

Protective Mechanisms of Limonoids from *Khaya grandifoliola* against Cisplatin-Toxicity in L-02 Hepatocytes: Targeting JNK Activation and Nuclear Translocation of Nrf2

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Abstract

Background: Cisplatin is one of the most effective antineoplastic drugs, but it has undesirable side effects such as hepatotoxicity. This study investigated *in vitro* the protective activity of three limonoids known as 17-epi-methyl-6-hydroxylangolensate, 7-deacetoxy-7-oxogedunin and deacetoxy-7R-hydroxygedunin, isolated from *Khaya grandifoliola* (Meliaceae) against cisplatin-induced hepatotoxicity and the possible mechanisms involved in this activity.

Methods: Normal human liver L-02 cells line were intoxicated with cisplatin or co-treated with the limonoids for 36h prior to evaluation of biochemical and molecular parameters through spectrophotometric assays, western blots and quantitative real-time polymerase chain reaction analysis.

Results: The studied limonoids prevented cisplatin-induced cell death and leakage of alanine aminotransferase (ALT) in the incubation medium in a concentration-dependent manner. Overproduction of intracellular reactive oxygen species (ROS), depletion of glutathione and lipid membrane peroxidation induced by cisplatin were inhibited by the limonoids. Cisplatin-induced phosphorylation of c-Jun N-terminal Kinase (JNK) and mitochondrial translocation of phospho-JNK was abrogated in limonoids co-treated cells. Interestingly, the studied limonoids increased the expression of mitogen-activated protein kinase phosphatase -1, an endogenous inhibitor of JNK phosphorylation, in cisplatin-intoxicated cells. Moreover, these compounds induced the nuclear translocation of nuclear factor erythroid 2-related factor 2 (Nrf2), decreased the expression of kelch-like ECH-associated protein-1, and increased the mRNA levels of Nrf2 target genes: catalase, superoxide dismutase-1 and glutathione-S-transferase.

Conclusions: The present findings suggest that limonoids from *K. grandifoliola* may constitute promising phytochemicals for alleviating cisplatin-induced hepatotoxicity.

Key words: *Khaya grandifoliola*; Limonoids; Cisplatin-toxicity; hepato-protection.

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Background

The most important form of toxic hepatic injury is caused by therapeutic agents. It is the case of cisplatin, one of the most effective antineoplastic drugs, which is particularly used for the treatment of various cancers [1–3]. Although the mechanisms of cisplatin-induced hepatotoxicity are not fully understood, many studies have provided evidences that its toxicity is associated to excessive generation of reactive oxygen species (ROS) which subsequently cause depletion of glutathione (GSH), inactivation of antioxidant enzymes, oxidation of biological molecules and eventually disruption of cellular integrity [4–6]. In addition, overproduction of ROS can activate the c-Jun N-terminal Kinase (JNK) which is highly associated to the pathophysiological action of numerous hepatic diseases including cisplatin-hepatotoxicity [7–10]. Consequently, several studies have examined exogenous or endogenous JNK inhibitor for their potential therapeutic use. It is the case of the mitogen-activated protein kinase phosphatase-1 (Mkp-1) [11], an endogenous inhibitor of JNK phosphorylation which was found to protects mice against acetaminophen-hepatotoxicity [12]. The liver possesses a range of antioxidant enzymes to neutralize ROS and their expression is regulated by the transcription factor Nrf2 [13, 14]. It has been demonstrated that Nrf2 protects from xenobiotics-induced liver damage through transcriptional up-regulation of an array of down-stream genes, such as superoxide dismutase (SOD), glutathione-S-transferase (GST) and Catalase (CAT) [15–18]; suggesting that activation of Nrf2 could also serve as a novel strategy to prevent or attenuate toxin or drug-induced liver injury.

There is an increasing interest in the use of medicinal plants and their active constituents for evaluating their capacity to protect the liver against the above-mentioned pathological processes [19]. One of these is *Khaya grandifoliola* (*K. grandifoliola*), a plant belonging to the family of Meliaceae, used in Cameroonian traditional medicine for the treatment of liver related diseases [20]. its hepatoprotective properties against carbon tetrachloride and acetaminophen-induced hepatotoxicity using *in vitro* as well as *in vivo* systems have been demonstrated [20, 21]. Interestingly, a fraction able to activate the nuclear translocation of Nrf2 has been isolated from the plant [22]. Moreover, three limonoids namely 17-epi-methyl-6-hydroxylangolensate, 7-deacetoxy-7-oxogedunin and deacetoxy-7R-hydroxygedunin have been purified from this active fraction and were found to protect normal human hepatocyte against acetaminophen-induced hepatotoxicity mainly through induction of Mkp-1 and nuclear translocation of Nrf2 [23]. These findings prompted us to investigate whether these limonoids can protect against cisplatin-evoked hepatotoxicity, which is a serious complication of cisplatin that may limits its therapeutic usage. To this end, investigation of the protective mechanisms of these three limonoids isolated from the active fraction of *K. grandifoliola* against cisplatin-induced oxidative injury in L-02 hepatocytes, a normal human hepatocyte cell line was the aim of the present study. The effects of these isolated compounds were compared to that of JNK inhibitor SP600125, an anthrapyrazolone [anthrax (1,6-c,d) pyrazole-6(2H)-one] [24]; which was found to protect mice against cisplatin-induced nephrotoxicity and acetaminophen-hepatotoxicity through inhibition of JNK phosphorylation and JNK-mediated cell death [25–28].

Methods

Chemicals compounds and reagents

Cisplatin, Thiazolyl Blue Tetrazolium Bromide, α -Keto-glutaric Acid, L-Alanine, Thiobarbituric Acid, Trichloroacetic Acid, 2'-7'-Dichlorodihydrofluorescein diacetate (H₂DCFDA), JNK inhibitor SP600125, MITOISO2 - Mitochondria Isolation Kit were purchased from Sigma-Aldrich (St Louis, USA); M-PER Mammalian Protein Extraction Reagent, NE-PER® Nuclear and Cytoplasmic Proteins Extraction Kit, Halt protease inhibitor cocktail EDTA-Free 100X, Pierce bicinchoninic acid (BCA) Proteins Assay Kit, SuperSignal West Pico Chemiluminescent Substrate were all purchased from Thermo Fisher Scientific (Rockford, USA). Rabbit polyclonal anti-phospho-JNK1/JNK2 and JNK2 antibodies (1:1000 dilution) were purchased from Signalway Antibody (Baltimore, USA); Rabbit polyclonal anti-MKP-1, Nrf2, Keap-1, and Lamin-B antibodies (all 1:1000 dilution) were purchased from Beijing Biosynthesis Biotechnology CO., LTD (Beijing, China); Mouse monoclonal anti- β actin primary antibody (1:5000 dilution), horseradish peroxidase-conjugated goat anti-rabbit and anti-mouse IgG AP-linked secondary antibodies (1:2000 dilution) were purchased from Santa Cruz Biotechnology (Ca., USA); TRIzol® Reagent was purchased from Ambion Lifes Technologies (Carlsbad, California, USA); First-Strand cDNA Synthesis Kit was purchased from Promega (Madison, USA); iTaq Universal SYBR Green Supermix Kit was purchased from Bio-Rad Laboratories (Ca., USA); All primers of the genes of interest were synthesized by TSINGKE Biological Technology Company (Beijing, China). All others reagent used in this study were of analytical grade.

Studied limonoids from *K. grandifoliola*

17-epi-methyl-6-hydroxylangolensate (C-A), 7-deacetoxy-7-oxogedunin (C-B) and deacetoxy-7R-hydroxygedunin (C-C) (Figure 1) were isolated from the active fraction of *K. grandifoliola* using chromatography (Column, Thin Layer and High Pressure Liquid Chromatography) and identified by structural analysis (High-Resolution Mass Spectra, Nuclear Magnetic Resonance) techniques as previously described [23].

Cells and culture conditions

The normal human liver cell line L-02 (Cell Bank, Type Culture Collection of Institute of Microbiology, Chinese Academy of Sciences, Beijing, China) were used. Cells were cultured in 100 mm dish and maintained in high glucose Dulbecco's Modified Eagle's Medium supplemented with 10 % fetal bovine serum, L-glutamine (2 mM), penicillin (100 IU/mL), streptomycin (100 μ g/mL) and amphotericin B (0.25 μ g/mL) in an atmosphere of 5 % CO₂ at 37°C.

General procedure of cells treatment

Cisplatin and isolated limonoids were diluted in 20 % DMSO (20 % DMSO in phosphate-buffer saline [PBS]). JNK-inhibitor SP600125 used as reference compound was dissolved in 100 % DMSO. L-02 cells (approximately 2.10⁵ cells/ml) in triplicate were seeded into 24-well plate labeled as control, cisplatin, standard and test (reference or isolated compound + cisplatin) and incubated for 24 h. Afterward, the medium was changed and cells were incubated with fresh medium in absence (control group) or in presence of cisplatin alone (cisplatin group), or in presence of cisplatin and JNK inhibitor SP600125 or isolated limonoids (test groups) for different time points (6; 12; 24 and 36 h) depending on the downstream analysis.

Determination of half toxic concentration of cisplatin to be used

L-02 cells were incubated in presence of cisplatin at the final concentrations of 0; 5; 10; 15; 20 and 30 μM . After 36 h, cell viability was determined using 3-(4, 5-dimethylthiosol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide kit (MTT; Sigma-Aldrich) according to the manufacturer's instructions and the cell membrane integrity was assessed by measuring alanine aminotransferase (ALT) activity released into the incubation medium using a calibration curve established with sodium pyruvate as described [29]. Cell viability was expressed as percentage of control and the half toxic concentration (TC_{50}) of cisplatin was determined using concentration-response curve and used as toxic concentration throughout the experiments.

Concentration-response study of hepatoprotective effect of the isolated limonoids against cisplatin-induced hepatotoxicity in L-02 hepatocytes

L-02 cells were treated simultaneously with cisplatin (TC_{50}) and isolated limonoids (0; 10; 20; 30; and 40 μM) or JNK inhibitor (0; 5; 10; 15 and 20 μM). After 36 h, cell viability and cell membrane integrity were assessed as above-mentioned and the half efficient concentration (EC_{50}) was determined.

Determination of intracellular ROS level and lipid membrane peroxidation

The effect of studied limonoids on the production of ROS in cisplatin-intoxicated hepatocytes was measured according to [30]. Briefly, L-02 cells were treated with 20 μM H_2DCFDA , cisplatin (TC_{50}) and isolated compounds or JNK inhibitor at the determined concentrations for 36 h. Following the treatment, supernatant was collected and cells were washed with PBS, lysed immediately in an appropriate lysis buffer, and the whole cell lysates were centrifuged (10,000g, 5 min, 4°C) and aliquot of lysate (100 μL) was used for fluorescence measurement at excitation of 485 ± 20 nm, and emission of 525 ± 20 nm in a black wall with clear bottom 96-well plate using a spectrophotometer (SpectraMax M5, Molecular Devices) and expressed as percentage of control. Lipid membrane peroxidation was evaluated through the malondialdehyde (MDA) level in the cellular supernatant by the thiobarbituric acid (TBA) method by using its molar extinction coefficient ($\epsilon_{\text{MDA}} = 1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$) as previously described [21].

Determination of cellular GSH content

Following the treatment of cells with cisplatin and isolated compounds or JNK inhibitor for 36 h at the indicated concentration, cells were harvested and lysed in lysis buffer. The whole cell lysates were centrifuged (10,000g, 5 min, 4°C). The supernatant was collected and the GSH concentration was determined by using its molar extinction coefficient ($\epsilon_{\text{GSH}} = 13,600 \text{ M}^{-1} \cdot \text{cm}^{-1}$) by the 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) assay according to Ellman [31].

Protein extraction and subcellular fractionation

L-02 hepatocytes were treated without cisplatin or simultaneously with cisplatin and studied limonoids or JNK inhibitor SP600125 at the determined concentration and incubated for different time points. Afterwards, total proteins were extracted using M-PER Mammalian Protein Extraction Reagent (Thermo Scientific) containing 0.2 % Halt protease inhibitor cocktail EDTA-Free 100X

(Thermo Scientific); cytosolic and nuclear protein were extracted using NE-PER® Nuclear and Cytoplasmic Proteins Extraction Kit (Thermo Scientific); mitochondrial and cytosolic fractions were prepared using MITOISO2 - Mitochondria Isolation Kit (Sigma-Aldrich); protein concentration in each sample was determined with Pierce BCA Proteins Assay Kit (Thermo Scientific). All procedures were performed according to each manufacturer's instructions.

Western blot analysis

Approximately 50 μg of protein in Laemmli loading buffer was subjected to 12 % Sodium-Dodecyl-Sulfate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) and electro-transferred into a Nitrocellulose Blotting Membrane (GE Healthcare, Life Science, Germany). The membrane was blocked with 5% w/v dehydrated skimmed milk in Tris-Buffered Saline Tween-20 (TBST: 10mM Tris-HCl; 150mM NaCl; 0.05% tween-20; pH 7.6); incubated overnight at 4°C with primary antibodies, rinsed, and then incubated for 1h at 25°C with horseradish peroxidase-conjugated secondary antibodies. Membrane was stained with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) and detected by Enhanced Chemiluminescent Method which combines MicroChemi Unit and GelCapture Software. Densitometry analysis of the protein bands was performed using ImageJ Software.

RNA isolation and cDNA synthesis

L-02 hepatocytes were treated without cisplatin or simultaneously with cisplatin and studied limonoids or JNK inhibitor SP600125 at the determined concentration for 36 h. Following the treatment, total RNA were extracted from cells using TRIzol® Reagent (Ambion, Life Technologies) according to the manufacturer's instruction. RNA concentration and purity were determined by reading the absorbance at 230; 260 and 280 nm using NanoDrop ND-2000 Spectrophotometer (Thermo Scientific). The 260/280 ratio of our RNA preparation ranged from 1.82-2.20 while the 260/230 ratio were greater than 2. These values indicate good RNA quality and therefore can be used for reverse transcription. First-strand synthesis cDNA was synthesized in the thermo cycler (Eastwin, Life Science) using Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT, Promega, Woods Hollow Road Madison, USA) as described by the manufacturer.

Quantitative real-time polymerase chain reaction (qRT-PCR) analysis

qRT-PCR was performed in Applied Biosystems 7500 System using iTaq™ Universal SYBR® Green Supermix (Bio-RAD Laboratories, Ca, USA) according to the manufacturer's instruction. Relative expression of interest genes was normalized to the endogenous gene (GAPDH) used as internal control, analyzed by the $2^{-\Delta\Delta\text{CT}}$ method using GenEX Software and given as ratio compared to the control group (cells incubated without cisplatin). The sequences of used primers are shown in the Table 1.

Statistical analysis

Results are presented as mean \pm standard deviation (SD) of three independent experiments in triplicate. Comparisons between the mean values of various treatments groups were analyzed by one-way analysis of variance (ANOVA) followed by the Bonferroni's *post hoc* test whenever significant differences were observed between the variances. Comparisons were made between untreated group (DMSO control group) and intoxicated group

(cisplatin-intoxicated group), and between cisplatin-intoxicated group and treated groups (cisplatin + studied compounds or JNK inhibitor SP600125). Differences between compared groups were considered significant for $p < 0.05$. Analyses were performed using Graph Pad Prism 5.03 statistical software (Graph Pad Inc.).

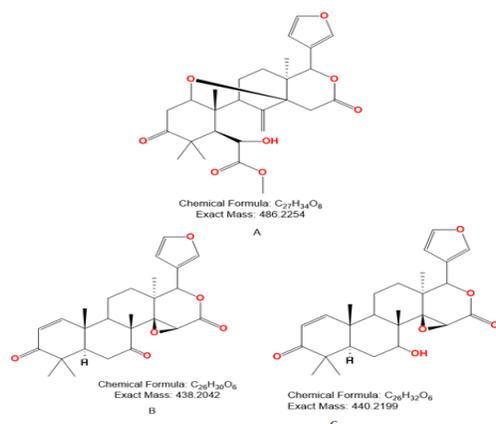


Figure 1. Chemical structures of studied limonoids. A: 17-epi-methyl-6-hydroxyangolensate; B: 7-deacetoxy-7-oxogedunin and C: 7-deacetoxy-7R-hydroxygedunin.

Results

Effect of cisplatin on cell viability and membrane integrity of L-02 hepatocytes

The toxicity of cisplatin in L-02 hepatocytes was evaluated by performing a concentration-dependent study with different concentration of cisplatin ranging from 5 to 30 μM . Incubation of L-02 cells with cisplatin for 36 h significantly ($p < 0.05$) decreased cell viability (Figure 2A) and increased extracellular ALT activity (Figure 2B) into the incubation medium in dose-dependent manner. The TC_{50} was $19.66 \pm 2.24 \mu\text{M}$, thus 20 μM of cisplatin was maintained as toxic concentration throughout the study.

Effect of isolated limonoids from *K. grandifoliola* on cisplatin-induced hepatotoxicity

The protective effect of the studied limonoids (17-epi-methyl-6-hydroxyangolensate, 7-deacetoxy-7-oxogedunin and 7-deacetoxy-7R-hydroxygedunin) (Figure 1) on cisplatin-induced hepatotoxicity was examined by quantifying the cell viability and the level of ALT activity leakage into the incubation medium. In cisplatin-treated-cell (20 μM , 36h), the cell viability decreased to approximately 50% (Figure 3A) while extracellular ALT activity increased by 291.67% (Figure 3B) compared to normal control (DMSO-treated cell). However, co-treatment with different concentrations of the tested limonoids (10; 20; 30 and 40 μM) or JNK inhibitor SP600125 (5; 10; 15 and 20 μM) resulted in a concentration-dependent protective and inhibitory effect on cell viability (Figure 3A) and extracellular ALT activity (Figure 3B). The EC_{50} were $22.53 \pm 2.37 \mu\text{M}$, $27.61 \pm 3.01 \mu\text{M}$, $26.78 \pm 2.57 \mu\text{M}$ and $14.23 \pm 2.95 \mu\text{M}$ respectively for 17-epi-methyl-6-hydroxyangolensate, 7-deacetoxy-7-oxogedunin, 7-deacetoxy-7R-hydroxygedunin and JNK inhibitor SP600125. Although the EC_{50} of JNK inhibitor was lower than those of isolated limonoids, there were no significant differences in cell viability and extracellular ALT activity in cells treated with

limonoids (40 μM) or JNK inhibitor SP600125 (20 μM) compared with control. These concentrations were therefore used to investigate the possible mechanism involved in this protective effect.

Effect of the isolated limonoids on cisplatin-induced ROS overproduction, GSH depletion and lipid membrane peroxidation

Cisplatin hepatotoxicity is associated with oxidative stress characterized by excessive generation of ROS. These ROS cause depletion of cellular GSH and trigger lipid membrane peroxidation. Therefore, the effect of isolated limonoids on intracellular ROS generation, cellular GSH content and MDA formation, an end product of lipid peroxidation process was evaluated after intoxication of cells with cisplatin. As shown in Figure 4A, Figure 4B and Figure 4C, incubation of L-02 hepatocytes in presence of cisplatin alone significantly ($p < 0.05$) increased intracellular ROS, decreased GSH content and increased MDA formation respectively, compared to untreated cells. In contrast, co-treatment of cells with isolated limonoids (40 μM) or JNK inhibitor SP600125 (20 μM) significantly ($p < 0.05$) inhibited excessive generation of intracellular ROS (Figure 4A), rescued cellular GSH (Figure 4B) and inhibited MDA production (Figure 4C), compared to cisplatin-treated cells.

Effect of limonoids from *K. grandifoliola* on cisplatin-induced JNK phosphorylation and mitochondrial translocation of p-JNK in L-02 hepatocytes

To investigate whether the protective effect of isolated limonoids is related to the activation of JNK/MAP kinase signaling pathway, protein samples obtained initially from total cell lysates were subjected to western blot analysis. As shown in Figure 5A and Figure 5B, although the un-phosphorylated form of JNK remained unchanged by cisplatin treatment whose administration led to a significant ($p < 0.05$) increase in the level of phosphorylated JNK1/2 compared to untreated cells. However, co-treatment of L-02 hepatocytes with isolated limonoids (40 μM) significantly ($p < 0.05$) suppressed JNK1/2 activation (Figure 5A and Figure 5B). Next, cytosolic and mitochondrial proteins fractions were prepared and JNK phosphorylation was detected 6 h and 12 h after treatment of cells to determine whether isolated limonoids affect p-JNK translocation into the mitochondria. Consistent to what was observed from the total cell lysates, administration of 20 μM of cisplatin alone led to JNK activation in the cytosol at 6 and 12 h (Figure 5C, Figure 5D and Figure 5E). A moderated phosphorylation of JNK was also observed in the in the mitochondria of cisplatin-treated cells 6 h after administration (Figure 5C) and massive phosphorylation at 12 h (Figure 5D). Co-treatment with the tested compounds significantly ($p < 0.05$) prevented JNK activation in the cytosol at 6 and 12 h and its translocation into the mitochondria (Figure 5C, Figure 5D and Figure 5E). These inhibitory activities were similar to those observed in JNK inhibitor SP600125 treated cells.

Effect of limonoids from *K. grandifoliola* on the expression of Mkp-1 in cisplatin-intoxicated L-02 cells

In order to determine the mechanism by which the isolated limonoids prevented JNK phosphorylation, their effect on the expression of Mkp-1, an endogenous inhibitor of JNK activation was determined by western blot after treatment of cells. Administration of cisplatin alone for 6 or 12 h significantly ($p < 0.05$) decreased the Mkp-1 expression level (Figure 6A and Figure 6C).

Co-treatment of cells with JNK inhibitor SP600125 (20 μ M) did not affect the expression of Mkp-1 (Figure 6A and Figure 6C). However, in co-treated cells with isolated limonoids (40 μ M), the Mkp-1 protein level markedly increased up-to 1.9-fold after 6 h (Figure 6A and Figure 6B) and up-to 2.6-fold after 12 h (Figure 6C and Figure 6D) as compared to the untreated cells.

Effect of limonoids from K. grandifoliola on nuclear translocation of Nrf2 in cisplatin-intoxicated cells

The ability of the limonoids to induce the nuclear translocation of Nrf2 was examined by immunoblotting. As shown in Fig. 7, co-treatment of cells with isolated limonoids (40 μ M) increased the Nrf2 protein level in the nucleus by up-to 2.1-fold and 3-fold respectively at 12 h (Figure 7A and Figure 7B) and 24 h (Figure 7C and Figure 7D) after treatment, as compared to untreated cells. There was no significant change in the nuclear Nrf2 protein level of cisplatin (20 μ M) or JNK inhibitor (20 μ M) treated cells. In addition, in the cytosol of limonoids co-treated cells, the nuclear translocation of Nrf2 was associated to a significant ($p < 0.05$) decrease of the expression of Keap-1 (Figure 7E and Figure 7F), a repressor of Nrf2 activation.

Effect of the isolated limonoids on the mRNA expression level of antioxidants enzymes in cisplatin-intoxicated L-02 hepatocytes

Following analysis of the capacity of isolated limonoids to induce nuclear translocation of Nrf2, the mRNA expression level of target genes of Nrf2 (SOD1, CAT and GST) in limonoids co-treated cells was quantified by qRT-PCR. Incubation of L-02 hepatocytes in presence of cisplatin (20 μ M) during 36 h significantly ($p < 0.05$) decreased the mRNA expression levels of SOD1, CAT and GST (Figure 8). In limonoids (40 μ M) co-treated cells, the mRNA expression levels of SOD1 (Figure 8A), CAT (Figure 8B) and GST (Figure 8C) were increased by up-to 2.4-fold as compared to the untreated or JNK inhibitor treated cells.

Discussion

Cisplatin is one of the most important chemotherapeutic agents used in the treatment of various forms of cancers. Unfortunately, it can produce undesired side effects in several tissues including the liver that interfere with its therapeutic efficacy. Clinical evidence of cisplatin-induced hepatic injury has been demonstrated by elevated activities of serum amino transaminase including ALT [32]. The rise in levels of ALT in the extracellular medium has been attributed to the disruption of structural integrity of hepatocytes, because this enzyme is normally located in the cytoplasm [5, 33]. Therefore, the protective effect of limonoids (17-epi-methyl-6-hydroxyangolensate, 7-deacetoxy-7-oxogedunin and 7-deacetoxy-7R-hydroxygedunin) isolated from *K. grandifoliola* on cisplatin-induced hepatotoxicity in L-02 hepatocytes was examined, not only by quantifying the level of ALT activity found in the incubation medium, but also by measuring cell viability after administration of cisplatin. As shown in Figure 2, administration of 20 μ M of cisplatin was found to be toxic for L-02 cells after 36 h of treatment, leading to a significant ($p < 0.05$) leakage of ALT in the incubation medium and to about 50% decrease in cell viability. Co-treatment of cells with isolated limonoids was protective in a concentration-dependent manner as indicated by the significant reduction in extracellular ALT activity (Figure 3B) and maintaining of cell viability (Figure 3A). This

protective effect was similar to that observed in JNK inhibitor treated cells.

Although the mechanism of cisplatin-induced hepatotoxicity is still unclear, several reports have shown that its hepatotoxicity is mediated via reactive oxygen species (ROS) generation and oxidative stress dependent mechanism [4, 6]. These ROS are highly reactive molecules mainly composed by superoxide radical ($O_2^{\bullet-}$), hydroxyl radical ($\bullet OH$) and hydrogen peroxide (H_2O_2). They can bind to the thiol group of cellular GSH resulting in the depletion of GSH stores and oxidation of biological molecules such as membrane lipid, leading to the loss of membrane integrity and eventually cell death [34]. In this study, overproduction of intracellular ROS (Figure 4A), GSH depletion (Figure 4B), and the formation of MDA (Figure 4C) a biomarker of lipid peroxidation, observed in cisplatin-treated cells were significantly ($p < 0.05$) abrogated by the co-treatment of cells with isolated limonoids or JNK inhibitor SP600125. These findings suggest that limonoids from *K. grandifoliola* prevent L-02 hepatocytes against oxidative damage induced by cisplatin.

ROS generated by cisplatin treatment can activate various downstream protein that mediate apoptosis and necrosis, in particular the MAP kinases family proteins [35]. The activities of different kinases lead to the phosphorylation of JNK and sustained activation of JNK have been reported to be involved in cisplatin-induced apoptosis and cell death [7]. Here, our results revealed the ability of cisplatin to induce the sustained activation of JNK and its translocation in the mitochondria of L-02 hepatocytes (Figure 5). These changes were abrogated when L-02 cells were co-treated with isolated limonoids and their effects were comparable to those observed in JNK inhibitor co-treated cells. These observations underlie the inhibition of JNK activation as a putative mechanism of the protective effect of the studied limonoids against cisplatin-hepatotoxicity.

The protective role of Mkp-1, an endogenous inhibitor of JNK, has been demonstrated against acetaminophen-hepatotoxicity [12, 23]. As cisplatin-hepatotoxicity is also associated to JNK phosphorylation, we evaluated the effect of the studied limonoids on the expression of Mkp-1. Our results showed that cisplatin treatment significantly ($p < 0.05$) decreased Mkp-1 protein level 6 h and 12 h (Figure 6) after cisplatin administration which correlates with overproduction of intracellular ROS and sustained phosphorylation of JNK. There was no obvious change in Mkp-1 protein level in JNK inhibitor co-treated cells. In contrast, Mkp-1 protein level increase by up-to 1.9-fold and 2.6-fold (Figure 6A and Figure 6B) respectively after 6 and 12 h of treatment. From these observations, it can be suggested that inhibition of JNK activation by the tested limonoids may be due to their up-regulating effect on the expression of Mkp-1 which could therefore contribute to their protective effect against cisplatin-toxicity in L-02 hepatocytes.

A major defense system of hepatocytes includes antioxidant and detoxification enzymes such as SOD, CAT and GST; and also antioxidant molecules as GSH which convert the toxic reactive metabolites from xenobiotic biotransformation into non-toxic compounds, scavenge and/or prevent generation of superoxide or hydroxyl radicals and therefore protect cellular constituents from oxidative damage [36]. The regulation of the gene expression of these antioxidant enzymes are mediated by Nrf2. Normally, Nrf2 is sequestered by its suppressor Keap1 in the cytoplasm when in an inactive state, but in an active state, Nrf2 translocates into the nucleus where it binds to the antioxidant response element and leads to the expression of anti-oxidant genes, upon cell stimulation [13]. Several studies demonstrated the protective role of Nrf2

against liver injury induced by various xenobiotics [15, 17, 37]. Here, western blot analysis revealed no significant change neither in nuclear Nrf2 protein level after 12 h or 24 h of treatment, nor in cytosolic Keap-1 protein level after 24 h in cells treated with cisplatin alone or co-treated with JNK inhibitor SP600125 (Figure 7). In contrast, in cells treated with isolated limonoids, the nuclear protein level of Nrf2 was increased by up to 2.1-fold and 3-fold respectively at 12 h and 24 h after treatment. This effect was associated with a significant ($p < 0.05$) decrease in the expression of Keap-1 in the cytosol (Figure 7). Similarly, SOD1, CAT and GST mRNA levels were significantly ($p < 0.05$) increased in limonoids co treated cells while the mRNA level of these antioxidant enzyme were significantly decreased ($p < 0.05$) in L-O2 hepatocytes in response to cisplatin administration alone (Figure 8). Based on these observations, it can be suggested that activation of Nrf2 by the limonoids from *K. grandifoliola* is one of the protective mechanisms by which these compounds prevent L-O2 hepatocytes from oxidative damage induced by cisplatin.

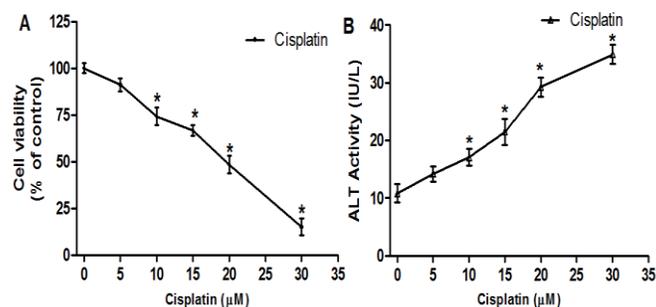


Figure 2. Cisplatin-induced cell death and disruption of membrane integrity in L-O2 hepatocytes.

Cells were treated with different concentration of cisplatin (5–30 μM) for 36 h. (A): Cell viability indicating dose-response of cisplatin toxicity; (B): Disruption of membrane integrity indicated by ALT activity found in the culture medium. Values are means \pm SD of three independent experiments in triplicate. Δ Values significantly different compared to control (0 μM) ($P < 0.05$) using Bonferroni's test.

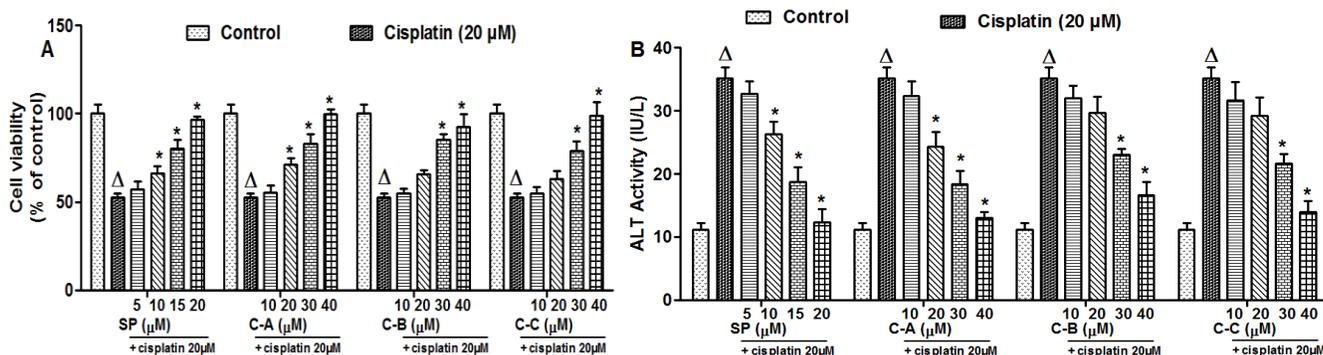


Figure 3. Protective effect of the isolated limonoids against cisplatin-induced cell death and loss of membrane integrity in L-O2 hepatocytes.

Cells were treated without or with cisplatin (20 μM), or co-treated with cisplatin (20 μM) and isolated limonoids (10; 20; 30 and 40 μM) or JNK inhibitor (5; 10; 15 and 20 μM) for 36 h. A and B: effect of isolated limonoids on cell viability and ALT leakage respectively. Values are means \pm SD of three independent experiments in triplicate. Δ Values significantly different compared to control group ($P < 0.05$); *Values significantly different compared to cisplatin-intoxicated group ($P < 0.05$) using Bonferroni's test. C-A: 17-epi-methyl-6-hydroxyangolensate; C-B: 7-deacetoxy-7-oxogedunin; C-C: 7-deacetoxy-7R-hydroxygedunin; SP: JNK inhibitor SP600125.

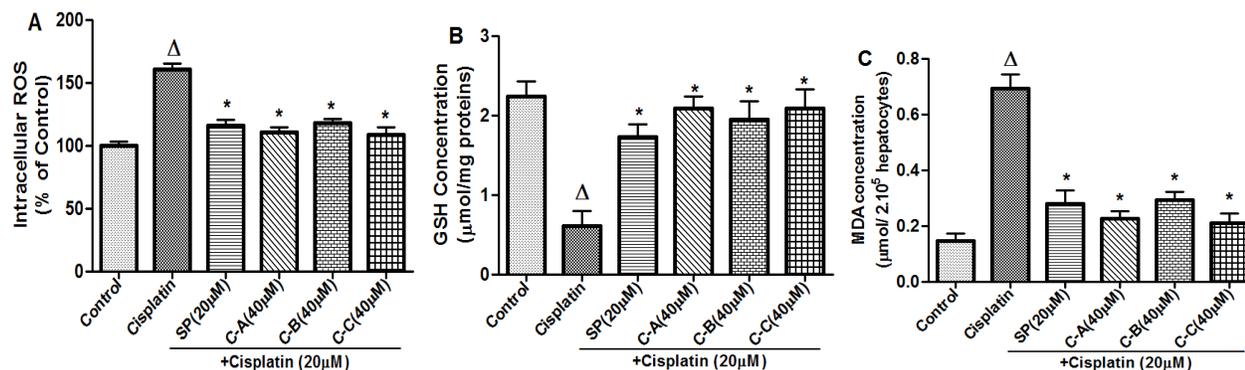


Figure 4. Preventive effect of the isolated limonoids on cisplatin-induced ROS overproduction, GSH depletion and lipid membrane peroxidation in L-O2 hepatocytes

Cells were treated without or with cisplatin (20 μM), or co-treated with cisplatin (20 μM) and isolated compounds (40 μM) or JNK inhibitor (20 μM) for 36 h. After treatment, intracellular ROS level (A), cellular GSH content (B) and MDA concentrations (B) in the incubation medium were measured. Values are means \pm SD of three independent experiments in triplicate. Δ Values significantly different compared to control group ($P < 0.05$); *Values significantly different compared to cisplatin-intoxicated group ($P < 0.05$) using Bonferroni's test. C-A: 17-epi-methyl-6-hydroxyangolensate; C-B: 7-deacetoxy-7-oxogedunin; C-C: 7-deacetoxy-7R-hydroxygedunin; SP: JNK inhibitor SP600125.

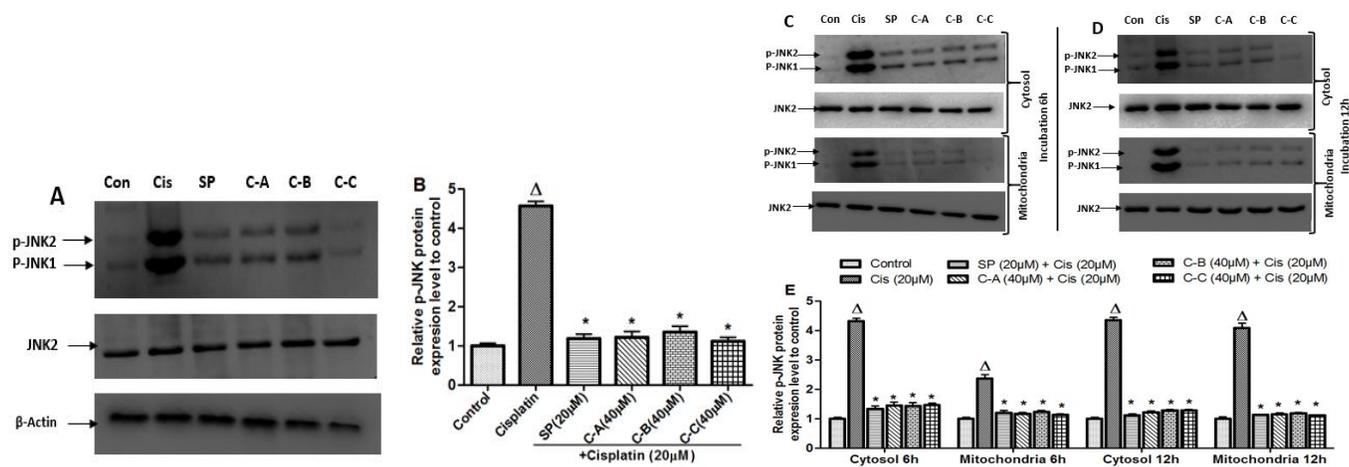


Figure 5. Inhibitory effect of the isolated limonoids on cisplatin-induced JNK phosphorylation and mitochondrial translocation of p-JNK in L-02 hepatocytes.

Cells were treated without or with cisplatin (20 μM), or co-treated with cisplatin (20 μM) and isolated compounds (40 μM) or JNK inhibitor (20 μM) for 6 h and 12 h. After treatment, p-JNK level was detected into total cell lysates, cytosolic and mitochondrial fractions by western blotting. JNK2 and β-actin were used as internal control. Each blot represents one of three independent experiments. **A:** effect of isolated limonoids on JNK activation in total cell lysate 6 h after treatment. **B:** densitometry analysis of blots observed in total cell lysate. **C and D:** effect of isolated limonoids on JNK activation in the cytosol and mitochondria respectively at 6 h and 12 h after treatment. **E:** densitometry analysis of blots observed in cytosolic and mitochondrial fractions. Values are means ± SD of three independent experiments in triplicate. ^ΔValues significantly different compared to control group (P<0.05); *Values significantly different compared to cisplatin-intoxicated group (P<0.05) using Bonferroni's test. Con: control; Cis: cisplatin; C-A: 17-epi-methyl-6-hydroxyangolensate; C-B: 7-deacetoxy-7-oxogedunin; C-C: 7-deacetoxy-7R-hydroxygedunin; SP: JNK inhibitor SP600125.

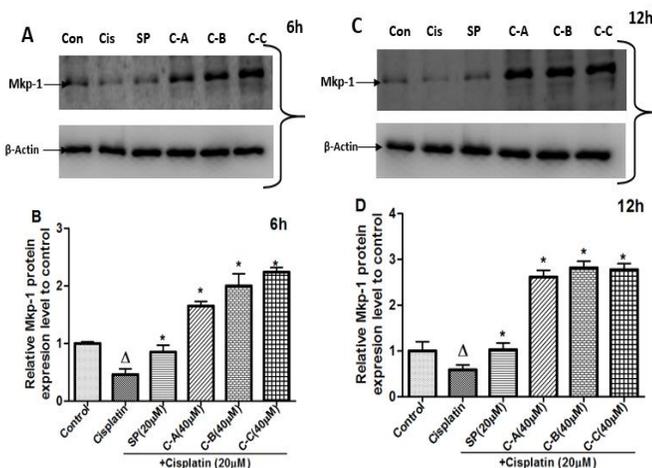


Figure 6. Up-regulating effect of the isolated limonoids on the expression of Mkp-1 in cisplatin-treated cells.

Cells were treated without or with cisplatin (20 μM), or co-treated with cisplatin (20 μM) and isolated compounds (40 μM) or JNK inhibitor (20 μM) for 6 h and 12 h. After treatment, total proteins were extracted from cells and Mkp-1 expression was determined by western blotting. β-actin was used as loading control. Each blot represents one of three independent experiments. **A and C:** effect isolated limonoids on Mkp-1 expression respectively at 6 h and 12 h. **B and D:** densitometry analysis of blots observed respectively at 6h and 12 h. Values are means ± SD of three independent experiments in triplicate. ^ΔValues significantly different compared to control group (P<0.05); *Values significantly different compared to cisplatin-intoxicated group (P<0.05) using Bonferroni's test. Con: control; Cis: cisplatin; C-A: 17-epi-methyl-6-hydroxyangolensate; C-B: 7-deacetoxy-7-oxogedunin; C-C: 7-deacetoxy-7R-hydroxygedunin; SP: JNK inhibitor SP600125.

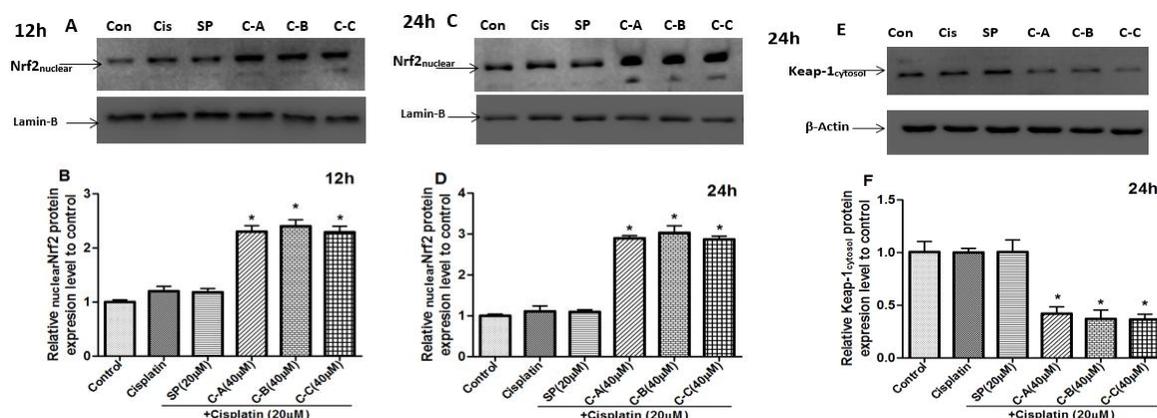


Figure 7. Up-regulating effect of the isolated limonoids on the nuclear translocation of Nrf2 in cisplatin-intoxicated cells. Cells were treated without or with cisplatin (20 μM), or co-treated with cisplatin (20 μM) and isolated compounds (40 μM) or JNK inhibitor (20 μM) for 12 h and 24 h. After treatment, Nrf2 level was detected into the nuclear fraction at 12 h and 24 h; and Keap-1 level was detected at 24 h into the cytosolic fractions by western blotting. Lamin-B and β-actin were used as loading control respectively for the nuclear and cytosolic fraction. Each blot represents one of three independent experiments. **A** and **C**: effect of isolated limonoids on Nrf2 expression in the nucleus respectively at 12 h and 24 h. **B** and **D**: densitometry analysis of blots observed respectively at 12 h and 24 h. **E**: effect of isolated limonoids on the expression of Keap-1 in the cytosol after 24 h. **F**: densitometry analysis of blot for Keap1 at 24 h after treatment. Values are means ± SD of three independent experiments in triplicate. ^ΔValues significantly different compared to control group (P<0.05); *Values significantly different compared to cisplatin-intoxicated group (P<0.05) using Bonferroni's test. Con: control; Cis: cisplatin; C-A: 17-epi-methyl-6-hydroxyangolensate; C-B: 7-deacetoxy-7-oxogedunin; C-C: 7-deacetoxy-7R-hydroxygedunin; SP: JNK inhibitor SP600125.

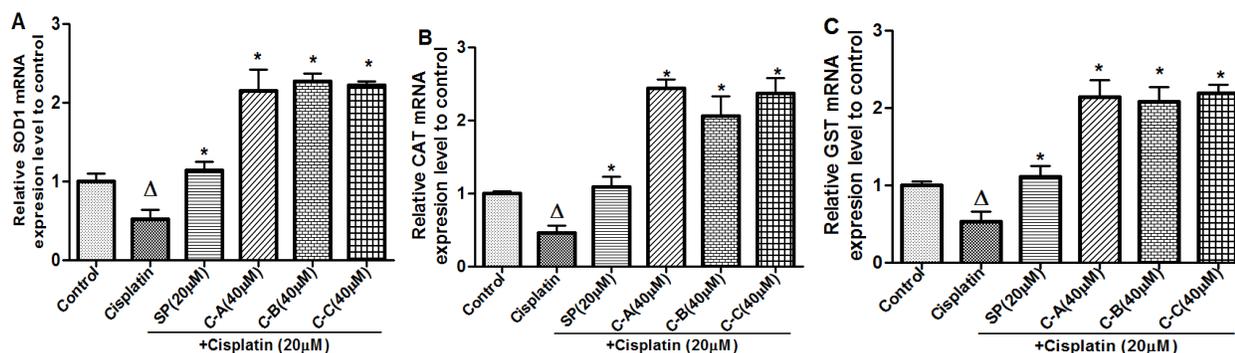


Figure 8. Up-regulating effect of the isolated limonoids on the mRNA expression of antioxidant enzymes in cisplatin-intoxicated cells. Cells were treated without or with cisplatin (20 μM), or co-treated with cisplatin (20 μM) and isolated compounds (40 μM) or JNK inhibitor (20 μM) for 36 h. After treatment, RNA was extracted from cells and relative mRNA expression level of SOD1 (**A**), CAT (**B**) and GST (**C**) were determined by qRT-PCR. GAPDH was used as internal control. Values are means ± SD of three independent experiments in triplicate. ^ΔValues significantly different compared to control group (P<0.05); *Values significantly different compared to cisplatin-intoxicated group (P<0.05) using Bonferroni's test. C-A: 17-epi-methyl-6-hydroxyangolensate; C-B: 7-deacetoxy-7-oxogedunin; C-C: 7-deacetoxy-7R-hydroxygedunin; SP: JNK inhibitor SP600125.

Table 1. Sequence of primers used for qRT-PCR analysis

Genes	Primer Sequence (5'___3')	
	Forward	Reverse
CAT	AGGCCAGTCTCTGACAAAATG	GAATCTCCGCACTTCTCCAG
SOD1	GAAGGTGTGGGGAAGCATTA	ACATTGCCCAAGTCTCCAAC
GST	TTGGCCTCTGTATTCTTG	AGCCAACCTGGATGCTGAGTT
GAPDH	CGACCACTTTGTCAAGCTCA	AGGGGTCTACATGGCAACTG

CAT: Catalase; SOD1: Superoxide dismutase1; GST: Glutathione-S-Transferase; MAT1A: Methionine adenosyltransferase 1A; GAPDH: Glyceraldehyde-3-phosphate deshydrogenase.

Conclusions

Our results demonstrated the protective action of three limonoids (17-epi-methyl-6-hydroxyangolensate, 7-deacetoxy-7-oxogedunin and deacetoxy-7R-hydroxygedunin) isolated from *K. grandifoliola* which was evidenced not only by their ability to alter some critical

events involved in cisplatin-cell death mechanism, but also by their ability to improve the cellular antioxidant defense system. This study also suggests that limonoids from *K. grandifoliola* may be promising phytochemicals to alleviate cisplatin-hepatotoxicity during platinum-treatment in cancer therapy.

Abbreviations

ALT: Alanine aminotransferase
 CAT: Catalase
 GSH: Reduced glutathione
 GST: glutathione-S-transferase
 H₂DCFDA: 2'-7'-Dichloro-dihydrofluorescein diacetate
 JNK: c-Jun N-terminal Kinase
 Keap-1: Kelch-like ECH-associated protein-1
 MAPK: Mitogen-activated protein kinase
 Mkp-1: Mitogen-activated protein kinase phosphatase-1
 Nrf2: Nuclear factor erythroid 2-related factor 2
 p-JNK: Phospho-JNK
 ROS: Reactive oxygen species
 SOD1: Superoxide dismutase-1
 SP: JNK inhibitor SP600125

Authors' Contribution

AFK, NFN and PFM defined the research subject and its aims, conceived and designed the experiments. FY, PFM provided facilities to perform the work; AFK, PM, SF, and BOO performed the experiments; AFK, NFN, FY, and PFM analyzed the data and wrote the paper. All the authors read and approved the final version of this manuscript.

Data availability statement

The datasets supporting the conclusions of this article are presented in this main paper. The supporting material can be obtained upon request via email to the corresponding author.

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Conflict of interest

The authors declare that they have no conflict of interests

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