Hepatoprotective activity of andrographolide possibly through antioxidative defense mechanism in Sprague-Dawley rats

Milon Mondal a, Chandan Sarkar a, Sushmita Saha b, Md Naim Hossain a, Roghayeh Norouzi c, Mohammad S. Mubarak d, Abolghasem Siyadatpanah e, f, Polrat Wilairatana g, h, Rajib Hossain a, i, Muhammad Torequl Islam a, Henrique Douglas Melo Coutinho b, c, f

a Department of Pharmacy, Life Science Faculty, Bangabandhu Sheikh Mujibur Rahman Science and Technology University, Gopalganj 8100, Bangladesh
b Department of Chemistry, The University of Jordan, Amman 11942, Jordan
c Ferdows School of Paramedical and Health, Birjand University of Medical Sciences, Birjand 9717853577, Iran
d Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand
e Laboratory of Microbiology and Molecular Biology (LMMB), Department of Biological Chemistry, Regional University of Cariri -URCA. Crato CE - 63105-000, Brazil

ARTICLE INFO

Keywords:
Andrographolide
Acetaminophen
Oxidative stress, Anti-oxidant, Hepatoprotective activity

ABSTRACT

The aims of this study to assess the efficiency of AGL against acetaminophen (APAP)-induced hepatic toxicity that was generated by mitochondrial oxidative stress and glutathione depletion. Free radical scavenging potentiality was analyzed by using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), hydrogen peroxide, nitric oxide, and hydroxyl radical scavenging assays. APAP-induced liver toxicity was formed at a dose level of 640 mg/kg mg/kg BW each, p.o. for 14 days for all experimental rats except the vehicle control group. AGL (5 and 10 mg/kg) were treated orally with negative control and negative control silymarin (50 mg/kg) group. To assess the protective effect, we looked at the levels of serum biochemical markers, liver histoarchitecture, and hepatic antioxidant enzyme activity. AGL showed in vitro anti-oxidant potentialsities by scavenging radicals in the respective assays. As evidenced by serum biochemical indicators and relative liver weight, AGL co-administration substantially reduced toxicant-induced hepatic damage. APAP-intoxication increased the malondialdehyde (MDA) level and declined in cellular endogenous antioxidant enzymes such as reduced catalase, superoxide dismutase, and glutathione, where, AGL treatment amended their level. In the same way, histopathological evaluation further verified that AGL protected the hepatocyte from APAP-induced damage. As AGL scavenges toxic free radicals, thereby protects mitochondria and other organelles from reactive oxygen and nitrogen species-mediated stress and its eventual consequence necrosis. Therefore, we propose the hepatoprotective activity of AGL through its antioxidant mechanism.

1. Introduction

The liver is an important organ that regulates xenobiotic metabolism, detoxification, and clearance. Among them, some toxicants distort these functions and initiate hepatic impairment by free radical generation [1–3]. Some earlier study reported that approximately 2.0 million fatalities per year around the world which are caused by liver diseases [4, 5]. Although there are several traditional hepatoprotective medicines on the market, some of them have possible adverse effects [6, 7]. Since research statistics suggest that the incidence continues to increase, therefore, there is a rising interest in alternative and complementary therapies that are both effective and safe for treating liver disorders [8, 9]. Some natural phytochemicals such as silymarin, curcumin, quercetin, and resveratrol are extensively used as protective agents against hepatic toxicity [10–12]. As a consequence, we designed our study to assess the hepatoprotective activity of andrographolide (AGL) in...
experimental animals, because, it is used as the main constituent of more than 26 Ayurvedic formulations to treat liver diseases [13,14].

AGL was isolated from *Andrographis paniculata* (Burm. F.) Wall and is a bitter, colorless, and crystalline compound. Ex Nees is a medicinal plant that is frequently used in Unani and Ayurvedic medicines [15]. This bioactive compound has anti-inflammatory, antiviral, anti-atherosclerotic, anti-thrombotic, anti-neoplastic [16], and neuropharmacological properties, among others [17–20]. Some earlier studies also reported the protective activity of AGL against hepatotoxins induced liver damage [21–23]. Acute and chronic liver damage can be produced by different in vivo models [24]. Because drug-induced liver damage is the most prevalent cause of severe liver failure around the world, we utilized drug-induced liver damage, where acetaminophen is mainly responsible for that [25,26].

Generally, the use of acetaminophen (APAP) is considered the standard antipyretic and analgesic drug, nonetheless, overdosing of APAP results in high levels of reactive metabolite n-acetyl para-phenyleneridine (NAPQI) formation via cytochrome P450 (CYP2E1 isomot) [1,27]. Endogenous glutathione (GSH) detoxifies NAPQI. An extreme amount of NAPQI reduces GSH store and then binds covalently with sulfhydryl groups of cellular proteins that causing excessive lipid peroxidation, oxidative stress, and the formation of free radicals [28,29]. The ultimate consequence is necrosis of hepatic cells and liver failure. However, hepatoprotective agents possess anti-radical defense mechanisms that counteracting the harmful effects of hepatotoxins [30–32]. Therefore, we assess the in vitro anti-radical activity of AGL and try to outline a defensive mechanism of AGL in contradiction of APAP-induced hepatic toxicity.

2. Materials and methods

2.1. Chemicals and reagents

Sodium nitroprusside, ascorbic acid, sodium pyruvate, trichloroacetic acid, 2-thiobarbituric acid, hydrogen peroxide, pyridine, and mannitol were purchased from a German multinational pharmaceutical, and life sciences company namely Merck. By contacting with Sigma-Aldrich Co., U.S.A, 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and Andrographolide (98% purity) were purchased. From Beximco Pharmaceuticals Ltd., Dhaka, Bangladesh, Acetaminophen (98% purity) was obtained. Other used reagents and chemicals were fallen under systematic grade.

2.2. In-vitro radical scavenging assay

2.2.1. 1-diphenyl-2-picrylhydrazyl (DPPH)-radical scavenging assay

This experiment was conducted according to Mondal et al.’s [33] protocol. Concisely, 1 ml of AGL or ascorbic acid solution with concentrations of (800, 400, 200, 100, 50, 25, 12.5, or 6.25 µg/ml) was placed in separate test tubes, and 2 ml of 0.004% DPPH solution was mixed to make a final volume of 3 ml. Incubation took 30 min at ambient temperature in a dark environment. After that, a UV spectrophotometer (Shimadzu UV PC-1600, Japan) was used to measure absorbance at 550 nm against control. The percentage inhibition (%) was calculated as \((A_0 - A) / A_0 \times 100\), where \(A_0\) represents the absorbance of the control and \(A\) represents the absorbance of the experimental solution. Using the regression analysis approach, the \(IC_{50}\) value was measured.

2.2.2. Nitric oxide (NO)-radical scavenging assay

This assay was performed in accordance with Hossain et al.’s [34] technique. In test tubes, 4.0 ml of each AGL or ascorbic acid solution at concentrations of (160, 80, 40, 20, 10 µg/ml) were added, then 1.0 ml of sodium nitroprusside (5 mM) solution was mixed and incubated at 25 °C for 30 min, followed by 1.5 ml of Griess reagent. Afterward, a UV spectrophotometer (Shimadzu UV PC-1600, Japan) was used to measure absorbance at 540 nm against control. The percentage inhibition (%) was calculated as \((A_0 - A_1) / A_0 \times 100\), where \(A_0\) represents the absorbance of the control and \(A_1\) represents the absorbance of the experimental solution. Using the regression analysis approach, the \(IC_{50}\) value was measured.

2.2.3. Hydrogen peroxide (H2O2)-radical scavenging and Hydroxyl (OH•)-radical scavenging assays

Different quantities (12.5, 25, 50, 100, 200, 500 µg/ml) of AGL and the reference chemical (sodium pyruvate) solution were produced with distilled water according to Mondal et al. [35], and then hydrogen peroxide (6 ml, 40 mM) solution was added. After that, three ml of phosphate buffer solution was combined with one ml of each combination in a test tube. All reagents except experimental compounds are present in a blank solution. After a 10-minute incubation period, the absorbance of \(H_2O_2\) was measured at 230 nm. The percentage inhibition (%) was calculated as \((A_0 - A_2) / A_0 \times 100\), where \(A_0\) represents the absorbance of the control and \(A_2\) represents the absorbance of the experimental solution. Using the regression analysis approach, the \(IC_{50}\) value was measured [35].

2.3. Animals

Male Sprague-Dawley rats (BW-140–180 g) were purchased from Jahangirnagar University’s Department of Pharmacy and acclimated to regular laboratory settings for one week before the experiment (temperature: 25 ± 2 °C, humidity: 55 ± 5%, and 12 h light/dark cycles). These were given a regular pellet meal and tap water, and the condition was maintained for an experimental period allowed by Jahangirnagar University’s Biosafety, Biosecurity, and Ethical Committee [permission number: BBEC, JU/M 2018 (1)3].

2.3.1. Experimental design

Thirty-five rats (Rattus norvegicus) were arbitrarily distributed into five groups, each with seven rats, and maintained under the following experimental conditions for 14 days:

- **Vehicle control**: Animals were given 0.05% tween 80 dissolved in 0.9% NaCl solution at 0.5 ml/rat along with their usual food.
- **Negative control (acetaminophen)**: Animals were given APAP dissolved in the vehicle at a dose of 640 mg/kg BW (p.o.).
- **Positive control (silymarin 50 mg/kg + APAP)**: Animals were given 50 mg/kg BW (p.o.) silymarin and 640 mg/kg BW (p.o.) APAP dissolved in the vehicle.
- **Treatment AGL 5 (andrographolide 5 mg/kg + APAP)**: AGL (5 mg/kg BW, p.o.) and APAP (640 mg/kg BW, p.o.) were dissolved in the vehicle and given to the animals.
- **Treatment AGL 10 (andrographolide 10 mg/kg + APAP)**: AGL (10 mg/kg BW, p.o.) and APAP (640 mg/kg BW, p.o.) were dissolved in the vehicle and given to the animals.

Two dosages of AGL were chosen for our experiment based on the results of a previous study [36], while the APAP dose was determined based on the findings of Janbaz and Gilani [37]. Blood samples and liver tissues were obtained after that for biochemical and histological examination, as described below [38].

2.3.2. Serum and liver tissue homogenate preparations

Using a heparinized syringe, blood samples (4–5 ml) were collected from the inferior vena cava of rats and put in plain tubes at room temperature for 30 min before centrifugation at 3000 rpm for 10 min to obtain serum for biochemical analysis [8]. Meanwhile, liver tissues were separated from surrounding tissues and rinsed in ice-cold phosphate buffer saline before being weighted. The tissue samples were then homogenized with phosphate buffer saline (25 mM, pH 7.4) by centrifugation (1700 rpm for 10 min) to generate a 10% (w/v) homogenate, and
the supernatant was collected before being stored at −20 °C until further analysis. A slice of the liver tissues was preserved in 10% formalin for histological examination. The liver’s relative organ weight growth was determined by dividing the liver weight by the rat’s final body weight using the formula below.

Relative organ weight (%) = (weight of organ/body weight) × 100

2.3.3. Analysis of biochemical parameters in serum

Biochemical parameters including alkaline phosphatase (ALP), aspartate transaminase (AST), alanine transaminase (ALT), globulin (GLB), albumin (ALB), γ-glutamyl-transferase (GGT), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), lactate dehydrogenase (LDH), total cholesterol (TC), triglyceride (TG), total protein (TP), and total bilirubin (TB) were analyzed with the help of an automated chemistry analyzer (Human commercial kits and Humalyzer 3500) [39].

2.3.4. Lipid peroxidation assay

A conventional experimental procedure was used to detect malondialdehyde (MDA), a major biomarker of lipid peroxidation (LPO) [8]. Concisely, liver homogenate tissue (0.2 ml) was mixed with 1.5 ml of thiobarbituric acid (8%), 1.5 ml of acetic acid (20%), and 0.2 ml of sodium dodecyl sulfate (8.1%). After that, distilled water (4 ml) was added, and the mixture was cooled in a water bath for 60 min at 95 °C. The liquid was then allowed to cool at room temperature before adding 5 ml of butanol: pyridine (15:1) combination. The contents of the mixture were then separated by centrifugation at 4 °C for 10 min at 3000 rpm for 10 min and the top organic layer was collected. The amount of MDA was reported as nmol of thiobarbituric acid reactive substances (TBARS) per mg of protein, and the absorbance was measured at 532 nm against a blank.

2.3.5. Evaluation of reduced glutathione (GSH) levels

With minor modifications, reduced glutathione (GSH) was identified using Ellman’s technique [40]. For the first 5 min, liver homogenate (1 ml) was combined with the same quantity of 10% TCA and incubated. The mixture was then separated by centrifugation at 4 °C for 10 min at 2000 rpm. In a test tube, 0.5 ml of supernatant was combined with 2 ml of 0.6 mM Ellman’s reagent (5, 5′-dithiobis-(2-nitrobenzoic acid)) and 5 ml of 0.2 M PBS (pH 8). The mixture was then vortexed, and the absorbance was measured at 412 nm using a UV–VIS spectrophotometer in fifteen minutes. Reduced glutathione concentrations were measured in nanomoles per milligram (nmol/mg) of protein.

2.3.6. Evaluation of superoxide dismutase (SOD) levels

The amount of superoxide dismutase (SOD) was tested using an older standard technique [41]. In brief, 0.1 ml of EDTA solution was added in 2.86 ml of 50 mM tris-buffer (pH 8.5). The reaction mixture was then added 0.02–0.08 ml of pyrogallol and 0.02 ml of homogenate sample. A UV spectrophotometer was used to measure absorbance at 420 nm against the blank. Units per milligram of protein were used to calculate the SOD levels.

2.3.7. Evaluation of catalase (CAT) levels

Following the Aebi technique, the catalase (CAT) enzyme’s activity was determined using a substrate hydrogen peroxide [42]. In a 3 ml cuvette, 0.1 ml of homogenate sample was mixed with 1.9 ml of PBS (pH 7.0), and the reaction was started with 1 ml of H2O2 (30 m/mL). A UV–VIS spectrophotometer was used to determine the absorbance at 240. Units per milligram of protein were used to calculate the CAT levels.

2.3.8. Evaluation of catalase (CAT) levels

The radical scavenging property of AGL was substantial, as indicated in Table 1. AGL displayed better results in OH• scavenging (IC50 value 23.82 µg/ml) among the radical scavenging assays than standard compound mannitol (IC50 value 71.35 µg/ml). In the DPPH radical scavenging experiment, AGL showed potential scavenging abilities (IC50 value 16.87 µg/ml), whereas conventional ascorbic acid had an IC50 value 109.34 µg/ml.

| Group     | Initial weight (g) | Final weight (g) | Weight gain (g) | Relative Liver weight (g) |
|-----------|--------------------|-----------------|----------------|-------------------------|
| Vehicle   | 150.75 ± 0.43      | 167.00 ± 78.15  | 16.25 ± 6.64   | 5.32 ± 0.18             |
| Control   | 12.66 ± 0.35       | 13.46 ± 0.28    | 1.79 ± 0.35    | 0.37 ± 0.37 •          |
| Negative  | 150.32 ± 0.43      | 158.25 ± 0.28   | 7.50 ± 0.18    | 8.58 ± 0.35 •          |
| Treatment | 160.75 ± 175.00    | 145.25 ± 1.79   | 14.25 ± 1.79   | 5.83 ± 0.37 * •        |
| Treatment | 175.00 ± 175.00    | 162.00 ± 17.75  | 17.50 ± 6.71   | 7.67 ± 0.11 *          |
| AGL 5     | 7.93 ± 4.61        | 7.56 ± 4.61     | 0.37 ± 0.21    | 0.21 ± 0.21 *          |
| Treatment | 156.50 ± 115.50    | 172.00 ± 5.18   | 15.50 ± 5.32   | 6.03 ± 0.03 *          |
| AGL 10    | 9.08 ± 8.19        | 8.19 ± 1.67     | 0.37 ± 0.21    | 0.21 ± 0.21 *          |

The data is provided as means with a standard error of the mean (means ± SEM) (n = 3). At p < 0.05, values in the same column with different superscripts differ considerably.

2.4. Histopathological evaluation

Liver tissues were fixed in 10% formalin for histological examinations. With the use of a rotary microtome (HM 325, Thermo Scientific, U. K.), liver tissue was cut to a thickness of 5 m and embedded in paraffin wax. Tissue slices were then stained with eosin and hematoxylin before being shot using an Olympus DP 72 microscope (Tokyo, Japan) [38].

2.5. Statistical analysis

All data is provided as a mean with standard error (SEM). SPSS (Statistical Packages for Social Science, version 20.0, IBM Corporation, New York, United States of America) and Microsoft Excel 2013 (Redmond, Washington, United States of America)were used to analyze the data. To analyze data sets, one-way analysis of variance (ANOVA) was used, and statistical analysis was performed using Tukey’s and Dunn’s multiple comparisons. At p < 0.001 and p < 0.05, differences were considered significant at 0.1% and 5% levels of significance.

3. Results

3.1. Radical scavenging capacity

The radical scavenging property of AGL was substantial, as indicated in Table 1. AGL displayed better results in OH• scavenging (IC50 value 23.82 µg/ml) among the radical scavenging assays than standard compound mannitol (IC50 value 71.35 µg/ml). In the DPPH radical scavenging experiment, AGL showed potential scavenging abilities (IC50 value 16.87 µg/ml), whereas conventional ascorbic acid had an IC50 value 109.34 µg/ml.
value of 12.15 µg/ml. In addition, when compared to the reference compounds, the experimental compound showed modest activity in two additional radical scavenging experiments.

### 3.2. Hepatoprotective activity

Table 2 shows the effects of AGL and controls on body weights and relative liver weights. The negative control (NC) rats were given APAP instead of the vehicle control (VC) group, which resulted in a modest decrease in body weight growth but a substantial rise in relative liver weight, indicating that the livers were enlarged as a result of APAP poisoning. However, positive control (PC) and treatment groups (AGL 5 and 10) increased the body weight in an insignificant manner, though, the AGL co-administration reduced relative liver weight significantly and, in a dose-response fashion, the PC group also compact it significantly.

When compared to the vehicle group, the NC substantially (p < 0.001) increased serum ALT, AST, ALP, GGT, and LDH levels (Table 3). On the other hand, the positive control group reversed the NC-mediated change in the animals with nearly identical values of such parameters as the VC group. Similarly, when compared to the NC group, both doses of AGL significantly (p < 0.001) lessened these parameters in the experimental animals.

In experimental mice, APAP therapy substantially (p < 0.001) raised blood TC, TG, and LDL levels, while decreasing HDL levels. On the other hand, the PC suggestively (p < 0.001) reversed all of these changes, bringing them closer to VC. Similarly, AGL reduced the APAP-induced changes in rats substantially (p < 0.05, p < 0.001) and dose-dependently (Fig. 1).

Serum GLB, ALB, TP, and TB are important indicators of hepatic function. The APAP-induced group had substantially higher blood globulin and bilirubin levels than the VC group. A considerable drop in serum ALB and TP was also seen in the NC group; however, the PC group and AGL dramatically (p < 0.001) reversed these biochemical parameters, bringing them closer to control values (Fig. 2).

In comparison to the vehicle group, APAP administration increased hepatic MDA levels while significantly lowering CAT, SOD, and GSH levels in rats. The PC-treated group improved the parameters in the animals considerably (p < 0.001). Likely, AGL inhibited APAP-induced Table 3

| Group       | ALT (U/L) | AST (U/L) | ALP (U/L) | GGT (U/L) | LDH (U/L) |
|-------------|-----------|-----------|-----------|-----------|-----------|
| Vehicle     | 68.03 ± 4.07 | 123.15 ± 3.03 | 195.42 ± 5.16 | 3.29 ± 0.06 | 54.16 ± 0.04 |
| Negative    | 138.30 ± 5.45 | 203.07 ± 6.15 | 324.25 ± 9.13 | 6.15 ± 0.09 | 91.37 ± 4.55 |
| Positive    | 76.50 ± 5.48 | 127.25 ± 5.94 | 197.55 ± 7.96 | 3.96 ± 0.09 | 53.69 ± 3.33 |
| Treatment   | 88.60 ± 5.58 | 157.83 ± 6.32 | 205.25 ± 8.64 | 6.15 ± 0.10 | 91.37 ± 4.55 |
| AGL 5       | 76.50 ± 5.45 | 156.65 ± 6.32 | 170.91 ± 8.84 | 4.11 ± 0.09 | 56.58 ± 4.38 |
| AGL 10      | 69.85 ± 5.58 | 124.65 ± 6.51 | 187.91 ± 7.75 | 4.11 ± 0.09 | 56.58 ± 4.38 |

The data is provided as means with a standard error of the mean (means ± SEM) (n = 7); p * < 0.05 compared to the vehicle control; p * * < 0.001 compared to the negative control.

Fig. 1. Effects of andrographolide and controls on serum lipid profile. (The data is provided as means with standard error of the mean (means ± SEM) (n = 7); p * < 0.001 compared to the vehicle control; p * < 0.05 and * * < 0.001 compared to the negative control).
liver damage in rats in a dose-dependent action. Moreover, the highest dose of AGL increased these endogenous anti-oxidants levels to a similar extent as compared to the PC group in the experimental animals (Fig. 3).

Fig. 4 reveals the typical cellular architecture with a specific arrangement of sinusoids lines, sinusoidal spaces, and central vein in the VC group (0.05% tween 80 dissolved in 0.9% NaCl) histological examination of hepatic tissues (Fig. 4(a)). On the other hand, APAP-intoxication caused the degeneration of hepatocytes with central and lobular necrosis, inflammatory cell infiltrations, and vascular edematous congestion (Fig. 4(b)). These pathological anomalies were significantly reduced by the PC (Fig. 4(c)) and AGL 10 mg/kg with mild inflammation and necrosis (Fig. 4(e)), while AGL 5 mg/kg showed moderate inflammation with vascular edematous congestion and inflammatory cell infiltrations (Fig. 4(d)).

4. Discussion

In general, humans are exposed to numerous toxic chemicals that cause direct or indirect injury to hepatic cells and alter normal hepatic function. Among them drug-induced hepatic damage is frequent and practically most of the potent medications can cause liver disorders. APAP is the definitive example of acute, dose-related drug-induced hepatic damage which is accounted for the largest number of fatal cases [25]. Though it is a safe antipyretic and analgesic, it can cause severe centrilobular liver necrosis at larger dosages. Previous researches have suggested that mitochondrial oxidative stress, coupled with lipid, DNA, and protein peroxidation, is the primary cellular event in APAP-induced liver damage [28,43]. In humans and animals, a lack of endogenous antioxidants and/or an excess of toxic free radicals (reactive oxygen species (ROS) and reactive nitrogen species (RNS)) generates oxidative stress, which plays a key role in the start of APAP-induced damage to proteins, DNA, and lipids [44,45]. At normal dosages, UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT) metabolize APAP to generate sulfated metabolites and glucuronidated, which are excreted through the urine system. A minute percentage is excreted as unchanged by the urine. Hepatic cytochrome CYP 2E1 (to a lesser degree CYP 1A2 and 3A4) metabolizes an extra amount of APAP (about 5–9%) to produce the highly reactive intermediate metabolite N-acetyl-p-benzoquinone imine (NAPQI) [46]. After APAP overdose produces significant depletion of GSH, which leads to covalent attachment of sulf/hydrl groups in proteins such as cellular and mitochondrial proteins, NAPQI is quickly removed by conjugating with glutathione (GSH) [47]. Consequently, mitochondrial oxidative stress and dysfunction, eventually necrosis may happen in hepatic cells. Furthermore, excessive quantities of NAPQI diminish the adenosine triphosphate (ATP) in mitochondria reduces the native antioxidant level as well as modifies the mitochondrial ATP-synthase α-subunit which ends with ineffectual ATP production [48]. Noxious NAPQI likewise affects the complex I/II of mitochondrial electron transport chain (ETC), instigating outflow of electrons from the ETC to molecular oxygen and thus produces superoxide radicals that are converted by manganese superoxide dismutase (Mn SOD) to form H_{2}O_{2} and molecular oxygen (O_{2}), and/or causes a reaction with endogenous NO to form peroxynitrite (ONOO\textsuperscript{-}) [48,49]. Most of the H_{2}O_{2} is neutralized by GSH or scavenged by endogenous hepatic antioxidant enzymes like peroxiredoxin (Prx), peroxidase (GPx), glutathione, and catalase [50]. As glutathione peroxidase cofactor glutathione level is dwindling that increased the intracellular peroxide levels by the Fenton mechanism which has been occupied in numerous toxicities [28]. On the other hand, Peroxynitrite is commonly detoxified by GSH. However, GSH depletion leads to a
buildup of ONOO\(^{-}\), which causes the development of nitro-tyrosine protein adducts and damage to mitochondrial DNA and results in ultimate necrotic cellular death [43]. Toxic free radicals caused by NAPQI stimulate c-Jun N-terminal kinase (JNK) signaling pathways [51]. Continuous JNK activation raises mitochondrial ROS levels and creates a self-sustaining stimulation loop [52]. Besides this, signaling pathways such as p53, nuclear factor erythroid 2-related factor 2 (Nrf2), adiponectin, and fibroblast growth factor 21 (FGF21) are activated in response to severe ROS-induced cellular stress. JNK also activates BAX, which leads to the opening of the mitochondrial permeability transition (MPT) pore, which causes the membrane potential to collapse and ATP to be depleted [53]. MPT expedites the discharge of mitochondrial inner-membrane proteins which translocate into the nucleus that causes DNA fragmentation and finally encourages the necrotic pathway [54]. As a result, ROS-induced oxidative stress is recognized as a dangerous event in APAP-induced hepatotoxicity. For this reason, a huge number of natural compounds have been examined by researchers and some of them exhibited potentiality to treat hepatic oxidative injury through their antioxidant capability which enhancing levels of GSH, SOD, CAT, and other antioxidant enzymes [43]. AGL is a natural diterpene lactone with antioxidant characteristics that directly neutralize harmful free radicals while also activating antioxidant enzymes, inhibiting pro-oxidant enzymes, and protecting mitochondrial integrity. AGL also regulates transcription factor Nrf2 that has a control mechanism over the antioxidant defense system [55]. Our in vitro radical scavenging assays revealed significant radical scavenging properties of AGL, which corroborated the earlier research findings [56]. AGL also recovers mitochondrial dysfunctions by reducing oxidative stress in both in vitro and in vivo experimental systems. Besides this, AGL abridged the superoxide anion formation and refurbished the mitochondrial membrane potential [57].

The serum levels of ALP, AST, and ALT are the most widely used indicators of the degree of liver damage. ALT catalyzes the transamination process, and an increase in ALT serum levels indicates liver injury. The skeletal muscle, liver, kidneys, brain, heart, and red blood cells all contain AST, an essential enzyme in amino acid metabolism. AST liberation into blood indicates liver damage. ALP is most likely a member of the hydrolase family, and an increase in this enzyme implies hepatobiliary problems [58]. As APAP impaired the hepatocytes by destroying the mitochondrial integrity and increasing membrane permeability of hepatocytes, therefore, elevated levels of serum, AST, ALT, and were observed [59]. Conversely, rats treated with both doses of AGL suggestively refurbished these enzyme levels in serum due to its protective activity. Lactate dehydrogenase (LDH) is an enzyme present in the cytoplasm of cells that catalyzes the conversion of lactate to pyruvate. The discharge of this enzyme indicated either the opening of the cell membrane or the cell’s demise [60]. Acetaminophen toxicity was informed to companion with the augmented discharge of LDH in experimental animals, while, its level in serum was alleviated by AGL [61]. The most powerful indication of liver illness is Gamma-glutamyl transferase (GGT), a microsomal enzyme presents in hepatocytes and biliary epithelial cells [62]. As observed in the control (NC) group, the membrane permeability produced by APAP toxicity increases the serum level of GGT, confirming the findings of Uchida et al. [63]. Conversely, The PC and AGL treated groups reduced serum GGT levels due to their defensive impact on the rat liver. Our outcome is validated by the earlier researcher’s results [61].

One of the most important markers of hepatic tissue injury is the lipid

---

Fig. 3. Effects of andrographolide and controls on hepatic oxidative markers. [The data is provided as means with standard error of the mean (means ± SEM) (n = 7); *p < 0.001 compared to the vehicle control; **p < 0.001 compared to the negative control].
profile. Oxidative stress disrupted lipid metabolism by compromising the integrity of cellular membranes and allowing certain membrane lipids to leak into the bloodstream [64]. When compared to the vehicle control group, the APAP treated group had a substantial rise in serum LDL-c, TG, and TC, but a significant decrease in serum HDL-c. In the AGL-treated groups, there was a decrease in TC, TG, and LDL-c levels and an increase in HDL-c, which was consistent with the findings of Al Batran et al. [65]. A combination of PC and APAP treatment resulted in a comparable effect.

Bilirubin is a consequence of hemolysis, which is transported to the liver by serum albumin and released into the bile following glucuronide conjugation. The rise in the serum bilirubin level designates the severity of necrosis in hepatocytes [66]. Most serum proteins are synthesized by a healthy liver, and their levels are reduced in most hepatic diseases. Therefore, hypo-proteinemia is a common indication in the APAP treated group in an experimental study [1]. Furthermore, Serum albumin and globulin have been identified as prognostic indicators for certain hepatocellular diseases [67]. Following administration of the APAP, there was a decrease in serum albumin levels, which is usually followed by an increase in globulin levels as a result of IgG and IgM [68]. However, AGL amended the levels of these biomarkers and ameliorated APAP-induced toxicity.

Lipid peroxidation (LPO) is a well-known cellular damage process and acceleration in the MDA or thiobarbituric acid reactive substances (TBARS) level is revealed at the beginning of oxidative stress in experimental animals that are exposed to detrimental toxicants [69]. The peroxidation process is initiated by HO• and HOO• which are produced from the Fenton reaction. Besides this, peroxynitrite-instigated LPO. Fatty acid radicals are produced by abstracting H• from lipid molecules [50]. All of these factors promote free-radical chain reactions, which result in the peroxidation of a wide variety of biological molecules, compromising cell membrane integrity, altering the activity of membrane-bound enzymes, and even causing DNA damage [70]. Previous research has shown that LPO causes APAP-induced hepatotoxicity in experimental animals, as well as the P450-mediated xenobiotic metabolism, which may create reactive ROS that controls APAP-induced LPO and liver damage [70,71]. Some natural compounds propose their notable hepatoprotective effect by diminishing lipid peroxidation significantly as evident in reduced MDA or TBARS levels in the liver. In this study, co-administration with AGL significantly reduced the levels of MDA or TBARS in APAP-treated rats, implying that it has anti-oxidant properties, which is consistent with the findings of Trivedi and colleagues, who proposed that AGL’s inhibitory effect on LPO is linked to membrane-stabilizing potentiality [62]. Endogenous hepatic enzymatic moieties metabolize and detoxify a variety of hazardous substances. GSH, an endogenous tri-peptide non-enzymatic antioxidant, is one of them. It plays an important role in xenobiotic metabolism and protects cells from oxidative stress-induced cellular toxicity by lowering H2O2 and scavenging ROS and RNS [72]. Usually, APAP toxic metabolite NAPQI conjugates with GSH for detoxification, however, a toxic dose of APAP produces a huge quantity of NAPQI which creates prompt depletion in GSH level. At the time of cellular GSH exhaustion, the remaining noxious NAPQI will covalently bind to cysteine residues of cellular proteins [73]. In APAP-treated experimental animals, a lower level of GSH is linked to an increase in lipid peroxidation. The combination of APAP and AGL significantly increased GSH levels in APAP-exposed rats. In the same way, antioxidant enzymes (CAT and SOD) are recognized as a first-line defense mechanism for preventing the production of ROS and RNS or scavenging these free radicals [33]. SOD converts the superoxide radical to H2O2 and O2, whereas, CAT transforms the H2O2 to O2 and H2O, therefore, eliminating the pro-oxidative influence of the harmful radicals [74]. On the other hand, the existence

Fig. 4. Effects of andrographolide and controls on histological examination of liver tissue against acetaminophen-induced histological changes in the investigational animals. (a): Vehicle control, (b): Negative control, (c): Positive control, (d): Treatment AGL 5, (e): Treatment AGL 10. [Magnification: 40X; scale bar: 20 µm]. (The red and yellow arrows denote necrosis in the peripheral area of the central vein and the lobule, respectively, while the blue arrow denotes congestion in the central vein associated with deep inflammatory infiltrates, and the green arrow denotes edema in various locations).
5. Conclusion

Co-admiration of AGL at both doses with APAP revealed substantial ameliorative effects on the liver in experimental animals. This consequence may be verified with the improvement of APAP-induced distress in oxidative stress in rat liver by elevating the levels of MDA and reducing the activities of GSH, SOD, and CAT as well as serum hepatic enzymes, lipid profiles, total bilirubin, total protein, albumin, globulin, and histopathological architecture. However, we further emphasize that more comprehensive pre-clinical and clinical studies are requisites for establishing the safety and efficacy of AGL as a therapeutic intervention against drug or toxicant-induced liver injury.

CRediT authorship contribution statement

M., M.S.M, R.H. and M.T.I.: Conceptualization. M.M., C.S., M.T.I. and R.N.: Data curation. S., R.H., A.S. and P.W.: Formal analysis. N., A.S., P.W. and R.H.: Funding acquisition. M., C.S, M.N.H., M.M.R. and M.T.I.: Investigation. M., P.W. and M.T.I.: Methodology. S., R.N, H.D.M.C. and M.T.I.: Project administration. S., R.H., A.S., P.W. and M.T.I.: Resources. H., C.S., M.N.H and R.N.: Software. M., R.H. and M.T.I.: Supervision. M., S.S. and M.T.I.: Validation. S., R.N, M.N.H., M.M.R. and M.T.I.: Visualization. M., C.S., S.S., R.H. and M.T.I.: Writing—original draft. S., R.N., P.W. and M.T.I.: Writing—review & editing.

All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.04.007.
