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Original Research Article

Glue Thistle Toxicology: In Vitro Assessment of Oxidative Stress Induced by the Active Compound Atractyloside in *Atractylis gummifera* L

Mohamed Habib Belmahi [a*] , Imane Mecheri [a], Henni Chader [b]

[a] *Toxicology Laboratory, Faculty of Medicine, Department of Pharmacy, University Salah Boubnider Constantine, Algeria*
[b] *Faculty of Pharmacy, University of Algiers 1, Algeria*

Abstract

Glue Thistle, scientifically known as *Atractylis gummifera* L., is commonly used in traditional medicine due to its topical wound-healing properties for conditions like syphilis, boils and abscesses. Additionally, it serves as an herbal infusion with diuretic, purgative, antipyretic, abortifacient and emetic qualities. The acute toxicity of this plant varies across animal species and administration routes, typically leading to hepatic necrosis and renal failure. The primary aim of our study is to assess the toxic potential of atractyloside, the primary active compound in Glue Thistle, in vitro. This is achieved by determining the inhibitory concentration 50 (IC50) on three different cell lines (RD, HEP2, and A549) and evaluating oxidative stress indicators, specifically, reduced glutathione (GSH), malondialdehyde (MDA) and catalase, in these cellular models. *In vitro* results indicated a significant increase in MDA levels, a decrease in reduced GSH concentration, resulting in a reduction in catalase activity ranging from 66% to 85%. These outcomes suggested oxidative stress induced by atractyloside, accompanied by an apoptotic process. Our research contributes to a comprehensive understanding of the acute toxicity of *Atractylis gummifera* L., offering insights for more effective preventive and treatment approaches.

Keywords:
Atractylis gummifera L., Glue Thistle, Atractyloside, In vitro, Oxidative stress, Toxicity.

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∗Corresponding author
Email address: habib.belmahi@univ-constantine3.dz (Mohamed Habib Belmahi)

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1. Introduction

Glue Thistle, which is widely distributed in Mediterranean basin countries, belongs to the Asteraceae family and is scientifically known as *Atractylis gummifera* L. [1].Its most commonvernacular name in the Maghreb region is "addad." The plant is frequently found in the wild. Due to its traditional use, the dried fragments of its rhizome can be found among herbalists [1]. In Algeria, this plant is omnipresent, ranging from coastal areas to the Tell region, across plains and mountains, at altitudes ranging from 1500 to 1900 meters, in various regions including EL-Kala, Annaba, Guelma, Constantine, Tizi Ouzou, Bejaia, Bouira, Alger, Miliana, Tiaret,Mostaganem, Sig, Sidi bel Abbes, and Oran [2,3]. The earliest botanical and chemical studies on Glue Thistle were initiated in Algeria in 1866, shedding light on its primary activecompound, atractyloside (ATR), the structure of which was determined later [4–6].

Intoxication by *Atractylis gummifera* L., often fatal and occasionally occurring in groups, frequently affects children who are drawn to the plant. Data from the Anti-Poison Center in Algiers (CAP) from 1991 to 2010 revealed that plant poisonings account for 3% of all poisonings, among which Glue Thistle contributes to 13% of cases, resulting in a 37% fatalityrate [7]. A survey conducted between 1992 and 2002 in the regions of Setif and Bordj Bou- Arreridj ranks Glue Thistle second among plant poisonings, representing 17.5% of cases [8]. In2014, a survey in Tlemcen reported an incidence of poisonings at 13% [9].

The toxicity of the plant is attributed to ATR and carboxyatractyloside (CATR), primarily present in the rhizome. These compounds inhibit oxidative phosphorylationand the Krebs cycle by disrupting the function of the adenine nucleotide translocator (ANT), amitochondrial protein responsible for the transport of ADP and ATP across the inner mitochondrial membrane. Additionally, ATR induces the opening of mitochondrial permeability transition pores, causing permeabilization of the membrane and the release of inner membrane proteins, including cytochrome C, which triggers apoptosis. Cells with abundant mitochondria, such as hepatic and renal cells, are particularly affected [10,11].

Currently, there is no specific treatment for Glue Thistle poisoning, and symptomatic treatment is often insufficient, necessitating emergency hospitalization. Studies have shown that certain compounds, such as N-acetylcysteine, may offer some protection against the toxiceffects of the plant, but only if administered before exposure to the plant [12]. A promising approach is immunotherapy, where specific antibodies against the toxic components of *Atractylis gummifera* L. are developed and utilized. This approach is akin to recent studies conducted on other toxic plants, such as Oleander (*Nerium oleander*), which have shown promising results [13]. However, it should be noted that these preventive treatments are still inthe research phase and are not widely available for clinical use. For now, prevention remains the only means to protect against poisoning by this plant or other toxic plants. This involves extensive information and awareness campaigns aimed at the population, which is not always aware of the risks involved.

The primary focus of our study is to evaluate the toxic potential of ATR. This will be achieved through *in vitro* tests, specifically by determining its IC50 on three distinct cell lines. Additionally, key indicators of oxidative stress, such as glutathione, MDA (malondialdehyde), and catalase, will be measured. These analyses aim to better characterize the toxic effects of ATR at the cellular level.

2. Material and Methods

2.1. Reagents and Biological Material

- **Reagents:** Atractyloside pure product 99% (Sigma Aldrich)**,** Trypsin (Gibco)-EDTA 0.25 (Sigma Aldrich), L-glutamine 200 mM in the culture medium (Gibco), Neutral red solution at 0.05 mg/mL in the culture medium (Gibco), Sterile distilled water (Gibco), Hydrophilic diluent: 0.9% sodium chloride (Saidal), Revelation solution: 1% glacial acetic acid (Sigma Aldrich) in ethanol (SigmaAldrich) at 50°C. Thio barbituric acid (TBA) at 0,8% (Sigma Aldrich); Acetic acid at 20% (VWR); Hydrogen peroxide (H2O2) at 0,2% (VWR); 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) (Sigma Aldrich); Sodiumdodecyl sulfate (SDS) at 8,1% (Fisher); Phosphate buffer at pH 7,4; Distilled water.
- **Biological Material**

Three distinct cell lines were used in this study:
-A549: Derived from human basal alveolar epithelial adenocarcinoma cells (12th passage, Pasteur Institute of Algeria - IPA).
-HEP2: Derived from human laryngeal carcinoma cells (34th passage, IPA).
-RD: Derived from human rhabdomyosarcoma cells (8th passage, IPA).
Cells were cultured in 75 cm^2 flasks using Dulbecco's Modified Eagle's Medium (DMEM) 1X (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 100 µg/ml penicillin (Gibco), and 100 µg/ml streptomycin (Gibco). The incubation conditions were set at 37°C in a humidified atmosphere (5% CO_2, 95% air), with media changes performed every two days.

2.2. Evaluation of ATR Cytotoxicity Using the Neutral Red Release Assay

In this section, cytotoxicity was evaluated by subjecting a monolayer of cells to varyingconcentrations of ATR for 24 and 48 hours. The parameter employed to assesscytotoxicity was the IC50 of ATR that induces 50% cell mortality. This determination was made indirectly by measuring the release of neutral red, a dye exclusively absorbed by viable cells, following the specified incubation periods.

Method: The experimental protocol was adapted based on standard cytotoxicity assays [14]:

- Cell seeding (Day -1): Cells are placed in two 96-well microplates per cell line oneday prior to cytotoxicity assessment. They are incubated for 24 h at 37°C, with 5%CO_2 concentration and 88% humidity.
- Preparation of ATR solutions (Day 0): ATR dilutions were prepared using 0.9% sodium chloride at increasing concentrations ranging from 0.05 to 6 mg/mL.
- ATR exposure to cells (Day 0): Wells are rinsed with PBS, then treated with 100 µL of each ATR concentration. The first microplate is incubated for 24 h, while thesecond one is incubated for 48 h.

- Preparation of the dye solution (Day 0 and Day 1): A 0.4% neutral red solution is diluted at 1/80 in culture medium and then incubated for 18 to 24 h under controlledconditions (37°C, 5% CO_2, and 88% humidity). The revelation solution is preparedon Day 0 and Day 1 for the 24 h and 48 h cytotoxicity assays, respectively.
- Preparation of the revelation solution (Day 1): A solution containing 1% glacial acetic acid in 50°C ethanol is prepared on the day of cytotoxicity revelation, specifically for the first microplate at Day 1. This solution can be stored for severalweeks.
- Cell staining (Day 1 and Day 2): After 24 or 48 h of ATR incubation with the test cells, the neutral red dye solution is centrifuged at 3000 g for ten minutes and addedat 0.1 mL per well. Subsequently, the microplate is placed for three hours in an incubator at 37°C, 5% CO_2 and 88% humidity. After this contact time, the dye solution in the wells is removed before proceeding with rinsing
- Rinsing: Five successive rinses are performed using 0,2 mL of PBS maintained at room temperature for each rinse.
- Cytotoxicity revelation: After completing the microplate treatment, 0.1 mL per wellof the revelation solution (1% acetic acid in 50°C ethanol) is added. The microplateis gently shaken for 15 minutes. Reading: The optical density (OD) of the neutral red in the revelation solution is measured using a spectrophotometer at 540 nm, compared to a blank (1% acetic acid in 50% ethanol).
- Expression and interpretation of results: The percentage of cell mortality is calculated for each ATR concentration using the following formula:
  % cell mortality = 100 * (Average OD of treated wells / Average OD of control wells)

$$\textbf{Cell mortality (\%)} = 100 \times \frac{\textbf{Average OD of treated wells}}{\textbf{Average OD of control wells}} \qquad \textbf{(1)}$$

A curve of cell mortality percentages as a function of ATR concentrations is plotted,and the IC50 causing 50% cell mortality is then calculated using linear regression analysis. To perform this curve fitting anddetermine the IC50, STATISTICA V.6 software is used.

2.3. *In vitro* Assessment of Oxidative Stress Induced by Atractyloside

Oxidative stress involves a complex set of parameters and cannot be highlighted by a single method. Therefore, we chose to measure three oxidative biomarkers. These biomarkers are categorized into two axes:

- Measurement of oxidative damage to lipids: Malondialdehyde (MDA).
- Determination of enzymatic and non-enzymatic antioxidants: catalase and Glutathion (GSH).

To determine the oxidative effect of ATR, these biomarkers are quantified in treated cells compared to untreated cells (negative control).

2.3.1. Malondialdehyde (MDA) Assay

The TBARS determination, following the modified YAGI method by SESS et al. (1992) [14], provides an indirect assessment of free radicals by quantifying substances resulting from lipid peroxidation that react with thiobarbituric acid (TBA). In this method, each molecule of Malondialdehyde (MDA) produced from lipid peroxidation reacts with two molecules of thiobarbituric acid (TBA). This reaction occurs under acidic and high-temperature conditions, leading to the formation of a pink-colored complex, measurable using spectrophotometry at 532nm. Despite the coloration representing all reacting substances (TBARS), the results are specifically expressed in terms of malondialdehyde (MDA) concentration [15].

- **Procedure:** The procedure involved the combination of 375 µL of TBA (0,8%), 375 µL of Acetic Acid (20%), 50 µL of SDS (8,1%), 100 µL of distilled water and 100 µL of cellular supernatant in screw-cap tubes. This mixture was vortexed for 10 s and thenincubated at 100 °C in a water bath for 1 h under acidic and high-temperature conditions.Subsequently, it was rapidly cooled to +4 °C to halt the reaction and then centrifuged at3000 rpm for 10 min. The absorbance at 532 nm was measured using a UV-Visible spectrophotometer.

  The MDA concentrations (C) in the solution were reported in µmol/L and calculated using the Beer-Lambert law ($A = \varepsilon.l.C$), where $\varepsilon MDA = 156\ mmol^{-1}.L.cm^{-1}$ and l (optical path length) = 0,625 cm.

2.3.2. Measurement of glutathione (GSH)

The quantification of glutathione (GSH) is conducted using the colorimetric method developed by Ellman (1959). The GSH determination relies on a reaction involving the sulfhydryl group of GSH. In this reaction, GSH reacts with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) to produce a yellow compound known as 5-thio-2-nitrobenzoic acid (TNB). Measuring its absorbance at 405 nm enables the determination of the GSH level in the sample [16].

- **Procedure:** In the procedure, 100 µL of GSH buffer (pH 7,1) and 50 µL of DTNB are added to each well of the microplate, followed by the addition of 50 µL of cellular supernatant. The mixture is agitated for 10 min and then incubated at 37 °C for 20 minutes. After incubation, the absorbance at 405 nm is measured. The GSH concentrations are reported in mmol/L, and the calculation is performed using the Beer-Lambert law: $A = \varepsilon.\ l.\ C$.

2.3.3. Evaluation of Catalase (CAT) Enzymatic Activity

The assessment of catalase (CAT) enzymatic activity is performed using the method developed by Clairborne (1985). The principle is grounded in the decomposition of hydrogen peroxide (H_2O_2) in the presence of the enzymatic source at 25°C [17].

- **Procedure:** For the evaluation of CAT enzymatic activity, a mixture is prepared by combining 950 µL of phosphate buffer (KH_2PO_4, 50 mM, pH 7.4), 50 µL of cellular supernatant, and 500 µL of H_2O_2, which is added just before the reading. The absorbance is recorded at 240 nm at 30 s intervals for a duration

of 2 minutes. Enzymaticactivity is quantified in terms of international units per minute per milliliter of liquid (UI/min/mL of liquid). The rate constant (K) is determined using the formula $K = 2,3/\Delta t \log A1/A2$, where Δt represents the time interval in minutes, A1 is the optical absorbance at time 1, and A2 is the optical absorbance at time 2. The catalase enzymaticactivity (U) is calculated as $U = K/V$, with V being the volume of supernatant utilized in milliliters.

3. Results

3.1. *In Vitro* cytotoxicity assay of atractyloside on RD, A549 and HEP2 Cells

Table 1 presents a summary of the results obtained from the cytotoxicity assay of ATR on various cell lines. The concentrations ranged from 0,05 mg/mL to 6,00 mg/mL, and the assays were conducted following 24-hour and 48-hour incubation periods on RD cells. Additionally, the Table provides an overview of the cytotoxicity assay results for ATR on HEP2 cells and summarizes the findings for A549 cells. Concentrations ranging from 0,05 mg/mL to 9,00 mg/mL were examined to assess their impact on cell cytotoxicity following both 24 h and 48 h incubation periods.

Table 1. Summary of ATR cytotoxicity results on different cell Lines

Concentrations (mg/mL)	Cytotoxicity (%) on RD Cells		Cytotoxicity (%) on A549 cells		Cytotoxicity (%) on HEP2 cells	
	Incubation Time (hours)		Incubation Time (hours)		Incubation Time (hours)	
	24	48	24	48	24	48
00	00	00	00	00	00	00
0.05	00	12,61	00	09,98	05,24	22,76
0.25	00	15,44	0,26	15,42	06,45	27,44
0.5	2,88	27,23	10,51	27,23	09,48	39,69
2.00	24,46	39,83	14,88	46,11	08,07	53,89
3.00	36,69	40,65	28,46	52,83	09,08	59,54
6.00	61,15	75,20	35,89	62,43	18,15	60,64
9.00	/	/	55,39	74,07	55,39	70,43

Figures 1 and 2 depict the cytotoxicity assay results of ATR on RD cells following 24 h and 48 h of incubation, respectively. Similarly, for A549 cells, Figures 3 and 4showcase the cytotoxicity assay outcomes after 24 h and 48 h of incubation. Furthermore, Figures 5 and 6 provide an insight into the cytotoxicity assay results for HEP2 cells following 24 h and 48 h of incubation, respectively.

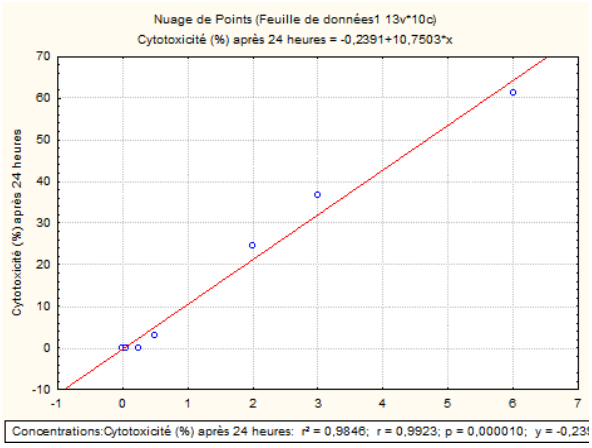

Figure 1. Cytotoxicity Curve of ATR on RDCells after 24 Hours of Incubation

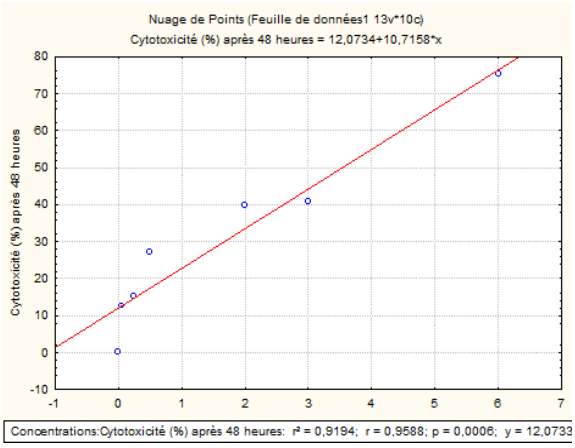

Figure 2. Cytotoxicity Curve of ATR onRD Cells after 48 Hours of Incubation

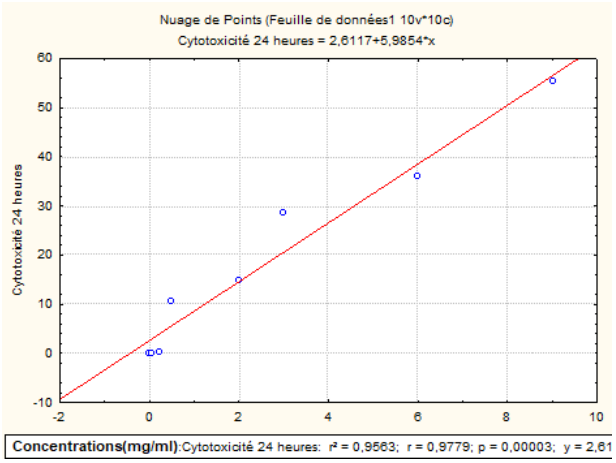

Figure 3. Cytotoxicity Curve of ATR on A549Cells after 24 Hours of Incubation

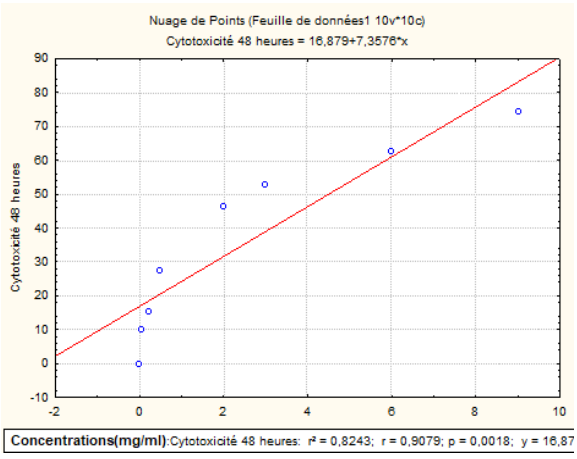

Figure 4. Cytotoxicity Curve of ATR onA549 Cells after 48 Hours of Incubation

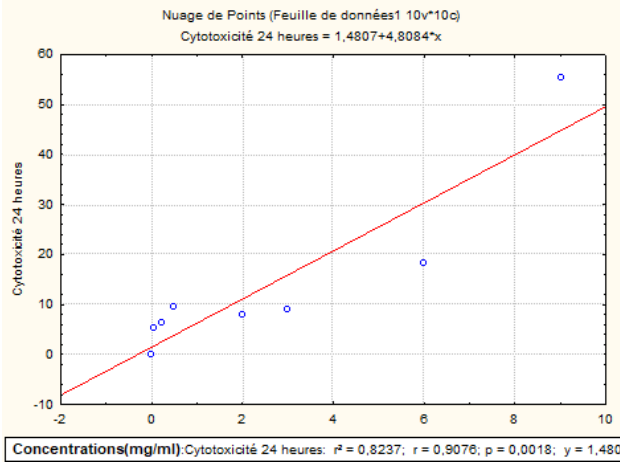

Figure 5. Cytotoxicity Curve of ATR onHEP2 Cells after 24 Hours of Incubation

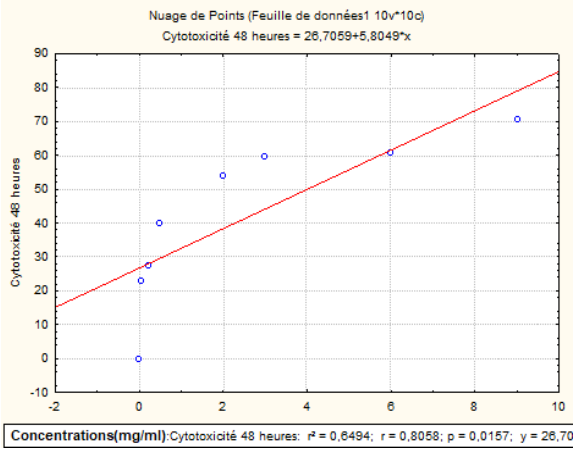

Figure 6. Cytotoxicity Curve of ATR onHEP2 Cells after 48 Hours of Incubation

The IC50 of ATR on RD, A549, and HEP2 cells was computed employing linear regression analysis with STATISTICA software. Table 2 consolidates the IC50 values for ATR at various incubation times, encompassing both 24 h and 48 h of incubation across the three cell lines. Prolonged exposure of RD, A549, andHEP2 cells to ATR resulted in an increased cytotoxic effect.

Table 2: Summary of the *in vitro* cytotoxicity assay results of ATR on different celllines.

Cell lines used in *in vitro* cytotoxicity assays.	**IC50 (mg/mL)**	
	Incubation Time (hours)	
	24	**48**
RD	4,67	3,54
A549	7,92	4,50
HEP2	10,01	4,01

3.2. Oxidative stress biomarker quantification

3.2.1. Changes in oxidative stress biomarkers in different cell lines (RD, A549 and HEP2 cells)

Table 3 encapsulates the variations in oxidative stress biomarkers (MDA, GSH and catalase) observed in different cell lines (RD, A549, and HEP2 cells) following 24 and 48 hoursof incubation with various concentrations of ATR.

Table 03**:** Summary Table of the evolution of oxidative stress biomarkers in different celllines after 24 and 48 hours of incubation with various ATR concentrations**.**

RD Cells						
ATR concentrations (mg/mL)	**MDA concentrations (moles/L)**		**Concentrations of reduced glutathione (moles/L)**		**Catalase activity (10^{-1} UI/mL)**	
	24 H	**48 H**	**24 H**	**48 H**	**24 H**	**48 H**
00	0,37	0,42	12,2	12,8	04,28	03,36
0,05	0,41	0,34	13,48	13,48	03,98	02,75
0,25	0,34	0,37	11,4	12,35	03,06	02,45
0,5	0,51	0,47	09,6	10,27	02,75	01,83
2,00	0,62	0,58	08,4	09,78	01,83	01,40
3,00	2,00	0,72	05,44	06,4	01,53	01,16
6,00	1,20	0,98	2,75	3,45	00,92	01,16
A549 Cells						
ATR concentrations (mg/mL)	**MDA concentrations (moles/L)**		**Concentrations of reduced glutathione (moles/L)**		**Catalase activity (10^{-1} UI/mL)**	
	24 H	**48 H**	**24 H**	**48 H**	**24 H**	**48 H**
00	0,37	0,34	10,7	11,3	03,98	03,06
0,05	0,36	0,34	11,0	11,3	02,14	01,53
0,25	0,44	0,37	11,4	10,35	02,14	01,47
0,5	0,53	0,5	08,6	10,27	00,92	01,22
2,00	0,87	0,68	08,1	08,4	00,92	00,92
3,00	0,9	0,87	05,1	06,19	00,92	00,92
6,00	1,4	0,95	2,66	3,76	00,61	00,61
HEP Cells						
ATR concentrations (mg/mL)	**MDA concentrations (moles/L)**		**Concentrations of reduced glutathione (moles/L)**		**Catalase activity (10^{-1} UI/mL)**	
	24 H	**48 H**	**24 H**	**48 H**	**24 H**	**48 H**
00	0,44	0,44	10,2	12,63	05,51	02,75
0,05	0,38	0,39	11,0	11,22	02,14	01,53
0,25	0,44	0,41	10,35	11,65	01,53	01,22
0,5	0,55	0,55	09,6	10,23	01,53	00,92
2,00	0,72	0,73	07,24	08,74	01,22	00,92
3,00	0,97	0,9	06,6	05,93	00,92	00,92
6,00	0,98	1,4	2,8	3,88	00,61	00,92

The concentration variation curves of the different markers as a function of ATR concentrations in different cells are presented in Figures 7-12.

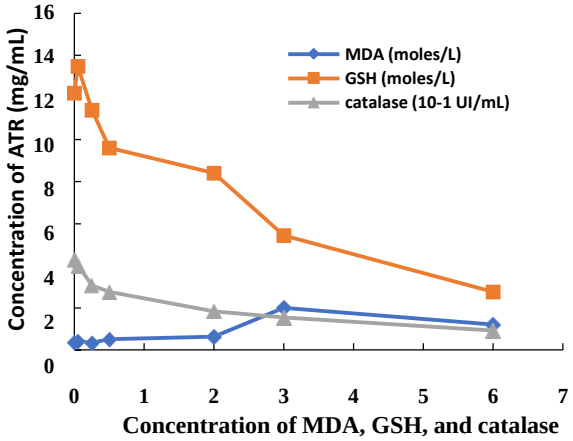

Figure 7. Changes in oxidative stress biomarkers inRD cells after 24 hours of incubation with ATR

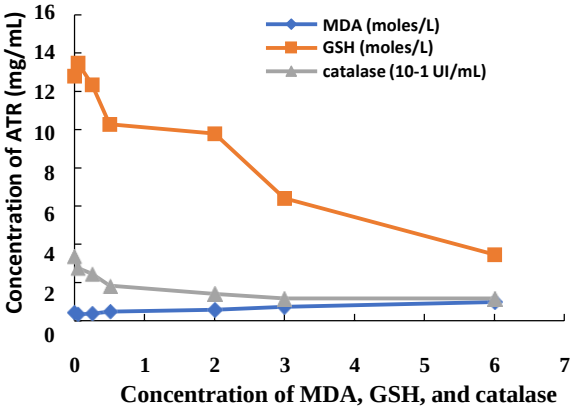

Figure 8. Changes in oxidative stress biomarkers inRD cells after 48 hours of incubation with ATR

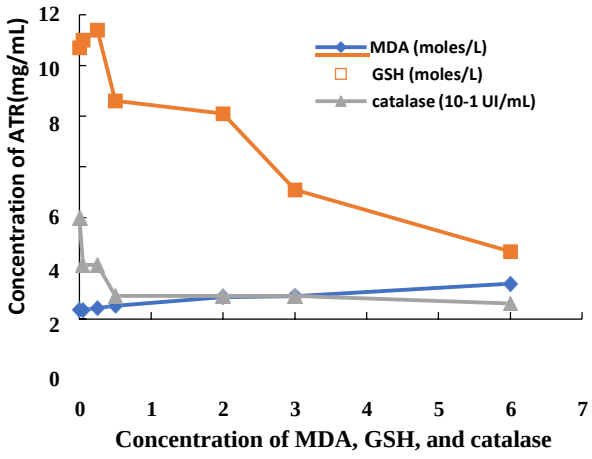

Figure 9. Changes in Oxidative Stress Biomarkersin A549 Cells after 24 Hours of Incubation with ATR

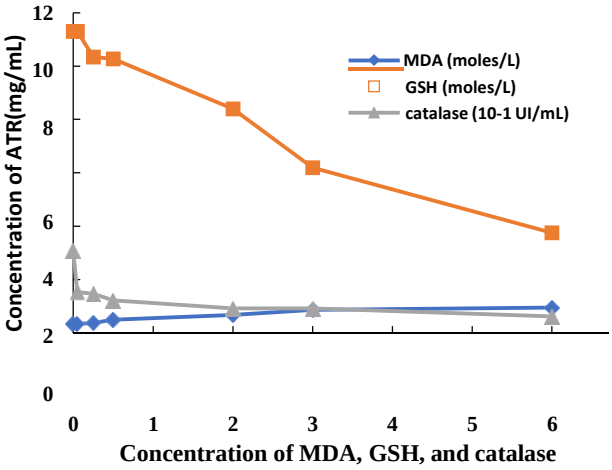

Figure 10. Changes in Oxidative Stress Biomarkers inA549 Cells after 48 Hours of Incubation with ATR

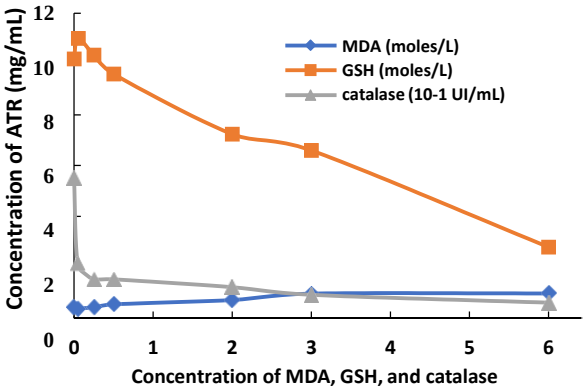

Figure 11. Changes in Oxidative Stress Biomarkersin HEP2 Cells after 24 Hours of Incubation with ATR

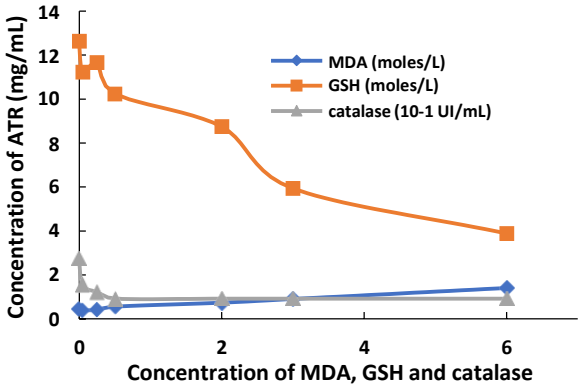

Figure 12. Changes in Oxidative Stress Biomarkers inHEP2 Cells after 48 Hours of Incubation with ATR

The *in vitro* assessment conducted on the three strains –RD, A549, and HEP2–unveiledthe following key findings:

- An escalation in the MDA level, exhibiting a range of 62% to 82%.
- A decline in the concentration of reduced glutathione (GSH), with variations ranging from 67% to 80%.
- A decrease in the activity of catalase and superoxide dismutase (SOD), demonstratinga range from 66% to 85%.

Discussion

These results indicate a shift in the antioxidant-pro-oxidant balance in favor ofoxidative stress. ATR accidentally causes damage to the cells, which, unable to repairthem, trigger pathological apoptosis, also known as intrinsic apoptosis, where the mitochondriaplays a significant role.

Studies conducted by Alpa Popat in 2001 were successful in uncovering the indirecttoxicity of ATR. A 24-hour incubation with the Impila extract (a plant with ATR as its active ingredient) demonstrated substantial toxicity on Hep G2 cells across all tested concentrations. Hep G2 hepatic cells serve as a model system for studies on hepatic metabolismand xenobiotic toxicity. The cytotoxicity observed is dose-dependent, with an LD50 of 3,5 mg/mL. Nearly 100% toxicity was noted at a concentration of 6,7 mg/mL. Oxidative stress assessment revealed that a 6-hour incubation led to the complete depletion of GSH in Hep G2 cells in a dose-dependent manner. This depletion occurred after only 3 hours of exposure, resulting in a 61% decrease compared to the control [18].

The work of JIAN YIN et al. further substantiates our hypothesis. Their study aimed to assess ATR-induced hepatotoxicity using rat and human hepatocytes in monoculture. Following a 48-hour treatment, ATR induced concentration-dependent hepatotoxicity, as evidenced by the cell viability rate and intracellular glutathione (GSH) content in rats. In summary, these findings suggest that *in vitro* hepatocytes, especially human hepatocytes, could serve as a valuable tool for screening herbal-based hepatotoxicity in vitro. A CI 100 in less than 6 hours on this type of cell indicates a necrotic effect following hepatic transformation [19]. In our case, prolonged mitochondrial permeability transition leads to a series of disturbances. The uncoupling of oxidative phosphorylation results in decreased ATP synthesis, which is further exacerbated by increased ATP hydrolysis, leading to severe intracellular ATP depletion. Additionally, the dissipation of the electrochemical proton gradient disrupts ionic homeostasis. These alterations contribute to the release of apoptotic factors, such as Apoptosis Inducing Factor (AIF) and cytochrome c, into the cytoplasm, triggering the formation of apoptosomes. Consequently, mitochondrial dysfunction can lead to necrotic cell death, while also inducing apoptosis through two distinct pathways: the caspase-dependent pathway activated by cytochrome c and the caspase-independent pathway mediated by AIF.

4. Conclusion

The *in vitro* study conducted on the three cell lines RD, A549, and HEP2 has yielded valuable insights into the effects of ATR exposure. The observed elevation in malondialdehyde (MDA) levels, accompanied by a decrease in the concentration of reduced glutathione (GSH) and catalase activity, highlights the presence of oxidative stress as a result of the apoptotic process initiated by ATR. These findings illuminate the intricate cellular responses to ATR and enhance our understanding of its cytotoxic mechanisms.

Declaration of Competing Interest

None of the authors have any conflicts of interest related to this manuscript.

Data availability

No data was used for the research described in the article.

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