# Contents

Evaluation of Neuroprotective Potential of Flavonoids In-Vitro.................................................. 117
12.1. Introduction ............................................................................................................................ 117
12.2. Materials and Methods ........................................................................................................ 118
12.2.1. Cell lines and maintenance ............................................................................................... 118
12.2.2. Chemicals ........................................................................................................................ 118
12.2.3. Apparatus ......................................................................................................................... 118
12.2.4. Treatments ....................................................................................................................... 118
12.2.5. Neuroprotection studies in-vitro....................................................................................... 118
12.2.5.1. In-vitro neuronal cell viability assays ............................................................................ 119
12.2.5.1.1. MTT assay .............................................................................................................. 119
12.2.5.1.2. Neuroprotection against DOX ................................................................................. 119
12.2.5.2. Cell cycle analysis by Flowcytometry ......................................................................... 119
12.2.5.3. Assessment of morphology and neurite length in differentiated IMR-32 cells .......... 119
12.2.5.4. Detection of apoptosis in IMR-32 cells by AO/EB staining and Annexin V assay by flowcytometry ................................................................................................................................................... 120
12.2.5.4.1. AO/EB staining ....................................................................................................... 120
12.2.5.4.2. Annexin V assay ..................................................................................................... 120
12.2.5.5. Intracellular ROS estimation ...................................................................................... 121
12.2.6. Organ protection studies in-vitro...................................................................................... 121
12.3. Results .................................................................................................................................... 121
12.3.1. In-vitro neuronal cell viability assays .............................................................................. 121
12.3.1.1. MTT assay ................................................................................................................. 121
12.3.1.2. Neuroprotection against DOX in IMR-32 and PC12 cell lines ................................ 122
12.3.2. Cell cycle analysis by flowcytometry in IMR-32 cells .................................................. 123
12.3.3. Assessment of morphology and neurite length in differentiated IMR-32 and PC12 ..... 123
12.3.4. Detection of apoptosis in IMR-32 or PC12 by AO/EB and Annexin V staining ............ 125
12.3.5. Intracellular ROS estimation ........................................................................................... 128
12.3.6. Major organ protection studies in-vitro .......................................................................... 129
12.4. Discussion ............................................................................................................................... 130
12.5. Conclusion .............................................................................................................................. 131
Bibliography .................................................................................................................................... 132
Evaluation of Neuroprotective Potential of Flavonoids In-Vitro

12.1. Introduction

Most accepted molecular mechanism underlying promising therapies against numerous neurodegenerative diseases involving cognitive decline is neuroprotection. This protective potential of promising therapies safeguards from neuronal damage and preserves the neuronal stores especially in hippocampus and frontal cortex anatomical structures, which thought to play a pivotal role in cognitive processing underlying neurobiology of learning and memory. Chemotherapy due to its nonspecific cytotoxic action on healthy living cells, can target neuronal cells in CNS. This can ultimately result in possible cognitive deterioration seen with chemobrain condition affecting the activities of daily living (ADL) in cancer survivor population.

We hypothesize that, the protection of flavonoids therapy against DOX-induced chemobrain may be attributed to their potential neuroprotective effect against DOX-induced neuronal damage. Hence the flavonoids, NAR and RUT were assessed for their neuroprotective ability against cytotoxic agent, DOX in-vitro in diverse neuronal cell lines using various in-vitro assays viz., cell viability assay (MTT), neurite outgrowth assay, detection of apoptosis by (AO/EB) staining, intracellular reactive oxygen species (ROS) assay and flowcytometric analysis were carried out to assess neuroprotective potential of test flavonoids against DOX-induced neurotoxicity in-vitro.

As DOX is well known for its dose limiting cardio and nephrotoxicities, additionally, we assessed protective effect of these flavonoids against DOX-induced toxicity in cardiac myoblasts (H9C2) and HEK (human embryonic kidney) cells for cardio and nephroprotective activities too. Although, DOX does not cross BBB significantly, the levels that reach CNS are more than sufficient to kill the most sensitive hippocampal neuronal stem cells in the region of dentate gyrus (DG). Furthermore, clinically lyophilized DOX formulations, viz. lipodox etc. are in current use for many forms of cancers (especially breast cancer) which can easily cross BBB and enter CNS with the resulting direct neurotoxic effects on very sensitive neuronal stem cell population. Hence, we assessed the protective effect of flavonoids against DOX in-vitro and demonstrated that, the protective nature of flavonoids against DOX-induced chemobrain is probably attributed to their potential neuroprotective effect.
12.2. Materials and Methods

12.2.1. Cell lines and maintenance

IMR-32 (human neuroblastoma cell line), PC12 (rat pheochromocytoma derived neuronal cell line), H9C2 (rat cardiac myoblasts) and HEK (Human embryonic kidney cells) were procured from National Centre for Cell Science (NCCS), Pune, India. IMR-32, H9C2 and HEK cells were maintained in DMEM supplemented with 10% FBS and suitable antibiotic, i.e. gentamycin (100 µg/ml) in T-25 culture flasks in CO2 incubator providing humidified environment comprising of 95% air and 5% CO2. PC12 cells were maintained in DMEM supplemented with 10% horse serum and gentamycin (100 µg/ml) in T-25 culture flasks.

12.2.2. Chemicals

DOX (Fresenius Kabi Oncology Ltd., Solan), NAR (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan) were procured. RUT, trypsin-EDTA, 3-[4,5dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), horse serum, retinoic acid (RA), acridine orange/ethidium bromide (AO/EB) were obtained from HiMedia Laboratories, Mumbai, India. Trypan blue (0.4% w/v), dichlorofluorescin diacetate (DCFDA), Dulbecco’s modified eagles medium (DMEM), Nerve growth factor (NGF) were purchased from Sigma-Aldrich Co. LLC, St. Louis, MO, USA. 96 and 6 well plates; 25 and 75 cm² tissue culture flasks (Tarsons) were obtained. All other chemicals used in this study were of reagent grade.

12.2.3. Apparatus

Vertical laminar air flow (Labline Instruments, Kochi, India), CO2 incubator (NuAire nu8500, USA), inverted microscope (Nikon, Eclipse TS-100, NY, USA), ELISA plate readers (ELx800 and FLx800; BioTek Instruments Inc., Winooski, VT, USA) for colorimetric as well as flourimetric analysis, flowcytometer (BD Accuri™ C6; BD Biosciences, San Jose, CA, USA) for cell cycle analysis were used in this study.

12.2.4. Treatments

NAR and RUT were made soluble at different concentrations in 0.1% v/v DMSO diluted with DMEM. DOX was prepared as clear red colored solution in DMEM. All the treatments were given as per the protocol accordingly.

12.2.5. Neuroprotection studies in-vitro

These studies were performed using two cell lines, IMR-32 and PC12 cells which are the widely used in-vitro neuronal research tools to study neuroprotection phenomenon (Hanstein et al., 2009; Huang et al., 2013; Kataria et al., 2012; Santos et al., 2015; Tabakman et al., 2002). IMR-32 is human derived and PC12 is derived cell line from tumor of rat adrenal gland, both were employed depending on the suitability of test to assess neuroprotection.
12.2.5.1. In-vitro neuronal cell viability assays

12.2.5.1.1. MTT assay

Cell viability was assessed using MTT assay. Cells (5 x 10^3) were seeded in 96 well plates and following 24 h of adherence, treatment was given with either test flavonoids at different concentrations (ranging from 50 to 500 µM) or DOX (ranging from 0.05 to 50 µM) and cell viability was assessed following 24 h of further incubation (Gerlier and Thomasset, 1986).

12.2.5.1.2. Neuroprotection against DOX

The neuroprotective potential of test flavonoids was assessed against DOX-induced neurotoxicity in IMR-32 and PC12 cells using MTT assay. Non-toxic concentrations of test flavonoids (50 and 100 µM) were selected. Following 2 h of incubation with flavonoids at predetermined concentrations, toxicant DOX (IC_{50}=1 µM) was added and incubated further for 24 h. Then, the intensity of purple color formed was noted using micro plate reader (ELx800; BioTek Instruments Inc., Winooski, VT, USA) at 540 nM which reflects the direct measurement of neuronal cell viability.

12.2.5.2. Cell cycle analysis by flowcytometry

Using flowcytometric analysis, one can assess the effect of test compounds on various phases of cell cycle. This assay was carried out only in IMR-32 cells, because the other cell line, i.e. PC12 cells morphologically forms cellular aggregates and clumps which may not result in single cell suspension required for flowcytometric analysis.

**Methodology**

IMR-32 cells were seeded in 6 well plates and after 24 h of inoculation, cells were incubated with test flavonoids at 100 µM for 2 h. Cells were then exposed to DOX (1 µM) and further incubated for 24 h. Then cells were fixed in 70% v/v ethanol for 4 h and trypsinised, centrifuged, suspended in PBS containing propidium iodide with RNAse and kept in dark for 20 min. Samples were analyzed by using a flow cytometer (BD Accuri™ C6; BD Biosciences, San Jose, CA, USA) (Shoja et al., 2015).

12.2.5.3. Assessment of morphology and neurite length in differentiated IMR-32 cells

Neuroprotective ability of test compounds was determined through examination of morphological features of differentiating and matured neuronal structures, i.e. formation of elongated neurite outgrowth and clear nucleus with formation of dendrite structures. In contrast, neurotoxic agents can inhibit this process of differentiation and formation of mature neurons. Hence the protective potential of test flavonoids was assessed using this morphological examination against DOX-induced neurotoxicity.
Chapter 12
Evaluation of Neuroprotective Potential of Flavonoids In-Vitro

Methodology

IMR-32 cells were treated with retinoic acid (10 µM) to induce differentiation into matured neurons. Then they were treated with flavonoids at 100 µM for 2 h followed by DOX exposure and further incubated for 24 h. Cells were examined using an inverted microscope (Eclipse TS100F; Nikon Instruments Inc., Melville, NY, USA). For measuring neurite length, roughly 60-70 images were captured randomly from each treatment well and length of each neurite drawn was measured using Image J software supplemented with Neuron J plug-in by National Institute of Health (NIH) (Meijering et al., 2004; Nampoothiri et al., 2014).

12.2.5.4. Detection of apoptosis in IMR-32 cells by AO/EB staining and Annexin V assay by flowcytometry

To identify and recognize the cellular death produced, i.e. apoptosis (programmed cell death), AO/EB and Annexin V staining method was used. We assessed the percentage of apoptotic cells produced by DOX and possible protection by flavonoids.

Methodology

12.2.5.4.1. AO/EB staining

Differentiated IMR-32 cells were incubated with either DOX (1 µM) alone or in combination with test flavonoids (at 100 µM, 2 h incubation prior to DOX exposure) in 6 well plates. Following 24 hours of incubation, wells were washed with PBS, then cells were fixed using 1 ml of ice cold ethanol (100%) for 10 min at room temperature. Cells were again washed with PBS and 1 ml AO/EB reagent stain was added to each well and incubated at 37º C for 10 min. Individual cells were observed under an inverted fluorescent microscope. Morphology of apoptotic and necrotic cells was recognized based on the staining pattern as described elsewhere (Ribble et al., 2005).

12.2.5.4.2. Annexin V assay

RUT was further tested for apoptotic assessment as it showed promising effect with AO/EB staining. The Annexin V apoptosis assay was performed as per the manufacturer’s instruction manual. Briefly, 2×10^4 differentiated cells/well seeded in a six-well tissue culture plate, were treated with 1 µM DOX alone or a combination of DOX (1 µM) and RUT (50 and 100 µM) after 12 hours of seeding and then incubated for 48 hours. After 48 hours, the cells were trypsinised and centrifuged. After centrifugation, the cell pellets were resuspended in 100 µl of media containing 10% FBS. Approximately 100 µl Annexin V was added to the cell suspension and incubated for 20 minutes. The apoptotic profile of the cells was analyzed using Muse Cell Analyzer (Model Number 0500-3115, Merck Millipore, Billerica, MA, USA).
12.2.5.5. Intracellular ROS estimation

It is a well-known fact that most of the chemotherapeutic drugs including DOX act by producing the reactive oxygen free radicals which are highly lethal to cellular populations. Hence we assessed this intracellular ROS formation with DOX treatment and also explored whether the test flavonoids, NAR and RUT were able to prevent DOX-induced ROS, so as to protect from oxidative stress-induced cellular damage.

Methodology

IMR-32 or PC12 cells in black 96 well plate were incubated with flavonoids, NAR or RUT at 100 µM for 2 h and then subjected to DOX (1 µM) exposure. After 24 h of incubation, culture supernatants were discarded and replaced with 100 µl of dichlorofluorescein diacetate (DCFDA, 100 µM). Following 1 h incubation with fluorescence dye, cells were washed with sterile Hank’s balanced salt solution (HBSS). Fluorescence intensity was noted with the help of fluorescence micro plate reader (FLx800; BioTek Instruments Inc., Winooski, VT, USA). The ROS level was measured and calculated with respect to media control (Wang and Joseph, 1999).

12.2.6. Organ protection studies in-vitro

Cardioprotective and nephroprotective studies were conducted in-vitro using H9C2 and HEK cell lines respectively. Cells were seeded in 96 well plates and DOX, test flavonoid treatments were given as per the protocol for assessment of cell viability as mentioned on page 119.

Methodology

Non-toxic concentrations of test flavonoids i.e. 50 and 100 µM were selected. Following 2 h of incubation with flavonoids at pre-determined concentrations, toxicant DOX (IC50=1 µM) was added and incubated further for 24 h. Then, the intensity of purple color formed was assessed at 540 nM which reflects the cell viability.

12.3. Results

12.3.1. In-vitro neuronal cell viability assays

12.3.1.1. MTT assay

From the preliminary studies in our lab, IC50 for DOX was noticed at 1 µM in IMR-32 as well as PC12 cells. Hence this concentration was used for all further studies to assess neuroprotective ability of test flavonoids. IC50 values of all the test flavonoids was found to be in the range of 250-400 µM in both the cell lines. Two concentrations of flavonoids that showed more than 70% viability were selected for assessing neuroprotective ability against DOX-induced toxicity. For flavonoids, 50 and 100 µM concentrations were found to produce
about 70% viability. Hence these concentrations were used for assessing neuroprotective ability against DOX (1 µM). For studies, viz., flow cytometry, AO/EB staining etc., we used only one concentration at 100 µM as it showed better neuroprotection profile at this level.

**12.3.1.2. Neuroprotection against DOX in IMR-32 and PC12 cell lines**

Significant reduction in cell viability was noted with DOX treatment as compared to media control. However prior treatment with flavonoids resulted in significant improvement of cell viability in contrast to DOX control which indicates the neuroprotective nature of these natural flavonoids against DOX. Pretreatment with flavonoids significantly attenuated DOX-induced neuronal cell death in a dose dependent manner, the most effective neuroprotective agent was found to be RUT at 50 and 100 µM (Fig.12.1).

![Graph showing effect of flavonoids on DOX-induced neurotoxicity in IMR32 cells using MTT assay. Data represents mean ± SEM of % cell viability, **p<0.01 and ***p<0.001 vs. DOX, (n=6).](image)

In case of PC12 cell line also, DOX (1 µM) significantly reduced cell viability as compared to media control. However prior treatment with either NAR or RUT dose dependently inhibited DOX-induced neurotoxicity, significantly improved cell viability as compared to DOX control (Fig.12.2).

![Graph showing effect of NAR and RUT on DOX-induced neurotoxicity in PC12 cells using MTT assay. Data represents mean ± SEM of % cell viability, *p<0.05, **p<0.01 and ***p<0.001 vs. DOX, 1 µM (n=6).](image)
12.3.2. Cell cycle analysis by flowcytometry in IMR-32 cells

DOX arrested IMR-32 cells at G2/M and S phases. Hence the % population was increased at G2/M (M2) and S (M3) phase whereas the population at G0/G1 (M1) was remarkably reduced due to inhibition of the cell cycle progression as compared to normal control.

![Flowcytometry analysis](image)

Fig. 12.3. Effect of flavonoids on various phases of cell cycle using flowcytometric analysis in IMR-32 cells. Histogram a, whole cell population with cell debris; b, gated for actual cell population; c, normal control (NC); d, DOX (1 µM) treated; e, NAR and f, RUT treated at 100 µM respectively prior to DOX. M1 represents population percentage in G0/G1 phase, M2 represents cell population in G2/M phase and M3 reflects the cellular population in S phase of cell cycle.

12.3.3. Assessment of morphology and neurite length in differentiated IMR-32 and PC12 cells

Treatment with DOX resulted in morphological alterations in neuronal cells. The nucleus was condensed and cell membrane was fragmented due to toxicant insult, whereas prior treatment with flavonoids showed most of the cells comparable to that of media control. DOX exposure significantly \( (p<0.001) \) inhibited the development of neurite outgrowth in differentiated IMR-32 cells compared to media control.
Chapter 12

Evaluation of Neuroprotective Potential of Flavonoids In-Vitro

Fig. 12.4. Effect of test flavonoids on neurite outgrowth and morphology. Illustration shows representative images of (a) Normal media control, (b) DOX control, (c) Treatment with NAR prior to DOX, (d) Treatment with RUT prior to DOX (40X-Magnification, arrow marks indicates development of the neurite).

However, treatment with test flavonoids at 100 µM showed prominent establishment of neurite outgrowth and hence, significantly \((p<0.001)\) averted inhibitory effect of DOX. Hence this indicates that flavonoids protected the differentiated neuronal cells in due course of neurite development against the neurotoxic insult produced by DOX. The most effective treatment in promoting the neurite outgrowth against DOX was found to be RUT. The neurite development and its length were illustrated in Fig. 12.4 & Fig. 12.5.

Fig. 12.5. Effect of test flavonoids against DOX-induced inhibition of neurite length in IMR-32 cells. Data represents mean ± SEM of neurite length, *\(p<0.001\) vs. media control, #\(p<0.001\) vs. DOX control (n=50).
Chapter 12  Evaluation of Neuroprotective Potential of Flavonoids In-Vitro

In case of PC12 cell line also, NAR and RUT were tested for their neurite promoting ability against inhibitory action of DOX. It was noted that, DOX (1 µM) significantly attenuated the neurite outgrowth and length as compared to media control. However prior treatment with either NAR or RUT significantly improved neurite development as compared to DOX control. Most effective treatment was found to be RUT at 100 µM (Fig. 12.6 & 12.7).

Fig. 12.6. Effect of test flavonoids on neurite outgrowth and morphology in PC12 neuronal cells. Illustration shows representative images of media control, DOX control, treatment with either NAR or RUT prior to DOX (40X-Magnification).

Fig. 12.7. Effect of test flavonoids against DOX-induced inhibition of neurite outgrowth in PC12 cells. Data represents mean ± SEM of neurite length, *p<0.001 vs. media control, #p<0.001 vs. DOX control (n=50).

12.3.4. Detection of apoptosis in IMR-32 or PC12 by AO/EB and Annexin V staining

Apoptosis by AO/EB staining showed that DOX produced marked apoptotic cell death in IMR-32 cells with the observation of bright condensed nuclei and fragmented cellular
Chapter 12  Evaluation of Neuroprotective Potential of Flavonoids In-Vitro

structures (Fig. 12.8). The percentage of cells with apoptotic morphology were noted. The percentage of apoptotic cells was significantly ($p<0.01$) high in DOX group when compared to the normal media control (Fig. 12.9). RUT (but not NAR) prior treatment reversed DOX-induced elevation in apoptotic cell death and produced significant decline ($p<0.01$) in percentage of apoptotic cells. This showed that neuroprotective potential of flavonoids may be due to inhibitory action on apoptotic pathways. Most effective was found to be RUT at a tested concentration of 100 µM.

![Fig. 12.8. Detection of apoptosis using AO/EB staining method in IMR-32 cells. Illustration shows representative images of a) Normal media control, b) DOX control, c) Treatment with NAR prior to DOX, d) Treatment with RUT prior to DOX.]

![Fig. 12.9. Effect of flavonoids on % of apoptotic cells in IMR-32 cells. Data represents mean ± SEM of % of apoptotic cells, *$p<0.01$ vs. media control, #$$p<0.01$$ vs. DOX control. Annexin V assay was conducted with RUT. RUT dose dependently reduced the number of apoptotic cells. Treatment with DOX (1 µM) induced early and late apoptosis in IMR-32]
cells (Fig. 12.10). Pretreatment with RUT (50 and 100 µM) decreased the number of apoptotic cells induced by DOX, indicating the neuroprotective effect of RUT. Furthermore, RUT prevented early, rather than late apoptosis in IMR-32 cell lines challenged with DOX.

![Figure 12.10](image1.png)

**Fig. 12.10.** Effect of RUT on apoptosis profile in IMR-32 cells by Annexin V staining. (a) Normal control, (b) DOX (1 µM), (c) DOX (1 µM) + RUT (50 µM) and (d) DOX (1 µM) + RUT (100 µM).

With PC12 cell line also, DOX significantly (p<0.001) elevated the number of apoptotic cells and prior treatment with flavonoids RUT at 100 µM significantly (p<0.01) reduced the number of apoptotic cells (Fig.12.11 & Fig. 12.12). However, NAR showed a comparative decrease in apoptotic cells without any significant difference as compared to DOX control.

![Figure 12.11](image2.png)

**Fig. 12.11.** Detection of apoptotic cells using AO/EB staining method in PC12 cells. Illustration shows representative images of media control, DOX control and treatment with either NAR or RUT prior to DOX.
Chapter 12  Evaluation of Neuroprotective Potential of Flavonoids In-Vitro

![Bar chart showing effect of flavonoids on percentage of apoptotic cells in PC12 cell line.](image)

**Fig. 12.12.** Effect of flavonoids on percentage of apoptotic cells in PC12 cell line. Data represents mean ± SEM of percentage of apoptotic cells, ***p<0.001** vs. media control, **##p<0.01** vs. DOX control.

**12.3.5. Intracellular ROS estimation**

DOX produced significant (p<0.001) elevation of intracellular ROS as compared to the normal media control. However, prior treatment with flavonoids significantly (p<0.001) inhibited the ROS generation by DOX which shows the potential antioxidant role of these flavonoids and thereby scavenging the intracellular ROS that was generated by DOX (Fig. 12.13).

![Graph showing effect of flavonoids on intracellular ROS generation in IMR-32 cells.](image)

**Fig.12.13.** Effect of test flavonoids on intracellular ROS generation in IMR-32 cells. Data represents mean ± SEM of ROS as percentage of control, ***p<0.001** vs. media control, **##p<0.01, ###p<0.001** vs. DOX control (n=6).
12.3.6. Major organ protection studies in-vitro

Test flavonoids significantly protected both H9C2 and HEK cell lines from DOX-induced cardio and nephrotoxicity respectively.

![Cardioprotection in H9C2](image1)

**Fig.12.14.** Effect of flavonoids on DOX-induced cardiotoxicity in cardiac myoblasts. Data represents mean ± SEM of percentage of viable cells considering DMSO control as 100% viability, *p<0.05, ***p<0.001 vs. DOX control (n=6).

Both the flavonoids have protected the cell lines from DOX-induced toxicity in a dose dependent manner at two tested doses, i.e. 50 and 100 μM (Fig. 12.14 & Fig. 12.15). However, the most effective treatment in both the cases was found to be RUT.

![Nephroprotection in HEK](image2)

**Fig.12.15.** Effect of flavonoids on DOX-induced nephrotoxicity in human embryonic kidney cells. Data represents mean ± SEM of percentage of viable cells considering DMSO control as 100% viability, *p<0.05, **p<0.01, ***p<0.001 vs. DOX control.
Chapter 12 Evaluation of Neuroprotective Potential of Flavonoids In Vitro

12.4. Discussion

In this chapter, we focused on evaluating the possible neuroprotective role of flavonoids, NAR and RUT against DOX in various cell lines in-vitro which could be likely a molecular mechanism underlying their protective potential against DOX-induced chemobrain in-vivo.

Neuronal cell viability was assessed using MTT assay and we found that, the IC$_{50}$ of DOX was 1 $\mu$M in IMR-32 and PC12 cell lines. Hence this IC$_{50}$ value was used in neuroprotective studies of flavonoids against DOX-induced neurotoxicity in all further studies. IC$_{50}$ values of flavonoids were found to be in the range of 250-400 $\mu$M. The concentrations of flavonoids which allowed growth of around 70% of cells as compared to DMSO control, i.e. 50 and 100 $\mu$M were tested to assess their neuroprotective potential against DOX.

Prior treatment with test flavonoids resulted in significant protection of either IMR-32 or PC12 neuronal cells in a dose dependent manner against DOX-induced neurotoxicity. Hence, wherever feasible in all further studies, highest concentration was used i.e., 100 $\mu$M as in case of flowcytometric analysis, detection of apoptosis by AO/EB staining, morphology and neuritogenic assay etc.

In flowcytometric analysis using IMR-32 cells, DOX was found to arrest the cell cycle in G$_2$/M phase whereas prior treatment with test flavonoids NAR and RUT did not prevent the changes as a result of DOX. Flavonoids are converted into pharmacologically active moieties in-vivo, hence this could be the reason for lack activity in flowcytomtetry and the active aglycone parts may show neuroprotective potential in this assay.

Establishing the neurite outgrowth is essential for making synapses and connections with other neuronal cells so as to develop and maintain synaptic plasticity and thereby the neurobiological mechanism involved in learning and memory, i.e. long-term potentiation (LTP) (Cao et al., 2009). It was identified that synaptic activity in developing neurons further promotes dendritic arbor elaboration and stabilizes dendritic structure, which is critical for synaptic remodeling during memory processing (Cline, 2001; Jan and Jan, 2001). With the morphological assessment and neurite outgrowth of IMR-32 cells and PC12 cells, we observed that DOX inhibited the development of neurite whereas pretreatment with test flavonoids resulted in significant protection against DOX through enhancement of neurite outgrowth. Here, in view of neuritogenic potential, the most effective treatment was found to be RUT at 100 $\mu$M compared to NAR.

Detection of apoptosis in differentiated neuronal cells by AO/EB staining revealed that, DOX significantly increased the number of apoptotic cells as compared to media control in IMR-32 and PC12 cell lines. However prior treatment with RUT but not NAR significantly (p<0.01)
Chapter 12  Evaluation of Neuroprotective Potential of Flavonoids In-Vitro

averted this DOX-induced increase in apoptotic cells in IMR-32 cells. With Annexin V assay, RUT dose dependently averted the number of apoptotic cells undergoing apoptosis. Similarly, with PC12 cell line, RUT but not NAR was able to diminish DOX-induced elevation of percentage of apoptotic cells significantly (p<0.01). Hence inhibiting the apoptotic path ways (preventing the proapoptotic factors, e.g. BAX, BAD, BIM, caspase-8 and caspase 10 etc. and enhancing the activity of anti-apoptotic factors, e.g. Bcl2, Bcl-xl etc.) could be the mechanism of action attributed to the neuroprotective ability of test flavonoids against DOX-induced neurotoxicity.

Further, DOX exposure has elevated the intracellular ROS as compared to the media control whereas prior treatment with test flavonoids at 100 µM has resulted in significant inhibition of ROS generation intracellularly. This proves the potential antioxidant and free radical scavenging ability of natural flavonoids against oxidative damage to the cellular structures. The test flavonoids significantly alleviated the DOX-induced cell death either in cardiac myoblasts (H9C2) or human embryonic kidney (HEK) cell lines dose dependently which proved their potential cardioprotective and nephroprotective effects respectively. This could be the possible mechanism by which the DOX-induced organ toxicity that was found in-vivo during chemobrain animal models was efficiently averted by flavonoid co-treatment.

12.5. Conclusion

NAR and RUT offered significant protection against DOX-induced neuronal toxicity in two diverse neuronal cell lines, the most effective being RUT. Inhibition of apoptosis, moderate effects on DOX-induced cell cycle changes, neurite promoting effect and also the potential antioxidant effects would have possibly underlie the neuroprotective effect of test flavonoids. Further, they offered significant organ protective effects in-vitro for DOX-induced cardiotoxicity and nephrotoxicity which further supported their therapeutic potential to alleviate DOX-induced systemic organ toxicity along with the preventing effect on chemobrain.
Chapter 12 Evaluation of Neuroprotective Potential of Flavonoids In-Vitro

Bibliography


Chapter 12  Evaluation of Neuroprotective Potential of Flavonoids In-Vitro

