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Acute toxicity evaluation of aminomethylnaphthoquinone (AMNQ 1) in BALB/c mice

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Abstract

We report the first preclinical tests showing that aminomethylnaphthoquinone - 3-[*N*-(n-butyl) amino-2,4-diclorobenzyl]-2-hydroxy-1,4-naphthoquinone (AMNQ 1) has low cytotoxicity and anti-HSV-1 activity *in vitro* in Vero cells. The aim of this study was to evaluate the acute toxicity of AMNQ **1** in BALB/c mice. We used BALB/c female mice to evaluate the median lethal dose (LD_{50}) of AMNQ 1 with 2000 mg/kg body weight and other three concentrations using 550, 175 and 55 mg/kg. We compared this to acyclovir (ACV-50 mg/kg) and 10% dimethyl sulfoxide (10% DMSO) over a 14-day period. The BALB/c mice received a single oral dose by gavage. There were no deaths in either group and no change in the murine clinical signs. The hematological and biochemical analyses showed some changes that returned to reference levels without impairment of hemostasis. The AMNQ 1 treatment did not induce untoward changes in organs as shown by histological studies. The *in vivo* results showed that AMNQ **1** has low toxicity. In conclusion, AMNQ **1** is safe and can be potentially used as an anti-HSV-1 agent in future studies.
Keywords: Aminomethylnaphthoquinone, acute toxicity, preclinical tests, BALB/c mice

1. Introduction

Natural naphthoquinones are widely found and may be extracted from trees belonging to the Bignoniaceae family, which includes the important native tree known as *Tabebuia impetiginosa* (Mart ex DC.) or Purple Ipê. Naphthoquinones may also be found in microorganisms and animals. Naphthoquinones have several pharmacological activities, e.g. antibacterial, antiviral and antifungal activities [1-3]. These compounds are a source of free radicals and can form irreversible complexes with nucleophilic amino acids of proteins [3, 4]. Some of these molecules, e.g. rifampicin, mitomycin and other intermediates, are used to construct biologically important compounds containing amine groups. These have been associated with a wide range of biological properties [5, 6].

We studied AMNQ 1 and compared it to acyclovir (ACV) using *in vitro* assays with Vero cells. The AMNQ 1 cytotoxicity was CC_{50}=2654±135.7 μM, the effective anti-HSV-1 was $EC_{50=}$ 1.74±0.22 μM, and the selective indices was SI=1325.2. These corresponding values for ACV were: CC_{50}= 960±1.56 μM, $EC_{50=}$ 1.09±0.25 μM and SI= 880. This suggests that AMNQ 1 could be used to treat Herpes Simplex Virus Type 1 (HSV-1) [7, 8].

The HSV-1 is an important human pathogen that can cause latent infections. It is an important cause of morbidity especially in immunocompromised patients [9-12]. Acyclovir (ACV) it is one of the most commonly used antiviral drugs and is primarily used to treat HSV-1 infections. However, acyclovir-resistant HSV infections have emerged from immunocompromised patients including organ-transplant and AIDS patients [13, 10-12].

These results encourage additional studies to evaluate the acute toxicity of AMNQ 1 in experimental animals. Our department is studying various substances with antiviral potential, and AMNQ 1 inhibits the HSV-1 virus with low toxicity.

2. Materials and Methods

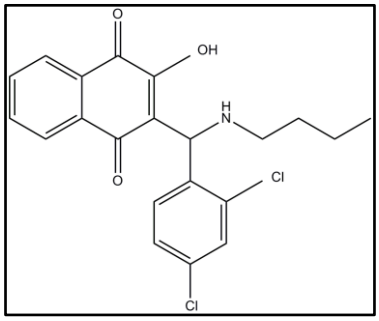
2.1 Animals

Three-month-old female BALB/c mice (n=34) weighing 19-25 g were acquired from the Nucleus of Laboratory Animals (NAL) of the Federal Fluminense University (UFF). The mice were first acclimated to the environment of the UFF Biomedicine animal facility for 15 days prior to the experiments. The animals were kept in polypropylene cages at 25±2°C, humidity 55±10%, under a 12 h/12 h light/dark cycle with free access to food and water. This research was approved by the Use of Animal Ethics Committee of UFF (CEUA-UFF- certificate number 255/12).

2.2 Compounds

The 3-[*N*-(n-butyl) amino-2,4-diclorobenzyl]-2-hydroxy-1,4-naphthoquinone (AMNQ **1**) (Figure 1) was synthesized as previously [14, 15, 2]. The AMNQ **1** was dissolved in 10% DMSO (v/w) (dimethyl sulfoxide - Merck®- 99.9%). The positive control was acyclovir (ACV) (Sigma).

Figure 1: Structure of AMNQ 1 - (3-[N-(n-butyl) amino-2,4 -diclorobenzyl]-2-hydroxy-1,4- naphthoquinone



2.3 Experimental protocol: Lethal Dose (LD) and Acute Toxicity (14 days)

In the LD50 test, the animals were divided into two groups: AMNQ 1 group – 2000 mg/kg (n = 5) and 10% DMSO group (control-vehicle) (n = 5). These two groups were administered 2000 µL (500 µL/hour for 4 hours) orally and observed for 48 hours. Immediately after, the animals were euthanized by anesthetic overdose (ketamine + xylazine), and the organs were removed for histology. To evaluate the acute toxicity over 14 days, the animals were divided into five groups: I) AMNQ 1 – 550 mg/kg (n = 5); II) AMNQ 1 - 175 mg/kg (n = 5); III) AMNQ 1 - 55 mg/kg (n = 5); IV) ACV-50 mg/kg – positive control (n = 5) and V) 10% DMSO - control (n = 4). These were administered a single oral dose of 500 µL. The animal behavior was observed throughout both experiments. At the end of the experimental period (14 days), the animals were euthanized by anesthetic overdose. The protocol and concentrations used in this study were based on the OECD 423 guidelines [16].

2.3.1 Hematological and biochemical parameters

Blood samples were collected on day zero (D0) and day 14 (D14). Hematological tests used samples collected in EDTA-BD-Microtainer® vials and analyzed with an auto-hematology analyzer (BS-2800-Bioclin) for red blood cells (RBC), hematocrit (HCT), hemoglobin (HGB), white blood cell leukocytes (WBC), lymphocytes (LYC), monocytes (MNC), granulocytes (GNC), and platelets (PLT).

Biochemical parameters were assessed following collection in BD-Microtainer® (Clot Activator/SSTTM Gel–Amber) vials to analyze: alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (AP), blood urea nitrogen (BUN), creatinine (CRE), totals protein (TP) and albumin (ALB). The results were obtained with an automatic biochemistry meter (BS-210-Bioclin).

The reference values for analysis were determined in all samples before animal treatment (D0, n=24).

2.3.2 Histological analysis

The organs, liver, kidneys, duodenum, stomach, spleen and heart were fixed in 10% Carson formalin [17, 18] for histological analyses [19]. Staining used hematoxylin and eosin (H&E). Histological analysis used values of: 0.5, not significant or not observed; 1, low; 2, moderate; and 3, severe.

2.4 Statistical Analyses

The data were expressed as the mean ± SD and were subjected to one way analysis of variance (ANOVA) followed by the Tukey post-test. Tests used the GraphPad Prisma 5.01 software and PRISMA graphic. For statistical values, $p < 0.05$ was considered to be statically significant.

3. Results

3.1 Acute toxicity analyses - LD50 and over 14 days

The administration of 2000 mg/kg of AMNQ 1 to BALB/c mice did not induce death or adverse clinical signs in animals. Our results showed that the LD_{50} of AMNQ 1 was greater than 2000 mg/kg.

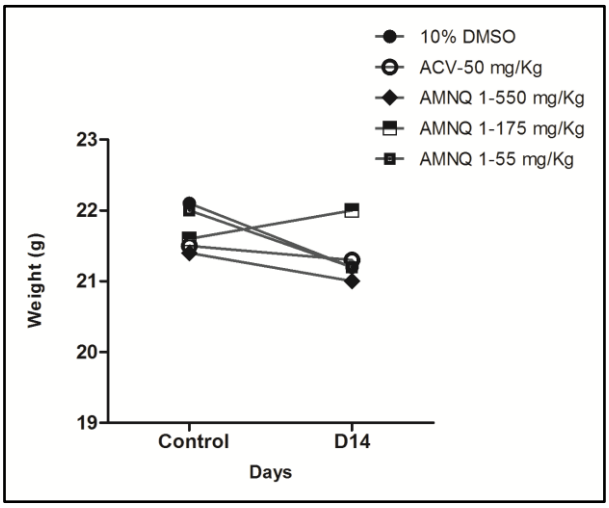
3.2 Histology analyses - LD_{50}

The tissues of the organs liver, kidney, duodenum, spleen and stomach of animals administered AMNQ 1 were compared to those administered with 10% DMSO (control-vehicle). The architecture and cellularity of organs from all animals (n = 10) were maintained and comparable to controls. The duodenum of the animals administered AMNQ 1 and control substances showed a reduced number of villi and increased lymphocyte cells. There were no histological changes in the stomachs or spleens in treated animals relative to controls. The livers of the animals treated with AMNQ 1 presented interstitial edema and focal coagulation necrosis. The kidneys of the animals treated with AMNQ 1 presented multifocal hemorrhages, tubular necrosis and dilatation of proximal and distal tubules as well as normal glomeruli and interstitial edema.

3.3 Weight and Clinical Signs (over 14 days)

All animals treated with AMNQ 1, 10% DMSO and ACV survived for 14 days. There were no significant adverse clinical signs or changes in body weights ($p > 0.05$) (Figure 2).

Figure 2: Body weight of BALB/c mice treated with AMNQ 1 and controls (10% DMSO and ACV-50 mg/kg) on days 0 (Control) and D14.



Average AMNQ 1 (n =5), ACV (n=5) and 10% DMSO (n = 4). p>0.05. Tukey test

3.4 Hematological analyses (over 14 days)

The hematological analyzes (Table 1) showed some significant decreases in the RBC and HGB levels between control animals and animals treated with AMNQ 1 groups 550 mg/kg and 175 mg/Kg on D14 ($p < 0.05$). The platelets there was significant increase group ACV ($p<0.01$). All analyses were compared with reference values (D0, n=24)

Table 1: Effect AMNQ 1 on hematological analyses in acute toxicity study in BALB/c mice

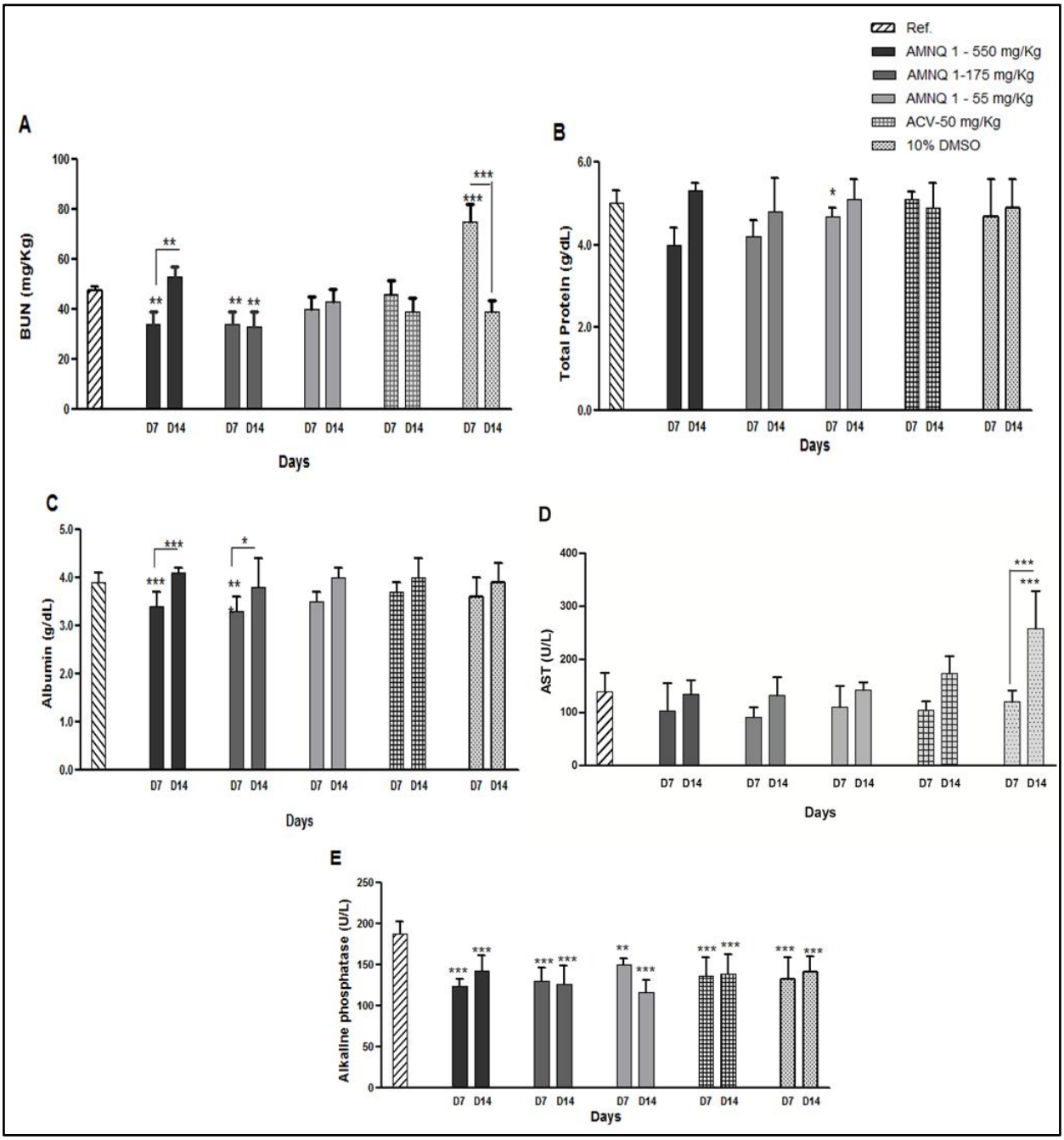
Parameters hematology	Reference (D0)	AMNQ1 550 mg/Kg	AMNQ1 175 mg/Kg	AMNQ1 55 mg/Kg	ACV 50 mg/Kg	10% DMSO
RBC (x 10^6 mm^{-3})	8.3 ±0.28	7.5±0.61*	7.5 ±0.30*	8.5±0.11	7.8 ±0.27	7.8 ±0.20
HCT (%)	43.5 ±1.6	41.3 ±3.57	40.3 ±1.58	45.7±0.82	42.5±2.11	43.0 ±1.79
HGB (g/dL)	13.9 ±0.97	12.8 ±1.14	12.5 ±0.61*	13.7±0.24	12.9 ±0.62	13.2 ±0.44
PLT (x 10^3 mm^{-3})	274 ±47	268 ±26.9	282±27.6	327 ±50.5	361 ±29.3**	313±17.2
WBC (x 10^6 mm^{-3})	9.8 ±1.0	10.1±1.48	7.8 ±1.45	10.0 ±0.79	8.7±1.84	8.6 ±0.73
LYC (%)	78.1 ±2.51	76.3±2.8	74.3±2.4	75.7±5.3	78.0±3.2	77.5 ±3.7
MNC (%)	4.3 ±0.7	4.8 ±0.7	5.1 ±0.8	4.4 ±1.2	5.0±0.8	4.3 ±1.2
GRC (%)	17.5 ±2.5	18.8±2.3	20.6 ±1.9	19.8 ±4.3	17.0±2.7	18.2 ±2.8

The data expressed as Mean ±SD (n=5), 10% DMSO (n=4) and Reference (D0, n=24). * $p<0.05$; ** $p<0.01$. *Tukey test*

3.5 Biochemical Analyses

At the end of the 14-day experiment, there were no significant changes in any of the groups for creatinine and ALT. The BUN test showed that in the AMNQ **1**-550 group there was a significant decrease in D_7 ($p < 0.01$). In the 10% DMSO group there was an increase in D7 ($p<0.001$), but on D14 both (AMNQ 1-550 and 10% DMSO) returned to levels of the reference group (ref.). In the AMNQ 1 – 175 group, there was a decrease on D14 relative to the reference group ($p < 0.01$) (Figure 3A). The total protein and albumin tests of all groups returned levels of the reference group on D14 (Figure 3B and C). In the AST test, the 10% DMSO control group on D14 showed a significant increase compared the reference group ($p < 0.01$) (Figure 3D). The AP enzyme showed in all groups on D_{14} a significant decrease compared the reference group ($p < 0.001$) (Figure 3E).

Figure 3: Effect AMNQ 1 on biochemical analyses in acute toxicity study in BALB/c mice female. (A) BUN; (B) total protein; (C) albumin; (D) AST and (E) AP

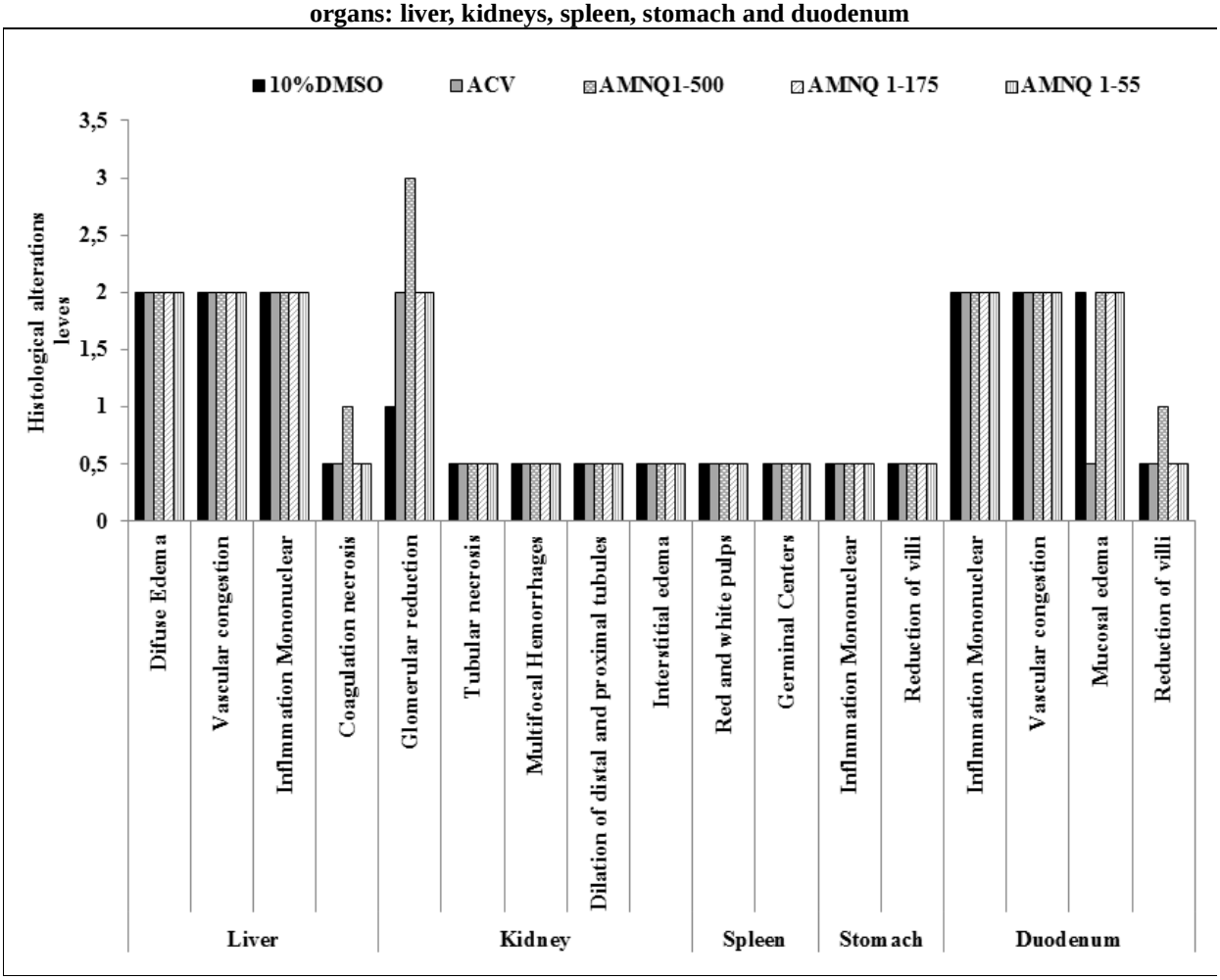


The data expressed as Mean ±SD (n=5), 10% DMSO (n=4) and Ref. (D0, n=24). *p<0.05; **p<0.01; ***p<0.001. *Tukey test*

3.6 Histological Analyses

The stomach and duodenum of the animals were observed and no significant changes were noted except for the AMNQ **1**-550 group (n = 4) that had reduced villi. The other AMNQ **1** (n = 15) and 10% DMSO (n = 4) groups showed moderate sub-mucosal edema, vascular congestion and a mononuclear inflammatory process. The ACV group (n = 5) showed normal villi. The liver of all groups showed moderate diffuse edema, vascular congestion and a mononuclear inflammatory process. The AMNQ **1**-550 group (n = 5) had focused coagulation necrosis. In all groups, the renal cortex showed reduced glomeruli. This was more evident in the AMNQ **1**-550 group. The controls also showed moderate changes. All alterations are described in Figure 5.

Figure 4: Histological changes in acute toxicity over 14 days. Graph summarizing the changes in BALB/c mice organs: liver, kidneys, spleen, stomach and duodenum



4. Discussion

Our studies of the acute toxicity of AMNQ **1** at a dose of 2000 mg/kg administered orally showed that this compound presents low toxicity in BALB/c mice with LD_{50} value > 2000 mg/kg. This was determined based on a scale proposed by Lorke [20]. This compound can be included in Category 5 (low toxicity) according to the OECD Guideline 423 [16].

A 14-day trial with oral administration (gavage) indicated that AMNQ **1** did not produce death or any clinical signs of toxicity in mice at any dose (OECD, 2001) [16]. There was no significant difference among the groups in terms of body weight of the mice during the 14-day experiment (Figure 2).

The hematology analyses did not reveal any dose-dependent changes with toxicological significance. Araujo's work showed reference dates using the same mice strain to interpret biochemical and hematological analysis [21]. Other work using plants extracts with mice showed similar reference values in all AMNQ 1 groups and control group on D14 [22, 1].

The animals treated with AMNQ 1 had low levels of ALT and AST enzymes despite the presence of cellular liver changes [1, 23]. These enzymes produce cellular changes in the liver, but these levels were within the reference range at all AMNQ1 concentrations were within the normal reference [21, 22, 1]. The AP enzyme also is an indicator of changes in liver enzymes in addition to bone and the biliary tract. In our study, a significant decrease in alkaline phosphatase was observed at all doses indicating that AMNQ **1** did not induce any biochemical changes indicative of liver disease in mouse serum [24, 25].

The kidneys presented some significant alterations in the glomerulus in the AMNQ **1**-550. However, on the 14th day, the BUN levels were within the reference levels. Creatinine levels are an important indicator of changes in kidney function, and they did not show any significant changes. This suggests that the compound has low toxicity [26]. Studies on the biotransformation of acyclovir (control drug) in mice showed alterations, but this is considered low toxicity [27]. Some studies using experimental animals have shown biochemical parameters that corroborate our results at D0 and on the 14th day. This facilitates comparison between baseline levels and subsequent time points [28]. Thus, some changes were found

in the AMNQ **1**-treated animals as seen in this preclinical acute toxicity testing; however, they did not cause any deaths or behavioral changes. Thus, AMNQ **1** can be considered a low toxicity compound based on OECD Guidelines 423 [16] and in relation to the behavior of animals.

In conclusion, the LD_{50} of this drug was over 2000 mg/kg (48 h). This shows that AMNQ **1** is safe and a Category 5 drug (low toxicity) according to the OECD for acute toxicity. Tests using single dose oral routes at three different concentrations (550, 175 and 55 mg/kg) were observed for 14 days and showed significant changes in hematological, biochemical and histological parameters including the controls (10% DMSO and ACV). This acute toxicity preclinical study showed few changes and no deaths. However, further tests are required to assess the toxic potential of aminomethylnaphthoquinone AMNQ **1**.

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