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# Neurogenic Impact of Quercetin and Rosmarinic Acid on Neuroblastoma Cells

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**Running Title**: Combinational efficacy of quercetin and rosmarinic acid in neurogenesis in In Vitro system.

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## Abstract

Neurogenesis, the process of generating new neurons, plays a critical role in brain development, learning, memory, and overall brain health. Natural compounds with neurodegenerative properties have gained significant attention in recent years as potential therapeutic agents for promoting neurogenesis. This study investigates the effects of rosmarinic acid and quercetin, two natural compounds with known neuroprotective and antioxidant properties, on neurogenesis. Using SH-SY5Y cells, a human neuroblastoma cell line commonly employed as a model for neurogenesis research. In our present study, we used a combination of herbal compounds Rosmarinic acid (RA) and quercetin (Q) to see the combined effect on the *in vitro* system. We treated SH- SY5Y cells with 10 µM concentration of each compound (Q+RA) and found the upregulation of neuronal marks viz, NFM (1.534 ± 0.03049;**p<0.01), Nestin (1.390 ± 0.2031;*p<0.05) and β-III tubulin (1.459 ± 0.04676***p<0.001) at the protein level. Reactive oxygen species (ROS) study on neuroblstoma cells also shows the anti-oxidative nature of Q and RA. The combined therapy also displays low Malondialdehyde (MDA) and Nitric oxide (NO) production in co-exposed cell, which strongly supports the neurogenic nature of Q+RA in comparison to control cells. Therefore, we can state that our results support the therapeutic efficacy of combined herbal therapy for neurogenic impact. Our findings demonstrate that the combination of rosmarinic acid and quercetin synergistically enhances neurogenesis in SH-SY5Y cells.

**Key-words**: Rosmarinic Acid, Quercetin, SH- SY5Y, Neurogenesis, Phytochemicals.

## Introduction

The nervous system is exceedingly vulnerable to a variety of damaging stimuli, including oxidative stress, excitotoxicity, injury, and is especially vulnerable to irreparable damage due to its limited regeneration ability [1]. The sensitivity of the brain and neurons make neuronal complications the most sensitive health issue. With ageing, compromised lifestyle and chemical exposure, the neuronal loss and damage is very fast and unavoidable phenomena. There is commercial therapy to restore the neurons with high expense and long-term side effects. Therefore, natural therapy with easy availability and the least side effects is needed to cure neuronal complications [2]. Phytochemicals are key neuroprotective agents that function primarily as antioxidants in the brain, scavenging stress-induced free radicals [3]. Phytochemicals from medicinal plants, such as Rosmarinic acid (RA) and quercetin (Q), target neurotrophins and assist in maintaining the chemical balance of the brain. Many antioxidants, including Rosmarinic acid and quercetin, which are derived from natural products, can potentially prevent toxic neuronal damage and protect neurons [4]. Natural plant pigments present in a variety of meals, including fruits,

cereals, herbs, and beverages, are the source of phytochemicals. These have been shown to possess neuroprotective, cardioprotective, anti-oxidant, and anti-cancer qualities [5, 6]. In a range of neurogenic impact, polyphenolic compounds, in particular, have a critical neuroprotective role. The average daily intake of quercetin (3,5,7,3′,4′-pentahydroxyflavone) is 25 mg. This typical natural substance may be found in a variety of fruits and vegetables, including onions, berries, and apples [7]. Quercetin has anti-cancer, antithrombotic, anti-inflammatory, and anti-apoptotic characteristics, among other biological effects. Quercetin reduces the release of proinflammatory cytokines by glial cells, making it anti-inflammatory [8]. Research has indicated that quercetin inhibits neuroinflammation by reducing NO production in microglial cells, delaying NF-κB activation, and minimizing neuronal injury [9]. By suppressing neuroinflammation-mediated neurodegeneration, quercetin has been found to attenuate manganese-induced neurotoxicity. It does this by altering the nuclear factor kappa B (NF-κB) and heme oxygenase-1 (HO-1)/ Nuclear factor erythroid 2-related factor 2 (Nrf2) pathways [10]. Quercetin's neuroprotective impact against a variety of neurodegenerative diseases has also been described and verified *in vitro* and *in vivo* models [11]. In a study looking at quercetin's transport across the blood-brain barrier (BBB), it was found that giving quercetin to rats under prolonged stress from swimming lowered the amount of oxidative stress in their brains by using its antioxidant properties. When quercetin and catechin's anti-oxidative properties were assessed in cocoa liquor, it was shown that quercetin have shown anti-oxidative activity [12].
Rosmarinic acid(-O-caffeyl-3,4-dihydroxyphenyl lactic acid;) is an antibiotic, anti-virus, anti-inflammatory, and immunomodulatory compound that is present in herbs like rosemary, sweet basil, and perilla [13,14]. Rosmarinic acid (RA) is a common example of a hydroxyl cinnamic acid. It is renowned for its anti-aging and diabetes prevention properties [12]. It is a powerful antioxidant that may directly scavenge free radicals. To protect neurons, it works in concert with precise signaling pathways and neurotransmitter activity [15]. In PC12 cells, altering the signal transduction pathways linked to extracellular signal-regulated kinase 1/2 (ERK1/2) and mitogen-activated protein kinase (MAPK) has also been shown to have neurotrophic effects and raise cholinergic activity [16]. Also, RA demonstrated an increase in the number of glutathione transferases. The reduction in B lymphocytes triggered by nuclear factor kappa light chain enhancer (NF-κB) is accompanied by an increase in anti-Bcl-2 activity [17]. Also, RA has been observed to consume reactive nitrogen and oxygen species, such as the peroxy nitrates and various types of reactive oxygen species (ROS) [18]. Bioactive compounds from natural resources have been discovered to enhance neuritogenesis in cultured neurons by synergistically interacting with neurotrophic factors. Natural phytochemicals are easily available with the least side effects. These compounds may also hold more potential than conventional drugs. Alone activity is already investigated but combined efficacy of natural compound needs to be explored. Therefore, our current study was intended to explore the combined efficacy of Rosmarinic acid and quercetin as neurogenic agents on neuroblastoma SH-SY5Y cell lines by measuring the oxidative stress and alteration of expression in neuronal markers (NFM, Nestin and β-III tubulin) at a transcriptional and translational level as well. For this study, we have used nerve growth factor (NGF) 50 ng/mL [19] as positive control to compare the neuronal differentiation efficacy of phytochemicals (Q+RA) used for our study. Our present study showed that SH- SY5Y cells with 10 µM concentration of each compound (Q+RA) and found the upregulation of neuronal marks viz, NFM (1.534 ± 0.03049;**p<0.01), Nestin (1.390 ± 0.2031;*p<0.05) and β-III tubulin (1.459 ± 0.04676***p<0.001)at the protein level. ROS study on neuroblastoma cells also shows the anti-oxidative nature of Q and RA. The combined therapy also showed low MDA and NO production in co-exposed cell, which strongly supports the protective nature of Q+RA in comparison to control cells. Therefore, we can state that our results support the therapeutic efficacy of combined herbal therapy.

## Purpose of the research

The purpose of the research is to explore and understand the consequences of Quercetin and Rosmarinic acid on neurogenesis in neuroblastoma cells. The researchers aim to determine whether these two compounds have a neurogenic impact, meaning whether they can promote the generation of new neurons in neuroblastoma cells. The study involves assessing the expression of neuronal markers, evaluating cell viability and proliferation, and exploring the underlying mechanisms by which Quercetin and Rosmarinic acid may influence neurogenesis in these cells.

## Materials and Methods

### Consumables and Reagents

Chemical reagents used were procured from Sisco research laboratories (India) and sigma (USA). Essential DMEM/F12 medium (#12400024), FBS (#10439024) and D-PBS (#10010023) were procured from Thermo Fisher Scientific. Millipore provided all of the primary antibodies. (MAP2#05-346, β-III tubulin # MAB3408, NFM# AMAB91027). We bought secondary antibodies from Sigma-Aldrich in the United States (#114038001A, #114068001A). Plastic and culture wares were purchased from Thermo Fisher Scientific and Corning Inc. All experiments were conducted with Autoclaved Milli Q water. Rosmarinic acid (CAS No. 20283-92-5) was purchased from sigma and quercetin (CAS No. 117-39-5) from TCI chemicals (India) Pvt Ltd.

**Cell Culture**

The human neuroblastoma cells (SH-SY5Y) were obtained from the American Type Culture Collection (ATCC) and were preserved and grown on Dulbecco's modified Eagle's medium (DMEM)/F12 supplemented with heat-inactivated 10% fetal bovine serum (FBS), 0.2% sodium bicarbonate, and antibiotic/anti-mycotic solution (100X, 1mL per 100 mL of medium solution) [20]. The cells were incubated at a $CO_2$ concentration of 5% in a 37°C incubator that was humidified. The cells were passaged and used at $9^{th}$ passage. The cells were evaluated for mycoplasma contamination using a PCR method. The Trypan blue dye exclusion test was employed to assess the viability of the cells. The cells with a viability of over 98 percent were used in the investigations. The SH-SY5Ycells were exposed to the fresh serum-free medium supplemented with different doses of respective compounds. Following incubation, the cells were separated and examined using immunoblotting, immunocytochemistry, and reverse transcriptase-polymerase chain reaction (RT-PCR) methods for the analysis of neurogenic properties and morphological changes.

**MTT Assay**

The percentage of live cells was ascertained using the dye reduction assay, also known as 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) dye [21]. The cells were grown in 96-well plates ($1\times10^4$ cells/well) and given 24 hours at 37°C in a $CO_2$ incubator. Following the incubation period, each well that contained 100 µL of cell suspension received 10 µL of MTT (5 mg/mL), the plates were then incubated for an additional 4 hours. The crystals of formazan inside the cell were dissolved by adding 200 µL of DMSO after the supernatant had been discarded. Following the correct mixing, an ELISA reader (BIOTEK, USA) was used to measure the absorbance at 550 nm. In order to provide a control, untreated sets were also tested in the same circumstances. Relative cell viability was given as a proportion of the group that received no treatment.

**Morphological Examination**

Morphological changes in SH-SY5Ycells exposed to different concentrations of phytochemicals were shown to ascertain the protection-mediated alterations induced by the compounds. Every cell was subjected to the fixed concentration of compounds as mentioned above for a predetermined time interval. Using an inverted phase microscope with a 20X magnification, cell images were captured.

**Cellular ROS Measurement**

The extent of reactive oxygen species released in cells after exposure to the pre-mentioned concentration of herbal compounds was measured according to the previously defined protocol [22]. Briefly, after the treatment of compounds, cells were harvested by trypsinization and washed with PBS. The cells were then incubated after adding a cell-permeable agent, 2', 7' –dichlorofluorescein diacetate (DCFDA), for half an hour without light. The fluorescence intensity was measured, and data accumulation and analysis were done using a fluorimeter. The untreated cells were also maintained in parallel and treated as control.

**Biochemical Estimations**

SH-SY5Ycells were seeded into 6-well plate. Following cell culture with respective phytochemicals for the defined time interval, cells were scraped using ice-cold phosphate buffer saline, followed by ultra-sonication for lysis. Further, the cell lysate was centrifuged for five minutes at 4 °C at 10,000 g. Supernatants from cells were extracted and stored for further biochemical estimations [23, 24].

**Lipid Peroxidation Estimation**

Malondialdehyde (MDA) concentrations were measured in order to calculate lipid peroxidation [25]. In short, 2% thiobarbituric acid and 30% trichloro acetic acid were combined with the cell supernatant; then, the combination was incubated for 15 minutes at 90°C. Using a sigma centrifuge, the mixture was spun for 10 minutes at 10,000 rpm. ELISA was used to determine the MDA content was given in nanomoles per milligramme of protein, and 532 nm was used to measure the absorbance of the pink-tinted supernatant.

**Evaluation of Nitrite (NO) Content**

Nitrite concentration of SH-SY5Ycells was determined using a procedure described in earlier publications [26]. The cells supernatant was centrifuged for 10 minutes at 1600g, and the next, equal volumes of Griess reagent (100 µL) and recovered supernatant (100 µL) were added and then incubated for 20 minutes. An ELISA reader was used to perform a spectrophotometric analysis of the aforementioned combination at 570 nm.

**Quantitative reverse transcription-PCR is used for RNA extraction and gene expression analysis.**

TRIZOL's reagent (Sigma Aldrich, India) was used to separate total RNA from untreated and treated SH-SY5Y human neuroblastoma cells, as described previously [27]. Using the Roche, USA's Light Cycler 480 and SYBR green chemistry, the expression of the targeted gene was tested in triplicate (Thermo Scientific, USA). The relative cycle threshold approach was used to analyze gene expression, the fold change was computed in relation to the control after being normalised to β-actin expression. The primer list is included in the supplemental material (table S1).

**Protein Levels Analysis by Immunoblotting**

***Immunocytochemistry (ICC)***

With a few minor modifications, the ICC proceeded according to the same methodology. For two hours at 4°C, 0.1% concentrations of primary antibodies (NFM, Nestin, and β-III tubulin) were applied to the cells. 400 nM of Alexa Fluor-conjugated secondary antibodies that were specific for the target protein were incubated with the cells for one hour in blocking buffer. Cells on glass slides that were treated with Sigma Aldrich's (USA) DAPI mounting solution after first being cleaned in PBS. A Leica microscope with a digital camera (Leica, Wetzlar, Germany) and the ImageJ programme were used to record the fluorescence intensity (NIH, USA).

***Statistical Analysis***

GraphPad's statistical software version 5.01 (San Diego, CA, USA) was used to do the statistical analysis of the data. One-way/ two-way ANOVA was used to estimate the average difference between experimental groups, which was then followed by the student's t-test and the Bonferroni post hoc test. A two-way repeated measures ANOVA with F values was used to assess the immunocytochemical analysis results, and the Tukey's multiple comparison test was used to ascertain the individual differences between the treatment groups. The *$p<0.05$ threshold was used to determine the significance of differences.

## Results and Discussion

### 1. Cell viability assessment

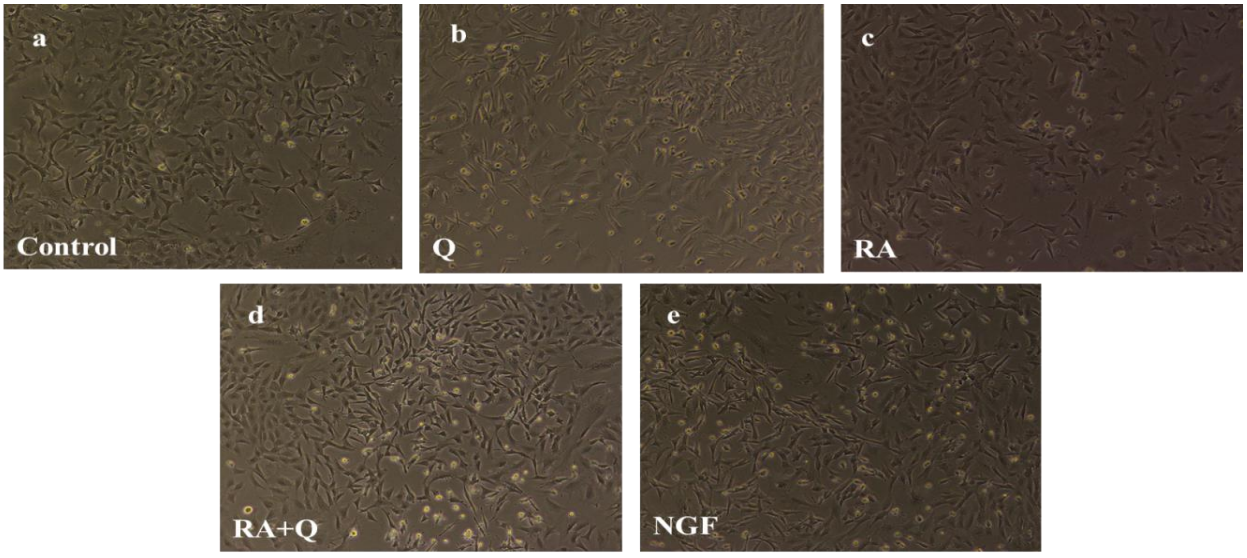
The cell viability and IC50 concentration of respective herbal compounds were determined using an MTT reagent using various concentration of quercetin (Q) and Rosmarinic acid (RA) at four distinctive time point 24h/48h/72h/96h (Supplementary Figures S1 and Figure S2.). The sample size in each group was n= 6 approximately. The cell viability was found to be decreased by approximately 50% compared to controls after exposure of 20 µM dose of quercetin and 100 µM of Rosmarinic acid to SH-SY5Ycells for 48h. Further exposure of cells with 10 µM dose of quercetin and 10 µM dose of Rosmarinic acid over 48 hours to the control, there was

no discernible cell death; instead, cell viability continuously decreased across the concentration range. Based on the findings, we have selected a concentration of 10 µM of quercetin as IC50 for the rest of the study. Therefore, 10 µM for quercetin and 10µM Rosmarinic acid was used in combination for the experimental work of this study.

### 1.1 Morphological Alterations

The morphology of SH-SY5Ycells was largely sharply edged, fusiform in shape, with long neurites and exiguous dendrites, while SH-SY5Ycells had far fewer and shorter neurites. (Figure. 1b). Exposure of quercetin to SH-SY5Ycells resulted in the slight degeneration of neuroblastoma cells and decreased proliferative capacity. Although, a significantly improved morphology of SH-SY5Ycell line was observed after treatment with Rosmarinic acid (RA), with a significant increase in proliferative and differentiative potency of neuro-blastoma cells (Figure 1c). Further, the combination of RA and quercetin (Q) again together in combination improved the neurogenic characteristics of cells in comparison to control, as it could be depicted in Figure 1d. NGF 50 ng/mL is taken as a positive control to show neuronal differentiation.

**Figure 1:** Treatment of RA with Q alone or in combination with (RA+Q): A). (a) Control of morphology, (b) Changes in morphology following exposure to Q, (c) Alterations in morphology, recovery, and protection following exposure to RA, and (d) Morphological changes after RA+Q co-exposure. (e). NGF 50ng/mL is used as a positive control to demonstrate neuronal differentiation. The bio-logical duplicates are shown by phase-contrast microscopy in all photographs.




### 2 Alteration in biochemical parameters in SH-SY5Ycells

Next, we measured the levels of oxidative stress burden by estimating the content of ROS (Figure 2 a) generated, MDA (Malondialdehyde) (Figure 2 b) and NO (nitrite) (Figure 2 c) in SH-SY5Ycells after exposure to a decided concentration of RA and Q. Surprisingly, the release of ROS in SH-SY5Ycells was found to be slightly elevated in NGF exposed cells but in a non-significant manner (2.027±0.34) which was further notably ameliorated with the treatment of quercetin at a concentration of 10 µM significantly (*$p<0.05$; 0.889± 0.03249) as observed after quantification represented through bar graph (Figure 2 a.). Further, we also observed that ROS levels were similar to control (1.824 ± 0.05931) and NGF-treated cells when exposed to Rosmarinic acid alone (1.758± 0.1426) and Rosmarinic acid in combination with quercetin (2.062± 0.09803). Moreover, the values of MDA showed no significant differences between the control (untreated cells) (15.830 ± 1.215) and the other treated cells with quercetin (16.760± 1.877), Rosmarinic acid (15.110 ± 0.05721) and their combination (14.940± 0.3114) (Figure

2 b). Similar results were observed for NO in a non- significant manner between the control (untreated cells) (7.981± 0.6956) and the other treated cells with quercetin (8.686 ± 0.6434), Rosmarinic acid (7.936 ± 0.7977) and their combination (7.181 ± 0.3232) (Figure 2 c). The changes among all the groups were found to be non-significant otherwise mentioned in the figure. These results indicate that RA and Q did not elevate any cellular stress after treatment. The calculations were done by taking mean± SEM, where SEM represents standard error of mean.

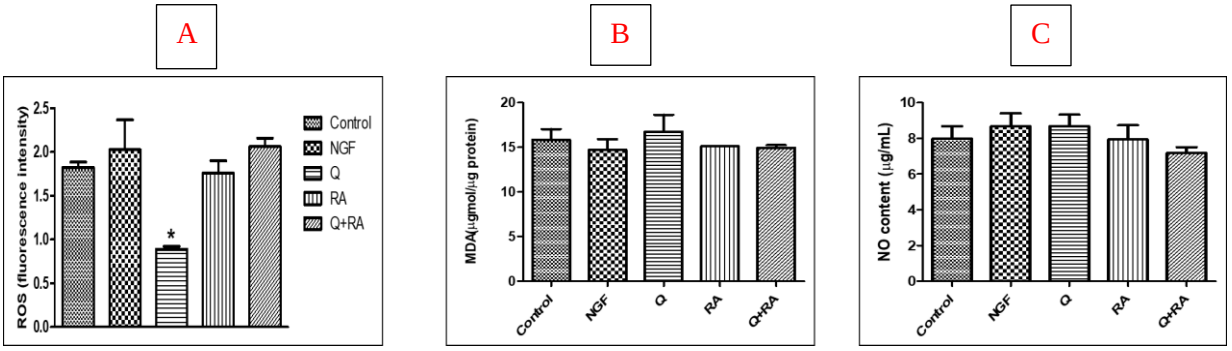



**Figure 2**: (A) ROS production, (B) MDA production, and (C) NO generation in SH-SY5Y cells after being exposed to different exposure groups to Q, RA, Q+RA, and NGF. Based on three independent experiment duplicates conducted in n = 3 (biological repetitions), the mean ±SEM is used to indicate significant differences from the unexposed control group.

**3. Alteration in the gene expression of genes linked to neurogenesis**

We further estimated whether quercetin and Rosmarinic acid exert proliferative or differentiation efficacy in SH-SY5Ycells by studying the gene expression of NFM, Nestin and β-III tubulin alongside β-actin as an internal control. After considering the assessment, we observed that the cells that were exposed to the NGF exhibited a significant increase in the expression of NFM (327.52±112.17), Nestin (300.38 ± 84.46) and β III tubulin (458.18 ± 66.30) (Figure 3). A similar augmentation was further noted when the cells were exposed to Rosmarinic acid alone (NFM (239.00 ± 31.02), Nestin (215.00 ± 79.79) and β III tubulin (407.00 ± 27.25) and in combination with quercetin (NFM, 293 ± 69.06; Nestin, 272 ± 38.54; β III tubulin, 442 ± 23.68). Surprisingly, quercetin alone treatment didn't show the promising results as expected. Although combined with Rosmarinic acid, it shows the enhanced neurogenesis as compared to quercetin and rosmarinic acid alone treatment groups.



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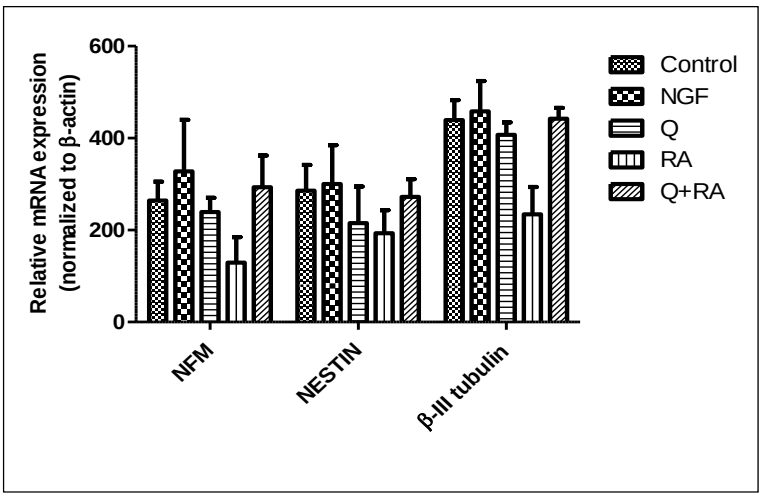


**Figure 3:** Q, RA, Q+RA, and NGF (positive control) were used to treat the cells. According to the materials and techniques, RNA was extracted. RT-qPCR was used to quantify the gene expression levels. The data that was shown was changed to β-actin. The mean ± SEM was used to express the data. It was found that none of the group's modifications were statistically significant.

***4 Quercetin and Rosmarinic acid in combination improved the proliferation, differentiation and neurogenesis***

Further, we also examined the changes in protein expression of NFM, Nestin and β-III tubulin using the immunoblotting technique. Expectedly, a significant surge in protein expression of NFM (1.375 ± 0.1359;*p<0.05), Nestin (1.291 ± 0.06580;*p<0.05) and β-III tubulin (0.962 ± 0.008555;**p<0.01) was observed in NGF-treated SH-SY5Ycells. The protein expression was insignificantly decreased with the exposure to quercetin. This downregulated gene expression was further upregulated remarkably NFM (1.534 ± 0.03049;**p<0.01), Nestin (1.390 ± 0.2031;*p<0.05) and β-III tubulin (1.459 ± 0.04676***p<0.001) when quercetin was co-administered with Rosmarinic acid to SH-SY5Ycells at a defined concentration. However, the levels were found to be in a similar manner when Rosmarinic acid (NFM,0.871± 0.1085; Nestin, 0.947± 0.1451; β III tubulin, (1.049± 0.03185) was exposed to neuroblastoma cells alone but in a non-significant fashion Figure 4.

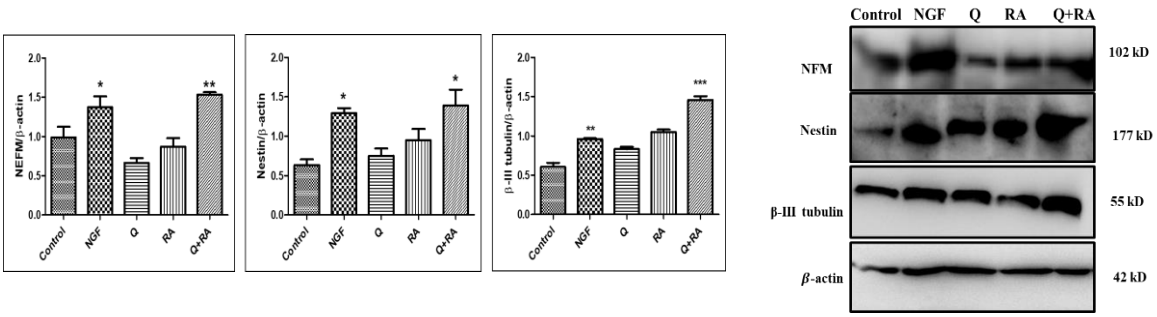


**Figure 4:** Changes in the neural markers' expression in SH-SY5Y cells subjected to a 48-hour Q, RA, or Q+RA treatment. As an internal control, β-actin was used to standardise the data. The relative protein level was determined using the densitometry reading for each band, and it was then normalised using the internal control. The mean ± SEM was used to display the data ± SEM. *p < 0.05, **p < 0.01, ***p < 0.001.

**Quercetin and Rosmarinic acid in combination enhanced the expression of microtubule-associated protein-2 and β-III tubulin**

Furthermore, upon assessment of neuron-specific cytoskeletal proteins MAP-2, it was found that quercetin and Rosmarinic acid combination showed a similar effect as NGF treated cells. As expected, the expression of MAP 2 was found to be significantly upregulated in NGF treated cells (5168.± 515.63) when compared to control (2413 ± 113.46;***p<0.001) (Figure 5). RA when co-administered with Q exerted an enhanced neurogenic potency with significant change in MAP2 content (3808 ± 178.26, **p<0.01) upon comparison to control. Although the individual treatment with both the phytochemicals (quercetin; 441.4 ± 34.97 and Rosmarinic acid; 1154.2 ± 52.86) did not show any significantly elevated expression instead, the expression of MAP-2 protein was found to be downregulated. Next, the levels of β-III tubulin were also found to be remarkably increased in the case of NGF treated cells (4146.8 ± 379.83) and the cells exposed with quercetin and Rosmarinic acid in combination (3464.4 ± 221.38; ***p<0.001) were found to have a significant difference when compared to untreated cells (2671.6 ± 173.79), and NGF treated cells (Figure 6). Also, when exposed alone, there was no significant change in the quercetin (885.4 ± 47.27) or Rosmarinic acid (1261.6 ± 69.67) exposed cells. However, the expression in alone treated cells was downregulated when compared with the cells treated in combination with both the phytochemicals.

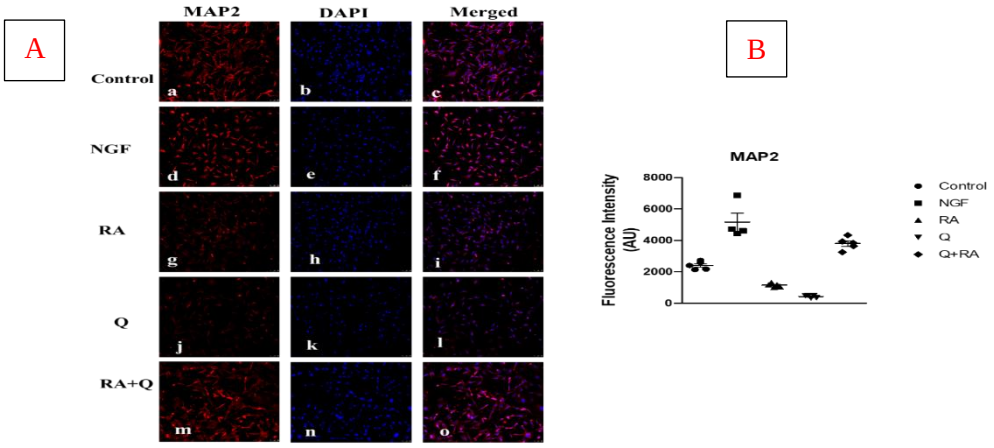


**Figure 5.** RA and Q have amplified the neurogenic marker MAP2 when exposed to cells in combination.(A) Immunocytochemistry (ICC) images of SH-SY5Y demonstrate the expression of functioning neuronal marker MAP2. The cells bearing labels (a-c) are control cells that are not exposed to the label, the cells treated with NGF (d-f) are examples of this type, only the cells exposed to RA (g-i) are examples of this type, and only the cells treated with Q (j-l) are examples of this type. Images were acquired with an Invitrogen EVOS FL fluorescent microscope. One hundred and millimeters is the distance bar. (B) A Leica microscope that is inverted; it has a digital camera that can detect fluorescence (Leica, Wetzlar, Germany). Images were subsequently created in graphical format. The fluorescence intensity was determined using the ImageJ program (NIH, USA). The results are reported as the mean SEM of two studies with n = 2 participants (biological replicates).

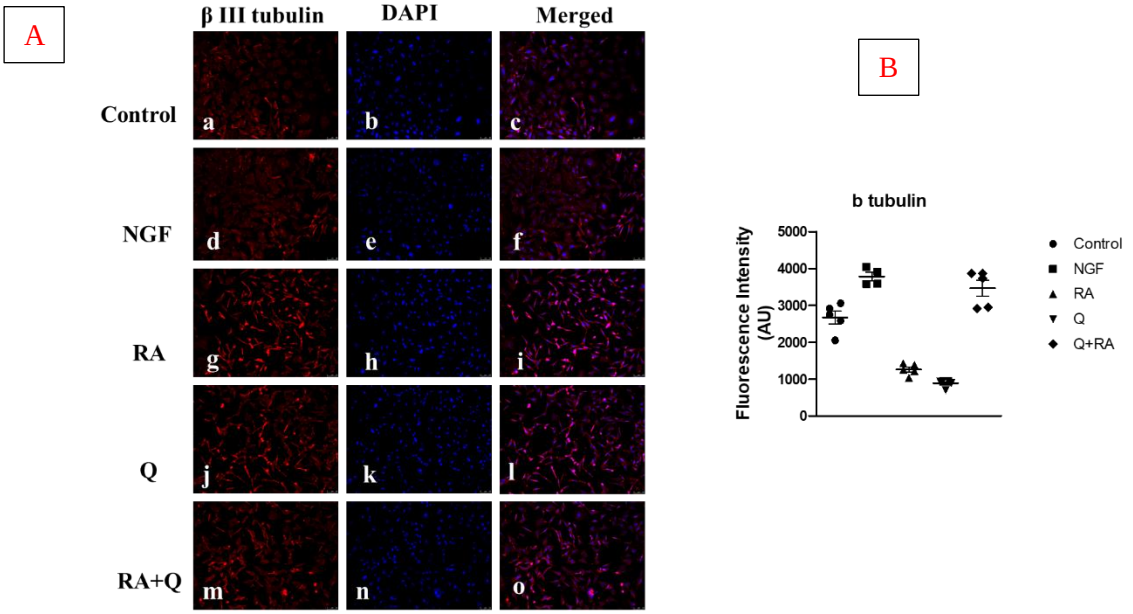


**Figure 6.** RA and Q have intensified the neurogenic marker β III tubulin expression when exposed to cells in combination. (A) Immunocytochemistry (ICC) images of SH-SY5Y reveal the expression of functioning neuronal marker β III tubulin. (a-c) control or unexposed cells, (d-f) NGF treated cells, (g-i), only RA exposed cells (j-l) only Q treated cells and (m-o) RA with Q co-treatment to cells. An Invitrogen EVOS FL fluorescent microscope was used to capture the images. The scale bar is 100 millimeters. (B) Fluorescence intensity was measured using Digital CCD camera with Leica inverted fluorescence microscope (DMI 6000) (Leica, Wetzlar, Germany), and images were rendered in graph format using ImageJ software (NIH, USA). The results are reported as the mean ± SEM of two studies with n = 2 (biological replicates).

## Discussion

A range of neurodegenerative illnesses is caused by neuronal damage and degeneration, defined by the destruction of brain cells and their axons, which results in functional deficiencies [28]. Because of the damaged neurons' inability to regenerate, altering the growth of neurites is of paramount importance in developing methods to enhance the regeneration of axons and dendrites following brain trauma and in diseases that have a degenerative nature [29]. Many recent studies have concentrated on the precise components of active herbs, and the therapeutic effects of well-known active substances, particularly their impact on neurodegenerative diseases, need to be dissected.

In previous study, it was evaluated that RA significantly increased the expression of the neuronal marker (nestin and β-III tubulin) in the Wharton's jelly stem cells. One study reported that RA protects the cells against $H_2O_2$ damage in N2A cells [30]. Therefore, based on the existing literature, in this research, we have explored the neurogenic capacity of plant-based well-known active compounds RA and Q in combination in neuroblastoma cells. The effects of RA and Q were compared with combined concentrations, and NGF was taken as a positive control. SH-SY5Y cell line, a widely used neuroblastoma cells in vitro neuron-like cell culture model, is widely used in *in vitro* to study the modifications in neurons induced by the toxicant and/or protective agents and neurogenic efficacy. Therefore, our study has reported the neuronal differentiation on morphology analysis when treated with 10 µM (for each) Q+RA combination for 48 h. Also, the effects of 10 µM RA alone on neuronal differentiation were higher than 10 µM Q alone. Immunofluorescence cell imaging provided additional support for these findings. We also have studied the cellular ROS generation in cells when treated with herbal compounds and it is found that Q and RA do not elevate ROS generation alone and in combined way. The same pattern we have reported when cells were studied for NO and MDA. Therefore, we can state from our study that RA and Q

will for sure be proved as anti-oxidative, anti-stress herbal compounds. Previous studies also support the neurogenic and protective efficacy of herbal compounds when co exposed with toxicant. RA was also reported to provide protection in *'In vivo'* system against chronic ethanol-induced learning and memory impairments in rats and scopolamine-induced memory impairment. RA has been examined in an experimental animal model as a potential treatment for ischemic stroke [31]. The impact of RA on the hippocampal antioxidant capacity was investigated in one study by measuring SOD and MDA levels [32]. Another study discovered that RA has anti-inflammatory properties in BV2 cells treated with LPS could be attributed to the inhibition of COX-2 and iNOS production, inhibiting inflammatory processes [33]. The effectiveness of chitosan-coated RA emulsion as a protective agent against memory loss, inflammation, and oxidative stress in rats is documented for the first time, with the decresease in TBARS levels. [34]. In our study, RA showed no cellular ROS generation which strongly indicates the antioxidative properties of herbal compounds (Q, RA). The combined RA+Q did not significantly impact on neuronal markers at transcriptional level but we reported significant upregulations in neuronal markers at translational level, which can be a possible promising outcome of neuroprotective efficacy.

## Conclusion

One of the most popular cell lines in the field of neurosciences is the SH-SY5Y human neuroblastoma cell line. The expression of various adult neuronal markers, including β-III tubulin, neurofilament proteins (NFM), and microtubule associated protein 2 (MAP2), is either triggered or enhanced with differentiation [35]. Quercetin, a bioactive molecule that has been demonstrated to have healing properties in-vivo animal models of neurodegeneration or neurotoxicity [36]. RA, another phytoactive constituent has also manifested some promising therapeutic effects in the field of neuroscience. But the combination of both phytoactive molecules have not yet been investigated. The present study henceforth targeted the enhanced effect of both phytoactive constituents in combination upon neurogenesis using human neuroblastoma cell line. The results showed some optimistic indications towards the usage of quercetin and Rosmarinic acid in combination. The enhanced potency towards neurogenesis could be observed with the enhanced expression of β-III tubulin and MAP-2 post exposure of cells with both RA and Q. Therefore, it could be suggested that the combination of both Q and RA would be a promising therapeutic agent for neuronal differentiation. Although exploration of plausible signaling pathways involved in the neuronal markers' upregulations with significant/non-significant impact on in RA or Q treated cells either alone or in combination is needed. In our future study, we will focus upon the signaling cascade molecules associated with the neuronal differentiation and neuronal markers upregulations in combination therapy of these two key herbal compounds Q+RA.

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**Data Availability:** Upon reasonable request, the corresponding author will provide the data supporting the study's findings.

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