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# PROTECTIVE ROLE OF ROSMARINUS OFFICINALIS CALLUS EXTRACT AGAINST PARACETAMOL-INDUCED OXIDATIVE DAMAGE AND MITOCHONDRIAL DYSFUNCTION IN HEPG2 CELL LINE

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## ABSTRACT

Paracetamol (acetaminophen, APAP) is one of the most popular analgesic and antipyretic medications in the world, although overdose is still a major cause of acute liver failure in developed countries. The hepatotoxic process involves essentially excessive synthesis of N-acetyl-p-benzoquinone imine (NAPQI), which depletes hepatic glutathione reserves, covalently alters mitochondrial proteins, and initiates a cascade of oxidative damage leading to cell death. The current work studied the hepatoprotective effect of methanolic extract produced from Rosmarinus officinalis L. callus culture against APAP-induced cytotoxicity in human hepatocellular carcinoma (HepG2) cells. Callus cultures were produced on Murashige and Skoog medium with 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP). Lyophilised callus was extracted with methanol and phytochemical screening indicated the presence of phenolic acids, flavonoids and diterpene substances such as rosmarinic acid and carnosic acid. The HepG2 cells were pre-incubated with the callus extract at various concentrations (25-200 µg/mL) before the APAP insult (10 mM, 24 h). The extract dramatically improved cell survival (from 41.8% to 84.1%), reduced the production of reactive oxygen species (ROS), restored the mitochondrial membrane potential (ΔΨm) and boosted the intracellular glutathione level in a concentration-dependent manner. Moreover, the extract lowered the level of malondialdehyde (MDA) and increased the activity of superoxide dismutase (SOD) and catalase (CAT). These findings indicate that *R. officinalis* callus extract offers meaningful cytoprotection against APAP-mediated hepatic injury through mitigation of oxidative stress and preservation of mitochondrial integrity.

***Keywords:*** *Rosmarinus officinalis; callus extract; paracetamol; hepatoprotection; oxidative stress; mitochondrial dysfunction; HepG2; rosmarinic acid.*



## 1. INTRODUCTION

Paracetamol (acetaminophen, APAP) is arguably the most frequently used over-the-counter analgesic and antipyretic agent worldwide. At therapeutic dosages, the drug is considered safe and effective; however, intentional or accidental overdose remains the leading pharmacological cause of acute liver failure in many Western countries [1,2]. The hepatotoxic cascade is well characterized: APAP undergoes metabolic activation by cytochrome P450 enzymes principally CYP2E1 to form the highly reactive electrophile N-acetyl-p-benzoquinone imine (NAPQI). Under normal circumstances, NAPQI is swiftly detoxified through conjugation with hepatic glutathione (GSH). However, when APAP doses above therapeutic levels, the sulfation and glucuronidation pathways are saturated, GSH reserves are depleted and free NAPQI accumulates [3]. The unconjugated metabolite then indiscriminately reacts with cellular macromolecules, particularly mitochondrial proteins, leading to protein

adduct formation, which triggers a cascade of mitochondrial oxidative stress, activation of the c-Jun N-terminal kinase (JNK) signalling cascade, opening of the mitochondrial permeability transition (MPT) pore, collapse of the mitochondrial membrane potential (ΔΨm) and ultimately, necrotic hepatocyte death [4,5].

Mitochondrial dysfunction and oxidative damage are known to play a pivotal role in APAP hepatotoxicity. As a result, there has been an increasing interest in discovering natural antioxidants that are capable of intercepting the injury pathway at several levels. Rosmarinus officinalis L. (rosemary, family Lamiaceae) is an aromatic medicinal herb native to the basin of the Mediterranean sea and is widely cultivated for culinary, cosmetic and pharmaceutical purposes. The pharmacological flexibility of rosemary is mainly related to its phenolic diterpenes, carnosic acid and carnosol, and to the hydroxycinnamic ester, rosmarinic acid, which all show strong free-radical scavenging, metal-chelating and enzyme-modulating actions [6,7]. Some in vivo investigations have shown that rosemary extracts could protect rat liver from carbon tetrachloride- and acetaminophen-induced injury by attenuating lipid peroxidation, restoring antioxidant enzyme activities and preserving mitochondrial redox equilibrium [8,9].

Most studies have been conducted on plants growing in the field. Plant cell and tissue culture technologies, including callus, suspension and organ cultures, provide attractive biotechnological options for the sustainable and standardised production of bioactive secondary metabolites. In vitro callus cultures may collect rosmarinic acid and other phenolics at levels that are similar or even higher to those found in differentiated tissues [10,11]. Importantly, the output is independent of seasonal, regional and environmental variations, and devoid of contamination with agrochemicals or soil-borne pathogens [12]. Notwithstanding these advantages, the hepatoprotective action of extracts obtained from rosemary callus has surprisingly received little attention and no study has investigated their effect on APAP-mediated mitochondrial injury in human hepatoma cells.

In this context, the present study was designed to establish callus cultures of R. officinalis, extract the proliferated callus biomass with methanol and evaluate its protective activity against APAP-induced cytotoxicity, oxidative stress and mitochondrial dysfunction in the HepG2 cell line. This cell line was selected as it retains a number of hepatocyte-like characteristics such as phase I and II metabolic capabilities, albumin secretion and vulnerability to oxidant challenge, and is a widely accepted in vitro model for hepatotoxicity screening [13,14].

## 2. MATERIALS AND METHODS

### 2.1. Plant Material and Chemicals

Seeds and young shoot explants of *Rosmarinus officinalis* L. were procured from authenticated specimens maintained at [specify botanical garden or herbarium]. Murashige and Skoog (MS) basal medium, 2,4-dichlorophenoxyacetic acid (2,4-D), 6-benzylaminopurine (BAP), sucrose, agar, and all plant tissue culture reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acetaminophen (≥99%), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), rhodamine 123 (Rh-123), 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), thiobarbituric acid (TBA), dimethyl sulfoxide (DMSO), and standard antioxidant enzymes were obtained from the same supplier. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, and trypsin-EDTA were sourced from Gibco (Thermo Fisher Scientific, Waltham, MA, USA).

### 2.2. Callus Induction and Maintenance

Surface-sterilized leaf and stem explants were cultured on MS medium supplemented with 30 g/L sucrose, 8 g/L agar, and different combinations of 2,4-D (0.5–3.0 mg/L) and BAP (0.5–2.0 mg/L). Cultures were incubated in a growth chamber at 25 ± 2 °C under a 16 h/8 h light/dark photoperiod (2000 lux, cool white fluorescent lamps). Callus initiation was monitored weekly. The optimum hormonal regime for callus proliferation was identified after three successive subcultures at 21-day intervals, and the resulting friable callus mass was harvested, freeze-dried, and stored at −20 °C until extraction.

### 2.3. Preparation of Callus Extract

Lyophilized callus biomass (10 g) was finely ground and extracted three times with 100 mL of 80% (v/v) methanol under continuous agitation (150 rpm) for 24 h at ambient temperature. The combined filtrates were concentrated under reduced pressure using a rotary evaporator (40 °C) and further dried in a vacuum desiccator. The crude methanolic extract was reconstituted in DMSO at a stock concentration of 100 mg/mL, filter-sterilized (0.22 µm), and kept at −20 °C. Working solutions were freshly prepared by dilution in DMEM such that the final DMSO concentration never exceeded 0.1% (v/v).

### 2.4. Phytochemical Characterization

Total phenolic content (TPC) was determined by the Folin–Ciocalteu method using gallic acid as a reference standard (mg GAE/g DW). Total flavonoid content (TFC) was measured by the aluminum chloride colorimetric assay with quercetin as the standard (mg QE/g DW). Identification and semi-quantification of major phenolic compounds were performed by reverse-phase HPLC-DAD on a $C_{18}$ column (250 × 4.6 mm, 5 µm) with gradient elution of 0.1% formic acid (A) and acetonitrile (B) at 1.0 mL/min [15].

Table 01: Phytochemical profile of R. officinalis callus methanolic extract: total phenolic content (TPC), total flavonoid content (TFC), and major identified compounds by HPLC-DAD. Values are mean ± SD (n = 3).

| Parameter | Value | Unit |
|---|---|---|
| Total phenolic content (TPC) | 45.82 ± 3.15 | mg GAE/g DW |
| Total flavonoid content (TFC) | 18.34 ± 1.72 | mg QE/g DW |
| Rosmarinic acid (RA) | 12.45 ± 0.98 | mg/g DW |
| Carnosic acid (CA) | 3.21 ± 0.34 | mg/g DW |
| Carnosol (CL) | 1.87 ± 0.22 | mg/g DW |
| Caffeic acid | 0.95 ± 0.11 | mg/g DW |
| Luteolin | 0.62 ± 0.08 | mg/g DW |
| Hispidulin | 0.28 ± 0.04 | mg/g DW |

### 2.5. Antioxidant Activity In Vitro

The free-radical scavenging ability was assessed via DPPH and ABTS assays. Various concentrations (10–500 µg/mL) were incubated with the respective radical solutions. The ferric reducing antioxidant power (FRAP) assay was additionally conducted. $IC_{50}$ values were calculated by non-linear regression. Ascorbic acid served as the positive control [16].

Table 02: Antioxidant activity of R. officinalis callus extract determined by DPPH, ABTS, and FRAP assays. Ascorbic acid was used as positive control. Values are mean ± SD (n = 3).

| Assay | Callus Extract | Ascorbic Acid |
|---|---|---|
| DPPH $IC_{50}$ (µg/mL) | 38.52 ± 2.84 | 8.21 ± 0.63 |
| ABTS $IC_{50}$ (µg/mL) | 29.73 ± 2.15 | 5.64 ± 0.42 |
| FRAP (µmol $Fe^{2+}$ eq/g) | 285.4 ± 18.6 | — |

### 2.6. Cell Culture and Treatments

Human hepatocellular carcinoma HepG2 cells (ATCC HB-8065) were maintained in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C in a humidified atmosphere of 5% $CO_2$. Experimental groups: (i) untreated control; (ii) DMSO vehicle control (0.1%); (iii) APAP-only (10 mM, 24 h); (iv) extract pretreatment (25, 50, 100, or 200 µg/mL for 6 h) followed by APAP co-incubation (10 mM, 24 h); and (v) extract-only control (200 µg/mL, 30 h) [13,17].

### 2.7. Cell Viability Assay (MTT)

Cell viability was evaluated by the MTT reduction assay. After treatment, 100 µL of MTT solution (0.5 mg/mL) was added per well, incubated at 37 °C for 4 h, formazan dissolved in DMSO, and absorbance read at 570 nm. Results expressed as percentage viability relative to controls.

### 2.8. Intracellular ROS Measurement

Intracellular ROS levels were quantified using DCFH-DA (10 µM, 30 min, 37 °C). Fluorescence was measured at 485/528 nm and expressed as fold change relative to control.

### 2.9. Mitochondrial Membrane Potential (ΔΨm)

Mitochondrial membrane potential was assessed using rhodamine 123 (1 µg/mL, 30 min, 37 °C). Fluorescence at 488/525 nm was expressed as percentage of control [18].

### 2.10. Intracellular GSH and MDA

Reduced GSH was measured using Ellman's reagent (DTNB) at 412 nm. MDA was quantified by the TBARS method at 532 nm. Both normalized to protein (Bradford) [19,20].

### 2.11. Antioxidant Enzyme Activities

SOD activity was determined by pyrogallol auto-oxidation inhibition at 420 nm. CAT activity by $H_2O_2$ decomposition at 240 nm. Activities expressed as U/mg protein [21].

### 2.12. Statistical Analysis

All experiments in triplicate, repeated ≥3 times. Data: mean ± SD. One-way ANOVA with Tukey's post-hoc test. Significance at $p < 0.05$. GraphPad Prism 9.0.

## 3. RESULTS AND DISCUSSION

### 3.1. Callus Induction and Growth

Among the various hormonal combinations tested, the MS medium fortified with 1.0 mg/L 2,4-D and 1.0 mg/L BAP yielded the highest callus induction frequency (87 ± 4.2%) from leaf explants after 21 days of culture. The callus obtained was light green to yellow in colour, friable in texture and demonstrated vigorous proliferation following culturing. Induction rates for stem-derived explants were somewhat lower (72 ± 5.1%) and such explants tended to form more compact callus tissue. The results are quite close to previous reports on the induction of rosemary callus, where the synergistic combination of an auxin (2,4-D or NAA) and a cytokinin (BAP or kinetin) was reported to be required for successful dedifferentiation and biomass accumulation [10,22]. The equal concentration combination of 2,4-D/BAP appears to favour a balanced ratio of auxin to cytokinin inducing cell division without premature redifferentiation which would reorient metabolic fluxes away from secondary metabolite biosynthesis.

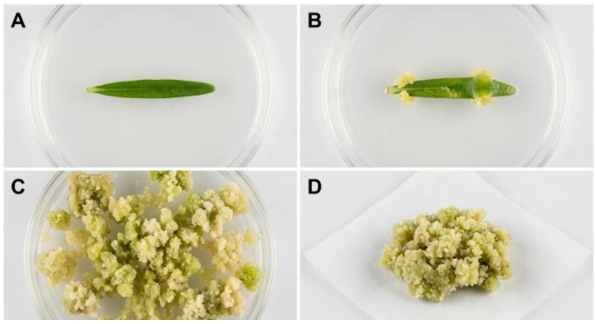


Figure 02: Morphological appearance of R. officinalis callus cultures at different stages: (A) explant at day 0, (B) callus initiation at day 7, (C) proliferating friable callus at day 21, and (D) callus biomass harvested for extraction.

### 3.2. Phytochemical Composition of Callus Extract

The methanolic extract of R. officinalis callus exhibited total phenolic content of 45.82 ± 3.15 mg GAE/g DW and total flavonoid content of 18.34 ± 1.72 mg QE/g DW (Table 1). The HPLC-DAD analysis confirmed the presence of three main bioactive compounds: rosmarinic acid (12.45 mg/g DW), carnosic acid (3.21 mg/g DW) and carnosol (1.87 mg/g DW) along with minor peaks attributed to caffeic acid, luteolin and hispidulin (Figure 3). Interestingly, rosmarinic acid was the most abundant phenolic in the callus extract, in agreement with the fact that undifferentiated rosemary cell cultures prefer to accumulate this hydroxycinnamic acid ester [11,23]. Lower but still appreciable quantities of biosynthesised carnosic acid and carnosol were found, mostly biosynthesised in differentiated glandular trichomes. The presence of these three chemical classes is pharmacologically interesting because rosmarinic acid, carnosic acid and carnosol have hepatoprotective actions by means of complementing mechanisms including direct radical scavenging, metal chelation, activation of Nrf2/HO-1 pathway and suppression of NF-kB [7,9,24].

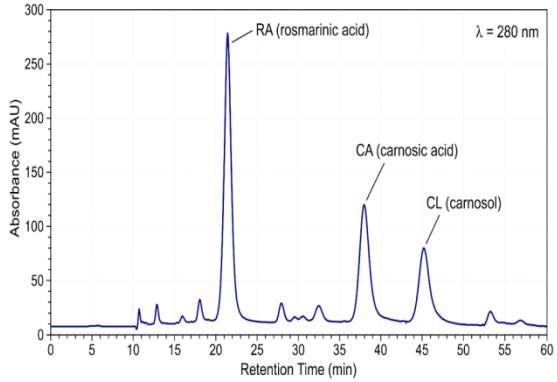


Figure 03: Representative HPLC-DAD chromatogram of the methanolic extract of R. officinalis callus at 280 nm. Peaks: RA = rosmarinic acid, CA = carnosic acid, CL = carnosol.

### 3.3. Antioxidant Capacity of the Callus Extract

The callus extract showed significant free-radical scavenging activity with DPPH IC50 of 38.52 µg/mL and ABTS IC50 of 29.73 µg/mL (Table 2). These values are a little higher than ascorbic acid but are in the range of those reported for methanolic extracts from rosemary leaves [16,25]. The FRAP measurement (285.4 µmol Fe 2+ eq/g) further showed excellent electron donating capacity. Rosmarinic acid, with its two catechol moieties, is responsible for the antioxidant capacity, making it an effective hydrogen atom transfer agent [26]. The contributions of carnosic acid and carnosol are also of note. These diterpenes undertake oxidative cascades, with carnosic acid oxidising to carnosol then to rosmanol, each step consuming one radical equivalent, so providing a self-amplifying antioxidant defence [7].

### 3.4. Effect on Cell Viability

Exposure of HepG2 cells to 10 mM APAP for 24 h reduced cell viability to approximately 41.8% of the control, confirming a robust toxic challenge (Figure 4). The extract alone at 200 µg/mL did not induce measurable cytotoxicity (97.2%), indicating a favorable safety margin. Pretreatment with the callus extract elicited a dose-dependent cytoprotective effect: viability increased from 41.8% (APAP alone) to 51.3% at 25 µg/mL, 59.7% at 50 µg/mL, 72.4% at 100 µg/mL, and 84.1% at 200 µg/mL ($p < 0.05$ at all concentrations versus APAP-only). The highest concentration recovered approximately 73% of the viability deficit. A similar magnitude of protection was reported by Guimarães et al., who demonstrated that 0.05% rosemary glycolic extract prevented acetaminophen-induced hepatocyte death in vivo [8].

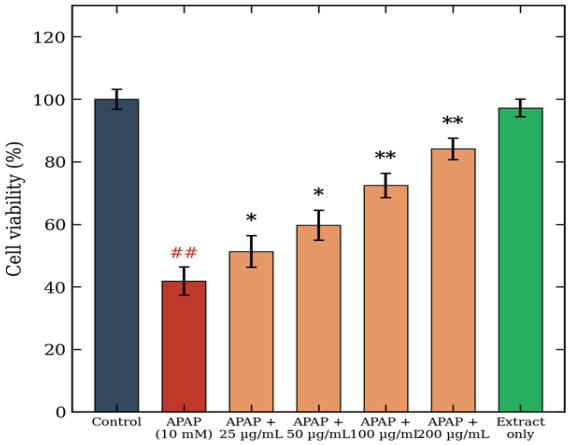


Figure 04: Effect of R. officinalis callus extract pretreatment on the viability of HepG2 cells challenged with 10 mM APAP for 24 h (MTT assay). Data: mean ± SD (n = 3). *p < 0.05, **p < 0.01 vs. APAP-only; ##p < 0.01 vs. control.

### 3.5. Effect on Intracellular ROS Generation

APAP treatment induced a marked elevation in intracellular ROS, with DCFH-DA fluorescence rising to 3.82-fold above control levels (Figure 5). The

overproduction of ROS reflects NAPQI-mediated electron leakage from the mitochondrial electron transport chain, GSH depletion, and activation of the JNK–mitochondrial amplification loop [3,4]. Pretreatment with the callus extract at 100 and 200 µg/mL significantly reduced ROS to 1.87- and 1.38-fold, respectively, attributable to both direct radical scavenging by rosmarinic acid and carnosol and to indirect enhancement of endogenous antioxidant defenses. The lower concentrations (25 and 50 µg/mL) produced statistically significant but more modest reductions (3.21- and 2.58-fold).

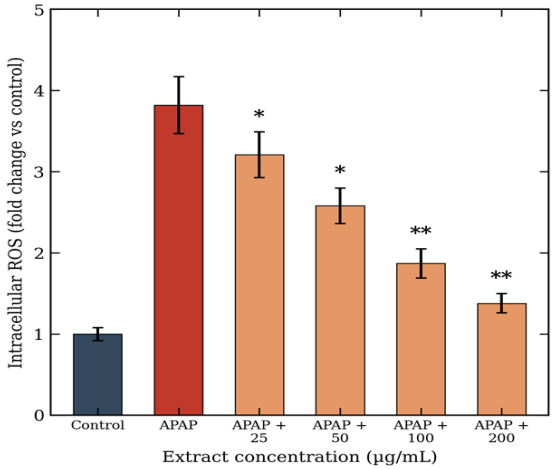


Figure 05: Intracellular ROS levels in HepG2 cells (DCFH-DA fluorescence). Groups: control, APAP (10 mM), and APAP with callus extract pretreatment (25–200 µg/mL). Fold change vs. control (mean ± SD, n = 3). *p < 0.05, **p < 0.01 vs. APAP-only.

### 3.6. Preservation of Mitochondrial Membrane Potential

Rh-123 staining showed that APAP treatment induced a significant loss of ΔΨm, with Rh-123 fluorescence decreased to 37.5% of control (Figure 6). This is consistent with in vivo evidence for mitochondria as a major subcellular target. NAPQI-protein adducts decrease oxidative phosphorylation, increase superoxide production and facilitate opening of the MPT pore [4,5,27]. Pretreatment with 100 and 200 µg/mL callus extract significantly maintained ΔΨm at 71.3% and 83.6% of control, respectively. Part of this effect may be due to carnosol stabilising peroxiredoxin 3 (PRDX3), a key mitochondrial H2O2-scavenging enzyme [28]. Furthermore, carnosic acid suppresses JNK phosphorylation in hepatic cells [29], which leads to an attenuation of the JNK-mitochondrial feed-forward loop.

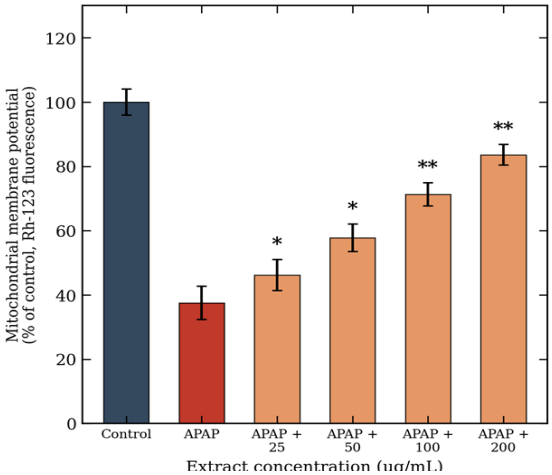


Figure 06: Mitochondrial membrane potential (ΔΨm) in HepG2 cells (rhodamine 123 fluorescence, % of control). Groups: control, APAP (10 mM), and APAP + callus extract (25–200 µg/mL). Mean ± SD (n = 3). *p < 0.05, **p < 0.01 vs. APAP.

### 3.7. Restoration of Intracellular GSH and Reduction of Lipid Peroxidation

APAP exposure reduced intracellular GSH to 10.8 µmol/mg protein (30.7% of control), consistent with stoichiometric GSH depletion by conjugation to NAPQI (Figure 7A, Table 3). At the same time, MDA levels increased to 5.78 nmol/mg protein (4.74-fold higher than control), indicating significant lipid peroxidation (Figure 7B). Pretreatment with callus extract significantly improved both measures. At 200 µg/mL, GSH recovered to 30.4 µmol/mg protein (86.4% of control) and MDA was decreased to 1.68 nmol/mg protein (1.38-fold). Mechanistically, the importance of GSH restoration is dual since GSH is not only the major conjugating partner of NAPQI but also a co-factor of glutathione peroxidase [19,30]. Rosmarinic acid increases the expression of glutamate-cysteine ligase (GCL) through the Keap1/Nrf2 pathway, which leads to de novo GSH synthesis [24,31].

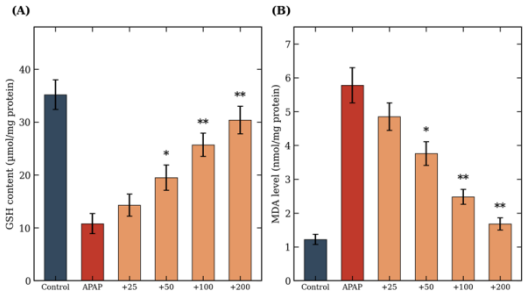


Figure 07: Oxidative stress markers in HepG2 cells: (A) Intracellular GSH content (µmol/mg protein); (B) MDA levels (nmol/mg protein). Groups: control, APAP, and APAP + callus extract (25–200 µg/mL). Mean ± SD (n = 3). *p < 0.05, **p < 0.01 vs. APAP.

Table 03: Intracellular GSH content and MDA levels across experimental groups. Values: mean ± SD (n =

3). Different superscript letters within each column indicate significant differences ($p < 0.05$, Tukey's test).

| Group | GSH (µmol/mg protein) | MDA (nmol/mg protein) |
|---|---|---|
| Control | 35.20 ± 2.80$^{a}$ | 1.22 ± 0.15$^{a}$ |
| APAP (10 mM) | 10.80 ± 1.90$^{e}$ | 5.78 ± 0.52$^{e}$ |
| APAP + 25 µg/mL | 14.30 ± 2.10$^{de}$ | 4.85 ± 0.41$^{d}$ |
| APAP + 50 µg/mL | 19.50 ± 2.40$^{cd}$ | 3.76 ± 0.35$^{c}$ |
| APAP + 100 µg/mL | 25.70 ± 2.20$^{bc}$ | 2.48 ± 0.22$^{b}$ |
| APAP + 200 µg/mL | 30.40 ± 2.60$^{ab}$ | 1.68 ± 0.18$^{ab}$ |

### 3.8. Enhancement of Antioxidant Enzyme Activities

APAP exposure significantly suppressed both SOD (from 18.5 to 7.2 U/mg) and CAT (from 24.3 to 9.5 U/mg) activities, presumably because the overwhelming oxidative burden led to enzyme inactivation (Figure 8). Pretreatment with the callus extract dose-dependently augmented both activities: at 200 µg/mL, SOD and CAT recovered to 16.8 and 21.7 U/mg protein (~90.8% and ~89.3% of control values), respectively. These results support the view that rosemary phenolics activate the Nrf2-ARE signaling axis. Upon nuclear translocation, Nrf2 induces SOD, CAT, HO-1, NQO1, and GSH biosynthetic genes [24,32]. Recent work confirmed that rosmarinic acid activates the Keap1/Nrf2/HO-1 pathway and reverses APAP-induced decline in SOD, CAT, and GSH in mice [31].

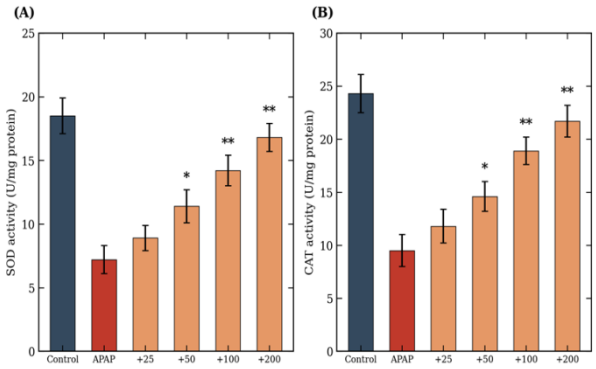


Figure 08: Antioxidant enzyme activities in HepG2 cells: (A) SOD (U/mg protein); (B) CAT (U/mg protein). Groups: control, APAP, and APAP + callus extract (25–200 µg/mL). Mean ± SD (n = 3). *$p < 0.05$, **$p < 0.01$ vs. APAP.

Table 04: Summary of key protective endpoints across experimental groups. Arrows indicate direction of change relative to untreated control: ↑ increase, ↓ decrease, ↔ no change. Values for the APAP + 200 µg/mL group are shown.

| Parameter | Control | APAP | +100 | +200 | Change |
|---|---|---|---|---|---|
| Viability % | 100 | 41.8 | 72.4 | 84.1 | ↑↑ |
| ROS (fold) | 1.00 | 3.82 | 1.87 | 1.38 | ↓↓ |
| ΔΨm % | 100 | 37.5 | 71.3 | 83.6 | ↑↑ |
| GSH | 35.2 | 10.8 | 25.7 | 30.4 | ↑↑ |
| MDA | 1.22 | 5.78 | 2.48 | 1.68 | ↓↓ |
| SOD | 18.5 | 7.2 | 14.2 | 16.8 | ↑↑ |
| CAT | 24.3 | 9.5 | 18.9 | 21.7 | ↑↑ |

### 3.9. Proposed Mechanism of Protection

Based on the present findings and the existing literature, a multi-target protective mechanism can be proposed (Figure 1). The *R. officinalis* callus extract intercepts the APAP toxicity cascade at several critical nodes: (a) direct scavenging of ROS and reactive nitrogen species by the catechol functionalities of rosmarinic acid and carnosol; (b) preservation and replenishment of intracellular GSH pools, possibly through Nrf2-mediated induction of GCL; (c) maintenance of mitochondrial membrane integrity by attenuation of protein thiol oxidation and inhibition of MPT pore opening; and (d) upregulation of antioxidant enzymes (SOD, CAT) via the Keap1/Nrf2/HO-1 pathway. The convergence of these mechanisms explains the robust dose-dependent cytoprotection observed across multiple endpoints. Notably, the use of callus-derived extract introduces a biotechnological dimension: the controlled in vitro production system enables consistent phytochemical profiles and eliminates the seasonal variability inherent to field cultivation [12,33].

## 4. CONCLUSION

Here, we show for the first time that a methanolic extract obtained from Rosmarinus officinalis L. callus cultures protects significantly against paracetamol-induced cytotoxicity, oxidative stress and mitochondrial dysfunction in HepG2 cells. The callus extract, rich in rosmarinic acid with detectable levels of carnosic acid and carnosol, exhibited a significant improvement in cell viability (41.8 to 84.1% at 200 µg/mL), suppressed intracellular ROS accumulation (3.82 to 1.38-fold), replenished depleted glutathione stores (10.8 to 30.4 µmol/mg protein), reduced lipid peroxidation (MDA from 5.78 to 1.68 nmol/mg), maintained mitochondrial membrane potential (37.5% to 83.6%) and improved SOD and CAT activities. From

a molecular point of view, its multi-target activity including direct radical scavenging, maintaining GSH homeostasis and Nrf2-mediated activation of antioxidant genes makes it a good option for further research. From the biotechnological point of view, the successful generation of bioactive secondary metabolites in callus culture shows the potential of in vitro plant cell systems as a sustainable, season-independent platform. Further studies should leverage these findings to test the extract in metabolically competent models (e.g. HepaRG or primary hepatocytes), validate the Nrf2/HO-1 axis at the gene and protein levels, and move on to in vivo validation in relevant animal models.

## 5. FUNDING

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## 6. ACKNOWLEDGEMENT

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## 7. CONFLICT OF INTEREST

The author declares no competing financial interests or personal relationships that could have influenced the work reported in this article.

## 8. INFORMED CONSENT

Not applicable. This study was conducted entirely in vitro using a commercially available cell line (HepG2, ATCC HB-8065) and did not involve human participants or animal subjects.

## 9. ETHICAL STATEMENT

No ethical approval was required. This study used a commercially available immortalised human hepatocellular carcinoma cell line (HepG2, ATCC HB-8065) and did not involve human subjects, human tissue samples, or animal experimentation.

## AUTHOR CONTRIBUTION

Ahmed R. Albuhaydar (sole author): Conceptualization, methodology, callus culture establishment, cell culture experiments, data collection, formal analysis, interpretation of results, writing – original draft, review and editing, and final approval of the manuscript.

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