JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

IMPACT OF *ROSMARINUS OFFICINALIS* ON THE MITOCHONDRIAL-DEPENDENT APOPTOTIC PATHWAY IN NICKEL-INDUCED HEPATOTOXICITY IN RATS

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https://doi.org/10.55251/jmbfs.11234

ARTICLE INFO

Received 12. 4. 2024
Revised 20. 11. 2024
Accepted 2. 12. 2024
Published 1. 2. 2025

Regular article

OPEN ACCESS

ABSTRACT

Nickel and its compounds are common environmental pollutants that may induce hepatotoxicity. Oxidation stress has been proposed as a possible mechanism implicated in this toxicity. The aim of this study was to evaluate the protective effect of *Rosmarinus officinalis* (RO) extract against $NiCl_2$-induced hepatotoxicity by inhibiting mitochondrial-dependent apoptosis. To achieve this objective, 24 male rats were divided into four groups, each containing six rats. The first group was used as a control, the second was treated with RO extract (RO-E), the third was treated with $NiCL_2$ (10 mg/kg b.w.), and the fourth group was pre-treated with RO-E and then re-treated with $NiCl_2$ after 2 hours for 28 days. Oral administration of $NiCl_2$ disrupts the redox state of liver tissue through the generation of reactive oxygen species (ROS), depletion of glutathione (GSH), breakdown of mitochondrial membrane potential ($\Delta\Psi m$), and release of cytochrome c. This leads to the activation of the mitochondrial apoptotic pathway. The activity of proteins from the BcL-2 family can be modulated by inhibiting the expression of the anti-apoptotic protein BcL-2 and increasing the expression of the pro-apoptotic proteins Bax and Bad. This leads to an increase in the activity of caspase-3 and 9, and alters the membrane integrity of hepatocytes, resulting in histohepatic changes and an increase in AST, ALT and LDH levels. Pre-treatment with *Rosmarinus officinalis* extract can ameliorate oxidative hepatotoxicity and $NiCl_2$-induced apoptosis due to its antioxidant capacity. These findings suggest that *Rosmarinus officinalis* has the potential to protect liver tissue and could be used as a preventive agent against oxidative stress and $NiCl_2$-induced apoptosis.

Keywords: Apoptosis, BcL-2 family protein, mitochondrial membrane potential ($\Delta\Psi m$), nickel chloride ($NiCl_2$), *Rosmarinus officinalis*, Oxidative stress

INTRODUCTION

Nickel (Ni) is a widely abundant chemical element found in the earth's crust with an average concentration of approximately 75 µg/g **(Klein et Costa., 2020; Mustafa *et al.*, 2023)**. Nickel and its compounds have diverse mechanical and commercial applications, the material is extensively utilised in electronic and medical industries, predominantly in electroplating and the manufacturing of batteries, electronic devices, and stainless steel. The increase in industrialisation has led to their growing release into the environment **(Shrestha *et al.*, 2021)**. Due to the high availability of Ni in the environment, plants readily absorb Ni, which then enters the food chain, posing significant health risks to both animals and humans **(Altaf *et al.*, 2022).** The main source of nickel exposure in the general population comes from foods such as chocolate, coffee, tea, legumes and nuts, which tend to have higher concentrations of Ni, and to a lesser extent drinking water, this suggests that oral exposure was the primary route of exposure **(Buxton *et al.*, 2019)**. The liver is one of the most important organs in the body and plays a key role in metabolism of various substrates **(Chang *et al.*, 2017)**, Nickel administered orally can be distributed to various organs and metabolized in the liver, which can be subject to the detrimental effects of this element **(Genchi *et al.*, 2020)**. Nickel can induce cytotoxicity by changing the redox state of hepatocytes and producing reactive oxygen species (ROS) **(Renu *et al.*, 2021; Teschke, 2022)**. The findings of **(Akinwumi *et al.*, 2020)** indicate that both ROS and the mitochondrial pathway play significant roles in the induction of apoptosis in the liver of rats due to nickel chloride (NiCl2) **(Guo *et al.*, 2015)**, These molecules can modify various cellular compartments by altering the mitochondrial membrane potential (MMP) **(Mutlu Gençkal *et al.*, 2020)**, ultimately affecting the regeneration rate of ATP. Additionally, reduces the overall content of glutathione (tGSH) **(Wei *et al.*, 2022)**. This process induces oxidative stress in the celular rodox system, which then triggers the mitochondrial apoptotic pathway **(Guo *et al.*, 2015)**, this occurs through the modulation of BcL-2 family proteins, including the expression and release of pro-apoptotic molecules into the cytosol (Bad, Bax, cytochrome c) and the inhibition of anti-apoptotic molecules such as BcL-2, resulting in the activation of cell death enzymes caspase-9 and caspase-3 **(Genchi *et al.*, 2020; Salimi *et al.*, 2020)**. Vascular damage, Kupffer cells aggregation, immune cells infiltration and even necrosis occur as a result of all these damaging changes in the liver, it can even lead to an inflammatory reaction, genotoxicity and carcinoma **(Renu *et al.*, 2021)**.

Since ancient times, medicinal herbs have been used to treat a range of ailments. Today, there is a growing interest in the potential of natural bioactive compounds found in these herbs for creating effective and affordable treatments **(Widayanti *et al.*, 2020)**. Additionally, researchers are investigating the bioactive power of these compounds, particularly flavonoids and phenolic compounds, these have been shown to reduce ROS and to maintain the balance between pro-oxidants and antioxidants, which could help to limit complications related to oxidative stress **(Fernando *et al.*, 2019; Topal et Gulcin., 2022; Benkhedir *et al.*, 2023)**. It is hoped that could provide a promising new avenue for the development of therapeutic treatments. Phenolic compounds are present in various plant species, each with varying levels and types. The Rosmarinus officinalis is among the plants containing these compounds **(Kong *et al.*, 2022)**.

Rosmarinus officinalis has been extensively studied in numerous academic sources, which have documented its bioactive properties, including antioxidant, metal-chelating, anti-inflammatory, and anti-microbial effects (particularly in vitro), as demonstrated across various forms such as essential oil, methanolic extract, and aqueous solutions **(Salehi *et al.*, 2020; Saker *et al.*, 2023)**. Moreover, there is currently no research have clearly demonstrated the impact of Rosmarinus officinalis on hepatotoxicity induced by NiCl2 in vivo. Additionally, no studies have explored how this herb affects the relationship between Reactive Oxygen Species and the mitochondrial apoptotic pathway, the proteins of the BcL2 family (anti-apoptotic and pro-apoptotic), as well as the apoptotic enzymes (caspase-3 and 9).

The study aimed to assess the impact of the phenolic extract on the parameters associated with the mitochondrial apoptotic pathway in NiCl2-induced hepatotoxicity in rats. The impact of Rosmarinus officinalis extract (RO-E) was examined on numerous biological parameters, including the concentration of ROS, MMP levels, tGSH levels, ATP levels, as well as the activity and expression of various enzymes and apoptotic proteins such as caspase-3, caspase-9, and cytochrome c. Additionally, we evaluated the impact of RO-E on the expression of anti-apoptotic protein BcL-2 and pro-apoptotic proteins Bad and Bax. To visualise the findings, we implemented histological sections.

MATERIAL AND METHODS

Chemicals

Nickel chloride (R: 339350-50G) was acquired from Sigma-Aldrich, while all other products, including RIPA buffer, polyvinylidene difluoride membrane, protease and phosphatase inhibitor cocktail, BSA, ATP quantification kit, ECL Detection Kit, 5,5′-Dithio-bis - 2-nitrobenzoic acid "DTNB", 2′,7′-dichlorodihydrofluorescein diacetate "DCFH-DA", Rhodamine 123, were sourced from the same supplier. The AST, ALT and LDH analysis kit, on the other hand, was obtained from SPINREACT ®.

Rosmarinus Officinalis extract preparation

Rosemary was collected in April 2023 from the eastern part of Algeria during the flowering period. The identification of the plant was performed by a botanist. Afterwards, it was cleaned and dried in the shade. The voucher specimen (05-2023) has been deposited in the herbarium of the Department of Applied Biology at Larbi-Tebessi University in Tebessa, Algeria.
The Rosmarinus officinalis extract (RO-E) was obtained following (Markham, 1982) protocol, with adjustments proposed by (Bruneton, 1993). Our study only investigates the solid-liquid phase of this method.

Animals and experimental

A total of 24 male Wistar rats (Rattus norvegicus) were acquired from the Pasteur Institute, the rats, aged 2 months, weighed between 200 and 220g and received no prior drug treatment. The rats were exclusively used for the present study. Prior to the experiment, the rats were acclimatized to laboratory hygienic conditions for 10 days. The temperature conditions were maintained throughout the experiment 23 c° ± 2 c° and natural photoperiod: 12h light and 12h dark, they were fed with pellet diet (ONAB-Elharouche, Skikda-Algeria), and water avail.

Treatments with NiCL2 and RO-E

The 24 rats were randomly allocated into 4 groups of 6 rats, and the doses administered were selected based on prior research with slight modification **(Kayashima *et al.*, 2020; Iqbal *et al.*, 2020; Saker *et al.*, 2023)**, as shown in the table below:

Group 01:	Control rats (vehicule 0,5 ml/100 g b.w)
Group 02:	RO-E (100 mg/kg b.w)
Group 03:	$NiCL_2$ (10 mg/kg b.w)
Group 04:	RO-E + $NiCL_2$ (the mixture of the 2 previous doses)

The extract was dissolved in a vehicle (1% Tween 80), NiCL2 was dissolved in water saline 0.9% w/v. The animals received their doses orally via a number 7 gavage tube. Group 01 received only the vehicle solution (Tween 80 dose 0.5 ml/100 g b.w.), group 02 received a dose of RO-E (100 mg/kg b.w.), group 03 received a dose of NiCL2 (10 mg/kg b.w.) and group 04 received the first dose of RO-E, then after 2 h the dose of NiCL2, these treatments were applied for 28 days with food and water intake within a fixed time.

Collection of samples

After 28 days of treatment, the rats were anaesthetised and sacrificed by cervical decapitation. Serum was collected by centrifuging the blood at 1500×g for 10 min for the biochemical determination of Serum marker enzyme activities (AST, ALT and LDH). The liver, which was the subject of our study, was retrieved directly in 10% formalin solution and immediately sent for histological examination. After rinsing the liver with ice-cold saline, a section was initially homogenised with a 10% w/v solution of 0.1 M Tris-HCL buffer at pH 7.4. It was then centrifuged at 10,000 × g for 15 minutes at 4°C. The resulting supernatant was used to determine the levels of ATP, tGSH, and the enzymatic activity of caspase-3 and 9. Additionally, Western blot analysis was performed for the proteins BcL-2, Bad, Bax, Cyt c, Casp-3, 9 and β-actin.
The other part of the liver was utilized to isolate the mitochondrion, and the mitochondrial membrane potential (MMP) was measured by employing the method outlined in **(Kun *et al.*, 1979)** with slight modifications performed by **(Baracca *et al.*, 2013)**. The tissue was homogenised using a solution of 0.24 M manitol, 0.08 M sucrose, 0.02 M HEPS, 12 mM K-EDTA, 0.1 mM K-EGTA at pH 7.4, and 0.4% albumin without the addition of digitonin. After centrifugation at 2000 × g for 10 minutes followed by another centrifugation at 10000 × g for 10 minutes, a supernatant containing mitochondrial fragments was obtained. The supernatant was then rinsed with a solution of (0. 25 mM sucrose, 0.02 mM HEPES, 1 mM K-EDTA, and 0.1 mM K-EGTA at pH 7.4 were utilized. K-EGTA was chosen to prevent potential interference with the mitochondrial membrane potential caused by Ca^{2+} contamination.

Serum marker enzyme activities

Aminotransferase (AST, ALT) and Lactate dehydrogenase (LDH) enzyme leakage only occurs when the cytoplasmic membrane of the liver is ruptured. The serum enzyme concentration was assessed through an autoanalyser (400 COBAS INTEGRA®) using a commercial kit of AST, ALT, LDH, (SPINREACT®, Ref: 1001160, Ref: 41280 Ref: 1001260) in accordance with manufacturer's instructions.

Activity of apoptotic enzymes (caspase-3 and 9)

The method of Hayami (1999) was used to measure the activities of caspases-3-9 in the liver homogenate. This involved centrifuging the homogenate at 15,000 g for 5 minutes, followed by dilution of the supernatant with PBS. A volume of enzyme solution was added to the mixture (100 mM Tris-Hcl, 2 mM EDTA and 20 mM EGTA), the resulting solution was added to 1 mM dithiotreitol, then was followed by adding 50 mM fluorogenic substrates (Ac-DEVD-AFC for caspase-3 assay and Ac-LEHD-AFC for caspase-9 assay), this reaction was incubated at 37 C°, PH=7. 5, then an aliquot of the reaction mixture was taken after 10 and 20 min and 5 % HCLO4 was added to complete the reaction. After centrifugation at 15,000 g for 5 min, the fluorescence of the supernatant containing free AFC was measured using a fluorescence microplate reader (excitation 380 nm, emission 460 nm).

Evaluation of the total (tGSH), reduced (GSH) and oxidised (GSSG) glutathione

The GSH and GSSG levels in the liver supernatant were quantified using the DTNB-GSSG reductase recycling assay, following the procedure outlined by Anderson (1985). In summary, the supernatant was neutralized by adding an equivalent volume of 0,76 M ($KHCO_3$) were centrifuged at 15000 g for 5 minutes at 4°C, and the supernatant was retrieved. The total glutathione (tGSH) was measured by adding the recovered supernatant, standard, and blank to a 96-well microplate, and subsequently injecting a pre-made reagent containing 24 mM NADPH and 1.3 DTNB were dissolved in a phosphate buffer of 63.5 mM Na_2HPO_4, 63.5 mM NaH_2PO_4 and 0.65 mM EDTA (pH 7.5). After incubating the microplate for 15 minutes at 30°C, the reaction was initiated by the addition of glutathione reductase solution. The formation of TNB was monitored at 415 nm every 10 seconds for 3 minutes. The tGSH content in the samples was calculated through interpolation from a standard curve. GSSG measurement involved adding a solution of 2-vinylpyridine to the recovered supernatant, which was then mixed for 1 hour at 4°C for group (SH) derivatisation. Like how tGSH was measured, GSSG was also measured and data normalised to the protein amount for each sample. Reduced glutathione (GSH) was calculated using the formula: GSH = tGSH - (2 × GSSG). Values are expressed as nmol GSH/mg protein.

Determination of reactive oxygen species (ROS) levels

Measurement of ROS regeneration in liver supernatant was carried out using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) probe, which is hydrolysed by the cell to form 2′,7′-dichlorodihydrofluorescein (DCFH). This can react with the ROS present in the cell to form the fluorescent dichlorofluorescein (DCF) as per Crow's method (1997). Briefly, the supernatant was dispensed into a 96-well microplate, then incubated with 20 µm of DCFH-DA for 30 minutes at 37 °C. The plate was read using a fluorescence microplate reader (PirkinElmer Enspire) with excitation and emission wavelengths (485/530 nm).

Evaluation of ATP content

ATP quantification in the liver supernatant was determined through the D-luciferin-luciferase technique. This method catalyses D-luciferin + ATP + O_2 to produce oxyluciferin + Pi + AMP + CO_2 + light. The light was measured using a luminescence microplate reader. In summary, the liver tissue was weighed and homogenised with 5% TCA. The resulting homogenate was centrifuged at 5000 g, 4°C for 5 minutes (pellet proteins was quantified). The supernatant was then neutralised with Tris-acetate buffer and the pH adjusted to 7.5. The ATP quantity was subsequently measured in a 96-well microplate using the Sigma kit (119107-1KIT) in accordance with the manufacturer's protocol. The quantity of ATP correlates with the light produced. This was measured using a luminescence microplate reader (Enspire from PerkinElmer). To determine the ATP concentration (nmol/mg of protein), an ATP standard curve was established.

Evaluation Assessment of mitochondrial membrane potential (Δψm)

Mitochondrial membrane potential (MMP) was rapidly assessed using the method described by (Baracca *et al.* 2003) on mitochondria isolated from liver homogenate. Changes in mitochondrial potential (ΔΨm) were estimated by measuring the quenching of the fluorescent cationic dye Rhodamine 123 (Sigma, R: R8004-5MG). Breifly liver mitochondrial isolate was incubated with Rhodamine 123 (10 µM) for 15 min in a microplate, and centrifuged (16,000 × g,

5 min, 4 °C), then the fluorescence of the supernatant was measured using a microplate fluorescence reader (490/520 nm excitation and emission wavelength).

Western blot

Liver tissue lysate from the control and treated groups (RO-E, Ni, RO-E+Ni) was prepared using RIPA buffer (sigma- R: 0278) with a protease and phosphatase inhibitor cocktail (Sigma-R: PPC1010), and subsequently stored at -80°C. Protein quantification was performed by the Bradford method (1976). In short, an equal amount of protein (15 µg/well) was taken from each sample, separated by electrophoresis in a 10% SDS-polyacrylamide gel, and subsequently transferred onto a polyvinylidene difluoride (PVDF) membrane (Sigma-Immobilon®-R: IPVH08100). The nonspecific sites of the membrane were then blocked with 5% low-fat milk in saline solution buffered with PB-T (PBS and 1% Tween 20) for 1 hour at room temperature. Next, the membrane was incubated overnight at 4°C with primary antibodies diluted (1:500) of rabbit anti-BcL-2 (R: SAB4500003), Bad (R: SAB5701290), Bax (R: SAB5700071), Cytochrome c (R: SAB5701571), Caspase-3 (R: SAB5700196), Caspase-9 (R: PRS2515), β-actin (R: ZRB1312-4X25UL) from (Sigma antibodies). Then the membrane was washed with PBS-T three times, followed by incubation with secondary antibodies diluted (1:2000) of Goat-anti Rabbit IgG antibody HRP conjugate (Sigma-R: 12-348). The samples were incubated at room temperature for 1 h, washed three times with PBS-T, and visualized using the ECL Detection Kit (Sigma-R: GERPN2232), the chemiluminescent signal was observed with (FUSION FX Spectra system), and densitometric analysis was carried out utilizing the Image J software.

Determination of proteins content

Protein quantification from the homogenate was determined based on the protocol outlined by Bradford (1976).

Statistical analysis

The results are presented as means ± standard deviation (n = 6). The difference between groups was evaluated using One Way ANOVA, followed by a Tukey post-hoc test utilizing Graph Pad Prism 8.0.1 software, and significance was set at $P < 0.05$.

RESULTS

Serum marker enzyme activities

The study reveals that administering NiCL2 to rats led to significant increase in serum enzyme levels (P<0.05) in comparison to the control group. However, in rats treated with NiCL2, pre-treatment with RO extract resulted in a significant return of serum enzyme levels to normal (P<0.05) when compared to the group treated with NiCL2 alone. In regards to the RO-E treated group, there was no significant change in serum enzymes when compared to the control group (Fig. 1).

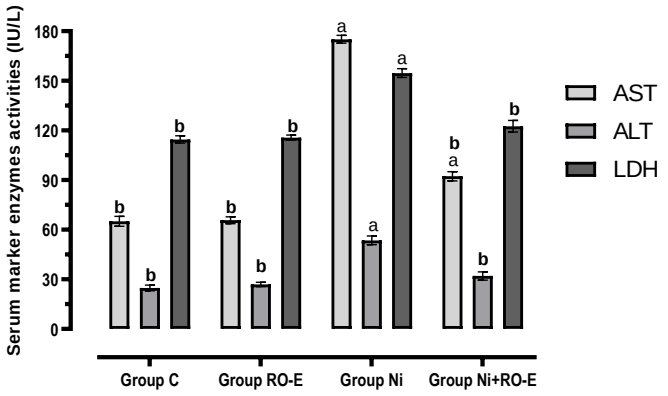

Figure 1 Effect of RO-E on serum marker enzymes activities in control and experimental rats. *Values were expressed as means ± SD (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to group Control, [b] in respect to group Ni, (ANOVA followed with Tukey test), C; control, Ni; Nickel chloride, RO-E; Rosmarinus officinalis extract, AST; aspartate transaminase, ALT; alanine transaminase, LDH; lactate dehydrogenase*

Activity of apoptotic enzymes (caspase-3 and 9)

The findings indicate a significant increase in the activity of caspase-3, -9 enzymes in rats exposed to NiCL2 in comparison to control rats (P<0.05). In contrast, rats that were pretreated with RO extract and then treated with NiCL2 showed a significant decrease in apoptotic enzyme activity (P<0.05), whereas no significant difference in enzyme activities was observed between RO extract-treated and control rats (Fig. 2).

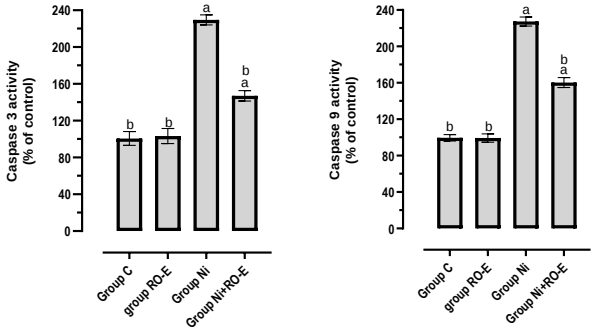

Figure 2 Effect of RO-E on Activity of apoptotic enzymes (caspase-3 and 9) in control and experimental rats. *Values were expressed as means ± SD (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to group Control, [b] in respect to group Ni, (ANOVA followed with Tukey test), C; control, Ni; Nickel chloride, RO-E; Rosmarinus officinalis extract.*

Evaluation of the total (tGSH) reduced (GSH) and oxidised (GSSG) glutathione.

Exposure of laboratory rats to NiCL2 resulted in significant changes to the liver's redox status. These alterations were associated with the amount of tGSH, which was depleted of GSH (29.87±1.8 nmol tGSH/mg prot) and an increased concentration of GSSG (7.5±1.32 nmol tGSH/mg prot) compared to the control group (GSH; 59.9±1.85 and GSSG; 1.23±0.21 nmol tGSH/mg prot) (P<0.05). On the other hand, the combination of RO-E and NiCL2 led to a positive improvement in the tGSH level. This was evidenced by significant restoration in the levels of GSH and GSSG (46.97±2.05 and 2.4±0.53 nmol tGSH/mg prot) when compared to rats treated with NiCL2 only. It is noteworthy that the levels of GSSG were reduced without significant difference when compared to control rats. Moreover, there was no significant difference in the rats treated with only RO extract when compared to control rats (Fig. 3).

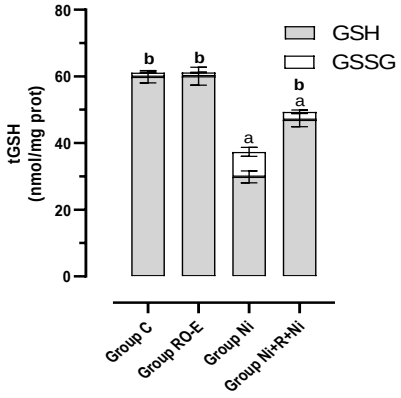

Figure 3 Effect of RO-E on tGSH levels in control and experimental rats. *Values were expressed as means ± SD (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to group Control, [b] in respect to group Ni, (ANOVA followed with Tukey test), C; control, Ni; Nickel chloride, RO-E; Rosmarinus officinalis extract, GSH; reduced glutathione, GSSG; oxidized glutathione.*

Determination of reactive oxygen species (ROS) levels

An analysis of ROS generation rates, evaluated using DCFH-DA dyes as a fluorescence probe, revealed that rats treated with NiCL2 exhibited a significant increase in ROS levels (224.6 ±4.86 DFC%) compared to the control (P<0.05). Conversely, pretreatment with RO extract for rats treated with NiCL2 resulted in a significant decrease (142±5.65 DFC%) in ROS formation compared to rats treated with NiCL2 (P<0.05). No significant difference was observed in the rats solely treated with the RO extract in comparison to the control rats (Fig. 4).

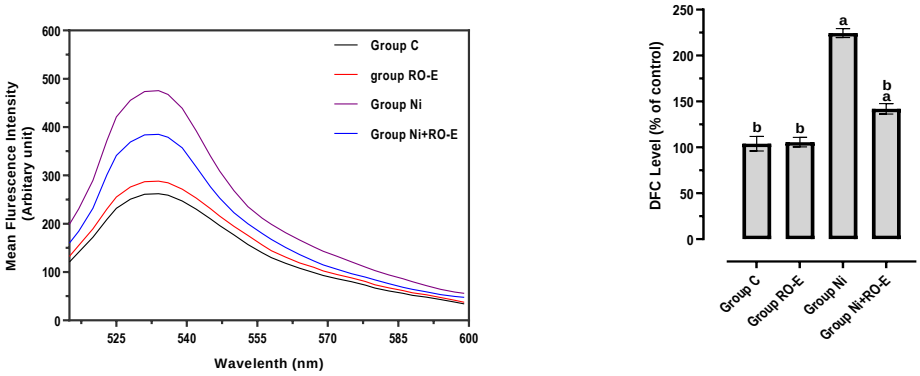

Figure 4 Effect of RO-E on ROS generation in the liver of control and experimental rats. *Values were expressed as means ± SD (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to group Control, [b] in respect to group Ni, (ANOVA followed with Tukey test), C; control, Ni; Nickel chloride, RO-E; Rosmarinus officinalis extract, DFC; dichlorofluorescein.*

Evaluation of ATP content

Analysis of the results from (Fig. 5) showed the effect of the RO extract on hepatocyte ATP levels. The treatment of rats with $NiCl_2$ resulted a significant reduction in ATP levels compared to the control group (P<0.05). The protective effect of the extract against $NiCl_2$ was observed as significant restoration of ATP levels compared to rats treated only with $NiCl_2$ (P<0.05). Conversely no significant difference was observed between the rats treated with the RO extract and the control group.

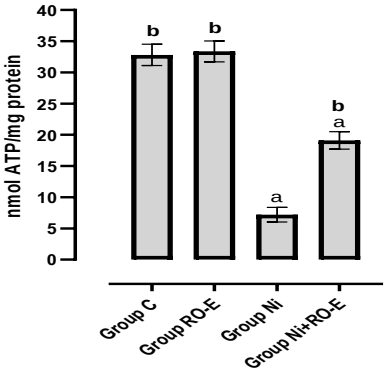

Figure 5 Effect of the RO extract on liver ATP levels in control and experimental rats. *Values were expressed as means ± SD (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to group Control, [b] in respect to group Ni, (ANOVA followed with Tukey test), C; control, Ni; Nickel chloride, RO-E; Rosmarinus officinalis extract.*

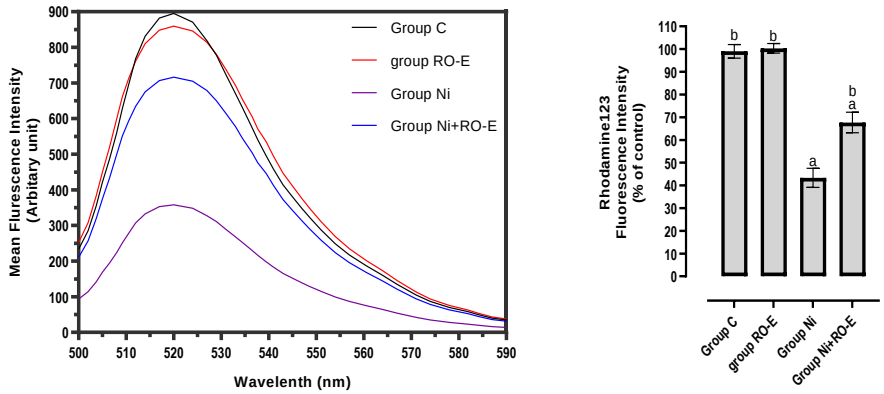

Figure 6 Effect of the RO extract on liver mitochondrial membrane potential (Δψm) in control and experimental rats. *Values were expressed as means ± SD (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to group Control, [b] in respect to group Ni, (ANOVA followed with Tukey test), C; control, Ni; Nickel chloride, RO-E; Rosmarinus officinalis extract.*

Assessment of mitochondrial membrane potential (Δψm)

The collapse percentage of ΔΨm in experimental and control rats was measured by recording the fluorescence intensity of the mitochondrial dye rhodamine (Rh 123), (Fig. 6) shows the results, which indicate a significant decrease in Rh 123 fluorescence intensity in the livers of rats treated with NiCL2 compared to the control group (P<0.05). Rats treated with a combination of RO extract and NiCL2 exhibited a notable reduction in mitochondrial membrane potential (P<0.05). In contrast, no significant variance in ΔΨm was observed between the group treated with RO-E and the control group.

Western blot

The BcL-2 protein family has a regulatory function in controlling the mitochondrial apoptotic pathway. Western blot analysis (Fig. 7) revealed a significant down-regulation in the expression of BcL-2 protein in rats treated with NiCL2 (4,5 fold over control), whereas a significant upsurge in BAX and bad expression was observed relative to control rats (2,5 and 4,3 fold over control) (P<0.05). All the variations in apoptotic protein expression were significantly reversed following prior administration of RO extract in rats treated with NiCL2 in comparison with rats treated only with NiCL2 (2- 1,4- 1,3 fold over control consecutively) (P<0.05). Nonetheless, there was no significant difference between the rats treated with the RO extract and the control.

The decrease in ΔΨm will ultimately result in the liberation of cytochrome c from mitochondria. This subsequently triggers caspase-3 and 9, initiating the process of apoptosis. Analysis of western blot results (Fig. 7) revealed a significant rise in the

expression of cyt c, Casp-3 and 9 (2,3- 4,6 and 8,5 fold over control consecutively) in NiCL2-treated rats compared to control rats (P<0.05). Preventive treatment with Ro extract for NiCl2-treated rats resulted in a significant improvement in the expression of apoptotic proteins (1,4- 1,6 and 2,8 fold over control consecutively) compared to rats treated with NiCl2 alone (P<0.05). However, no significant difference was observed between the RO-E group and the control.

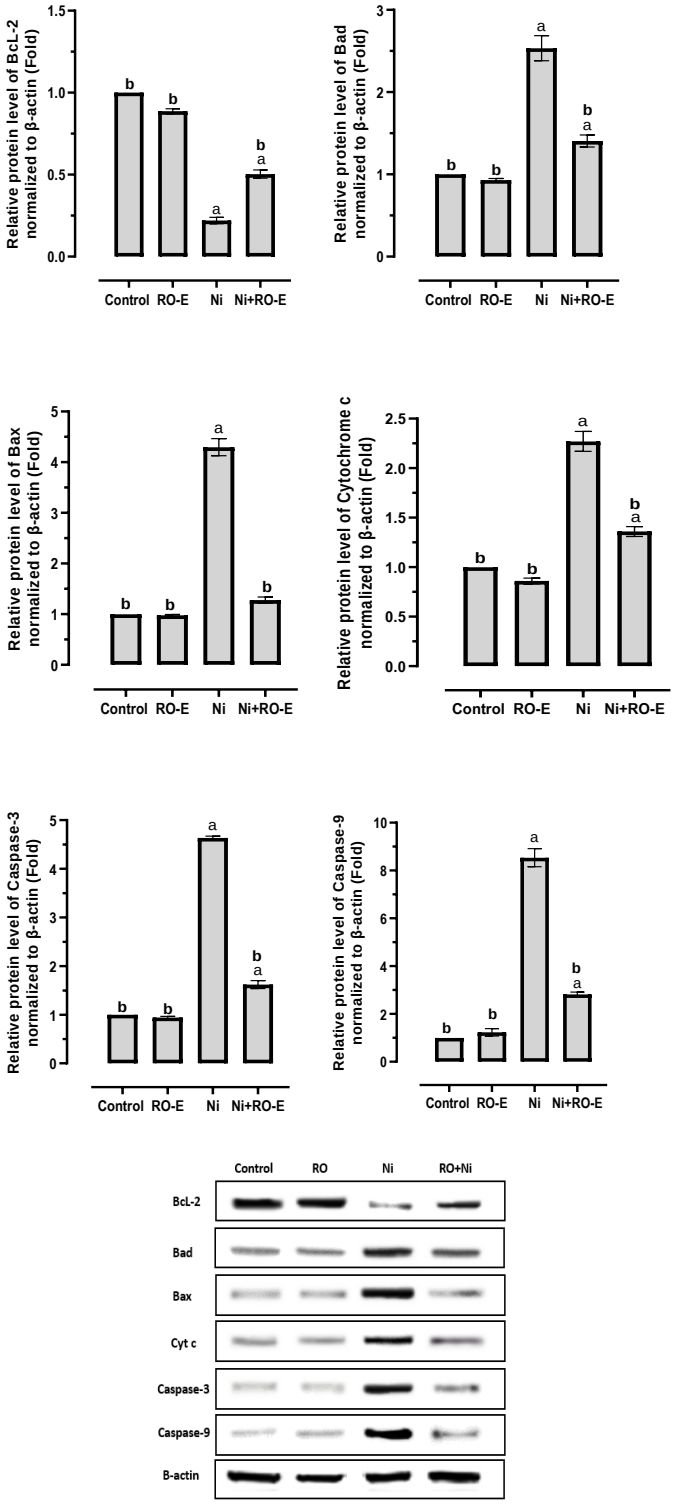

Figure 7 Effect of the RO extract on levels expression of proteins apoptotic in control and experimental rats. *Values were expressed as means ± SD (n = 6), minimal significant level; P< 0,05, significantly difference; [a] in respect to group Control, [b] in respect to group Ni, (ANOVA followed with Tukey test), C; control, Ni; Nickel chloride, RO-E; Rosmarinus officinalis extract.*

Histology

The histological sections of liver tissue stained with H&E after 28 days of treatment in experimental and control rats are shown in (Fig 8). Control rats and rats treated with RO extract displayed regular hepatocyte and nucleus architecture, along with sinusoids and central vein lined with intact flat endothelial cells. Treatment of rats with NiCL2 resulted in changes to the distribution of hepatocytes, degeneration of the nucleus, piknotyc nucleus, development of necrotic zones around the centrilobular vein with infiltration of inflammatory cells, invasion by kupffer cells and degeneration of sinusoids. However, rats who received a preventive treatment with RO extract combined with NiCL2 showed significantly reduced alterations in these areas.

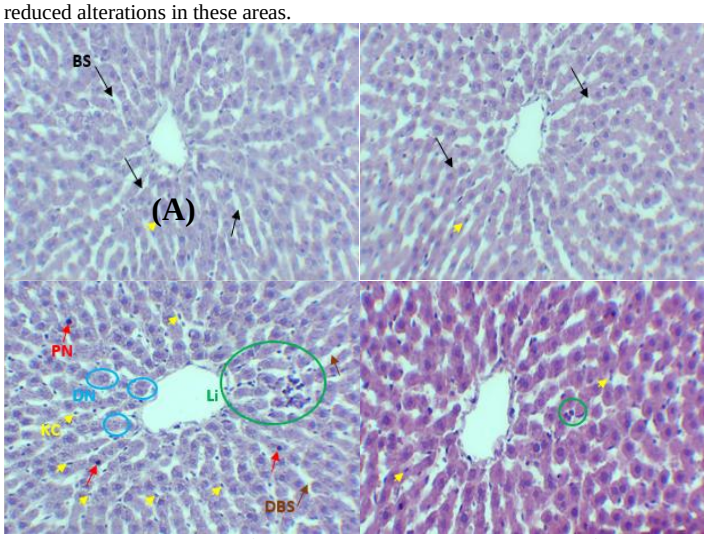

Figure 8 Liver histology from control and experimental rats after 28 days treatment (40×). (A); control rats, (B); RO-E, (C); Ni, (D); Ni+RO-E, BS; basal sinosoid, KC; kupffer cells, PN; Pyknotic nuclei, Li; lymphatic infiltration, DN; degenerated nuclei, DBS; degenerated basal sinosoid.

DISCUSSION

Most of the toxic effects induced by NiCL2 are apparent in various organs **(Tyagi *et al.*, 2013; Xu *et al.*, 2023)**. Among these organs, the liver is an essential site for bioaccumulation and detoxification **(Renu *et al.*, 2021)**. Consequently, the liver is more vulnerable to the adverse effects of NiCL2 that can lead to oxidative stress. This stress generates ROS, which are involved in the mitochondrial pathway that induces apoptosis by modulating the expression of apoptotic proteins **(Iqbal *et al.*, 2021; Salah *et al.*, 2021)**. An experimental model of liver damage caused by NiCl2 was employed to investigate the protective impact of *Rosmarinus officinalis* extract on the diverse parameters altered by NiCl2. In this study, high levels of transaminase and LDH were observed in the serum of rats treated with NiCl2. These results indicate the occurrence of cell leakage and loss of functional integrity in the liver cell membrane, implying the destruction of hepatocytes **(Derbal *et al.*, 2020)**. Therefore, these findings reflect the hepatotoxic effect of this metal **(Akinwumi *et al.*, 2020)**. The pretreatment of RO extract to NiCL2-treated rats results in a hepatoprotective effect through the reduction of serum AST, ALT, and LDH activity. The research indicates that the extract from RO has distinct bioactive properties with high flavonoid and phenolic acid levels. In particular, Rosmarinic acid and quercetin have a strong antioxidant effect due to their ability to scavenge free radicals. These molecules assist in maintaining the integrity of the hepatic cell membranes by stabilising these enzymes **(Abou Zeid *et al.*, 2017; Aoiadni *et al.*, 2021)**.

NiCl2 can enhance the function of various apoptotic enzymes including caspase-3 and 9, which have a direct role in the stimulation of apoptosis in liver cells. Administration of NiCL2 to rats significantly increases the activity of caspases 3 and 9, and this increase is significantly related to changes in the cellular rodox system due to overproduction of reactive oxygen species (ROS). The latter can activate the apoptotic enzyme caspase-3 and 9 through the intrinsic mitochondria-dependent pathway **(Zhang *et al.*, 2022; Elderdery *et al.*, 2023)**. Pretreatment of rats with rosemary (RO) extract significantly reduced the activity of apoptotic enzymes, caspase-3 and 9, in rats treated with NiCl2. RO extract was discovered to contain high levels of carnosic acid, a molecule with substantial antioxidant properties that can scavenge ROS, thereby preventing the activation of apoptotic enzymes via the mitochondrial pathway **(de Oliveira *et al.*, 2015)**. The study observed an augmentation in intracellular ROS and a decrease in GSH levels, accompanied by a minor upsurge in GSSG concentrations in rats treated with NiCL2, where the ROS rise is attributable to changes in the prooxidant-antioxidant equilibrium **(Rahman et Rahman., 2021)**. Previous studies have demonstrated that the mitochondrion is the primary location responsible for the excessive generation of oxygenated reactive species. Mutant cells lacking mitochondrial DNA have evidenced a reduction in ROS and increased resistance to Ni **(Sousa *et al.*, 2018)**, supporting the proposition that the respiratory chain is involved in

overproducing ROS and serves as the initial source of O_2- and H_2O_2. Moreover, recent findings suggest that Ni may catalyse the creation of OH- radicals via the Waber-Wies reaction **(Chen *et al.*, 2003; de Oliveira *et al.*, 2015; Derbal *et al.*, 2022)**. Reactive oxygen species (ROS) are efficiently regulated by an antioxidant system consisting of enzymatic and non-enzymatic components. Among these components, GSH is involved in the neutralization of cellular ROS. However, studies suggest that the depletion of GSH may be attributed to the sulfhydryl group of cysteine from GSH, which has a high affinity for Ni. It has been reported that cells lacking the GSH gene show an elevated ROS level and increased sensitivity to Ni **(de Oliveira *et al.*, 2015; Ali *et al.*, 2019; Kakavand., 2021)**. Also other studies have reported a slight elevation of GSSG levels, which is caused by the effect of Ni in creating an imbalance between GSH/GSSG through the formation of a bond with the thiol group of GSSG **(De Luca *et al.*, 2007; Saleh *et al.*, 2019)**. All these changes were significantly improved when the application of a preventative dose of RO extract at 100 mg/kg b.w., this improvement is due to the antioxidant properties of RO, which act against various reactive species that consume the quantity of cellular GSH during oxidative stress **(El-Demerdash *et al.*, 2021)**. A new compound, Rosm1, was recently isolated from the methanolic fraction of RO, which demonstrated the most potent antioxidant power among the compounds of the RO extract when compared to vitamin E. It was also demonstrated that Rosm1 protects the different cellular constituent from oxidative stress **(de Macedo *et al.*, 2020)**. Another study demonstrated the efficacy of carnosic acid extracted from rosemary in the internalisation of H_2O_2 and enhancement of cellular GSH levels **(de Oliveira *et al.*, 2015)**.

Based on the aforementioned results, it has been determined that $NiCL_2$ has a significant impact on increasing cellular ROS. It has also been discovered that $NiCL_2$ inhibits mitochondrial succinate dehydrogenase **(Sousa *et al.*, 2018)**. These findings prompted us to assess the change in ΔΨm, which can also affect cellular energy reserves (ATP). The group administered with NiCL2 displayed a noteworthy modification in ΔΨm, resulting in a reduction in the quantity of ATP. As we said earlier, nickel inhibits the activity of succinate dehydrogenase in the mitochondrial respiratory chain. This, in turn, can cause a reduction in ΔΨm alongside an increase in reactive oxygen species. It has been posited that ΔΨm is utilised to move protons to the ATPase pump to generate ATP. Any change in ΔΨm levels could decrease the quantity of ATP within the cell **(Xu *et al.*, 2010; Suski *et al.*, 2018; Zorova *et al.*, 2018)**. All these alterations were significantly reduced during pre-treatment with RO extract. This reduction could be due to the presence of distinct bioactive compounds in the plant, including apigenin, which has a notably high level of antioxidant power **(Tian *et al.*, 2021)**. It has been noted that apigenin stabilises ΔΨm, which ultimately increases the functionality of the ATPase pump, leading to an improvement in cellular ATP levels. Studies have revealed that apigenin's capacity to eliminate free radicals is attributed to its possession of over 3 OH functions and a double bond between carbon 2 and 3, providing this substance with a remarkably elevated antioxidant capability **(Ahmad et *et al.*, 2019; Wang *et al.*, 2020)**. Another study demonstrated that RO carnosic acid has the ability to preserve ΔΨm by internalizing ROS molecules generated by the respiratory chain. Additionally, it promotes the activity of mitochondrial antioxidant enzymes against these oxygenated reactive species **(Park *et al.*, 2010; de Oliveira *et al.*, 2015; de Oliveira, 2018)**.

Based on our aforementioned results and other research it has been established that $NiCl_2$ leads to a collapse in ΔΨm, as mitochondria play a key role in the activation of the intrinsic apoptotic pathway **(Salimi *et al.* 2020)**, a loss of ΔΨm is considered as a trigger for the release of cytochrome c. the release of the latter is regulated by the proteins of BcL-2 family **(Aghaei *et al.*, 2020)**, which can be classified into two groups: anti-apoptotic (e.g. BcL-2) which inhibits the release of cytochrome c and pro-apoptotic (e.g. Bax and Bad) **(Opferman et Kothari, 2018)**. During the collapse of ΔΨm, the Bax protein translocates to the mitochondrial membrane, increasing its permeability **(Zhang *et al.*, 2022)**. Subsequently, Bad suppresses BcL-2 protein, causing the release of cytochrome c to the cytosol, leading to the activation of caspase-3 and 9 and eventually cell death **(Chota *et al.*, 2021)**. Numerous studies have reported these changes induced by NiCl2 **(Guo *et al.*, 2021; Xu *et al.*, 2023)**. Caspase-3 and 9 activity has been observed in NiCL2-treated rats. The caspase activity was confirmed by performing Western blot analysis which showed a significant decrease in BcL-2 expression and significant increase in the expression of Bax, Bad, cytochrome c, caspase-3 and 9 proteins. These changes were significantly positively improved during pretreatment with Ro extract. This improvement may be attributed to the plant's ability to inhibit the mitochondrial apoptotic pathway related to the downregulation of proapoptotic protein expression **(Park *et al.*, 2010; Moore *et al.*, 2016)**. Carnosic acid is widely known to reduce the levels of caspase-3 and 9 at low doses **(Meng *et al.*, 2015)**. It also limits the release of cytochrome c by decreasing the expression of BcL-2 protein and stabilising the ΔΨm **(de Oliveira *et al.*, 2016; de Oliveira *et al.*, 2018)**. The histological analysis of the liver validates the aforementioned changes observed in rats treated with NiCL2, as identified in numerous studies **(Hashem *et al.*, 2019; Saleem *et al.*, 2022)**. The alterations present notable disparities in the distribution of hepatocytes, degradation of the nucleus, pyknotic nucleus, and the emergence of necrotic areas. These hepatic alterations involve first degree oxidative stress in their development, and which will subsequently progress towards failure of this organ. The results indicate that prior administration of the RO extract to rats mitigated liver tissue damage to almost normal levels. The attributed cause of this positive outcome is potentially due to the hepatoprotective actions of carnosic acid, carnosol, apigenin and rosmarinic acid **(Azab et Albasha, 2018; Das *et al.*, 2018; Yang *et al.*, 2022; Guimarães *et al.*, 2023)**.

CONCLUSION

In conclusion, administering Rosmarinus officinalis extract (100 mg/kg b.w.) to rats with NiCl2-induced hepatotoxicity demonstrated a substantial effect. This effect was revealed for the first time and was found to modulate the redox state of hepatocytes by attenuating ROS and increasing the level of cellular GSH. Additionally, it prevented the induction of the intrinsic mitochondrial apoptotic pathway by stabilizing mitochondrial membrane potential ΔΨm. It preserved ATP levels, limited cytochrome c release, which in turn attenuated caspase-3 and -9 activity and expression of Bax and Bad proteins, the plant increased BcL-2 expression, and protected hepatocyte membrane integrity by reducing AST, ALT, and LDH serum levels. we can suggest that this plant can be used as a preventive agent against NiCl2. Further studies are required to investigate the impact of this plant on other extrinsic apoptotic pathways induced by NiCL2.

Funding: This research has been funded by the Algerian Ministry of Higher Education and Scientific Research and the General Directorate of Scientific Research and Technological Development (DGRSDT). Technological Development (DGRSDT).

Conflicts of interest/competing interests: The authors declare that they have no conflicts of interest.

Ethics approval: All experimental protocols were approved and carried out by the Ethics Committee of the Pasteur Institute of Algeria (PIA) in accordance with the Ethics Code: No. 2812/5.2018.

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