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***Original Research***
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# Neuroprotective Effect of Naringin on Acrylamide-induced Cytotoxicity in U87MG Cells and Wistar Rats: An *in-vitro* and *in-vivo* Study

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## Abstract

Acrylamide (ACR) is a potent neurotoxic to humans and animals. Neuroprotective effect of naringin was evaluated on ACR induced cytotoxicity using U87MG cells as *in-vitro* model and rat as *in vivo* model. ACR (50 mg/kg, i.p.) and Naringin (50 and 100 mg/kg) were administered to rats for 4 weeks. After 4 weeks, rats were sacrificed and sciatic nerves were isolated to determine the biochemical and apoptotic parameters. The exposure of U87MG cells to ACR reduced cell viability. Pretreatment of cells with 5–300 µg/ml naringin before ACR treatment significantly attenuated ACR cytotoxicity in a dose-dependent manner. Naringin down-regulated the Bax and up-regulated the Bcl2 protein expression levels and also scavenged ACR induced free radicals in rats. In conclusion, our results indicated that pretreatment with naringin protected cells and rats from ACR-induced cytotoxicity and the neuroprotective effect was due to its antiapoptotic and antioxidant potential.

**Keywords:** Acrylamide, Apoptosis, Naringin, Neurotoxicity, Oxidative Stress, U87MG Cells

## 1. Introduction

Nowadays food is the risk factor as it has toxic chemicals due to food processing or microbes or combination of both. One such risk factor present in food is Acrylamide. Acrylamide (ACR) with the molecular formula of CH2=CH–CO–NH2, IUPAC name of 2-propenamid, and also chemical names such as prop-2-enamide, 2-propenamide, acrylic amide or ethylene carboxamide; is easily dissolved in water and organic solvents such as methanol or ethanol[1,2]. ACR is formed during frying, deep frying and baking (at the temperature exceeding 120°C via Millard's reaction) in high-carbohydrate foods and especially in asparagine amino acid-rich foods[3]. Europeans and Americans mean daily intake of ACR is between 0.14 and 1.31µg/kg and 0.43 to 1.1 µg/kg, respectively[4]. But acceptable daily intake limit of ACR is 1 µg/kg[5]. Daily consumption of processed food is the main source of ACR in the general population.

Although polyacrylamides are non-toxic, ACR monomer is a potent neurotoxic and damages the central and the peripheral nervous system in humans and animals[6]. ACR induces ataxia, skeletal muscle weakness, and weight loss in both occupationally exposed humans and experimental animal models[7]. Currently, there is no ideal drug available for the treatment of neuropathy, phytochemicals are a safe alternative to prevent or treat neuropathy.

Naringin (4, 5, 7-trihydroxy-flavonone-7-rhamnogluco side) is a major flavonone glycoside present in grapefruits and citrus fruits (Citrus paradise, Citrus sinensis, Citrus unshiu, and Artemisia selengensis, as well as fruits of Pon cirus), and is responsible for the characteristic sour

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flavor of fruits8. Naringin has multiple pharmacological properties, including antioxidannt[9,10], anti-inflammatory[11], anti-apoptotic[12], antihypercholesterolaemic[13], and neuro protective effects[14]. The present study is designed to investigate neuroprotective effects of Naringin in Uppsala 87 Malignant Glioma cells (U87 MG cells) as an *in-vitro* model and ACR induced Wistar rat model as *in-vivo* neurotoxicity model.

## 2. Material and Methods

Naringin was purchased from Sisco Research Laboratory, Bangalore, India. Acrylamide was obtained from Merck, India. All other chemicals used were of analytical grade.

### 2.1 Cell Culture

U87MG cell line was obtained from National Center for Cell Science (NCCS), Pune, India. Cells were maintained in a humidified atmosphere of 5% $CO_2$ at 37$^0$C. Cells were grown in Dulbecco's Modified Eagles medium (Gibco Invitrogen, Paisley, UK) supplemented with 10 % fetal bovine serum, 1 % non-essential amino acids, 1 % penicillin (1000U/mL), 1 % streptomycin (1000 µg/mL) and 1 % amphotericin (250 U/mL). The cells were passaged enzymatically with 0.25 % trypsin- 1 mM EDTA and sub-cultured on 75 $cm^2$ plastic flasks at a density of 2.2 x 104 cells/$cm^2$. Culture medium was replaced every 2 days. Cell confluence (80 %) was confirmed by microscopic observance. Treatment was performed 12 hours post-seeding to prevent cell differentiation.

### 2.2 Cell Viability

The viability of cultured cells was determined by assaying the reduction of 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) to formazan. Briefly, U87MG cells were cultured in a 96- well microtiter plate at a density of 105cells/well. After pretreatment with Naringin (5, 50, 100, 150, 200, 250, 300 µg/ml) for 24 h, ACR at a concentration of 5.46 mM was added to each well to know the neuroprotective effect of naringin on ACR cytotoxicity. The cells were then incubated for 48 and 72hrs and then treated with MTT solution (0.5 mg/ml PBS) at 37$^0$C until intracellular purple formazan crystals were visible under a microscope. 100 µL of the dimethyl sulfoxide (DMSO) was added to each well and mixed gently on an orbital shaker for one hour at room temperature. The volume of DMSO was adjusted depending on the volume of cell culture. Absorbance was measured at 570 nm for each well on an absorbance plate reader.

### 2.3 Animals

Male rats of Wistar strain weighing 280–300 g were used for the study. The rats were maintained under 12 h light/dark cycle and had free access to food and water. The study protocol was approved by the Institutional Animal Ethics Committee (No. 1529/PO/Re/11/CPCSEA./CHIPS/IAEC7/PRO-7/2019-20).

### 2.4 Experimental Design

Rats were randomly divided into 5 groups (n=6 in each group). Group-I rats served as control and received the vehicle Carboxy methyl cellulose. Group II, III, IV and V rats were administered with ACR (50 mg/kg, i.p.) for 4 weeks. After one hour of ACR administration, group II rats received the vehicle, group III rats received pregabalin 10 mg/kg, group IV rats received naringin 50 mg/kg and group V rats received naringin 100 mg/kg orally for 4 weeks. Rats were monitored regularly for the manifestation of neuropathy. Finally, rats were sacrificed by cervical dislocation; the sciatic nerves (SN) were isolated and processed for biochemical analysis.

### 2.5 Estimation of Superoxide Dismutase (SOD)

SOD activity was estimated according to the method of Misra and Fridovich., 1972. In brief, the homogenate was centrifuged at 10,000 rpm for the enzyme assay. 100µl of sciatic nerve homogenate was added to 880 µl of carbonate buffer (0.05M, pH -10.2, containing 0.1mM EDTA), and 20 µl of 30 mM epinephrine (in 0.05% acetic acid) was added to the mixture and the optical density values were measured at 480 nm for 4 min on a UV-Vis Spectrophotometer. One unit of activity is expressed as the amount of enzyme that inhibits the oxidation of epinephrine by 50%[15].

### 2.6 Assessment of Lipid Peroxidation (LPO)

LPO was assessed by measuring the formation of thiobarbituric acid reactive substances (TBARS). The reaction mixture contained 0.2 ml of sciatic nerve homogenate, 1.5 ml of acetic acid (pH 3.5, 20 %), 1.5 ml of 0.8 % thiobarbituric acid (0.8 % w/v) and 0.2 ml Sodium dodecyl sulphate (SDS) (8 % w/v). The mixture was heated to boiling for 45 min and TBARS adducts were extracted into 3 ml of 1-butanol and its absorbance was measured at 532 nm and quantified as malondialdehyde (MDA) equivalents using 1,1,3,3-tetra methoxy propane as the standard[16].

### 2.7 Reduced Glutathione (GSH)

Reduced glutathione was measured according to the method of Ellman GL., 1959[17]. Equal quantity of sciatic nerve homogenate was mixed with 10% trichloro acetic acid and centrifuged to separate proteins. To 0.01ml of this supernatant, 2ml of phosphate buffer (pH 8.4), 0.5 ml of 5, 5- dithio, bis (2- nitrobenzoic acid) and 0.4 ml double-distilled water were added. The mixture was vortexed and the absorbance was read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as nmol/mg of protein.

## 2.8 Estimation of Total Calcium

A 0.5 ml of the sample was added to 4.5 ml of deproteinated buffer in a glass centrifuge tube and placed in a water bath for 3 minutes. Tubes were centrifuged while they were still hot, 0.5 ml of each supernatant and standard were transferred into clean test tubes. For the reagent blank, 0.5 ml of blank solution was prepared by mixing 9 volumes of deproteinization buffer with one volume of water. 5 ml of working colouring reagent was added to each tube, mixed well and then read at 570 nm[18].

## 2.9 Detection of Bcl-2 and Bax Expression by Western Blotting

The sciatic nerve from each experimental group was minced and homogenized in an ice-cold lysis buffer. Homogenates were centrifuged at 4,000×g for 10 min to remove cellular debris. The cytosolic fractions of the proteins were obtained by collecting the supernatant and centrifuged at 16,000×g for 30 min at $4^0$C to maximize protein extraction. The membrane fraction was obtained by treating the pellet with a lysis buffer supplemented with 1 % Triton-X followed by centrifugation at 16,000×g. Protein concentrations were determined using a modified Lowry, 1951 method[19]. Proteins were denatured with sodium dodecyl sulfate (SDS) sample buffer and epitopes were exposed by boiling the protein samples at $100^0$C for 5 min. A 50 µg of protein was loaded and separated by electrophoresis on 12 % (w/v) SDS-polyacrylamide gel electrophoresis and proteins were transferred to a nitrocellulose membrane. Immuno blotting was carried out by incubating the membrane in blocking solution [5% dry milk in Tris-buffered saline–Tween 20 (TBST) buffer for 1 h] and then with specific polyclonal antibodies, i.e., Bcl2 and Bax (1:100, Santa Cruz Biotechnology, Inc., USA) for 12 h at $40^0$C. Membranes were washed three times with TBST buffer and incubated with Horseradish Peroxidase conjugated secondary antibody (1:5,000, Santa Cruz Biotechnology, Inc., USA) for 1 h at room temperature followed by washing three times with TBST buffer. Bands were visualized on the Odyssey infrared scanner (Biosciences, USA) and quantitatively analyzed by densitometry with Quantity One software (BioRad).

## 2.10 Statistical Analysis

Data were expressed as mean ± S.E.M (n=6) and was analyzed using one-way analysis of variance for antioxidant parameters and two-way analysis of variance (ANOVA) followed by Dunnet's T-test by using Graph pad prism 8.0. A value of P < 0.05 was considered to be statistically significant.

# 3. Results

## 3.1 Effect of Naringin on ACR Induced Cyto toxicity in U87MG Cells

Pretreatment of cells with Naringin (5, 50, 100, 150, 200, 250, 300 µg/ml) significantly (p<0.001) decreased the ACR-mediated cytotoxicity in a dose-dependent manner in comparison to acrylamide group (Figure 1a). Treatment with ACR reduced the number of U87MG cells and narin-gin treatment restored the number of cells and morphology observed after 48hr of narinigin (Figure 1b).

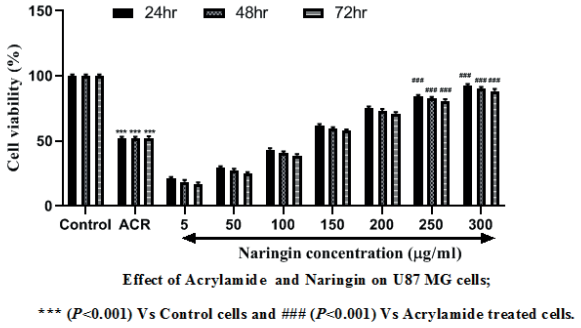
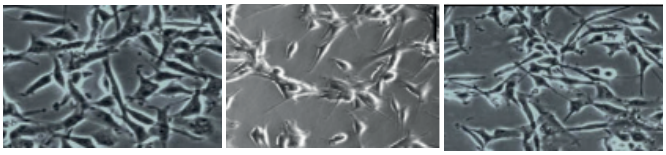



**Figure 1a.** Effect of ACR and Naringin on U87MG Cell viability after 24, 48 and 72hr pretreatment.



Control ACR Naringin 300µg/ml

**Figure 1b.** Morphology of U87MG cells at 48hr time interval.

## 3.2 Effect of Naringin on ACR Induced Oxidative Stress Markers and Calcium in Sciatic Nerve

ACR treatment significantly (p<0.001) decreased the SOD and GSH levels in the sciatic nerve as compared to the control group. Whereas Naringin treatment significantly (p<0.001) restored SOD and GSH levels when compared to the ACR group. MDA levels were significantly increased in ACR administered rats (p< 0.001) when compared to the control group. Naringin treatment significantly (p<0.001) attenuated MDA levels when compared to the ACR group (Figure 2). Calcium levels were significantly increased in ACR administered rats (p< 0.001) when compared to the control group. Naringin treatment significantly (p<0.001) attenuated Calcium levels when compared to the ACR group (Figure 2).

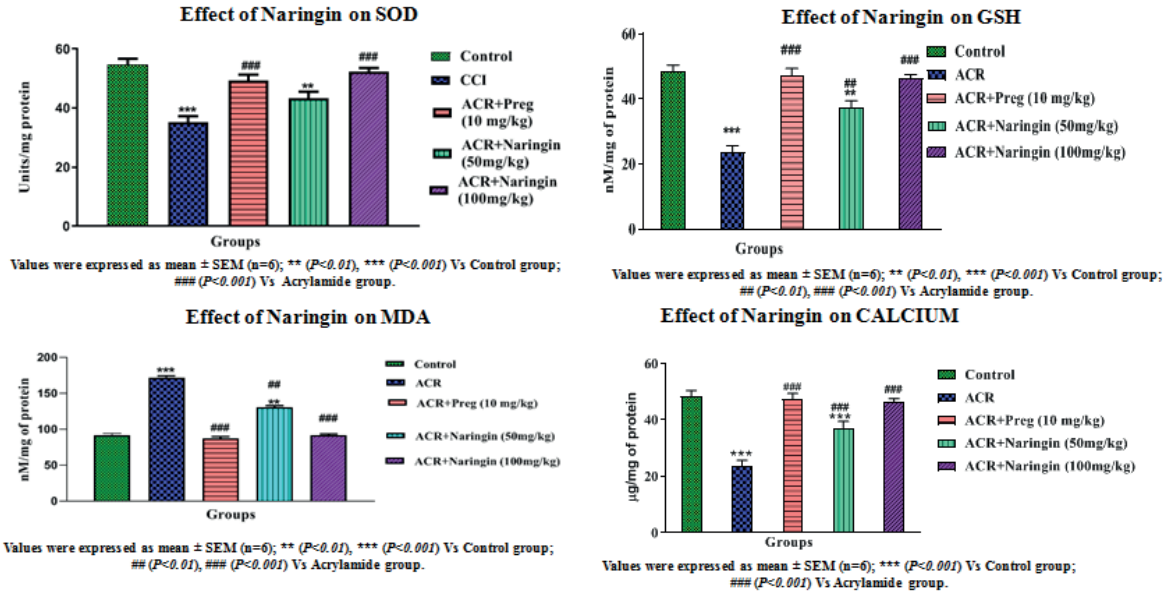



**Figure 2.** Effect of Naringin on oxidative stress markers and Calcium.

## 3.3 Effect of Naringin on Bax and Bcl-2 Protein Expressions

ACR treatment significantly (p<0.001) up-regulated the Bax and down-regulated the Bcl2 protein expression when compared with a control group and naringin reversed significantly (p<0.001) these alterations in the expressions of Bax and Bcl-2 (Figure 3).

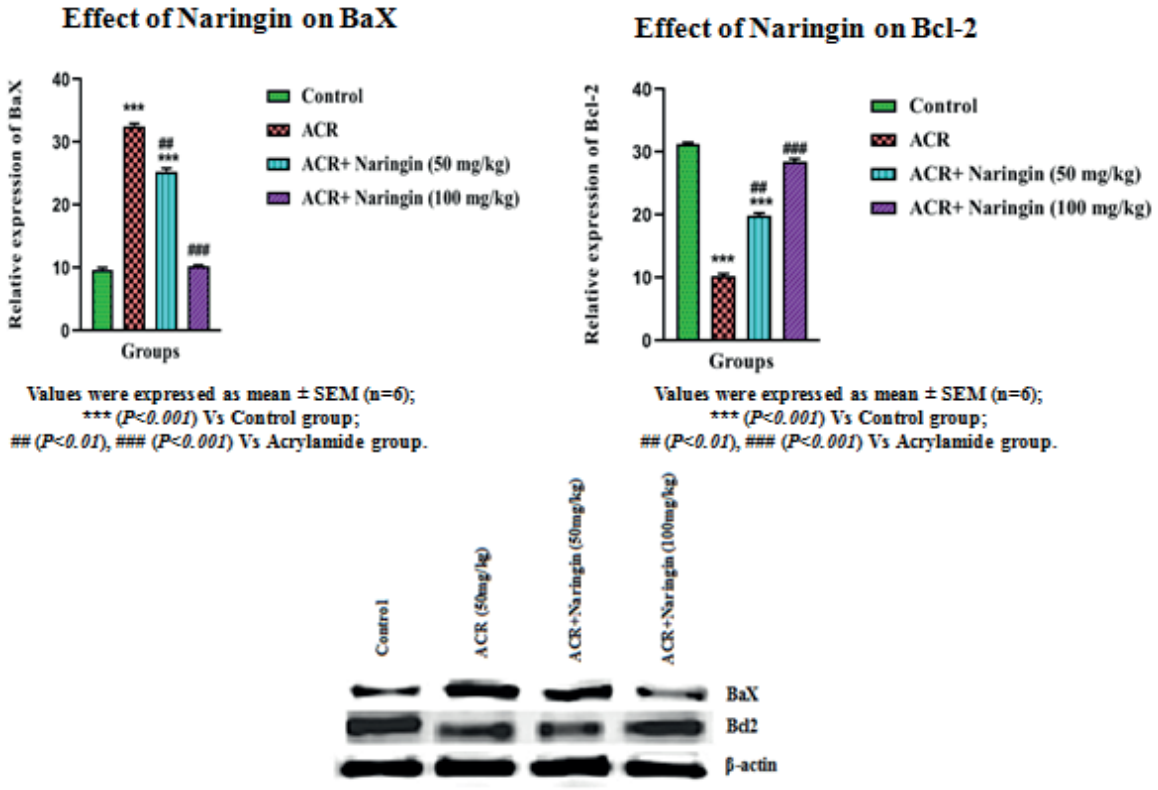



**Figure 3.** Effect of Naringin on the expression of apoptotic markers.

## 4. Discussion

Neurotoxicity detection induced by chemicals represents a major challenge due to the physiological and morphological complexity of the Central Nervous System (CNS) and Peripheral Nervous System (PNS). The simplest *in-vitro* model, the cell line, is a population of cells which are maintained in culture for an extended period. Neuronal cell lines normally originate from a single common ancestor cell (clonal) and are often derived from tumours, e.g., adrenal medullary tumour cell - PC12 cell line for studying pheochromocytoma[20] and for neuroblastomas[21] human SH-SY5Y and U87MG cell lines. Pretreatment of PC12 cells with Epigallocatechin gallate, epicatechin gallate[22], Fasudil[23] and Crocin[20] attenuated ACR-induced Cytotoxicity. In the present study also, the morphological observation of U87MG cells indicated that Naringin attenuated ACR induced cytotoxicity of U87MG cells.

ACR-induced neurotoxicity is mediated through different mechanisms but mainly through induction of oxidative stress and apoptosis[6,7]. Reactive Oxygen Species (ROS) play important role in brain ischemia/reperfusion injury[24] and neurodegenerative diseases including neuropathy[25]. Several fundamental cellular signal transduction pathways, induced by intracellular ROS generation, contribute to cell apoptosis and death[26–29]. Treatment of rats with ACR significantly reduced the activity of enzymatic antioxidants and the content of reduced glutathione in the cerebral cortex[30]. ACR increased lipid peroxidation levels and reduced antioxidant capacity in nervous tissue particularly in the sciatic nerve[31]. Previous reports suggested that antioxidant agents could rescue neurotoxicity induced by ACR via increasing antioxidant activity[32–34]. In the present study, ACR altered oxidative stress markers (SOD, MDA and GSH) and naringin decreased the oxidative stress by restoring GSH and SOD. The previous report suggested that calcium play a vital role in the ACR induced neurotoxicity. ACR increased *in vitro* calcium and calmodulin-dependent kinase-med iated phosphorylation of rat brain and spinal cord neurofilament proteins[35]. In this study also ACR increased calcium levels in the sciatic nerve where it was normalized by naringin treatment.

Bax and Bcl-2 proteins are the members of the Bcl-2 family which contradict each other. The elevation in the proapoptotic protein, Bax, shows an increase in the tissue apoptosis. Also, the elevation in the anti-apoptosis protein, Bcl-2 protein, shows anti-apoptotic activity. Thus, alterations in Bax and Bcl-2 proteins indicate the apoptosis level of tissue cells[36]. Morphological observation suggested that ACR induced apoptosis in various models including *in-vitro* studies (e.g. neuroblastoma SH-SY5Y and human liver embryo L-02[37,38] and *in-vivo* studies (e.g. CNS and PNS tissues in Wistar rats and Sprague-Dawley rats)[39,40]. It was evinced that the main damage induced by ACR is incorporated in neurons[41]. The expression levels of apoptotic genes including Bax, Bcl-2, and caspase-3 are increased in ACR-intoxicated Leydig (TM3) and Sertoli (TM4) cells[42]. As a result, apoptosis is the most important features of ACR-induced neurotoxicity which was inhibited by many phytochemicals through their antioxidant capacity. Previous studies evidenced that ACR caused elevation in Bax and reduction in Bcl-2 protein expression which were reversed by Crocin[43], Taurine[44], Epigallocatechin-3-gallate[45], Thymoquinine[46], Fausidil[23] and Ellagic acid[47]. The results of the current study indicated that Bax protein expression increased and Bcl-2 protein expression decreased in the sciatic nerve of ACR-treated rats. Naringin at the dose of 100 mg/kg was able to reverse these alterations. Hence, Naringin (100 mg/kg) could prevent apoptosis induced by ACR via reducing the Bax and elevating the Bcl-2 protein expressions through its antioxidant activity. Therefore, Naringin can render neuroprotection due to its antioxidant and anti-apoptotic properties. The results of the effect of naringin on ACR induced neuropathy are tabulated in Figure 4.

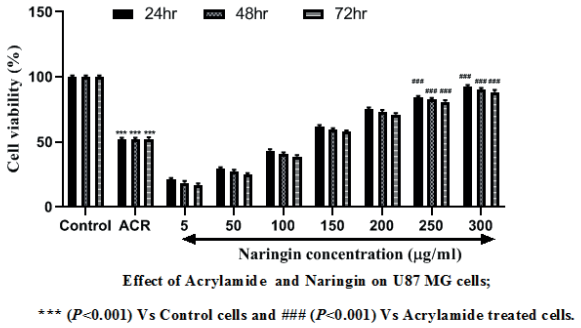



**Figure 4.** Indicates the influence of ACR and ACR along with Naringin on antioxidant parameters (SOD, MDA and GSH), Calcium and apoptotic markers.

## 5. Conclusion

In summary, the current study demonstrated that Naringin was effective in attenuating ACR-induced neurotoxicity both in *in vitro* and *in vivo*. Naringin decreased oxidative stress via reducing the levels of MDA and increased the capacity of endogenous antioxidant system such as SOD and GSH levels against ACR induced neurotoxicity. And also Naringin treatment ameliorated the neuronal apoptosis by decline of Bcl-2, and the increase in Bax activity induced by ACR in the sciatic nerve. Therefore, our results suggest that Naringin treatment could be a novel candidate against ACR-induced neuropathy.

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