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ORIGINAL ARTICLE

Naringin Ameliorates Doxorubicin-induced Neurotoxicity *In vitro* and Cognitive Dysfunction *In vivo*

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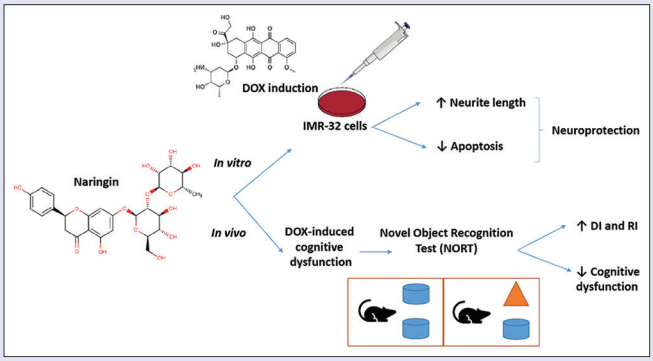
ABSTRACT

Objectives: The primary objective of the study was to study the neuroprotective potential of naringin (NAR) against doxorubicin (DOX)-induced neurotoxicity *in vitro* and DOX-induced cognitive deficits (chemobrain) *in vivo*. **Materials and Methods:** *In vitro* methods, viz., 3-[4,5dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay, flow cytometry, acridine orange/ethidium bromide staining, and neuritogenic and reactive oxygen species (ROS) assays, assessed neuroprotective potential of NAR and its aglycone naringenin (NGN) in IMR-32 cells. Chemobrain was developed in Wistar rats on chronic administration of ten cycles of DOX, and episodic memory was assessed using novel object recognition task. Serum cortisol, locomotor activity, and hematological biochemical and histological analysis were carried out. **Results:** A protective effect of NAR or NGN was observed upon pretreatment with the respective compounds in IMR-32 cells challenged with DOX. Flow cytometry revealed that flavonoids reduced cell cycle changes produced by DOX. In addition, an increase in apoptosis, intracellular ROS generation, and inhibition of neurite growth was noticed in IMR-32 cells with DOX treatment, which was significantly prevented by NAR or NGN pretreatment. Interestingly, NAR (50 mg/kg, p.o.) significantly ameliorated episodic memory deficit associated with DOX without influencing locomotion, upon chronic treatment. NAR also prevented histological changes to major organs observed with DOX. **Conclusion:** NAR showed neuroprotective potential and may be used as an adjuvant therapy for amelioration of neurocognitive complications associated with chemotherapy in cancer survivors.

Key words: Breast cancer, chemobrain, cognitive impairment, episodic memory, novel object recognition test

SUMMARY

- Chemobrain is a condition affecting the cognitive function of individuals who have undergone chemotherapeutic treatment for cancer. It is especially observed in long-term survivors of breast cancer. Many chemotherapeutic agents such as cyclophosphamide, doxorubicin (DOX), 5-fluorouracil have been identified to cause this debilitating condition at acute and high doses. This study focuses on the protective effect of the flavonoid, naringin (NAR), and its aglycone, naringenin, against neuronal toxicity *in vitro* and against chemobrain condition induced by the chronic administration of DOX *in vivo* which causes oxidative/nitrosative stress and cytokine dysregulation in the brain. The present study revealed that NAR exerts protective effect against DOX-induced chemobrain by attenuating cell cycle arrest, neuronal development, and improving cell viability in IMR-32 cells. Administration of the flavonoid also improved the performance of DOX-treated animals in episodic memory tasks. This study therefore projects NAR as a potential adjuvant therapy for protection against the development of chemobrain.



Abbreviations used: CKL: Creatine kinase level; COX: Cyclooxygenase; DMEM: Dulbecco's modified eagle media; DOX: Doxorubicin; FBS: Fetal bovine serum; MTT: 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide; NAR: Naringin; NGN: Naringenin; NORT: Novel object recognition task; NOS: Nitric oxide synthase; QOL: Quality of life; RA: retinoic acid.

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INTRODUCTION

Chemobrain is a debilitating condition affecting cognition, observed especially in populations of long-term breast cancer survivors. This results in cognitive dysfunction that negatively impacts the quality of life (QOL) in survivors.[1,2] Cognitive deficits comprise alteration in the diverse components of visual, verbal, episodic, spatial, and working memories including lack of concentration, difficulty in multitasking, attention, planning, and reduced processing speed with an impaired executive function.[3,4] Furthermore, the occurrence of breast cancer is more alarming especially in the early thirties (WHO). A recent review reported that several clinical or preclinical studies worked on interventions, viz., modafinil, methylphenidate, ginkgobiloba, resveratrol, N-acetyl cysteine, and nonsteroidal anti-inflammatory drugs, were found to be not up to the mark to alleviate the memory complications.[5,6]

Various mechanisms have been proposed for the cytotoxic agents induced chemobrain condition, viz., reduced gray and white matter,

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direct neurotoxic injury, inhibition of adult hippocampal neurogenesis, reduced blood flow with diminished metabolic activity, increased brain oxidative stress, cytokine dysregulation, microglial activation, and induced neuro-inflammation, which can ultimately lead to neurotoxicity.[1,6]

To the best of our knowledge, researchers developing chemobrain animal models have focused on effect of acute and high-dose chemotherapeutic agents such as cyclophosphamide, doxorubicin (DOX), 5-fluorouracil, and methotrexate either alone or in combination in rodent models for various cognitive components.[7-10] However, not much attention has been paid on the influence of chronic administration (which is comparable to that of human chemotherapeutic regimen) of a widely used (for almost all forms of neoplasia) chemotherapeutic agent (DOX) on cognitive function in rodents. Our prior work confirmed that DOX can produce chemobrain through the dysregulation of cytokines and induction of oxidative/ nitrosative stress.[11]

A recent report showed that DOX reaches rat brain in cerebral hemispheres, cerebellum, and brainstem when given alone as a single dose.[12] Although DOX reaches brain in less quantities, the concentration reached may be sufficient to produce direct neurotoxicity. One of the earlier studies reported the effect of a single injection of DOX at different dose levels in an inhibitory avoidance paradigm on an acute treatment basis.[13] Our study demonstrated a validated protocol for chronic DOX-induced chemobrain-like condition in Wistar rats and established a neurocognitive animal model for assessing episodic-like memory using an object recognition task.

Till date, no satisfactory intervention is available to prevent or treat chemobrain symptoms. Therefore, there is a requirement for the development of therapeutic interventions to prevent such deficits in patients undergoing chemotherapy, such that there is improvement of QOL in these patients. Alternative and traditional therapeutic approaches have become one of the major sources of new drugs for cure or prevention of most of the human diseases.[14] Animal studies provide supporting data which point to the ability of flavonoids to improve the various components of cognitive processes through neuronal differentiation, long-term potentiation, and also enhancing the synaptic plasticity.[15-17]

Naringin (NAR) is one such flavonoid with naringenin (NGN) as aglycone possessing antioxidant, cardioprotective, anti-inflammatory, anti-apoptotic, blood-forming, anti-depressant, and neuroprotective effects.[18,19] Earlier works revealed that NAR prevented the lipopolysaccharide-induced pro-inflammatory cytokine responses and gene expression of COX and NOS *in vitro*[18,20] as well as *in vivo*.[21] In kainic acid-induced model of epilepsy, NAR has been reported to attenuate associated behavioral changes.[22] Similar effects have been reported in an animal model of Huntington's disease using 3-nitropropionic acid.[23] NAR administration also improved cognitive deficits in colchicine[24] and D-galactose[25] induced learning and memory deficits in rats. At the same time, it is interesting to note that NAR has been reported to exhibit neuroprotective effects in cerebral injury caused by ischemia-reperfusion through decreased oxidative damage and mitochondrial dysfunction as well as neurological impairments.[26] Hence, in the present study, we have investigated the possible neuroprotective role of NAR to alleviate chemobrain symptoms through its pleiotropic pharmacological activities.

No information is available on the efficacy of NAR or its aglycone (active metabolite) NGN in protecting the human neuroblastoma cells (IMR-32) against DOX-induced neurotoxicity and also its potential to ameliorate the cognitive deficits associated with DOX-induced chemofog-like condition in rodents. Hence, in our present study, we have evaluated neuroprotective potential of this flavonoid glycoside NAR along with its aglycone moiety on IMR-32 against DOX-induced neurotoxicity *in vitro* followed by evaluation of possible mechanisms underlying neuroprotection. Then, we have proceeded with the evaluation of flavonoid glycoside NAR *in vivo* for its possible ability to prevent chemobrain using novel object recognition task (NORT) in a DOX-induced model of chemofog that is associated with episodic memory deficit.

MATERIALS AND METHODS

Chemicals and apparatus

DOX (Fresenius Kabi Oncology Ltd., Solan, Himachal Pradesh), NAR (Tokyo Chemical Industry Co. Ltd., Tokyo, Japan), NGN (Sigma-Aldrich Co. LLC, St. Louis, MO, USA) were acquired. Trypsin-EDTA (0.25–0.025% w/v in sterile PBS), dipotassium EDTA, 3-[4,5dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), retinoic acid (RA), acridine orange/ethidium bromide (AO/EB) were obtained from HiMedia Laboratories, Mumbai, India. Trypan blue (0.4% w/v), dichlorofluorescein diacetate (DCFDA), and Dulbecco's modified eagles medium (DMEM) were purchased from Sigma-Aldrich Co. LLC, St. Louis, MO, USA. 96-well and 6-well plates and 25 cm^2 and 75 cm^2 tissue culture flasks (Tarsons) were also used in this study. Rat cortisol ELISA kit was procured from Elabscience Biotechnology Co. Ltd., Wuhan, China.

Apparatus for NORT consisted of square boxes of dimension 49 cm (length × breadth × height) which are also called behavioral or observational arenas made with plywood. Inner portions of arenas were covered with black laminate. Behavioral observation of rats was monitored and recorded using a camera (Quickcam Pro9000, Logitech International S.A., Lausanne, Switzerland) that was mounted about 150 cm above the behavioral observation arenas. This apparatus was used to assess DOX-induced episodic memory deficits in NORT. Locomotor activity of animals was assessed using ANY-maze video tracking system (Software Package, Version 4.99 m, Stoelting Co., San Diego Instruments, USA).

Animals

Thirty-six healthy female Wistar rats of 10–12 weeks of age and of body weight ranging between 150 and 200 g were procured from the Central Animal Research Facility. All animal experimental protocols were approved by the Institutional Animal Ethics Committee. Animals were maintained at Central Animal Research Facility Annex (CARF-A) with a light and dark cycle of 12/12 h, a temperature of 23°C ± 2°C, and a relative humidity of 50% ± 5%. They were housed in sterile polypropylene cages (3 animals each) with sterile husk bedding and allowed access to food and water *ad libitum*. The animals were allowed to acclimatize to their surroundings before experimentation for 7 days.

Cell culture and maintenance

Human neuroblastoma cell line, i.e., IMR-32, was procured from National Centre for Cell Science, Pune, India, maintained in DMEM supplemented with 10% FBS and suitable antibiotic, i.e., gentamycin (100 µg/ml), in T-25 culture flasks in an incubator providing humidified environment with 95% air and 5% CO_2.

Cell viability and dose fixation for neuroprotective activity

Cell viability was assessed using MTT assay.[27] Twenty-four hours after seeding, the cells were exposed to various concentrations (50–500 µM)

of NAR or NGN and cell viability was assessed after 24 h incubation. Two concentrations that showed >80% viability were selected for assessment of neuroprotective activity.

Evaluation of *in vitro* neuroprotection on IMR-32 cells

Neuronal cell viability assessment using 3-[4,5dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay

Neuronal cell viability is an indirect measure of neuroprotection. Neuroprotective potential of NAR and NGN was evaluated against DOX in IMR-32 cells using MTT assay. Nontoxic concentrations of NAR and NGN, i.e., 50 and 100 μM, were selected. After 2 h of preincubation with either NAR or NGN, DOX (1 μM) was added and incubated for 24 h. Following incubation, intensity of purple color formed was measured at 540 nM using a microplate reader (ELx800; BioTek Instruments Inc., Winooski, VT, USA) and cell viability was calculated.[28]

Cell cycle analysis by flow cytometry

Approximately one million undifferentiated IMR-32 cells were seeded into 6-well plates in DMEM. After 24 h of seeding, cells were incubated with both test compounds for 2 h. Then, DOX (1 μM) was added in media and further incubated for 24 h. The cells were trypsinized following PBS wash and collected into microcentrifuge tube-containing PBS and centrifuged (4000 rpm for 5 min) and the cell pellet was collected. The pellet was resuspended in ice-cold 70% v/v ethanol and kept for freezing at −20°C for 4 h to fix the cells. The cells were then centrifuged and the resulting cell pellet was suspended in PBS-containing propidium iodide, RNAse solution and kept in dark for 20 min. The samples were subjected to flowcytometric analysis by using flow cytometer (BD Accuri™ C6; BD Biosciences, San Jose, CA, USA). Samples were analyzed for % population in various phases of cell cycle (G_1, S, and G_2/M) by using BD Accuri™ C6 software.[28]

Morphological changes and neurite length assessment in differentiated IMR-32 cells

IMR-32 cells were differentiated into neuron-like structures using RA (10 μM) supplemented in low serum-containing DMEM. Following differentiation, cells were exposed to NAR or NGN (100 μM for 2 h), following which DOX was added and incubated for 24 h. Suitable controls were maintained for the toxicant (DOX) and media. Next, cells were observed under an inverted microscope (Eclipse TS100F; Nikon Instruments Inc., Melville, NY, USA). For assessing the neurite length, 100 images were randomly captured by scanning each treatment well and length of each neurite drawn was measured using NIH Image J software supplemented with Neuron J plug-in.[28-30]

Detection of apoptosis/necrosis by acridine orange/ethidium bromide staining

IMR-32 cells were differentiated and further incubated with either DOX (1 μM) alone or in combination with NAR or NGN (100 μM, 2 h before DOX exposure) in 6-well plates. After 24 h of incubation, wells were washed with PBS and the cells fixed using 1 ml of ice-cold ethanol (100%) for 10 min at room temperature. Following fixing, 1 ml AO/EB reagent stain was added to each well and incubated for 10 min at 37°C. An inverted microscope was used to observe the fluorescence of individual cells. As described in earlier available report, apoptotic and necrotic cells were differentiated and identified on the basis of their staining pattern.[28,31]

Intracellular reactive oxygen species estimation

IMR-32 cells seeded in black 96-well plate were incubated with either NAR or NGN at concentrations of 50 and 100 μM for 2 h and then exposed to DOX (1 μM). After 24 h of incubation, culture supernatant was discarded and replaced with 100 μl of DCFDA (100 μM). Following 1 h incubation with DCFDA, wells were washed with sterile Hank's balanced salt solution at 37°C. The fluorescence intensity was measured using a fluorescence microplate reader (FLx800; BioTek Instruments Inc., Winooski, VT, USA) and reactive oxygen species (ROS) levels were calculated with respect to control.[28,32]

In vivo chemobrain study

Experimental design

Female Wistar rats were divided into four experimental groups (9 animals each). Group 1 rats were treated with normal saline, i.p., while Groups 2 and 3 were treated with DOX at a dose of 2.5 mg/kg, i.p., once in 5 days over a period of 50 days. In addition, Groups 2 and 3 were administered daily with sodium carboxymethyl cellulose (CMC) (0.25% w/v in WFI) and NAR (50 mg/kg prepared as suspension in CMC, p.o.), respectively, which was started 1 week before DOX and continued throughout the study period. Group 4 animals were treated with NAR alone (50 mg/kg, p.o.) to identify the chronic *per se* effect of this flavonoid on cognitive function and locomotor activity. The doses and the dosing intervals were selected based on the previous studies performed in our laboratory (unpublished). The entire dosing schedule continued for 60 days and a detailed protocol has been illustrated in Figure 1.

Novel object recognition task for episodic memory

Assessment of episodic-like memory in rodents is generally carried out using NORT. Procedures adopted were according to the previous reports with slight modifications.[33-37] Briefly, the experiment was conducted in a soundproof isolated room with a light intensity of 30–40 lux over a period of 2 days. Experiments consisted of three phases, viz., habituation, familiarization, and choice phases, and were performed between 09:00 AM and 04:00 PM.

Rats were acclimatized to the laboratory conditions. On day 1, animals were habituated to the behavioral observation arenas for 20 min. We have observed earlier that control animals were able to remember the familiar object up to an inter-trial interval (ITI) of 2 h in pilot studies. Therefore, on day 2, animals were subjected to familiarization and choice trials using an ITI of 2 h. In familiarization trial, animals were allowed to explore a pair of similar objects in the arenas. The investigatory behavior, i.e., exploration of rats directed toward objects, was recorded using a camera (Logitech Pro9000) mounted at a height from the behavioral observation arenas with two handheld stopwatches by an expert blinded observer.

In the choice trial, one of the familiar objects was replaced with a novel object and cumulative exploration time of each animal of 3 min was

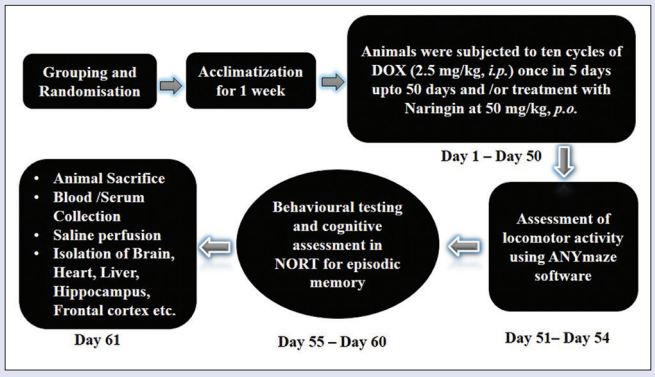

Figure 1: Validated and detailed protocol for doxorubicin-induced chemobrain in Wistar rats

noted for the familiar or novel. The objects as well as the arenas were thoroughly cleaned with 70% v/v ethanol solution to remove olfactory clues between the trials. Recognition index (RI) and discriminative index (DI) which is a measure of the animal's memory for the objects were determined according to the previous report.[38]

Body weight changes

Body weight was recorded once in 3 days and monitored throughout the study. The average body weight was calculated and compared among the treatment groups.

Open field test

Locomotion activity of all animals was assessed via an open field (OF) paradigm. The OF test was done using square arenas that were used for object recognition task. The procedures followed were according to earlier reports.[39,40] The rats were individually placed in OF boxes for 15 min, and their locomotor activity, i.e., distance (cm) traveled, and mean velocity (cm/s) during 15 min test were assessed using the Any-maze software with video tracking system.

Hematological analysis and organ index

Blood sampling was made by retro-orbital puncture. Complete hematological profile was measured using an automated veterinary blood cell counter (ERMA-PCE-210VET, Japan). The animals were anesthetized with ketamine-xylazine combination and whole-body perfusion was carried out with ice-cold saline. Organ collection was made for kidney, liver, brain, and heart. Organ index was calculated as the weight of each organ per 100 g body weight of the animal and compared.

Oxidative stress markers

Antioxidant parameters which include superoxide dismutase (SOD), catalase, glutathione (GSH) and total thiols levels were estimated in hippocampus and frontal cortex as per the standard procedures available in literature.[41-46]

Acetylcholinesterase activity in hippocampal and frontal cortex regions

Following hippocampal and frontal cortex isolation, samples were homogenized with phosphate buffer (pH 7.4), and supernatants were collected and stored in aliquots for further estimation. The method for acetylcholinesterase (AChE) estimation was as per the standard procedure given by Ellman *et al.*[47]

Serum cortisol estimation

Cortisol levels in serum were assessed using rat cortisol ELISA kit (Elabscience Biotechnology Co. Ltd., Wuhan, China) following the manufacturer's procedure.

Serum biochemistry

Biochemical analysis in serum was carried out for aspartate transaminase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), cholesterol, total protein, urea, creatine kinase level (CKL), creatinine, etc. with fully automated autoanalyzer (Cobas C111; Roche Diagnostics AG, Rotkreuz ZG, Switzerland) using the company's estimation kits following the manufacturer's standard protocol.

Histopathology

Histopathological analysis was performed for major organs such as liver, heart, kidney, and brain. Gross structural changes as a result of exposure to toxicant, DOX, and the ability of NAR to reverse these changes present (if any) were reported.

Statistical analysis

Statistical analysis was carried out using Prism 6.03 (trial version; GraphPad Software Inc., La Jolla, CA, USA) software package. All data are expressed as mean ± standard error of the mean of respective number of samples. Cell viability, neurite length, % apoptotic cells, intracellular ROS measures were analyzed using one-way ANOVA followed by Tukey's *post hoc* test. Data for episodic memory in chemobrain study are expressed as time in seconds spent by the animals exploring either familiar or novel objects and also as recognition and discriminative indices. Exploration time is compared by student's paired *t*-test within the group between the objects. RI and DI were analyzed by Kruskal–Wallis test followed by Dunn's *post hoc* test. Locomotor activity, organ index, AChE, biochemical and hematological parameters were analyzed by one-way ANOVA followed by Tukey's *post hoc* test. $P < 0.05$ was considered as statistically significant.

RESULTS

Cell viability and dose fixation for neuroprotective activity

The IC_{50} for NAR and its aglycone was observed at 300 and 250 μM, respectively, in IMR-32 cells. DOX showed a dose-dependent cytotoxic effect (0.01–5 μM), and IC_{50} of DOX was identified as 1 μM. Around 80% cell viability was observed at 50 and 100 μM; hence, the same concentration was used to test the neuroprotective ability of NAR and NGN.

Evaluation of *in vitro* neuroprotection on IMR-32 cells

Neuronal cell viability assessment using 3-[4,5dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay

DOX (1 μM) treatment has resulted in 53.47% cell death in IMR-32, whereas prior incubation with flavonoids (NAR or NGN at 50 and 100 μM produced 41.11%, 33.07% and 39.83%, 35.14% cell death, respectively) significantly ($P < 0.001$) prevented the DOX-induced cell death in a dose-dependent manner, the most effective being 100 μM. Since 100 μM concentration showed more protection, we have used the same concentrations for both NAR and NGN in the later studies [Table 1].

Cell cycle analysis by flow cytometry

In control group, percentage cell population at different phases (G_1:S: G_2/M) was 66.9:13.9:18.9, while in DOX sample, it was in the order of 40.9:34.7:23.1, which indicates that the DOX-treated cells were arrested in S phase and G_2/M phase. NAR at 100 μM reversed the cell

Table 1: Data represents mean±standard mean error of three test samples for protective effect of naringin and naringenin on cell viability against doxorubicin-induced neurotoxicity in 3-[4,5dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay on human neuroblastoma cell-32 cells

Treatment	Percentage viability
Media control	100.0±1.914*
DOX control, 1 μM	46.53±0.484
DOX, 1 μM + naringin 50 μM	58.89±0.948*
DOX, 1 μM + naringin 100 μM	66.93±0.841*
DOX, 1 μM + naringenin 50 μM	60.17±1.157*
DOX, 1 μM + naringenin 100 μM	64.86±2.339*

*$P<0.001$ compared to DOX control. DOX: Doxorubicin

cycle arrest in G_2/M phase by DOX (G_1:S: G_2/M = 42.4:37.3:19.5). NGN at 100 μM prevented cell cycle arrest by DOX prominently in S phase and moderately in G_2/M phase (G_1:S: G_2/M = 55.0:21.8:21.9). Hence, prior treatment with either NAR or NGN prevented DOX-induced fatal changes in cell cycle progression [Figure 2].

Morphological changes and assessment of neurite length in differentiated IMR-32 cells

DOX treatment significantly ($^{\#}P < 0.001$) inhibited the development of neurite outgrowth in differentiated IMR-32 cells as compared to normal control. However, prior treatment with either NAR or NGN at 100 μM showed prominent growth of neurite and therefore significantly ($^{*}P < 0.001$) averted inhibitory effect of DOX on neurite length [Figures 3 and 4].

Detection of apoptosis/necrosis by acridine orange/ethidium bromide staining

AO/EB staining revealed severe apoptotic cell death in IMR-32 (bright condensed nuclei and fragmented cellular structures) produced by DOX induction which was significantly high compared to normal control. The percentage of cells with apoptotic morphology is represented in Table 2. Prior treatment with NAR resulted in significant decline in percentage of apoptotic cells while NGN showed moderate decline [Figure 5 and Table 2].

Table 2: Data represent mean±standard error mean of three tests in triplicate for the effect of naringin and naringenin on cellular apoptosis against doxorubicin-induced neurotoxicity in differentiated human neuroblastoma cell-32 cells

Treatment	Percentage apoptotic cells
Media control	26.54±1.76
DOX control, 1 μM	70.78±4.56*
DOX, 1 μM + naringin 100 μM	42.22±3.76#
DOX, 1 μM + naringenin 100 μM	58.89±6.87

*P<0.001compared to media control, #P<0.001 compared to DOX control.
DOX: Doxorubicin

Intracellular reactive oxygen species estimation

DOX significantly ($^{***}P < 0.001$) elevated intracellular ROS levels as compared to control. Pretreatment with NAR or NGN significantly ($^{\#\#}P < 0.001$) inhibited the ROS generation by DOX in a dose-dependent manner which indicates the potential antioxidant role of flavonoids [Figure 6].

In vivo chemobrain study – Evaluation of naringin as a possible intervention

Novel object recognition task for episodic memory

Following an ITI of 2 h, animals treated with only vehicle were able to remember the familiar object. They were also able to discriminate novel object from familiar one. Animals treated with vehicle along with DOX (2.5 mg/kg, i.p.) were unable to discriminate between the objects. They spent an almost equal amount of time exploring both familiar and novel objects due to lack of episodic recognition memory for the familiar object. Chronic treatment with NAR (50 mg/kg, p.o.) prevented DOX-induced episodic-like deficits in NORT. We observed that the rats spent significantly more time exploring novel object compared to familiar object. Rats treated with NAR alone or in combination with DOX were able to discriminate the novel object from the familiar one observed as significant increase in recognition and discriminative indices compared to DOX control [Figure 7].

Body weight

We observed a gradual increase in body weight throughout the groups. However, it was comparatively less in DOX control group when compared to others though the difference observed was not statistically significant. Highest mean body weight was observed in groups treated with only vehicle or NAR. Treatment with NAR (50 mg/kg, p.o.) along with DOX for 60 days resulted in prevention of loss of body weight compared to DOX group [Figure 8].

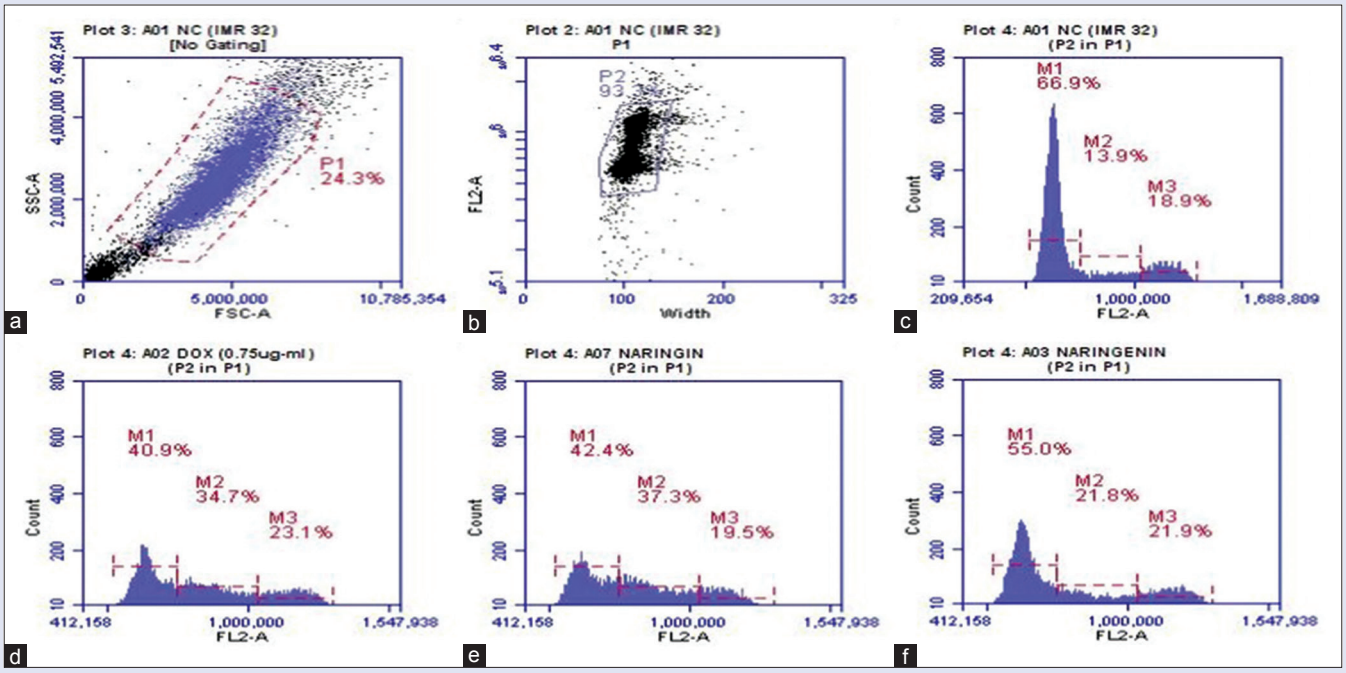

Figure 2: Histograms represents the effect of naringin and naringenin on doxorubicin-induced changes in percentage of cells in various phases of cell cycle in IMR-32 cells. (a) Whole-cell population with cell debris. (b) Gated for actual cell population after removing debris, doublet cells. (c) Normal control. (d) Doxorubicin treated. (e) Naringin treatment before doxorubicin. (f) Naringenin treatment before doxorubicin

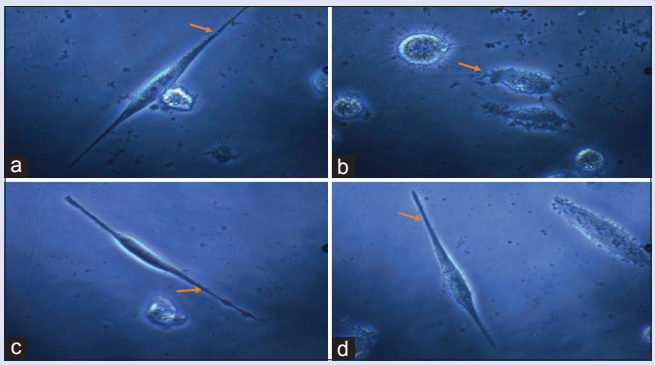

Figure 3: Effect of naringin and naringenin on neurite length and morphology of differentiated IMR-32 cells. (a) Normal control. (b) Doxorubicin treated. (c) Cells treated with naringin before doxorubicin. (d) Cells treated with naringenin before doxorubicin

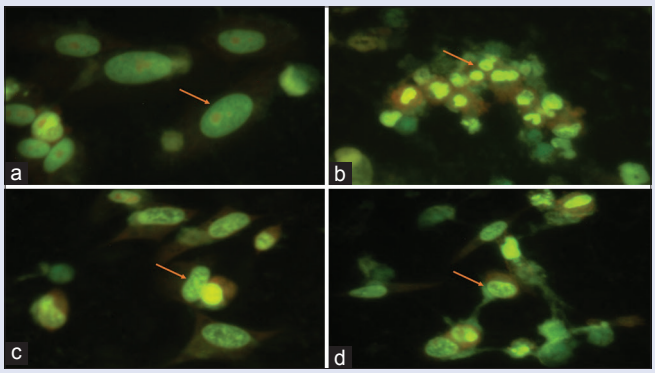

Figure 5: Illustration represents the effect of naringenin and naringin on doxorubicin-induced apoptosis in IMR-32 cells. (a) Normal control. (b) Doxorubicin treated. (c) Cells treated with naringin before doxorubicin. (d) Cells treated with naringenin before doxorubicin

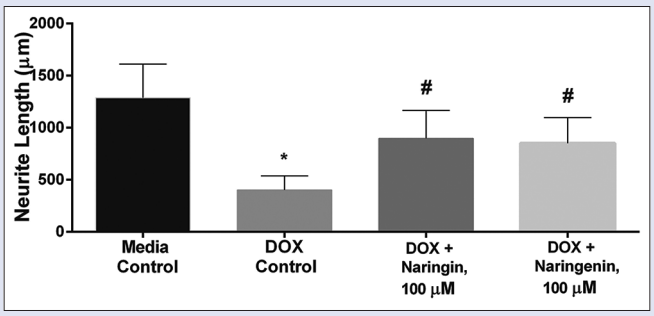

Figure 4: Illustration represents mean ± standard error of the mean of neurite length in micrometers for the protective effect of naringin and naringenin on doxorubicin-induced neurite inhibition in IMR-32, $^{*}P < 0.001$ versus media control, $^{\#}P < 0.001$ versus doxorubicin control

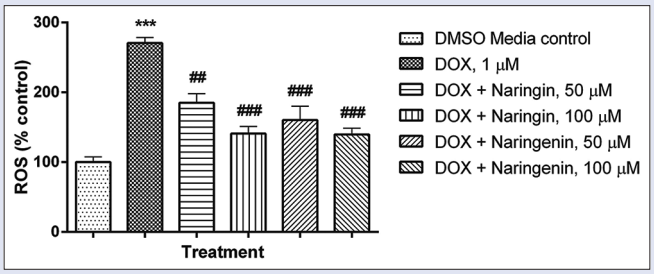

Figure 6: Illustration represents the effect of naringenin and naringin on doxorubicin-induced intracellular reactive oxygen species generation in IMR-32 cells in triplicates, $^{***}P < 0.001$ compared to media control, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ compared to doxorubicin control

Open field test

No significant difference was noted for either the distance traveled or the mean velocity among the four treatment groups. This proves the validity of NORT in assessing the episodic memory deficits associated with DOX chemotherapy [Figure 9].

Hematological analysis and organ index

Treatment with DOX for 50 days resulted in significant ($^{***}P < 0.001$) reduction in red blood cell (RBC), hemoglobin compared to vehicle control animals. This was completely reversed by NAR co-administration at a dose of 50 mg/kg, p.o. White blood cells (WBCs) were reduced in DOX group compared to vehicle though not statistically significant. Rats treated with only NAR showed improvement of RBC, hemoglobin compared to normal vehicle control which supports the NAR's blood-forming potential. Platelet count was unaffected by DOX treatment [Table 3]. No significant difference was observed for the organ index of heart, liver, brain, and kidney among the treatment groups (data not shown).

Oxidative stress markers

Catalase, SOD, GSH, and total thiols levels significantly ($P < 0.05$) declined in frontal cortex and hippocampal regions for DOX group as compared to control. However, these changes were prevented by co-administration of NAR at a dose of 50 mg/kg, p.o. Treatment with NAR alone did not affect above-mentioned antioxidant markers [Table 4].

Hippocampal and frontal cortex acetylcholinesterase activity

No significant difference in the level of AChE activity was observed among the treatment groups in either frontal cortex or hippocampus. It was confirmed that DOX or the flavonoid treatment did not produce any inhibition or activation of this enzyme activity [Table 5].

Serum cortisol levels

Serum cortisol levels were found to be insignificant among the treatment groups as the estimation of serum resulted in nondetectable range of cortisol concentration across the treatments (data not shown).

Serum biochemistry

It was found that CKL, urea, triglyceride, and total cholesterol were significantly elevated in DOX control group compared to control. NAR co-administration prevented the above changes observed with DOX alone. Other parameters, viz., glucose, AST, ALT, ALP, total bilirubin, total protein, albumin, and creatinine levels, were found to be unchanged among different groups [Table 6].

Histopathology

Histopathological analysis revealed that heart, kidney, liver, and brain samples of animals treated with DOX showed abnormal histological architecture and fatty changes compared to control animals. Treatment of rats with DOX along with NAR at 50 mg/kg, p.o. resulted in protection against the pathological changes observed in DOX-treated animals. This indicates that NAR was able to reverse the pathological abnormalities induced by DOX to the major organ systems [Figure 10].

DISCUSSION

There is an unmet need for animal studies to evaluate cognitive impairment objectively after cytostatic treatment and also to study the probable underlying mechanisms. This study was aimed at establishing an animal model for chemobrain and assessing a natural flavonoid for its potential to alleviate chemofog symptoms in the developed chemobrain animal model with an intention to elucidate its mechanism. It has been reported that DOX increases the susceptibility of brain mitochondria to calcium-induced permeability transition pore opening and oxidative stress which in turn predisposes neuronal cells to degeneration, as observed in rodent models.[48] The cognitive problems are reported in cancer patients, especially breast cancer patients undergoing diverse chemotherapeutic regimens, including DOX-based chemotherapy.[49-51] Further, it is surprising to note that the neurotoxic potential of DOX which leads to cognitive impairment has not attracted much attention, despite the widespread use of DOX to treat many forms of cancer. There is no published evidence on the influence of chronic DOX-induced chemofog on episodic memory in rodents. Hence, we focused on the effect of DOX on episodic memory using NORT. We were able to develop neurocognitive animal model for assessing episodic-like memory upon chronic DOX chemotherapy in Wistar rats by following a predetermined dosing schedule (based on the pilot studies).

The current study addressed the chemobrain-inducing potential of DOX in a developed animal model for replicating the episodic-like memory and the deficits associated with chemofog condition in cancer survivors. Furthermore, we hypothesized that natural flavonoid NAR can combat chemotherapy-related cognitive dysfunction through its pleiotropic actions (anti-inflammatory, antioxidant, blood-forming, cardioprotective, memory-enhancing, nephroprotective, and neuroprotective effects). Furthermore, we tried to identify the underlying mechanism involved in the protective potential of NAR against DOX-induced chemobrain. This was done by conducting various *in vitro* neuroprotection studies in human neuroblastoma (IMR-32) cell line as it is a widely used *in vitro* model for studying the neuroprotective potential.[52-55]

IMR-32 neuronal viability was assessed by MTT assay. Neuroprotective potential of NAR flavonoid along with its aglycone moiety NGN was assessed against DOX-induced neurotoxicity using cell viability assay. Both NAR and NGN were able to protect IMR-32 cells against DOX as we found significant increase in viability with flavonoid prior treatment.

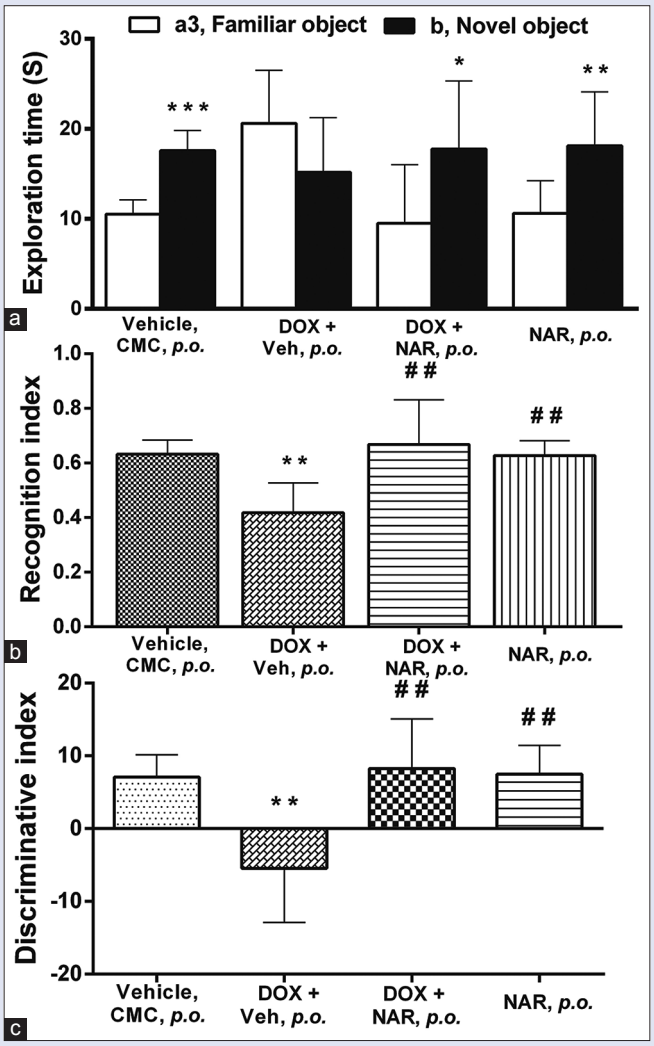

Figure 7: Illustration represents (a) exploration time of novel or familiar objects (***P < 0.001, **P < 0.01, *P < 0.05 vs. familiar object). (b) Recognition. (c) Discriminative indices (**P < 0.01 vs. vehicle control, ##P < 0.01 vs. doxorubicin control) of various groups of rats in choice trial in novel object recognition task (n = 7–9)

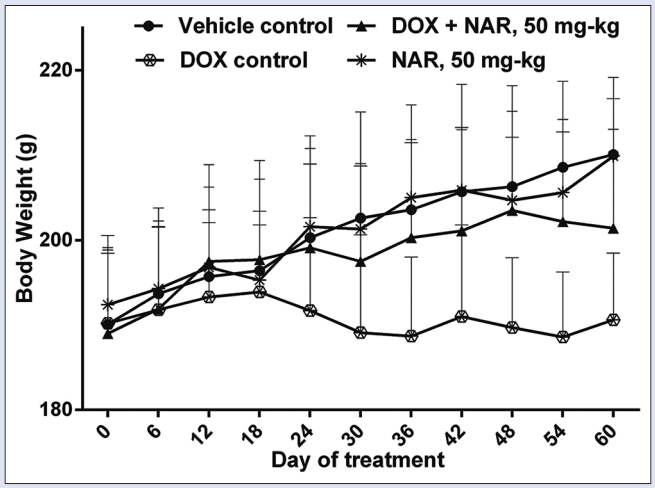

Figure 8: Illustration represents time course of change in body weight across the treatment groups (n = 9)

Table 3: Data represent mean±standard error mean of various hematological parameters

Treatment	RBC (×10⁶ cells/µl)	Hb % (g/dl)	WBC (×10³ cells/µl)	Granulocytes (×10³ cells/µl)	Lymphocytes (×10³ cells/µl)	Monocytes (×10³ cells/µl)	Platelets (×10³ cells/µl)
Vehicle control	7.94±0.15	12.86±0.17	8.68±0.68	1.18±0.10	8.04±0.55	1.11±0.10	538.11±23.55
DOX control	5.93±0.22***	10.81±0.16***	7.01±0.48	0.94±0.07	5.62±0.46	1.05±0.05	540.11±22.50
Naringin + DOX	8.75±0.42###	13.82±0.39###	7.32±0.68	1.22±0.15	7.33±0.27	1.11±0.12	533.88±31.02
Naringin	8.28±0.12###	13.14±0.28###	8.07±0.52	1.3±0.28	6.85±0.29	0.88±0.07	521.55±17.84

***P<0.001 compared to vehicle control, ###P<0.001 compared to DOX control. DOX: Doxorubicin; RBC: Red blood cell; WBC: White blood cell; Hb: Hemoglobin

Later, the flavonoids were evaluated for their influence on cell cycle analysis using flow cytometry which revealed that DOX arrests cells in S and G_2/M phases, and the flavonoid treatment has prevented these changes in cell cycle progression moderately.

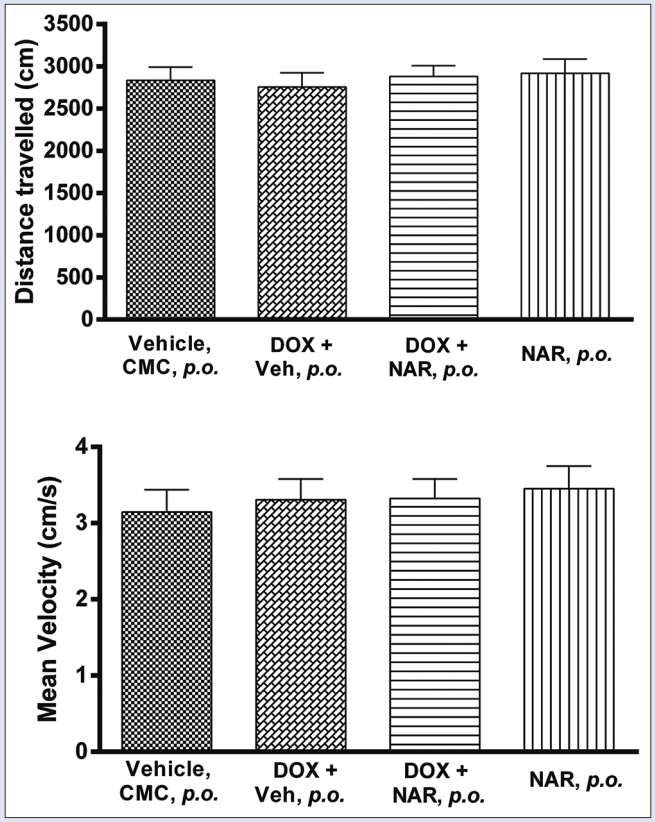

Figure 9: Illustration represents locomotor activity for the effect of naringin and doxorubicin on mean distance traveled and mean velocity in open field task ($n = 6$)

Table 4: Data represent mean±standard error mean of acetylcholinesterase enzyme activity in hippocampal and frontal cortex regions in brain (n=6)

Treatment	Acetylcholinesterase activity (μM of acetylthiocholine hydrolyzed/min/mg of protein)	
	Hippocampus	Frontal cortex
Vehicle control	7.075±0.303	8.819±0.414
DOX control	7.646±0.614	10.459±0.729
Naringin + DOX	6.773±0.267	9.100±0.189
Naringin	6.196±0.611	8.107±0.733

DOX: Doxorubicin

Further, we attempted to identify the mechanism underlying neuroprotective action by studying differentiated IMR-32 neurons for neurite-promoting effects. DOX significantly inhibited the neurite formation while prior treatment with flavonoids averted the inhibitory effect of DOX on neurite length. This indicates that NAR and NGN protected against neurotoxic (DOX) insult in differentiated neuronal cells in the course of the neurite development and formation of neuronal network. During the neuronal network creation, the first and foremost process that needs to take place is establishing neurite outgrowth implicated in synaptic plasticity and long-term potentiation. Mechanisms that regulate neurite outgrowth are tyrosine kinase receptor-mediated MEK/ERK and PI3K/Akt signaling pathways.[56] Compounds that show potent neurotrophic effects are of value in the treatment of brain injury and regeneration.[57] Chemotherapy as a result of brain neuronal damage may lead to cognitive dysfunction, and hence, the flavonoid NAR may be of great value in the treatment of chemobrain through its potential neurite-promoting effect.

We also found that prior treatment with NAR or NGN in cells resulted in significant decline in percentage apoptotic cell death induced by DOX was significantly reduced by of apoptotic cells, which indicates that neuroprotective potential of flavonoids is through inhibition of apoptotic pathways. Pretreatment with NAR or NGN led to significant ($^{\#\#}P < 0.001$) inhibition of DOX-induced ROS generation in a dose-dependent manner, which shows that they were able to scavenge the oxidative-free radicals produced by DOX in IMR-32 cells.

Since the flavonoid NAR contains the aglycone moiety NGN, it was also studied for *in vitro* neuroprotection in IMR-32 for any differentiating effects. However, the *in vivo* study evaluated the chemobrain preventive potential of flavonoid, NAR only as it already contains NGN as its aglycone portion.

Chronic treatment with cytotoxic agents can result in loss of body weight, and we noticed that DOX control animals showed comparatively low body weight with others; however, mean body weight is not significantly different. Stimulation or inhibition of locomotor activity will have confounding influences on behavioral assessment for evaluating cognitive function. In the present study, to know whether DOX and NAR treatments have influencing effects on locomotion, we have tested locomotion following the 50 days of DOX/NAR treatment. None of treatments had affected locomotor behavior, which supports the use of NORT assay for studying the chemofog.

One of the important cytotoxic mechanisms of DOX was generation of reactive oxygen species through its quinone redox cycling *in vivo*.[58] It was found that DOX has produced ROS which is indicated by significant reduction in antioxidant markers which would have been exhausted to balance the elevated oxidative stress. In hippocampal and frontal cortex regions for DOX control group, the antioxidant defense systems such as SOD, catalase, GSH, and total thiols levels were significantly reduced. The *in vitro* antioxidant potential of flavonoid NAR was correlated with *in vivo* antioxidant activity as NAR treatment produced a significant improvement of these defense systems compared to DOX control.

Table 5: Data represent mean±standard error mean of antioxidant markers in hippocampal and frontal cortex regions in brain (n=6)

Treatment	Hippocampal markers				Frontal cortex markers			
	Catalase (units/mg of protein)	SOD (units/mg of protein)	GSH (μg/mg of protein)	Total thiols (μg/mg of protein)	Catalase (units/mg of protein)	SOD (units/mg of protein)	GSH (μg/mg of protein)	Total thiols (μg/mg of protein)
Vehicle control	1.94±0.13	12.86±1.27	4.68±0.68	11.18±0.98	2.14±0.13	12.47±1.13	6.66±0.52	13.55±1.06
DOX control	0.93±0.12*	7.41±0.64 *	2.01±0.22*	5.54±0.73*	1.23±0.13*	5.41±0.97**	3.11±0.42*	6.66±0.96*
Naringin + DOX	1.85±0.42	11.08±1.23	4.12±0.62	10.82±1.15	1.95±0.22	13.08±1.23	5.92±0.96	12.95±2.66
Naringin	1.91±0.12	13.58±1.19	5.07±0.52	12.31±2.28	1.99±0.32	13.58±1.18	6.05±1.06	12.31±1.22

*P<0.05, **P<0.01 compared to vehicle control. SOD: Superoxide dismutase; GSH: Glutathione; DOX: Doxorubicin

Table 6: Data represents mean±standard error mean of biochemical parameters in serum (*n*=6)

Parameters	Vehicle control	DOX control	Naringin + DOX	Naringin
AST	129.72±8.46	123.53±4.95	123.50±8.26	115.68±8.15
ALT	43.60±3.03	45.47±3.19	49.77±2.53	41.10±2.38
ALP	104.52±5.80	105.98±7.55	98.83±9.16	93.73±6.29
CKL	375.05±41.37	801.83±167.91*	411.73±52.92$^{\#}$	338.18±47.80
Glucose	73.46±6.92	81.65±2.92	74.67±5.22	82.28±4.20
Urea	45.77±8.06	95.76±8.87***	43.54±7.82$^{\#\#\#}$	43.10±4.32
Total cholesterol	51.12±3.18	85.40±3.77***	62.54±2.17$^{\#\#\#}$	57.46±3.29
Triglyceride	81.98±4.67	147.58±7.53***	115.13±4.46$^{\#\#}$	78.733±4.41
Total bilirubin	0.10±0.00	0.10±0.00	0.08±0.02	0.08±0.02
Creatinine	32.32±2.17	31.23±1.98	30.65±0.55	31.53±2.01
Total protein	7.33±0.41	6.97±0.14	6.55±0.26	7.43±0.2
Albumin	4.35±0.30	4.32±0.23	3.98±0.37	4.21±0.15

*P<0.05, ***P<0.001 compared to control and $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001 compared to DOX control. DOX: Doxorubicin; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ALP: Alkaline phosphatase; CKL: Creatine kinase level

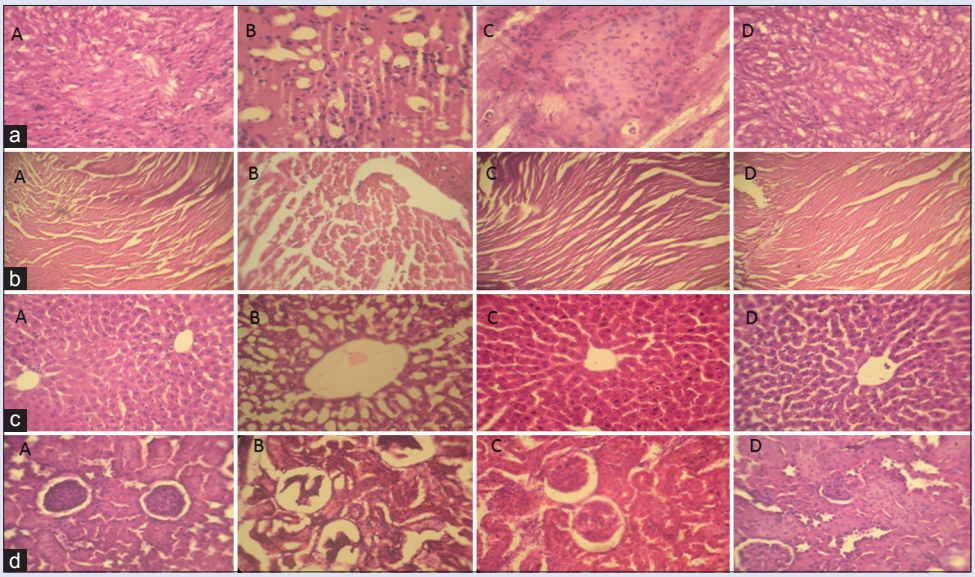

Figure 10: Histopathological analysis of major organs: (a) Cerebral cortex: (A) Normal control showing normal histological features, (B) doxorubicin control with abnormal and disrupted morphological features with numerous vacuoles and marked gliosis, (C) rats treated with doxorubicin and naringin (naringin; 50 mg/kg, p.o.) reflecting the slight morphological changes with reduced gliosis, (D) rats treated with only naringin showing the histological features as that of normal control. (b). Heart: (A) Normal control displaying healthy histological architecture of myocardial tissue, (B) doxorubicin control with numerous vacuoles and degeneration of normal tissue structures, (C) rats treated with doxorubicin and naringin (50 mg/kg, p.o.) showing slight histological changes in the architecture, (D) rats treated with naringin only showing no visible lesions as that of normal control. (c). Liver: (A) Normal control exhibiting histological architecture of normal hepatocyte parenchyma, central vein, (B) rats treated with doxorubicin showing degeneration of parenchyma and central vein dilation with necrosis, (C) naringin and doxorubicin-treated animals displaying slight modification of hepatocyte parenchymal and central vein morphology, (D) naringin *per se* group animals exhibiting liver histology compared to that of control. (d). Kidney: (A) Healthy control rats showing normal histological morphology of kidney with intact renal corpuscle and proximal tubules, (B) doxorubicin-treated rats showing abnormal histology with necrosis of renal corpuscles, proximal tubules, and medullary congestion, (C) rats treated with doxorubicin and naringin displaying healthy histological morphology as control, (D) naringin-treated group exhibiting normal kidney histology as that of control

To know whether chemotherapy or other treatments were associated with any depression-like states, we have studied cortisol levels in serum and found that levels were in nondetectable range across the groups, which shows no influencing effects of depression-like states in assessing the cognitive function in developed chemobrain model for assessing episodic memory. NAR or DOX treatment alone or in combination did not produce depression-like states which would otherwise have confounding influence on memory assessment.

AChE activity was assessed in the brain regions that are involved in the formation of memory, i.e., hippocampus and frontal cortex. Almost similar enzyme activity with no significant differences was observed in both the regions across the treatment groups. This shows that the pathological changes underlying chemobrain were different from neurodegenerative diseases such as Alzheimer's disease where the AChE inhibition seemed to be an effective yet purely symptomatic therapeutic strategy.

Episodic memory is defined as the memory of autobiographical events in relation to times and places and is the most useful form of memory for accomplishing day-to-day activities. The key player in encoding and retrieval of episodic memory is the prefrontal cortex.[59,60] In addition, other anatomical structures that are crucial for episodic memory are the amygdala, brainstem, and hippocampus. Declarative or explicit memory is one of the two major types of memory, consisting of episodic and semantic memories.[61] In the preliminary studies, we noticed that female rats were able to recognize and discriminate the novel object

from the familiar one at a trial delay (ITI) of 2 h. Hence, using the same ITI, we performed the NORT upon completion of DOX chemotherapy. In chemobrain *in vivo* study, DOX was found to impair the episodic memory whereas NAR coadministration significantly prevented episodic memory deficits and rats were able to discriminate between objects. This reflects that DOX has produced chemobrain condition with object recognition deficits, while NAR was able to attenuate these deficits when given with DOX.

Hematological profiling indicated that with comparison to vehicle-treated group, chronic DOX treatment caused a significant reduction in RBC and hemoglobin levels with moderate effects on WBC and platelets. On the other hand, chronic NAR treatment alone or in combination with DOX demonstrated an myeloprotective impact. Organ index was assessed and found to be of insignificant difference among the treated groups. Biochemical analysis revealed that chronic DOX treatment produced significant elevation of creatine kinase (marker of myocardial damage) while NAR treatment attenuated this increase in CK level showing cardioprotective effect. Furthermore, a significant increase in cholesterol, triglyceride, and urea was observed with DOX treatment; however, these changes were prevented by NAR treatment. This shows that NAR was able to prevent the metabolic changes associated with DOX.

Histopathological analysis also confirmed that chronic DOX produced gross structural abnormalities in major organs such as liver, heart, kidney, and cerebral cortex which were found to be reduced by treatment with the flavonoid glycoside NAR. This indicates the utility of NAR as potential cardio-, hepato-, neuro-, and nephro-protective agent against DOX toxicity.

The *in vitro* neuroprotective potential of flavonoid glycoside NAR has correlated with the antichemobrain activity of NAR *in vivo*. NAR through its neuroprotective and other pleiotropic effects, viz., anti-oxidant, anti-dementia, anti-inflammatory, and neuritogenic, was able to prevent the DOX-induced cognitive deficits for episodic memory in NORT in Wistar rats without any confounding effects on locomotion.

CONCLUSION

Chronic treatment of DOX produced episodic memory deficits in Wistar rats and flavonoid glycoside NAR was able to exert neuroprotective effect (neurotropic, antioxidant, anti-apoptotic, etc.) against these deficits associated with DOX, upon coadministration. Hence, NAR may be a potential adjuvant therapeutic intervention for alleviating the cognitive deficits associated with DOX-induced chemobrain condition *in vivo*. Further studies are warranted to assess the effect of DOX-induced chemobrain condition on other forms of memory, viz., working, spatial and emotional memories.

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Ahles TA, Saykin AJ. Candidate mechanisms for chemotherapy-induced cognitive changes. Nat Rev Cancer 2007;7:192-201.
2. Nelson CJ, Nandy N, Roth AJ. Chemotherapy and cognitive deficits: Mechanisms, findings, and potential interventions. Palliat Support Care 2007;5:273-80.
3. Berndt U, Leplow B, Kantelhardt E, Thomssen C. Cognitive effects of systemic therapy in patients with breast cancer. Breast Care (Basel) 2009;4:177-82.
4. Joly F, Rigal O, Noal S, Giffard B. Cognitive dysfunction and cancer: Which consequences in terms of disease management? Psychooncology 2011;20:1251-8.
5. Davis J, Ahlberg FM, Berk M, Ashley DM, Khasraw M. Emerging pharmacotherapy for cancer patients with cognitive dysfunction. BMC Neurol 2013;13:153.
6. Fardell JE, Vardy J, Johnston IN, Winocur G. Chemotherapy and cognitive impairment: Treatment options. Clin Pharmacol Ther 2011;90:366-76.
7. Konat GW, Kraszpulski M, James I, Zhang HT, Abraham J. Cognitive dysfunction induced by chronic administration of common cancer chemotherapeutics in rats. Metab Brain Dis 2008;23:325-33.
8. Macleod JE, DeLeo JA, Hickey WF, Ahles TA, Saykin AJ, Bucci DJ, *et al.* Cancer chemotherapy impairs contextual but not cue-specific fear memory. Behav Brain Res 2007;181:168-72.
9. Fardell JE, Vardy J, Shah JD, Johnston IN. Cognitive impairments caused by oxaliplatin and 5-fluorouracil chemotherapy are ameliorated by physical activity. Psychopharmacology (Berl) 2012;220:183-93.
10. Fardell JE, Vardy J, Logge W, Johnston I. Single high dose treatment with methotrexate causes long-lasting cognitive dysfunction in laboratory rodents. Pharmacol Biochem Behav 2010;97:333-9.
11. Aluise CD, Sultana R, Tangpong J, Vore M, St. Clair D, Moscow JA, *et al.* Chemo brain (chemo fog) as a potential side effect of doxorubicin administration: Role of cytokine-induced, oxidative/nitrosative stress in cognitive dysfunction. Adv Exp Med Biol 2010;678:147-56.
12. Sardi I, la Marca G, Giovannini MG, Malvagia S, Guerrini R, Genitori L, *et al.* Detection of doxorubicin hydrochloride accumulation in the rat brain after morphine treatment by mass spectrometry. Cancer Chemother Pharmacol 2011;67:1333-40.
13. Liedke PE, Reolon GK, Kilpp B, Brunetto AL, Roesler R, Schwartsmann G, *et al.* Systemic administration of doxorubicin impairs aversively motivated memory in rats. Pharmacol Biochem Behav 2009;94:239-43.
14. Farver D. The use of "natural products" in clinical medicine. South Dakota J Med 1996;49:129-30.
15. Williams RJ, Spencer JP, Rice-Evans C. Flavonoids: Antioxidants or signalling molecules? Free Radic Biol Med 2004;36:838-49.
16. Wang Y, Wang L, Wu J, Cai J. The *in vivo* synaptic plasticity mechanism of EGb 761-induced enhancement of spatial learning and memory in aged rats. Br J Pharmacol 2006;148:147-53.
17. Spencer JP. The impact of flavonoids on memory: Physiological and molecular considerations. Chem Soc Rev 2009;38:1152-61.
18. Kim HJ, Song JY, Park HJ, Park HK, Yun DH, Chung JH, *et al.* Naringin protects against rotenone-induced apoptosis in human neuroblastoma SH-SY5Y cells. Korean J Physiol Pharmacol 2009;13:281-5.
19. Raza SS, Khan MM, Ahmad A, Ashafaq M, Islam F, Wagner AP, *et al.* Neuroprotective effect of naringenin is mediated through suppression of NF-κB signaling pathway in experimental stroke. Neuroscience 2013;230:157-71.
20. Kanno S, Shouji A, Tomizawa A, Hiura T, Osanai Y, Ujibe M, *et al.* Inhibitory effect of naringin on lipopolysaccharide (LPS)-induced endotoxin shock in mice and nitric oxide production in RAW 264.7 macrophages. Life Sci 2006;78:673-81.
21. Liu Y, Wu H, Nie YC, Chen JL, Su WW, Li PB, *et al.* Naringin attenuates acute lung injury in LPS-treated mice by inhibiting NF-κB pathway. Int Immunopharmacol 2011;11:1606-12.
22. Golechha M, Chaudhry U, Bhatia J, Saluja D, Arya DS. Naringin protects against kainic acid-induced status epilepticus in rats: Evidence for an antioxidant, anti-inflammatory and neuroprotective intervention. Biol Pharm Bull 2011;34:360-5.
23. Kumar P, Kumar A. Protective effect of hesperidin and naringin against 3-nitropropionic acid induced huntington's like symptoms in rats: Possible role of nitric oxide. Behav Brain Res 2010;206:38-46.
24. Kumar A, Dogra S, Prakash A. Protective effect of naringin, a citrus flavonoid, against colchicine-induced cognitive dysfunction and oxidative damage in rats. J Med Food 2010;13:976-84.
25. Kumar A, Prakash A, Dogra S. Naringin alleviates cognitive impairment, mitochondrial dysfunction and oxidative stress induced by D-galactose in mice. Food Chem Toxicol 2010;48:626-32.
26. Gaur V, Aggarwal A, Kumar A. Protective effect of naringin against ischemic reperfusion cerebral injury: Possible neurobehavioral, biochemical and cellular alterations in rat brain. Eur J Pharmacol 2009;616:147-54.
27. Gerlier D, Thomasset N. Use of MTT colorimetric assay to measure cell activation. J Immunol Methods 1986;94:57-63.
28. Ramalingayya GV, Cheruku SP, Nayak PG, Kishore A, Shenoy R, Rao CM, *et al.* Rutin protects against neuronal damage *in vitro* and ameliorates doxorubicin-induced memory deficits *in vivo* in wistar rats. Drug Des Devel Ther 2017;11:1011-26.

29. Nampoothiri M, Reddy ND, John J, Kumar N, Kutty Nampurath G, Rao Chamallamudi M, *et al*. Insulin blocks glutamate-induced neurotoxicity in differentiated SH-SY5Y neuronal cells. Behav Neurol 2014;2014:674164.
30. Meijering E, Jacob M, Sarria JC, Steiner P, Hirling H, Unser M, *et al*. Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. Cytometry A 2004;58:167-76.
31. Ribble D, Goldstein NB, Norris DA, Shellman YG. A simple technique for quantifying apoptosis in 96-well plates. BMC Biotechnol 2005;5:12.
32. Wang H, Joseph JA. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Radic Biol Med 1999;27:612-6.
33. Ennaceur A, Delacour J. A new one-trial test for neurobiological studies of memory in rats 1: Behavioral data. Behav Brain Res 1988;31:47-59.
34. Ennaceur A, Meliani K. Effects of physostigmine and scopolamine on rats' performances in object-recognition and radial-maze tests. Psychopharmacology (Berl) 1992;109:321-30.
35. Verma T, Mallik SB, Ramalingayya GV, Nayak PG, Kishore A, Pai KSR, *et al*. Sodium valproate enhances doxorubicin-induced cognitive dysfunction in wistar rats. Biomed Pharmacother 2017;96:736-41.
36. Ramalingayya GV, Nampoothiri M, Nayak PG, Kishore A, Shenoy RR, Mallikarjuna Rao C, *et al*. Naringin and rutin alleviates episodic memory deficits in two differentially challenged object recognition tasks. Pharmacogn Mag 2016;12:S63-70.
37. Ramalingayya GV, Gourishetti K, Kishore A, Nandakumar K. Assessment of female rats for studying episodic memory and its deficit associated with doxorubicin-induced chemobrain. Clin Exp Pharmacol Physiol 2016;43:644-6.
38. Antunes M, Biala G. The novel object recognition memory: Neurobiology, test procedure, and its modifications. Cogn Process 2012;13:93-110.
39. Abel EL. Behavioral effects of isatin on open field activity and immobility in the forced swim test in rats. Physiol Behav 1995;57:611-3.
40. Prut L, Belzung C. The open field as a paradigm to measure the effects of drugs on anxiety-like behaviors: A review. Eur J Pharmacol 2003;463:3-3.
41. Moron MS, Depierre JW, Mannervik B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochim Biophys Acta 1979;582:67-78.
42. Misra HP, Fridovich I. The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. J Biol Chem 1972;247:3170-5.
43. Aebi H. Catalase. Methods of Enzymatic Analysis (Second Edition): Elsevier; 1974;2: p. 673-84.
44. Buege JA, Aust SD. Microsomal lipid peroxidation. Methods Enzymol 1978;52:302-10.
45. Sedlak J, Lindsay RH. Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with ellman's reagent. Anal Biochem 1968;25:192-205.
46. John J, Nampoothiri M, Kumar N, Mudgal J, Nampurath GK, Chamallamudi MR, *et al*. Sesamol, a lipid lowering agent, ameliorates aluminium chloride induced behavioral and biochemical alterations in rats. Pharmacogn Mag 2015;11:327-36.
47. Ellman GL, Courtney KD, Andres V Jr., Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95.
48. Cardoso S, Santos RX, Carvalho C, Correia S, Pereira GC, Pereira SS, *et al*. Doxorubicin increases the susceptibility of brain mitochondria to ca(2+)-induced permeability transition and oxidative damage. Free Radic Biol Med 2008;45:1395-402.
49. Freeman JR, Broshek DK. Assessing cognitive dysfunction in breast cancer: What are the tools? Clin Breast Cancer 2002;3 Suppl 3:S91-9.
50. Schagen SB, Hamburger HL, Muller MJ, Boogerd W, van Dam FS. Neurophysiological evaluation of late effects of adjuvant high-dose chemotherapy on cognitive function. J Neurooncol 2001;51:159-65.
51. Ahles TA, Saykin AJ, Furstenberg CT, Cole B, Mott LA, Skalla K, *et al*. Neuropsychologic impact of standard-dose systemic chemotherapy in long-term survivors of breast cancer and lymphoma. J Clin Oncol 2002;20:485-93.
52. Huang Y, Zitta K, Bein B, Scholz J, Steinfath M, Albrecht M, *et al*. Effect of propofol on hypoxia re-oxygenation induced neuronal cell damage *in vitro**. Anaesthesia 2013;68:31-9.
53. Wanakhachornkrai O, Pongrakhananon V, Chunhacha P, Wanasuntronwong A, Vattanajun A, Tantisira B, *et al*. Neuritogenic effect of standardized extract of centella asiatica ECa233 on human neuroblastoma cells. BMC Complement Altern Med 2013;13:204.
54. Hanstein R, Trotter J, Behl C, Clement AB. Increased connexin 43 expression as a potential mediator of the neuroprotective activity of the corticotropin-releasing hormone. Mol Endocrinol 2009;23:1479-93.
55. Kataria H, Wadhwa R, Kaul SC, Kaur G. Water extract from the leaves of *Withania somnifera* protect RA differentiated C6 and IMR-32 cells against glutamate-induced excitotoxicity. PLoS One 2012;7:e37080.
56. Kaplan DR, Miller FD. Neurotrophin signal transduction in the nervous system. Curr Opin Neurobiol 2000;10:381-91.
57. Horner PJ, Gage FH. Regenerating the damaged central nervous system. Nature 2000;407:963-70.
58. Bachur NR, Gordon SL, Gee MV. Anthracycline antibiotic augmentation of microsomal electron transport and free radical formation. Mol Pharmacol 1977;13:901-10.
59. Fletcher PC, Shallice T, Dolan RJ. The functional roles of prefrontal cortex in episodic memory. I. Encoding. Brain 1998;121(Pt 7):1239-48.
60. Fletcher PC, Shallice T, Frith CD, Frackowiak RS, Dolan RJ. The functional roles of prefrontal cortex in episodic memory. II. Retrieval. Brain 1998;121(Pt 7):1249-56.
61. Schacter DL, Gilbert DT, Wegner DM. Semantic and episodic memory. Psychology. 2nd ed. New York: Worth, Incorporated; 2011. p. 240-1.