Neuroprotective Synergy Of Quercetin And Rutin In Mitigating Aluminum Chloride-Induced Mitochondrial Oxidative Damage: An In Vitro Approach

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Abstract:

Aluminum (Al), the third most abundant element in the Earth's crust, induces neurotoxicity primarily through oxidative stress mechanisms and mitochondrial dysfunction. This study investigated the neuroprotective potential of two flavonoids, quercetin and rutin, against aluminum chloride ($AlCl_3$)-induced oxidative damage in isolated rat brain mitochondria. Brain mitochondria were isolated from five male Wistar rats and allocated to four experimental groups: control, $AlCl_3$-exposed (2 mg/L), $AlCl_3$ + quercetin (0.1 mg/L), and $AlCl_3$ + rutin (0.2 mg/L). All groups were incubated for 2, 6, and 20 hours to evaluate time-dependent effects.

After 20 hours of incubation, $AlCl_3$ exposure significantly increased lipid peroxidation (MDA levels, +104.28%) and decreased antioxidant enzyme activities (CAT: -21.98%, SOD: -34.43%, GST: -30.56%), indicating substantial oxidative stress induction. Mitochondrial respiratory chain analysis revealed marked impairment of complexes I/III (-76.60%) and IV (-37.00%). Co-administration of quercetin attenuated MDA elevation (+87.37% vs. +104.28% with $AlCl_3$ alone) while showing differential effects on antioxidant enzymes. Notably, quercetin significantly enhanced complex IV activity (+86.52%) despite further reduction in complexes I/III activity (-84.81%). Similarly, rutin treatment more effectively reduced lipid peroxidation (+50.31%) and provided partial protection of antioxidant enzyme activities, with moderate restoration of complex IV activity (+15.35%).

At earlier timepoints (2h, 6h), both flavonoids demonstrated distinct modulatory patterns on antioxidant enzymes, with transient upregulation of SOD and GST activities, suggesting dynamic temporal responses. These findings demonstrate that quercetin and rutin exert neuroprotective effects against aluminum-induced mitochondrial dysfunction through differential mechanisms, with quercetin showing stronger protection of complex IV activity while rutin more effectively mitigates lipid peroxidation.

1. Introduction

Aluminum (Al) is the third most abundant element in the Earth's crust, following oxygen and silicon [1]. Its ubiquitous presence in the environment stems from both natural processes—soil erosion and volcanic eruptions—and extensive anthropogenic activities. The dramatic increase in aluminum usage in recent decades has resulted in its incorporation into numerous everyday products, including food additives, pharmaceuticals (particularly antacids), cosmetics (deodorants, sunscreens), water treatment

processes, kitchenware, and industrial materials. Consequently, human exposure sources have multiplied, raising significant toxicological concerns [2,3].

While aluminum is only present in trace amounts in the human body under normal conditions and lacks established essential biological functions [1,3], elevated exposure induces pronounced prooxidant effects linked to its bioaccumulation. This culminates in enhanced free radical production [4], which, when exceeding the antioxidant capacity of biological systems, precipitates oxidative stress and mitochondrial dysfunction.

Mitochondrial oxidative stress is increasingly recognized as a pivotal factor in numerous pathological conditions, highlighting the importance of antioxidant compounds capable of penetrating cellular membranes to mitigate such stress [5]. Mitochondria serve as the primary energy generators in the brain, an organ with exceptionally high energy [6,7]. Beyond their bioenergetic function, mitochondria regulate the cell cycle, mediate redox signaling, and orchestrate apoptotic processes [8]. As the predominant endogenous source of reactive oxygen species (ROS), mitochondrial dysfunction—particularly within the central nervous system (CNS)—has been implicated in the pathogenesis of both acute ischemia-reperfusion injuries and chronic neurodegenerative disorders, including Alzheimer's and Parkinson's diseases [9].

Aluminum toxicity compromises mitochondrial function through multiple mechanisms, including disruption of the electron transport chain, augmentation of ROS production, and oxidative damage to mitochondrial DNA, proteins, and lipids. These effects ultimately precipitate the opening of the mitochondrial permeability transition pore and initiate cell death signaling cascades [10,11]. The resultant overwhelming oxidative stress can compromise natural antioxidant defense systems, leading to systemic disruption potentially affecting multiple organs, including the CNS [12]. Restoration of oxidative-antioxidant equilibrium is therefore critical for preserving physiological function, with antioxidant supplementation representing a promising therapeutic strategy.

Flavonoids have emerged as particularly noteworthy antioxidants with diverse pharmacological properties. These compounds exhibit neuroprotective effects by restricting free radical generation and modulating cellular signaling pathways involved in cell proliferation, survival, glutathione synthesis, and antioxidant protein expression [13].

Among flavonoids, quercetin and rutin have attracted substantial research interest due to their exceptional biological significance. Quercetin demonstrates potent anti-inflammatory, anticancer, and antioxidant properties, although it may exert pro-oxidant effects at high concentrations, particularly in neuronal contexts [14]. Rutin, a glycoside derivative of quercetin, possesses partially distinct physicochemical properties while sharing similar biological activities due to structural similarities in their active moieties.

At the mitochondrial level, both compounds influence signaling pathways regulating programmed cell apoptosis, rendering their investigation particularly relevant for preventing neurodegenerative conditions. Despite growing evidence supporting their neuroprotective potential, the comparative efficacy of quercetin and rutin against aluminum-induced mitochondrial dysfunction and their underlying mechanisms remain incompletely characterized.

The present study aimed to evaluate the protective capabilities of quercetin and rutin against aluminum chloride-induced oxidative damage in isolated cerebral mitochondria from Wistar rats. By examining temporal changes in oxidative stress parameters and mitochondrial respiratory chain function, this investigation sought to elucidate the differential neuroprotective mechanisms of these flavonoids.

2. Materials and Methods

2.1. Materials

Quercetin (≥95%), Rutin Hydrate (≥94%) and $AlCL_3$ (>99.9%), were from Sigma-Aldrich (St. Louis, MO, USA).

2.1.1. Animals and Experimental Design

Experimental procedures were conducted at the Laboratory of Experimental Biotoxicology, Bioremediation, and Phytoremediation at the University of Oran 1 Ahmed Ben Bella. Five male Wistar

rats (94-105 g) were housed under standard conditions (ambient temperature 22±2°C, regular light/dark cycle) with ad libitum access to food and water.

Following a four-week weaning period and six hours of fasting, rats were humanely sacrificed by decapitation. Brains were promptly removed, placed on ice, rinsed in phosphate buffer saline (PBS, 0.1 M, pH 7.2), dried, and weighed before mitochondrial isolation. The isolated mitochondrial suspensions were subsequently utilized for assessing oxidative stress parameters (protein content, catalase [CAT], Glutathione-S-transferase [GST], superoxide dismutase [SOD], and lipid peroxidation [MDA]) and evaluating enzymatic activities of the mitochondrial respiratory chain.

2.1.2. Preparation of Isolated Mitochondria

Brain hemispheres were placed in tubes containing 8 mL of homogenization buffer (PBS 0.1 M supplemented with 7.46 mM mannitol, 0.2 mM potassium chloride [KCl], and 0.2 mM magnesium chloride, pH 7.2). Tissues were homogenized using a WiseTis® HG-15A homogenizer. The resulting homogenate was centrifuged at 2000 g for 10 minutes, and the supernatant was collected and further centrifuged at 10,000 g for 10 minutes to remove cellular debris. The mitochondrial pellet was resuspended in 800 µL of buffer mixture comprising 100 µL isolation buffer (25 mM sucrose, 10 mM Tris-base, 1 mM EDTA, pH 7.4) and 700 µL respiration buffer (100 mM KCl, 10 mM KH_2PO_4, 75 mM mannitol, 5 mM $MgCl_2$, 10 mM Tris-HCl, 1 mM EDTA, 10 mM Tris-base, 0.2% BSA). The isolated mitochondria were stored at -20°C until analysis.

2.1.3. Incubation of Isolated Brain Mitochondria

Isolated brain mitochondria were allocated to four experimental groups as follows:

1. **Control:** 2 mL respiration buffer + 10 µL isolated mitochondria
2. **Intoxicated ($AlCl_3$):** 2 mL respiration buffer + 10 µL isolated mitochondria + 10 µL aluminum chloride (2 mg/L)
3. **Quercetin-treated ($AlCl_3$+Quer):** 2 mL respiration buffer + 10 µL isolated mitochondria + 10 µL aluminum chloride (2 mg/L) + 10 µL quercetin (0.1 mg/L)
4. **Rutin-treated ($AlCl_3$+Rut):** 2 mL respiration buffer + 10 µL isolated mitochondria + 10 µL aluminum chloride (2 mg/L) + 10 µL rutin (0.2 mg/L)

All samples were incubated at 37°C under controlled atmospheric conditions (5% CO_2) for different durations (2, 6, and 20 hours). Following each incubation period, metabolic activity was halted by overnight freezing. Samples were subsequently centrifuged at 15,000 rpm for 15 minutes to separate cellular debris from the supernatant. The resulting supernatants were collected and stored at -20°C pending analysis.

2.2. Biochemical Assays

2.2.1. Determination of Lipid Peroxidation (MDA)

Lipid peroxidation in mitochondria was quantified according to [15]. Briefly, 100 µL of mitochondrial supernatant was mixed with an equal volume of 8.1% sodium dodecyl sulfate (SDS), followed by 750 µL of 20% acetic acid (pH 3.5) and 750 µL of 0.8% thiobarbituric acid (TBA). The final volume was adjusted to 2 mL with distilled water. The mixture was heated at 90°C for 60 minutes, cooled in an ice bath for 5 minutes, and then supplemented with 500 µL distilled water and 2 mL n-butanol. After centrifugation at 4,000 rpm for 10 minutes, the optical density of the upper phase was measured spectrophotometrically at 532 nm. MDA concentration was calculated using the molar extinction coefficient ($\varepsilon = 156\ mM^{-1} \cdot cm^{-1}$) and expressed as nanomoles per milligram of protein.

2.2.2. Catalase Activity Assay

Catalase activity was assessed using the method described by [16] with modifications. The reaction mixture contained 100 µL mitochondrial supernatant, 900 µL phosphate buffer (0.01 mM, pH 7.4), and

100 µL hydrogen peroxide (H_2O_2, 0.2 M). After thorough mixing and incubation at 37°C for 15 minutes, 1.0 mL dichromate/acetic acid reagent (1:3 ratio of 5% sodium dichromate to glacial acetic acid) was added. The mixture was incubated at 90°C for 30 minutes, centrifuged at 1,600 rpm for 10 minutes, and absorbance was measured at 570 nm. Catalase activity was calculated using a molar extinction coefficient of 0.036 $mM^{-1} \cdot cm^{-1}$ and expressed as µmol of H_2O_2 decomposed per minute per mg protein.

2.2.3. Determination of Superoxide Dismutase (SOD)

SOD activity was determined according to [17] with minor modifications. The assay measures the inhibition of pyrogallol auto-oxidation by SOD. To 950 µL of Tris-HCl buffer (50 mM Tris-HCl with 1 mM EDTA, pH 8.2), 20 µL of mitochondrial supernatant and 50 µL of pyrogallol solution (2.5 mM) were added. Absorbance changes were measured at 420 nm at one-minute intervals over five minutes. Enzyme activity was expressed as units of SOD per mg protein, with one unit defined as the amount of enzyme causing 50% inhibition of pyrogallol auto-oxidation.

2.2.4. Glutathione-S-Transferase Assay (GST)

GST activity was measured according to [18]. The assay monitors the conjugation reaction between GST and 1-chloro-2,4-dinitrobenzene (CDNB) in the presence of reduced glutathione (GSH). The reaction mixture contained CDNB (1 mM), GSH (0.5 mM), 1 mL ethanol (pH 6), diluted in sodium phosphate buffer (1 mM, pH 6.5). A 200 µL volume of mitochondrial supernatant or buffer (blank) was mixed with 1.2 mL of reagent. Absorbance was recorded at 340 nm at one-minute intervals for five minutes. GST specific activity was calculated using the molar extinction coefficient of the GSH-CDNB conjugate and expressed as µM/min/mg protein.

2.2.5. Determination of NADH-Cytochrome c Oxidoreductase (Complex I and III) Activity:

The combined activity of mitochondrial Complexes I and III was assessed following [19]. The reaction mixture consisted of 0.5 mL potassium phosphate buffer (50 mM, pH 7.4) containing 1 mM EDTA, 0.1 mL sodium azide (NaN_3, 20 mM), 0.05 mL cytochrome c (50 µM), and 10 µg isolated mitochondria. After pre-incubation at 30°C for 1 minute, the reaction was initiated by adding 100 µL NADH (1 mM). Absorbance changes were recorded at 550 nm over 3 minutes, both with and without 0.01 mL rotenone (5 µM). Enzyme activity was expressed as nmol of cytochrome c reduced per minute per mg protein using a molar extinction coefficient of 19 $mmol^{-1} \cdot L \cdot cm^{-1}$.

2.2.6. Measurement of Cytochrome c Oxidase (Complex IV) Activity:

Complex IV activity was evaluated following [20], monitoring the oxidation of reduced cytochrome c at 550 nm. Reduced cytochrome c (50 µM, 0.998 mL) was incubated at 37°C for 3 minutes before adding 2 µL isolated mitochondria (2 µg/mL). Absorbance changes were recorded using a thermostated spectrophotometer at 37°C over 1 minute. Results were expressed as nmol of cytochrome c oxidized per minute per mg protein using a molar extinction coefficient of 18.5 $mmol^{-1} \cdot L \cdot cm^{-1}$.

2.3. Statistical Analysis

Data are presented as mean ± standard deviation (M ± SD) for five rats per group. Statistical comparisons between groups were performed using one-way analysis of variance (ANOVA) with IBM SPSS software version 21. Tukey's post hoc multiple comparison test was applied to identify specific intergroup differences. Statistical significance was defined as $p \leq 0.05$, with $p \leq 0.01$ considered highly significant and $p \leq 0.001$ extremely significant**.**

3. Results

3.1. Effect of Quercetin and Rutin on Lipid Peroxidation (MDA Levels)

Lipid peroxidation was assessed by measuring MDA levels across all experimental groups (Figure 1). $AlCl_3$ exposure produced time-dependent effects on MDA production in isolated brain mitochondria. After 2 hours of incubation, a non-significant decrease (-4.63%) in MDA levels was observed in the $AlCl_3$-treated group compared to controls. At 6 hours, MDA levels showed a marginal increase

(+3.02%), which intensified dramatically at 20 hours (+104.28%, $p < 0.001$), indicating substantial lipid peroxidation.

Co-administration of quercetin with $AlCl_3$ resulted in time-dependent changes in lipid peroxidation. At 2 and 6 hours, MDA levels increased by 28.12% and 19.10%, respectively, relative to controls (non-significant). By 20 hours, a marked elevation in MDA (+87.37%, $p < 0.01$) was observed, though this increase was less pronounced than with $AlCl_3$ alone, suggesting partial protective effects.

In the $AlCl_3$+Rut group, MDA levels decreased by 31.69% at 2 hours (non-significant), followed by moderate increases at 6 hours (+19.17%) and 20 hours (+50.31%, $p < 0.05$) compared to controls. Notably, the elevation at 20 hours was substantially lower than in both the $AlCl_3$ and $AlCl_3$+Quer groups, indicating superior protection against lipid peroxidation by rutin.

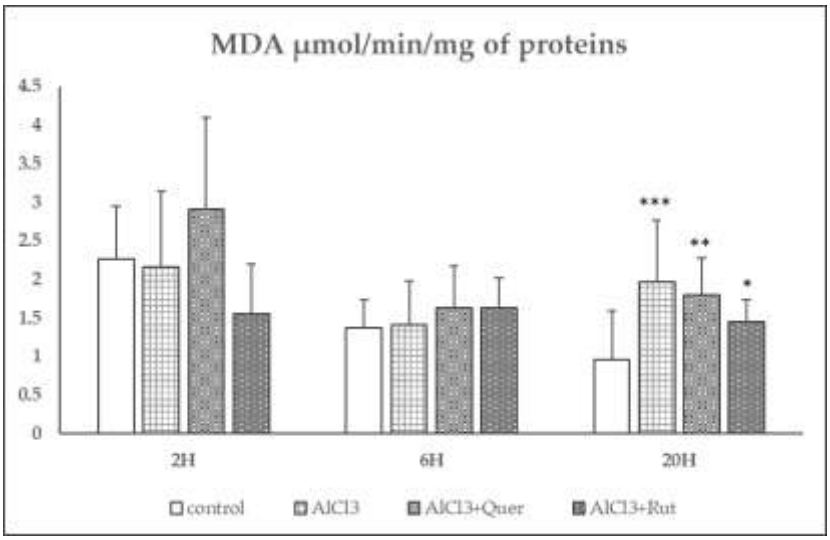

Figure 1: Variation in MDA Concentration in Isolated Rat Brain Mitochondria and Incubated with $AlCl_3$ and Treated with Quercetin ($AlCl_3$+Quer) and Rutin ($AlCl_3$+Rut) after 2h,6h and 20h. Data are presented as mean ± standard deviation (n = 5). Statistical analysis was conducted using One-Way ANOVA (*P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001 vs control)

3.2. Effect of Quercetin and Rutin on Antioxidant Enzyme Activities

3.2.1. Catalase (CAT) Activity

Aluminum chloride exposure induced a progressive decline in CAT activity over the incubation period (Figure 2). At 2 and 6 hours, modest reductions of 6.45% and 0.30% were observed, respectively. By 20 hours, CAT activity decreased significantly by 21.98% ($p < 0.05$) compared to controls, indicating compromised antioxidant capacity.

Treatment with quercetin following $AlCl_3$ exposure showed variable effects on CAT activity. A minimal decrease (-3.06%) was noted at 2 hours, while more substantial reductions occurred at 6 hours (-28.47%, $p < 0.05$) and 20 hours (-22.02%, $p < 0.05$) relative to controls, suggesting limited protection of this enzyme.

In contrast, rutin administration initially enhanced CAT activity by 36.20% at 2 hours ($p < 0.05$) compared to controls, demonstrating early antioxidant stimulation. However, this protective effect diminished over time, with decreases of 31.07% ($p < 0.05$) and 17.69% (non-significant) at 6 and 20 hours, respectively. Nevertheless, rutin maintained slightly higher CAT activity than $AlCl_3$ alone at 20 hours, indicating modest long-term protection.

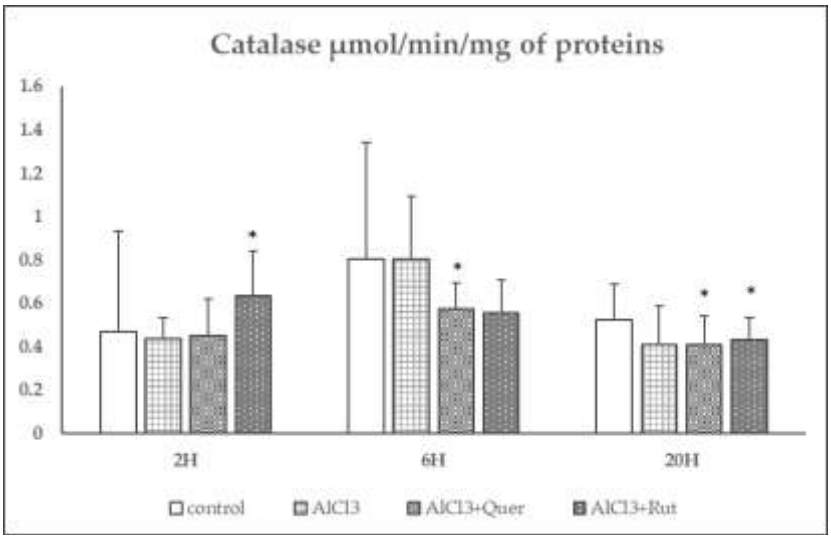

Figure 2: Modulation of Catalase Activity in Isolated Rat Brain Mitochondria and incubated Following $AlCl_3$ Exposure and Treatment with quercetin ($AlCl_3$+Quer) and Rutin ($AlCl_3$+Rut) after 2h, 6h and 20h**.** Data are presented as mean ± standard deviation (n = 5). Statistical analysis was conducted using One-Way ANOVA. (*P≤ 0.05 vs control)

3.2.2. Superoxide Dismutase (SOD) Activity

SOD activity exhibited dynamic temporal changes following aluminum exposure (Figure 3). In the $AlCl_3$ group, SOD activity initially increased by 48.35% (non-significant) at 2 hours and by 8.12% at 6 hours, potentially reflecting an early adaptive response. However, by 20 hours, a significant decrease of 34.43% ($p < 0.05$) was observed compared to controls, indicating eventual antioxidant system impairment.

Co-administration of quercetin with $AlCl_3$ resulted in decreased SOD activity (-27.84%) at 2 hours relative to controls. Interestingly, at 6 hours, a significant increase in SOD activity (+35.74%, $p < 0.05$) was observed, suggesting a delayed compensatory response. By 20 hours, SOD activity declined by 33.90% ($p < 0.05$), similar to the $AlCl_3$-only group.

In the $AlCl_3$+Rut group, SOD activity decreased by 45.24% (non-significant) at 2 hours, followed by a significant increase of 24.26% ($p < 0.05$) at 6 hours compared to controls. At 20 hours, activity decreased by 23.37% ($p < 0.05$), which was less pronounced than in both the $AlCl_3$ and $AlCl_3$+Quer groups, suggesting superior long-term protection with rutin.

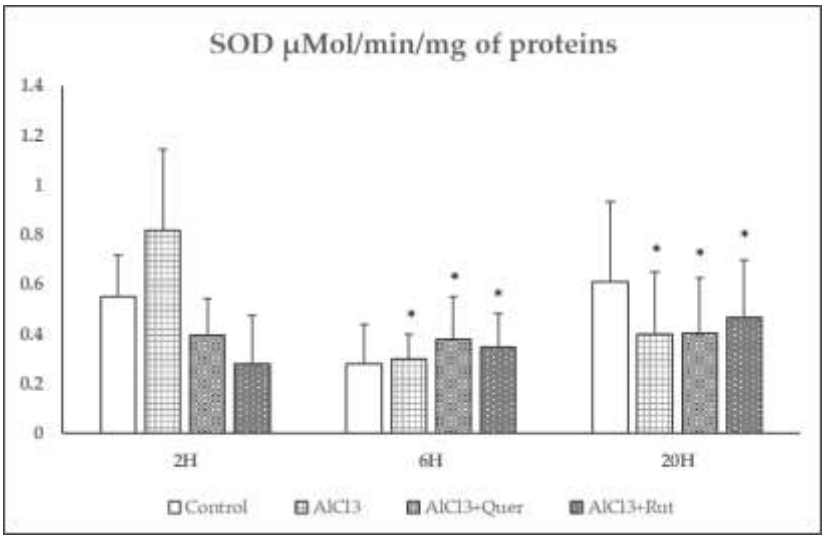

Figure 3: Variation in SOD levels in cerebral Isolated Rat mitochondria and incubated with $AlCl_3$ and Treated with Quercetin ($AlCl_3$+Quer) and Rutin ($AlCl_3$+Rut) After 2h, 6h and 20h. Data are presented as mean ± standard deviation (n = 5). Statistical analysis was conducted using One-Way ANOVA. (*P≤ 0.05 vs control)

3.2.3. Glutathione-S-Transferase (GST) Activity

GST activity in aluminum-exposed mitochondria showed biphasic changes (Figure 4). At 2 and 6 hours, GST activity increased by 39.11% and 18.78%, respectively, compared to controls (non-significant). By 20 hours, a significant decrease of 30.56% ($p < 0.05$) was observed, indicating compromised detoxification capacity.

Co-administration of quercetin with $AlCl_3$ significantly enhanced GST activity at 2 hours (+87.69%, $p < 0.05$) and maintained elevated levels at 6 hours (+37.02%, non-significant) compared to controls. By 20 hours, a moderate decrease of 16.08% (non-significant) was observed, which was significantly less pronounced than with $AlCl_3$ alone, demonstrating substantial protection.

Similarly, rutin treatment resulted in significantly increased GST activity at 2 hours (+44.93%, $p < 0.05$) and 6 hours (+53.43%, $p < 0.05$) compared to controls. At 20 hours, activity decreased by 28.91% ($p < 0.05$), which was comparable to the $AlCl_3$ group but more pronounced than with quercetin, suggesting differential long-term efficacy between the flavonoids.

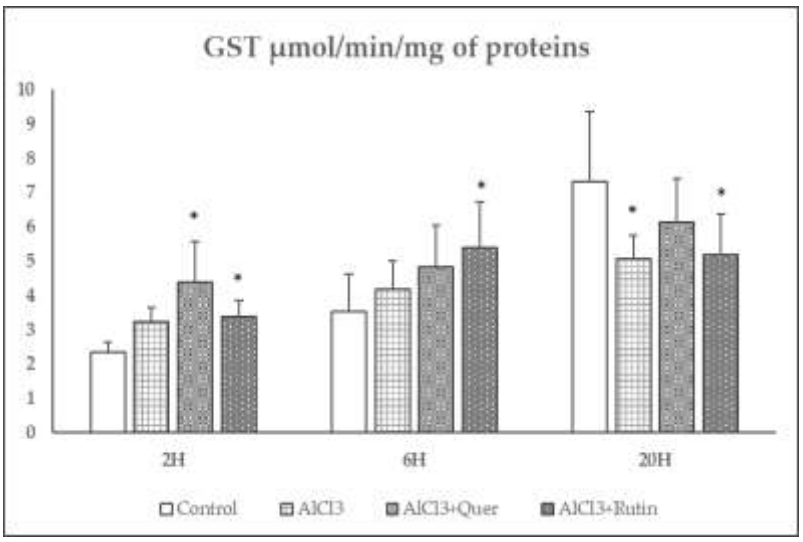

Figure 4: Concentration of GST in cerebral Isolated Rat mitochondria incubated for 2, 6, 20h in control group and treated by $AlCl_3$, $AlCl_3$+Quer, $AlCl_3$+Rut. Data are presented as mean ± standard deviation (n = 5). Statistical analysis was conducted using One-Way ANOVA. (*P≤ 0.05 vs control)

3.3. Effect of Quercetin and Rutin on Mitochondrial Respiratory Chain Activity:

3.3.1. NADH-Cytochrome c Oxidoreductase (Complex I and III) Activity:

The combined activity of Complexes I and III showed distinct temporal patterns across treatment groups (Figure 5). In the $AlCl_3$-exposed group, enzyme activity increased by 77.83% (non-significant) at 2 hours, possibly reflecting early compensatory mechanisms. By 6 hours, a significant decrease of 34.33% ($p < 0.05$) was observed, which intensified to 76.60% ($p < 0.01$) at 20 hours compared to controls, indicating severe impairment of electron transport.

Treatment with quercetin following $AlCl_3$ exposure significantly enhanced Complex I/III activity at 2 hours (+122.65%, $p < 0.05$) relative to controls, demonstrating early protective effects. At 6 hours, activity returned to near-control levels (-0.03%, non-significant). However, by 20 hours, a profound decrease of 84.81% ($p < 0.001$) was observed, which exceeded the reduction seen with $AlCl_3$ alone, suggesting potential long-term adverse effects.

In the $AlCl_3$+Rut group, Complex I/III activity remained near control levels at 2 hours (+0.24%, non-significant). At 6 hours, activity increased significantly by 9.71% ($p < 0.01$) compared to controls. By 20 hours, a substantial decrease of 68.09% ($p < 0.001$) was recorded, which was less severe than with quercetin but still pronounced compared to controls.

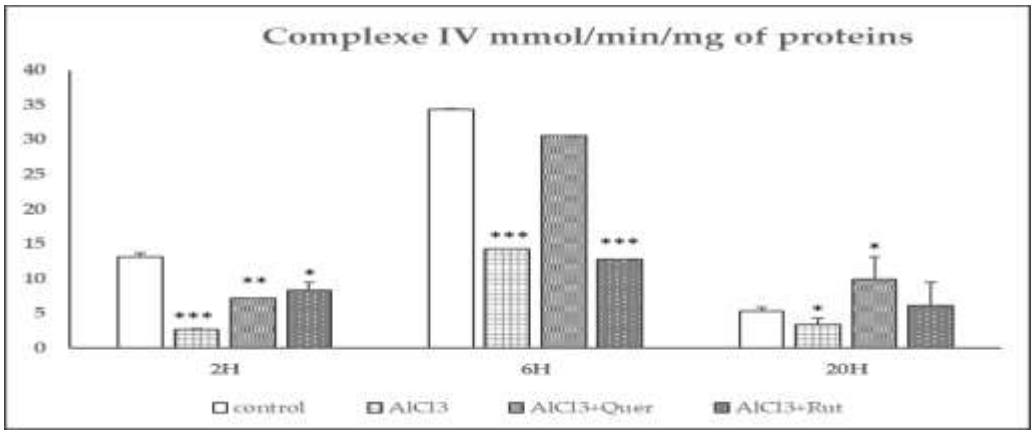

Figure 5: Enzymes activities in NADH-cytochrome c oxidoreductase combined (complex I and III) in isolated Rat Brain Mitochondria incubated for 2h,6h and 20h in control groups and treated with $AlCl_3$, $AlCl_3$+Quer and $AlCl_3$+Rut. Data are presented as mean ± standard deviation (n = 5). Statistical analysis was conducted using One-Way ANOVA (*P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001 vs control)

3.3.2. Cytochrome c Oxidase (Complex IV) Activity

Complex IV activity was markedly affected by aluminum exposure (Figure 6). In the $AlCl_3$ group, significant decreases of 80.17% ($p < 0.001$), 58.53% ($p < 0.001$), and 37.00% ($p < 0.05$) were observed at 2, 6, and 20 hours, respectively, compared to controls, indicating progressive impairment of terminal electron transfer.

Co-administration of quercetin with $AlCl_3$ initially resulted in decreased Complex IV activity at 2 hours (-45.95%, $p < 0.05$) and 6 hours (-11.09%, $p < 0.05$) relative to controls. Remarkably, by 20 hours, a substantial recovery was observed, with activity increasing by 86.52% ($p < 0.05$) compared to controls, demonstrating significant restoration of terminal electron transport capacity.

In the $AlCl_3$+Rut group, Complex IV activity decreased significantly at 2 hours (-36.95%, $p < 0.05$) and 6 hours (-62.96%, $p < 0.001$) compared to controls. By 20 hours, a modest increase of 15.35% (non-significant) was observed, indicating partial recovery, though less pronounced than with quercetin.

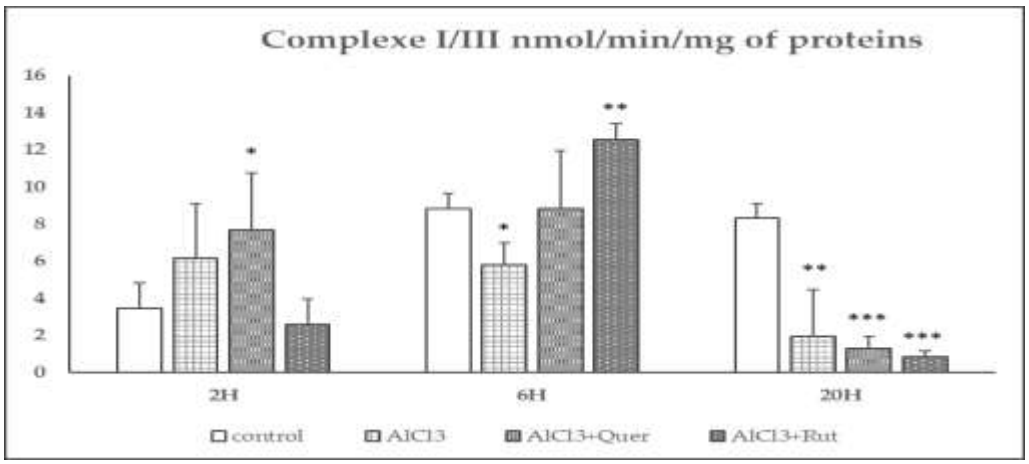

Figure 6: Determination of Cytochrome c oxidase (complex IV) activity in Isolated Rat Brain Mitochondria and Incubated at 2h, 6h, 20h with $AlCl_3$, $AlCl_3$+Quer and $AlCl_3$+Rut. Data are presented

as mean ± standard deviation (n = 5). Statistical analysis was conducted using One-Way ANOVA (*P≤ 0.05, ** P≤ 0.01, *** P≤ 0.001 vs control)

4. Discussion

Aluminum (Al), the third most abundant metal in the Earth's crust, has witnessed steadily increasing utilization across diverse industrial and biological applications [21]. The principal mechanism underlying aluminum-induced toxicity is its promotion of oxidative stress [4,22,23], predominantly through the generation of superoxide anions via Fenton-like reactions [24].

The brain exhibits particular vulnerability to oxidative damage due to several factors: its extraordinarily high oxygen consumption (approximately 20% of total body oxygen), abundance of polyunsaturated fatty acids within neuronal membranes, substantial iron content, and comparatively low antioxidant enzyme activities [25]. These characteristics render cerebral tissue especially susceptible to aluminum-induced neurotoxicity.

Rutin and its aglycone derivative, quercetin [26], are naturally occurring flavonoids found in various fruits, vegetables, grapes, citrus fruits, and tea. Beyond their established anticancer and anti-inflammatory properties, these flavonoids demonstrate potent antioxidant activity. They modulate oxidative stress parameters by enhancing key antioxidant enzyme activities (GPx, catalase, SOD), stimulating glutathione production, and attenuating lipid peroxidation. These effects strongly suggest a protective role against aluminum-induced oxidative stress in neural tissues [27].

4.1. Lipid Peroxidation and Oxidative Damage

Lipid peroxidation (LPO), the oxidative degradation of polyunsaturated fatty acids, compromises biological membrane integrity, resulting in structural disruption, loss of membrane fluidity, alterations in membrane potential, increased ionic permeability, and impaired receptor function [28,29,4]. The byproducts of lipid peroxidation represent principal biomarkers of oxidative stress, with the brain being particularly susceptible due to its high lipid content and elevated oxygen consumption [4].

Our results demonstrated a time-dependent increase in MDA levels within brain mitochondria of aluminum-intoxicated rats, culminating in a significant elevation (+104.28%) at 20 hours (figure 1). This observation aligns with previous studies reporting aluminum-induced enhancement of lipid peroxidation [30,31]. The mechanism likely involves disruption of iron homeostasis through aluminum binding to transferrin, displacing iron, and consequently increasing Fe^{2+} availability for Fenton reactions. The resultant hydroxyl radicals induce extensive lipid peroxidation, exacerbated by potential inactivation of endogenous antioxidant enzymes [32,33].

Co-administration of quercetin and rutin with aluminum significantly attenuated lipid peroxidation, with rutin exhibiting superior protective effects (MDA increase: +50.31% vs. +87.37% with quercetin) at 20 hours (figure 1). These findings corroborate previous research [34] demonstrating quercetin's ability to scavenge free radicals and chelate transition metal ions, thereby mitigating lipid peroxidation. Similarly, Sharma et al reported that quercetin administration reduced ROS production, lipid peroxidation, and protein oxidation in rats [35]. Rutin has likewise been shown to effectively lower MDA levels in models of neurotoxicity, reflecting its potent ROS-scavenging capacity through hydrogen atom donation to superoxide anions, peroxyl radicals, and hydroxyl radicals [36].

The limited effects of quercetin and rutin observed at earlier timepoints (2 and 6 hours) (figure 1) may be attributed to the disruptive effects of aluminum and/or the delayed onset of bioflavonoid activity, which may require longer exposure durations to manifest fully.

4.2. Antioxidant Enzyme Activities

The increase in lipid peroxidation and oxidative damage correlates closely with perturbations in key antioxidant enzyme activities [37]. These enzymes constitute the primary cellular defense against oxidative insults, playing crucial roles in preventing oxidative stress, maintaining cellular homeostasis, and facilitating ROS elimination.

Our findings revealed progressive reductions in the activities of catalase (CAT, -21.98%) (figure 2) and superoxide dismutase (SOD, -34.43%) (figure3) in brain mitochondria of aluminum-intoxicated rats compared to controls at 20 hours of incubation (figure 2, 3). This enzymatic decline may reflect exhaustion due to excessive utilization under oxidative stress conditions or direct inhibition of enzyme

expression. Both scenarios indicate disturbance in redox homeostasis and the presence of oxidative stress. Similar observations were reported by Bhrathi et al, suggesting that such changes could result from impaired axonal mitochondrial turnover, leading to the release of oxidative compounds and inhibition of antioxidant enzymes within neurons [38].

The observed reduction in catalase and SOD activities following aluminum exposure can be attributed to the depletion of essential cofactors including selenium, zinc, copper, and glutathione (GSH). These cofactors are vital for maintaining optimal antioxidant enzyme functionality. These findings align with previous studies [39,40,41] that similarly recorded a reduction in antioxidant enzyme activities following aluminum exposure. Furthermore, Guo et al, Lukyanenko et al, reported that aluminum chloride accumulation disrupts trace element absorption and homeostasis, thereby impairing the binding capacity of antioxidant enzymes [42,43]. More recent investigations by Laabbar et al, have demonstrated that aluminum ions interfere with enzyme active sites through displacement of essential metal cofactors, further compromising antioxidant defense systems [44].

Reduced glutathione (GSH) functions as a critical endogenous thiol that maintains intracellular redox balance and facilitates detoxification processes by acting as a co-substrate for antioxidant enzymes [45,39]. The decline in GSH levels observed after aluminum exposure may result from impaired GSH synthesis due to direct aluminum toxicity [39,4] or accelerated GSH degradation during free radical neutralization, where ROS readily interact with GSH thiol groups [46]. Moreover, the GST enzymatic system catalyzes the conjugation of GSH with oxidized molecules, forming glutathione-conjugated metabolites (GSSG), a finding corroborated by Alarifi et al [47]. LI et al, demonstrated a strong correlation between decreased GST activity and the onset of oxidative stress [48]**.** Viezeliene and al, showed that aluminum chloride exposure (25 mg/kg BW) significantly increased oxidized glutathione (GSSG) levels and decreased reduced GSH levels and GSH-dependent enzyme activities [49].

Aluminum chloride inhibits the activity of glutamyl-cysteine synthetase, the rate-limiting enzyme for GSH biosynthesis in the liver, and negatively affects glucose-6-phosphate dehydrogenase and NADP-isocitrate dehydrogenase, both essential for NADPH regeneration required for GSH recycling [50,3]. Recent metabolomic analyses by He et al, have identified additional aluminum-sensitive nodes in glutathione metabolism, highlighting the comprehensive disruption of this critical antioxidant pathway [51].

Co-administration of rutin and quercetin with aluminum effectively restored antioxidant enzyme activities (CAT, SOD, GST) in brain mitochondria compared to aluminum-intoxicated subjects. Celik et al, reported that rutin increases CAT, SOD, and GSH levels in the brain while functioning as a ROS scavenger by donating hydrogen atoms to superoxide anions, peroxyl, and hydroxyl radicals [52,36]. Additionally, rutin inhibits xanthine oxidase, a key enzyme involved in ROS generation, Enogieru et al and Sharma et al, similarly reported that both rutin suspension and nanoemulsion formulations improved antioxidant status by increasing GSH and SOD levels in the brain [53,54]. Follow-up research by Chen et al, has characterized the specific structural elements of rutin responsible for its metal-chelating properties, enhancing our understanding of its protective mechanisms [55].

Quercetin administration (10 mg/kg BW/day) significantly mitigates aluminum-induced oxidative stress by reducing ROS production and enhancing mitochondrial superoxide dismutase activity [35]**.** Al-Otaibi et al, found that quercetin pre-treatment restored reduced glutathione levels and normalized SOD activities by upregulating the MnSOD gene [56]. Recent work by Karabay et al, has demonstrated that quercetin also modulates multiple transcription factors involved in oxidative stress responses, including nuclear factor erythroid 2-related factor 2 (Nrf2) and nuclear factor-κB (NF-κB) [57].

An early increase in SOD activity at 2 and 6 hours (figure 3), and in GST activity at 2 hours post-intoxication (figure 4) was documented, potentially reflecting an initial disturbance in copper and zinc homeostasis that temporarily alters metal-dependent antioxidant enzyme function [58]. However, catalase activity showed no significant recovery at 6 and 20 hours after quercetin and rutin co-treatment (figure 2), suggesting a time-limited protective effect against aluminum-induced oxidative stress. Ahmed et al, have recently proposed a biphasic model of flavonoid protection that explains these temporal variations in antioxidant enzyme responses [59].

4.3. Mitochondrial Dysfunction in Aluminum Toxicity

Mitochondria constitute the primary site of energy production through the electron transport chain (ETC), culminating in ATP synthesis via oxidative phosphorylation [60]. Beyond their bioenergetic function, mitochondria regulate ROS metabolism, calcium homeostasis, and apoptotic signaling [61]. Dysregulation of mitochondrial homeostasis, particularly imbalances between ROS generation and antioxidant defense mechanisms, represents a critical factor in the development of neurodegenerative disorders and aging processes [62]. Recent advances in mitochondrial biology by Pinho et al, have further elaborated the complex interplay between mitochondrial function and cellular redox status under neurotoxic conditions [63].

The generation of mitochondrial superoxide radicals and other ROS occurs predominantly at Complex I and Complex III, with the latter representing the principal site of ROS production [64,65]. Aluminum exposure significantly impairs the enzymatic activity of mitochondrial complexes, particularly Complexes I, III, and IV. High-resolution respiratory chain analysis by Yu et al, has revealed specific electron transfer intermediates that are particularly vulnerable to aluminum-mediated disruption [66]. Mitochondrial-derived ROS are pivotal mediators in the release of cytochrome c and other pro-apoptotic factors, subsequently triggering caspase activation and apoptotic cell death, which may explain the reduced activity of cytochrome c oxidase (Complex IV) observed experimentally [52].

Iglesias-Gonzales et al, reported that Al^{3+} disrupts mitochondrial bioenergetics, promotes organelle uncoupling, alters membrane permeability and dynamics, affects maximal electron transport system capacity linked to Complex II, and diminishes the enzymatic activities of Complexes III and V in both in vitro and in vivo models [67]. Recent structural studies by Pathak and Sriram, have demonstrated that aluminum ions can directly bind to specific subunits of respiratory complexes, offering molecular insights into the mechanisms of aluminum-induced respiratory chain dysfunction [68].

Restoration of mitochondrial complex activities following quercetin and rutin treatment can be attributed to their potent free radical scavenging properties. These flavonoids attenuate free radical production through modulation of cell signaling pathways involved in cell proliferation, survival, glutathione synthesis, and antioxidant protein expression [13]. Using metabolomic approaches, Zheng et al, have identified specific metabolite signatures associated with flavonoid-mediated mitochondrial protection [69].

Selvakumar et al demonstrated that quercetin inhibits ROS-producing enzymes and exerts neuroprotective effects against oxidative stress-induced damage. Quercetin administration significantly reduces ROS levels, prevents neuronal apoptosis, inhibits aluminum-induced cytochrome c release, upregulates anti-apoptotic Bcl-2, downregulates pro-apoptotic Bax, p53, and caspase activation, and mitigates DNA damage [70]. Advanced imaging studies by Sharma et al. have visualized the subcellular distribution of quercetin in neuronal mitochondria, confirming its direct protective effects on these organelles [71].

Rutin exhibits strong antioxidant and free radical scavenging activities, lee et al and Singh et al found that rutin attenuates mitochondrial membrane potential loss [72,73]. The chemical structure of rutin enables it to directly scavenge ROS [54]. Recent computational modeling by Zazeri et al, has characterized the specific interactions between rutin and mitochondrial proteins, providing a structural basis for its protective effects on respiratory chain function [74].

Aluminum exposure significantly compromises mitochondrial electron transport chain functionality, particularly complexes I, III, and IV, leading to enhanced ROS production, severe oxidative stress, and neuronal apoptosis. Co-administration of quercetin and rutin effectively restores mitochondrial complex activity, reduces ROS levels, stabilizes mitochondrial membrane potential, and positively modulates cellular survival pathways. Given their potent antioxidant and anti-apoptotic properties, quercetin and rutin represent promising neuroprotective agents against aluminum-induced neurotoxicity.

5. Conclusions

Aluminum exposure significantly compromises mitochondrial electron transport chain functionality, particularly complexes I, III, and IV, leading to enhanced ROS production, severe oxidative stress, and neuronal apoptosis. Co-administration of quercetin and rutin effectively restores mitochondrial complex activity, reduces ROS levels, stabilizes mitochondrial membrane potential, and positively modulates cellular survival pathways. Given their potent antioxidant and anti-apoptotic properties, quercetin and rutin represent promising neuroprotective agents against aluminum-induced neurotoxicity.

Abbreviations

The following abbreviations are used in this manuscript:

$AlCl_3$	Aluminum chloride
MDA	Malondialdehyde
CAT	Catalase
SOD	Superoxide dismutase
CNS	Central Nervous System
GPx	Glutathione peroxidase
ROS	Reactive Oxygen Species
Quer	Quercetine
Rut	Rutine
GST	Glutathione S-transferase
GSH	Reduced Glutathione
GSSG	Oxidized Glutathione
BW	Body Weight
Nrf2	Nuclear factor erythroid 2-related factor 2
ETC	Electron Transport Chain
Bcl-2	B-cell lymphoma 2 (anti-apoptotic protein)
p53	Tumor protein p53
PBS	Phosphate Baffred Saline

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