20%, respectively, while increased antioxidant enzyme activities of CAT, T-SOD, GSH-Px, and T-AOC by 36.85, 30.27, 27.26, and 40% respectively, as compared to the AFB\(_1\) treated group (Figure 4A–F).
Antioxidant treatment prevents AFB1-induced liver damages in mice. (A) alkaline phosphate (ALP), (B) aspartate aminotransferase (AST), (C) Alanine aminotransferase (ALT), (D) gamma-glutamyl transferase (GGT), (E) albumin and (F) globulin. The results are presented as mean ± SD (n = 6). a–c Columns with different lowercase letters indicated significant differences between the compared groups (p < 0.05).

The histopathology of liver sections was stained with H&E staining. (A,C) Histological section of the liver from CON and LUTN group showed a normal histoarchitecture consisting of central vein surrounded by hepatocytes possessed sinusoids spaces. (B) The liver section from the AFB1 group showed microvesicular (black arrow) appearance of the abundant fatty droplets with small and large area of blood infiltration (red arrow) showed a hepatotoxicity. (D) Liver tissue from the group of mice treated with LUTN and challenged with AFB1 manifested recovered status of the liver from hepatotoxicity, as depicted small and rare patches of the lipids (black arrow) and the blood infiltration (red arrow) as compared to the AFB1 alone challenged group.

3.4. Luteolin Treatment Prevents AFB1-Induced Apoptosis in Mice Hepatocytes

Apoptosis rate in the hepatocytes was measured by flow cytometry (Figure 5). Apoptosis analysis revealed that AFB1 exposure considerably increased (p < 0.05) the proportion of apoptotic cells relative to the CON, LUTN, and AFB1 + LUTN groups, respectively. However, LUTN administration dramatically reduced (p < 0.05) the proportion of apoptotic cells in comparison to the AFB1 group.
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**Figure 4.** Luteolin treatment ameliorates oxidative damage in the liver of mice induced by AFB1. (A) Reactive oxygen species (ROS), (B) malondialdehyde (MDA) (C) total antioxidant capacity (T-AOC), (D) catalase (CAT), (E) glutathione peroxidase (GSH-Px) and (F) total superoxide dismutase (T-SOD). The results are presented as mean ± SD (n = 6). a–c Columns with different lowercase letters indicated significant differences between the compared groups (p < 0.05).

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**Figure 5.** Luteolin treatment prevents AFB1-induced apoptosis in mice hepatocytes. (A) The apoptosis rates of hepatocytes were measured using flow cytometry. (B) Statistical analysis of apoptosis rate. The results are presented as mean ± SD (n = 3). a–c Columns with different lowercase letters indicated significant differences between the compared groups (p < 0.05).

### 3.5. Luteolin Restrains AFB1-Induced Mitochondrial Apoptosis Pathway

Mitochondrial apoptosis-associated transcription and protein expressions were detected by qRT-PCR and western blotting. As depicted in Figure 6A–E, the transcripts levels of Bax, cytochrome-c, caspase-3, and caspase-9 were significantly (p < 0.05) increased, while Bcl-2 was decreased under AFB1 exposure. Contrastingly, LUTN administration significantly reversed the transcripts levels of these genes as comparative to the AFB1 exposed group. Moreover, we investigated the protein expression of Bcl-2, Bax and caspase-3 in the liver tissue of mice by western blotting (Figure 6F,G). Interestingly, the results of western blotting for Bcl-2, Bax and caspase-3 were consistent with the qRT-PCR results. Similarly, in the AFB1 exposed group, Bcl-2 protein expression was (p < 0.05) down-regulated, while Bax and caspase-3 protein expression was (p < 0.05) up-regulated as compared to the CON group. Conversely, LUTN treatment significantly alleviated the alterations in the Bcl-2, Bax and caspase-3 protein levels induced by AFB1.
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Figure 6. Luteolin treatment restrains AFB1-induced mitochondrial-mediated apoptosis pathway. The relative expression of mitochondrial-mediated apoptosis pathway transcripts was analyzed by qRT-PCR. (A–E) The relative mRNA expression of Bcl-2, Bax, Cyto-c, Cas-3 and Cas-9. The results are presented as mean ± SD (n = 6). Western blotting was used to detect the expression of mitochondrial apoptosis-associated proteins. (F) Western blotting analysis of Bcl-2, Bax and Cas-3. (G) Quantitative analysis of western blotting for Bcl-2, Bax and Cas-3. The results are presented as mean ± SD (n = 3). a–c Columns with different lowercase letters indicated significant differences between the compared groups (p < 0.05). B-cell lymphoma 2 (Bcl-2); Bcl-2-associated X protein (Bax); cytochrome-c (Cyt-c); caspase-3 (Cas-3); caspase-9 (Cas-9).

3.6. Luteolin Treatment Activates Nrf2 Signaling Pathway in the Liver of AFB1 Exposed Mice

To confirm our hypothesis that LUTN promotes the antioxidant capacity and alleviates hepatotoxicity in mice induced by AFB1 is associated with Nrf2 signaling pathway activation, the transcript levels and protein expression of Nrf2 and downstream targets were detected. As depicted in Figure 7A–E, AFB1 exposed group showed a significant (p < 0.05) decrease in the gene expression of Nrf2, HO-1, GCLC, NQO1 and SOD1 compared to the CON and other experimental groups. In contrast, LUTN treatment considerably improved the transcript expressions of Nrf2, HO-1, GCLC, NQO1 and SOD1 altered by AFB1.
(p < 0.05). Further, the protein expression of Nrf2 and its target genes were detected by western blotting and revealed the same tendency (Figure 7F,G). Similarly, protein expression of Nrf2 and its downstream targets, including HO-1, GCLC, and NQO1, were (p < 0.05) down-regulated in the AFB1 challenged group. However, LUTN treatment considerably up-regulated the Nrf2, NQO1, HO-1 and GCLC protein expressions as down-regulated by AFB1 (p < 0.05).

**Figure 7.** Luteolin treatment activates the Nrf2 signaling pathway in the liver of AFB1 exposed mice. The relative expression of Nrf2-mediated antioxidant signaling transcripts was analyzed by qRT-PCR. (A–E) The relative gene expression of Nrf2 and its downstream molecules, HO-1, GCLC, NQO1 and SOD1. The results are presented as mean ± SD (n = 6). The expression of Nrf2 and its associated proteins was detected by western blotting. (F) Western blots for Nrf2, HO-1, GCLC, and NQO1. (G) Quantification of western blots for Nrf2, HO-1, GCLC, and NQO1. The results are presented as mean ± SD (n = 3). a–c Columns with different lowercase letters indicated significant differences between the compared groups (p < 0.05). Nuclear erythroid-2-related factor (Nrf2); glutamate-cysteine ligase catalytic subunit (GCLC), heme oxygenase-1 (HO-1); superoxide dismutase 1 (SOD1); quinone oxidoreductase 1 (NQO1).

### 4. Discussion

Aflatoxins, threatening mycotoxins, are commonly found in cereals and animal forages and pose major health and economic risks to humans and animals [36]. Growth retardation is one of the most important symptoms of aflatoxins poisoning. In the present study, AFB1 exposure decreased ADG and ADFI in mice. The observed growth retardation may have resulted from anorexia, suppression of lipogenesis, and protein synthesis induced by AFB1 [37]. The liver is regarded as the main target organ of AFB1 poisoning. We found that AFB1 generated clinical and pathological symptoms of liver injury in mice, as evident from the increased haptic enzymes AST, ALT, ALP, GGT, and decreased globulin and albumin content as well as fatty droplets and hepatocytes infiltration with macro vesicles in the liver of mice. These findings imply that AFB1 can cause hepatotoxicity, consistent with previous findings [28,38,39]. Interestingly, luteolin (LUTN) treatment attenuated growth retardation and alleviated toxic effects on serum biochemical profile and pathological changes in the liver of mice induced by AFB1. The current results align with a previous study, which has reported that LUTN supplementation prevented acetaminophen-induced hepatic injury in rats [16]. The present findings strongly suggested that LUTN treatment can mitigate
AFB<sub>1</sub>-induced hepatic damage in mice. However, special clinical studies are warranted to know the hepatoprotective effects of LUTN on ongoing or established AFB<sub>1</sub> toxicity in humans or animals.

Oxidative stress is considered to be critical molecular process underlying cell damage [40]. Oxidative stress is associated with significant increase in the generation of reactive oxygen species (ROS) while decreased antioxidant capacity in the body [41]. Previous studies reported that excessive ROS production is a significant cause of AFB<sub>1</sub>-induced hepatotoxicity and apoptosis in mice [5,30,42]. Similarly, in the present study, AFB<sub>1</sub> exposure generated ROS and MDA accumulation and inhibited antioxidant enzyme activities such as T-SOD, CAT, GAH-Px and T-AOC, and induced apoptosis in the liver of mice. LUTN is considered a potent ROS scavenger, protecting cells from ROS accumulation and apoptosis induced by oxidative stress [43]. We found that LUTN treatment substantially suppressed oxidative stress induced by AFB<sub>1</sub> as evidenced by decreased ROS and MDA accumulation, strengthened antioxidant defense system (T-SOD, CAT, GAH-Px and T-AOC), and reduced apoptosis rate in the liver of mice.

Mitochondria have been identified as the primary targets for AFB<sub>1</sub> harmful effects in causing apoptosis [44]. The mitochondrial apoptosis pathway is regulated by anti-apoptotic and pro-apoptotic members of the Bcl-2 family [45]. Bcl-2 is an anti-apoptotic protein that impedes the release of apoptogenic molecules (Cyt-c), while Bax is a pro-apoptotic protein that promotes the release of Cyt-c into the cytoplasm by competing with Bcl-2, thereby causing cell death [46,47]. The release of Cyt-c into the cytoplasm, resulted with Bcl-2, thereby causing cell death [46,47]. The release of Cyt-c into the cytoplasm, resulted in caspase-3 and caspase-9 activation and the induction of apoptosis [48,49]. Previously, studies reported that AFB<sub>1</sub> exposure could cause apoptosis in various tissues and cells and the mechanism was linked with the mitochondrial apoptosis pathway [5,50–52]. Our results showed that expressions of Bax, Cyt-c caspase-3 and caspase-9 were considerably up-regulated, while Bcl-2 was down-regulated by AFB<sub>1</sub>. These findings demonstrated that AFB<sub>1</sub>-induced excessive apoptosis in the liver of mice is linked with the mitochondrial apoptosis pathway. Notably, LUTN treatment increased the Bcl-2 expression, inhibited mitochondrial Cyt-c release and reduced the activated caspase-3 and caspase-9 expression in the liver of mice under AFB<sub>1</sub> exposure. Furthermore, mitochondria are the primary target of ROS attacks, and superfluous ROS production can lead to oxidative stress and mitochondrial malfunctioning [31]. We observed that LUTN suppressed ROS generation induced by AFB<sub>1</sub>. As a result, we hypothesized that LUTN reduces AFB<sub>1</sub>-induced excessive apoptosis in mouse liver either directly or through the suppression of oxidative stress.

Nrf2 is a transcription factor that plays a significant role in the process of AFB<sub>1</sub>-induced cytotoxicity [53,54]. Under normal conditions, Nrf2 stays in the cytosol by its specific antagonist, Keap1, while under stimulation, it dissociates from Keap1 and translocates to the nucleus, where it binds to ARE and regulates the transcripts of downstream antioxidant genes (HO-1, NQO1, SOD, GCLC) [55]. Nrf2 and its targeted antioxidative genes, HO-1, NQO1, SOD, and GCLC, are critical components to maintain the redox system and have been shown to exhibit cytoprotective resistance against oxidative stress [56]. The present study revealed that AFB<sub>1</sub> exposure inhibited the expression of Nrf2 and its associated-target genes such as HO-1, NQO1, SOD1, and GCLC in the liver of mice. However, LUTN dramatically rescued these effects induced by AFB<sub>1</sub>. Previously, LUTN prevents the progression of liver fibrosis induced by carbon tetrachloride-(CCL4) through targeting AKT/mTOR/p70S6K and TGFβ/Smad signaling pathways [26]. The current results agree with previous investigations that AFB<sub>1</sub> exposure suppressed Nrf2 nuclear translocation [28,54], and LUTN could rescue cells from oxidative damage by activating Nrf2 and up-regulating the cellular antioxidant genes expression [57–59]. However, further research is needed to address the preventive effects of LUTN against ongoing and/or established AFB<sub>1</sub>-induced toxicity in human and/or animal.
5. Conclusions

The current study provides significant evidence on the potential protective effects of luteolin (LUTN) against AFB1-induced hepatotoxicity in mice. LUTN effectively rescued liver injury, as evident by the amelioration of toxic effects on serum biochemical profile and pathological alterations induced by AFB1. LUTN attenuates AFB1-induced excessive apoptosis by inhibiting the mitochondria-dependent apoptosis pathway. Additionally, LUTN suppressed AFB1-induced oxidative stress by scavenging ROS accumulation and enhancing antioxidant enzymes capacity via regulation of Nrf2 signaling. The current study suggested that the key mechanisms underlying the LUTN hepatoprotective effects were associated with the activation of the Nrf2 signaling pathway (Figure 8). The present study suggested that LUTN may serve as a potential mitigator against AFB1-induced liver injury and could be helpful for the development of novel treatment to combat liver diseases in humans and/or animals.

![Figure 8. Schematic diagram representing hepatoprotective mechanism of luteolin against AFB1-induced apoptosis and oxidative stress via the activation of Nrf2 signaling pathway in the liver of mice.](https://www.mdpi.com/article/10.3390/antiox10081268/s1)

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/antiox10081268/s1, Table S1: List of primers used in the present study for qRT-PCR analysis.

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