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ORIGINAL ARTICLE

Catechin ameliorates doxorubicin-induced neuronal cytotoxicity in in vitro and episodic memory deficit in in vivo in Wistar rats

Sri Pragnya Cheruku · Grandhi Venkata Ramalingayya · Mallikarjuna Rao Chamallamudi · Subhankar Biswas · Krishnadas Nandakumar · Madhavan Nampoothiri · Karthik Gourishetti · Nitesh Kumar

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Abstract Cognitive dysfunction by chemotherapy compromises the quality of life in cancer patients. Tea polyphenols are known chemopreventive agents. The present study was designed to evaluate the neuroprotective potential of (+) catechin hydrate (catechin), a tea polyphenol, in IMR-32 neuroblastoma cells in vitro and alleviation of episodic memory deficit in Wistar rats in vivo against a widely used chemotherapeutic agent, Doxorubicin (DOX). In vitro, neuroprotective studies were assessed in undifferentiated IMR-32 cells using percentage viability and in differentiated cells by neurite length. These studies showed catechin increased percentage viability of undifferentiated IMR-32 cells. Catechin pretreatment also showed an increase in neurite length of differentiated cells. In vivo neuroprotection of catechin was evaluated using novel object recognition task in time-induced memory deficit model at 50, 100 and 200 mg/kg dose and DOX-induced memory deficit models at 100 mg/kg dose. The latter model was developed by injection of DOX (2.5 mg/kg, *i.p.*) in 10 cycles over 50 days in Wistar rats. Catechin showed a significant reversal of time-induced memory deficit in a dose-dependent manner and prevention of DOX-induced memory deficit at 100 mg/kg. In addition, catechin treatment showed a significant decrease in oxidative stress, acetylcholine esterase and neuroinflammation in the hippocampus and cerebral cortex in DOX-induced toxicity model. Hence, catechin may be a potential adjuvant therapy for the amelioration of DOX-induced cognitive impairment which may improve the quality of life of cancer survivors. This improvement might be due to the elevation of antioxidant defense, prevention of neuroinflammation and inhibition of acetylcholine esterase enzyme.

Keywords Chemobrain · Doxorubicin · (+) Catechin hydrate · IMR-32 cells · Neuroprotection · Episodic memory

S. P. Cheruku · G. V. Ramalingayya · M. R. Chamallamudi · S. Biswas · K. Nandakumar · M. Nampoothiri · K. Gourishetti · N. Kumar (✉)
Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal University, Manipal, Karnataka 576104, India
e-mail: niteshkumar43@gmail.com; nitesh.kumar@manipal.edu

Introduction

Doxorubicin (DOX) is one of the most widely used broad-spectrum anthracycline anticancer drug either alone or in combination with other agents in the management of most forms of neoplastic diseases. The dose-dependent toxicity, especially the major organ (heart, kidney, liver, and brain) toxicity hinders the therapeutic efficacy of chemotherapy and limits its use. The major toxicities of DOX are myelosuppression, cardiotoxicity (Goodman 1996; Octavia et al.

2012; Rang 2007) and cognitive decline (Ahles and Saykin 2002, 2007; Aluise et al. 2010b). Among these toxicities, cognitive decline following cancer chemotherapy is one of the major problems which compromises the quality of life, even though the burden of cancer reduces. This significantly affects various aspects of survivor's day to day life (Goodman 1996; Octavia et al. 2012; Rang 2007). This behavioral and cognitive impairment after chemotherapy is known as Post Chemotherapy Cognitive Impairment (PCCI) or "Chemobrain" characterized by memory lapses, disorganized thinking, trouble in concentrating, remembering dates, names etc. along with hindered processing speed compared to the normal population (Boykoff et al. 2009). The reason for the cognitive decline is considered to be neuronal damage by promoting the formation of DNA double-strand breaks in neurons (Manchon et al. 2016). High level of DNA damage leads to accumulation/activation of P53 which in turn leads to cell cycle arrest and apoptosis (Deavall et al. 2012). Therefore, chemoprotective agents which not only alleviate the cytotoxicity but also alter the cell cycle arrest, are gaining importance for their potential role to alleviate the unwanted side effects of chemotherapeutic agents.

Plant-derived products are being used since a long time in herbal and traditional ayurvedic system of medicine. Some of the main constituents being polyphenols such as curcumin, garlic, alpha-lipoic acid, epigallocatechin gallate, alpha-tocopherol etc. which have shown chemoprotective effect against various toxic agents through their antioxidant properties (Dholwani et al. 2008; Schimmel et al. 2004). Since, tea is a widely consumed aromatic beverage in many countries, its main active constituent or derived flavonoid, catechin was selected for the present study. Catechins (chemically flavan-3-ol) such as (+) catechin hydrate (catechin), epicatechin, epigallocatechin gallate, natural polyphenols are present in high concentrations in tea leaves (Higdon and Frei 2003). They belong to the class of flavonoids and are known for their pleiotropic pharmacological actions, viz., antioxidant, anti-inflammatory and anticancer effects (Abd El-Aziz et al. 2012). Moreover, its antioxidant property shows neuroprotective effect against streptozotocin, 6-hydroxy dopamine (6-OHDA) and quinolinic acid (Ahmed et al. 2013; Braidy et al. 2010; Teixeira et al. 2013). Further, it was noted that catechins were synergistically effective along with the chemotherapy in alleviating various forms of neoplasia (Suganuma et al. 2011). A recent report on tea catechins indicated its preventive effect to neuron against mitochondrial damage during aging process (Assuncao and Andrade 2015) and HIV-associated neurocognitive disorder (Nath et al. 2012). Taking these points into consideration, the present study was designed to evaluate in vitro neuroprotective effect of (+) Catechin hydrate (catechin) against DOX-induced toxicity on IMR-32 cells and to assess its in vivo protective potential to alleviate DOX-induced cognitive decline in Wistar rats. An attempt was also made to elucidate the molecular pathways through which catechin exerts its neuroprotective potential.

Materials and methods

Chemicals and apparatus

Doxorubicin (Fresenius Kabi Oncology Ltd., Solan, India), Trypsin–EDTA, Dipotassium EDTA, 3-[4,5-dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), retinoic acid (RA) were acquired from Himedia Chemicals (Nagpur, Maharashtra, India). (+) Catechin hydrate (catechin) and DMEM was procured from Sigma Aldrich (St. Louis, MO, USA). Sterile 96 and 6 well plates, 25 and 75 cm^2 culture flasks were procured from Tarsons Product Pvt. Ltd. (Kolkata, West Bengal, India). Donepezil was procured as gift sample from Intas pharmaceuticals Ltd. (Ahmedabad, India). Acethylthiocholin iodide, and DTNB were procured from Sigma-Aldrich Inc.

Object recognition task (ORT) was carried out by using 49 cm (length × width × height) square arenas. The behavior of animals was monitored with the help of a camera (model: Quickcam Pro9000, Logitech International S.A., Lausanne, Switzerland) which was mounted 160 cm above the behavioral observation arenas. Two hand-held stopwatches and timer alarm were also used to observe the animals' behavior during the given trials.

Animals

Twelve weeks old, healthy male rats weighing 200–230 g were used for time-induced memory deficits model and DOX -induced chemobrain model.

Use of animals and the protocol was approved by the Institutional Animal Ethics Committee (Approval No. IAEC/KMC/80/2014) with adherence to Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) (New Delhi, India). Animals were maintained at Central Animal Research Facility of Manipal University, Manipal as per the directions specified by CPCSEA guidelines. A 12/12 h light and dark cycle was maintained with lights on at 07:00 AM. Standard rat pellet diet as well as potable water was given ad libitum.

In vitro studies

IMR-32 cell line is male Caucasian derived neuroblastoma cell line. It was procured from NCCS (Pune, India), subcultured in DMEM medium with 10% of fetal bovine serum. The cells were used for cell viability and cyto-protection studies. The treatments of catechin and DOX in the neuroprotection study were simultaneous (without changing the medium), where catechin was added 1 h prior to DOX addition.

MTT assay in undifferentiated IMR-32 cells

Five thousand cells per well were seeded in microplate consisting of 50 µl of medium and incubated for 24 h. After 24 h, 50 µl of catechin was added in a concentration ranging from 31.23 to 250 µg/ml in the wells for one hour. After that 50 µl of DOX (1 or 2 µg/ml) was added and incubated for 24 h. Followed by that 50 µl of MTT (2 mg/ml) was added and incubated at 37 °C for 3 h, after which the medium was removed and 100 µl of DMSO was added, and shaken for about 5 min on an orbital shaker. Formazan crystals formed were allowed to dissolve in DMSO. The absorbance of DMSO solubilized formazan was read at 540 nm (Shi et al. 2015). IC_{50} of catechin was calculated by fitting the data to non-linear regression using GraphPad Prism.

Neurite length of differentiated IMR-32 cells

The six well plates were seeded with 2 million cells per well for 24 h. After 24 h, fetal bovine serum was reduced from 10 to 5% initially for the first 3 days thereby starving the cells for promoting neuronal growth and 10 µM of all-trans-retinoic acid was also supplemented in the medium thereby promoting differentiation. Depending upon the extent of differentiation, the cells were allowed to differentiate for next 10 days (Kataria et al. 2012). Since differentiated cells were used for neurite length estimation, the concentration of DOX selected was 1.5 µg/ml, which was in between the tested dose of DOX in undifferentiated cells.

On day 13, the differentiated cells were observed for neurite growth. Cells were treated with catechin 40 µg/ml in 50 µl for one hour followed by addition of DOX at 1.5 mg/ml in 50 µl for 48 h. After 48 h of incubation, the medium was removed to stop the treatment, washed with phosphate buffer saline (PBS) and 3 ml PBS was added to observe the length of neurite under an inverted microscope at 40× objective and images were taken to assess the effect of treatments on the morphology of the neuronal cells. Approximately 100 images were taken from each treatment well. The neurite length was estimated by the public domain NIH Image J software. The length was defined as the straight-line distance from the tip of the neurite to the junction between the cell body and neurite base and expressed in µm.

Cell cycle analysis

Flow cytometric technique was used to evaluate the effect of catechin on DOX- induced alteration of cell cycle. One million differentiated cells were seeded in flasks for overnight and incubated with catechin (40 µg/ml) at 37 °C for 2 h, followed by DOX at 1.5 µg/ml for next 24 h. Cells were separated from the flask by trypsinization, washed with PBS with centrifugation. The cell pellets were collected and fixed with 70% ice-cold methanol and stored for 24 h at −20 °C. Then cell pellets were washed with PBS and added isotonic PI solution (25 µM propidium iodide, 0.03% NP-40 and 40 µg/ml RNase A) for staining. The stained cells were analyzed with Accuri C6 flow cytometer (BD Biosciences, San Jose, CA, USA) for cell cycle study at excitation wavelength 488 nm and emission wavelength 575/40 nm (Reddy et al. 2015; Simon et al. 2016).

In vivo studies

Selection of doses In the preliminary experiments for assessing the procognitive effect of catechin, the selected doses were 50, 100 and 200 mg/kg for dose–response analysis. In later studies for chemobrain i.e., DOX-induced memory deficit model, the dose of catechin selected was 100 mg/kg as it showed a

promising effect in preliminary studies and moreover, the treatment was on a chronic basis. The dose of DOX selected was 2.5 mg/kg according to the previous studies and standardized laboratory procedures (Steiniger et al. 2004; Swamy et al. 2011; Grandhi et al. 2016).

Preparation and administration of drugs In the preliminary study for assessing the nootropic effect of catechin using time induced memory deficit model, the doses were prepared at 50, 100, 200 mg/kg in 0.25% w/v sodium carboxy methylcellulose (CMC) and administered orally for 7 days prior to and during the experimental trials. Four experimental groups were used (n = 9 each) for one vehicle (CMC) and three groups of catechin (three doses).

For inducing neurotoxicity and systemic toxicity, DOX (Adriamycin at 2.5 mg/kg) was administered intraperitoneally in 10 cycles on every 5 days. Three experimental groups (n = 12 each) were used viz., vehicle control (0.25% w/v CMC), DOX alone and catechin (100 mg/kg in 0.25% CMC *p.o.*). Catechin was administered orally for 57 days including one-week treatment prior to the first cycle of DOX. Following the last cycle of DOX on day 57, i.e., on day 58, the behavioral study was conducted. All treatments, as well as the behavioral analysis, were carried out between 9 a.m. to 4 p.m. Body weight of the animals was taken once in 3 days throughout the study. During the experimental trials, the oral treatment was given 1 h before the familiarization trial.

Novel object recognition test

Episodic memory, the conscious memory of the past experiences was evaluated in this study. The study was carried out for 2–3 days depending on inter-trial interval (ITI) and type of the study. Animals were allowed to explore two similar objects in familiarization trial on day 2 (following a habituation session day 1) in behavioral arenas and after a predetermined ITI, rats were subjected to choice trial with one of the familiar object replaced by a novel object. All the trials lasted for 3 min duration. The exploratory behavior of the rats towards the individual objects during the experimental trials was assessed using two stopwatches by an expert observer who was blind to the treatment groups. Recognition and discriminative indices were calculated from the cumulative exploration time using formulae mentioned in earlier reports (Antunes and Biala 2012). Recognition index (RI) was the ratio of exploration of the novel object (b) to the total exploration time of the animal to both novel and familiar objects ($a_3 + b$) i.e., $RI = b/a_3 + b$. Discrimination index (DI) was a measure of the difference in the exploration time of the novel object (b) and familiar object (a_3) in test phase i.e., $DI = b - a_3$.

Initially, the procognitive effect of catechin was assessed in time-induced natural memory deficit model. With the promising effect in this basic cognitive model, one predetermined dose level was selected and proceeded with the evaluation of catechin effect in DOX-induced cognitive deficit model.

Time-induced natural memory deficits model

Episodic memory deficits can be induced in rats naturally by increasing the time delay between familiarization and choice trials. Hence the initial experiment was conducted to assess the effect of catechin on time -induced memory deficits by using an ITI of 24 h. In this test, rats were habituated to the arenas on day 1 and were subjected to familiarization trial on day 2. Then after an ITI of 24 h, i.e., on day 3, animals were subjected to recognition trial with one novel object replacing the familiar object. Four experimental groups were used. Rats were treated with either catechin (50, 100 and 200 mg/kg, *p.o.*) or CMC (10 ml/kg, *p.o.*) for 7 days before the trial initiation. During the experimental trials, treatment was given 1 h before the actual trial in familiarization and choice trials (Fig. 1).

Doxorubicin-induced memory deficits model

One day after completion of chemotherapy cycles, the behavioural assessment was carried out in novel object recognition task (NORT) assay. Following the habituation session on day 1, animals were subjected to familiarization and choice trials on day 2 with a predetermined ITI of 4 h from the pilot studies. In previous studies, we found that the rats could recognize the objects at an ITI of 4 h, hence we used this ITI. Only the chronic DOX treatment has been used as a challenge for producing memory deficits without any influencing effects of time delay on memory. After completion of the behavioral study in DOX-induced memory deficit model, blood was withdrawn under ketamine anesthesia by retro-orbital sinus puncture and after pericardial perfusion with 0.9% w/v saline.

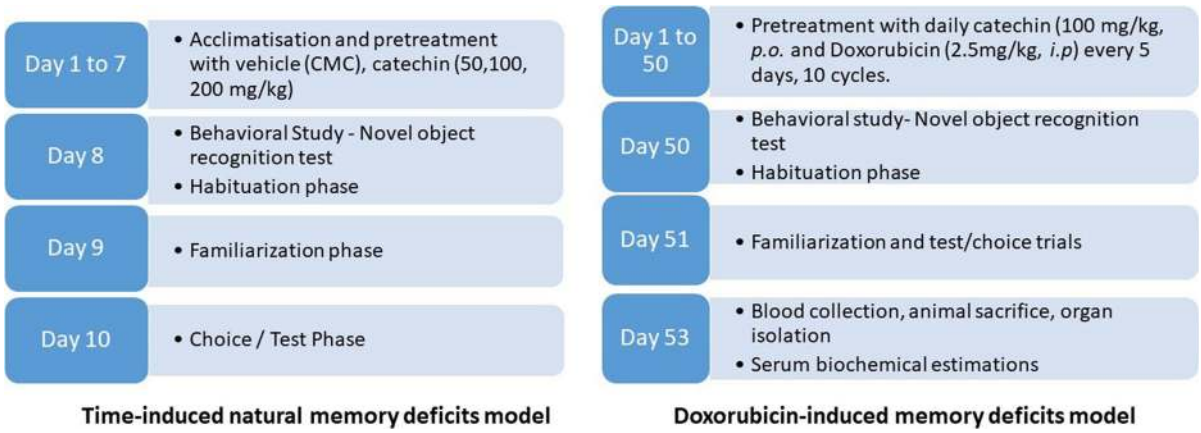

Fig. 1 Scheme of in vivo study

The hippocampal and frontal cortex brain regions were isolated and 10% homogenates were prepared in ice-cold 0.1 M PBS (pH 7.4) using Potter–Elvehjem type homogenizer fitted with Teflon plunger at a speed of 8000 rpm (Fig. 1).

Estimation of ex vivo and in vivo acetylcholinesterase activity

This method of estimation of acetylcholinesterase activity was done by Ellman's procedure using Ellman's reagent (Ellman et al. 1961; Khan et al. 2013). Ex vivo acetylcholinesterase activity was performed using untreated rat brain. The brain was isolated and homogenized using 0.1 M phosphate buffer. Different concentrations of donepezil and catechin ranging from 1 mM to 31.25 µM were prepared and incubated for 45 min with the brain tissue supernatant after which acetylcholinesterase activity estimated by UV spectrophotometric method. The result was expressed as micromoles of acetylthiocholine iodide hydrolyzed per minute per mg of protein.

The in vivo acetylcholinesterase study was performed with a modified method of Khan et al. (2013). Tissue supernatant (0.4 ml) was added directly to a mixture of 0.02 ml of acetylthiocholine iodide, 0.1 ml of DTNB, 2.6 ml of phosphate buffer (pH 8) in the cuvette. The change in the absorbance was recorded at 412 nm at a time interval of 60 s for 4 min. The result was expressed as micromoles of acetylthiocholine iodide hydrolyzed per minute per mg of protein.

Estimation of oxidative stress, inflammatory markers, and total protein levels

Antioxidant parameters namely catalase (Aebi 1984), SOD (Misra and Fridovich 1972), GSH (Hu 1994), lipid peroxidation (Konings and Drijver 1979) were estimated in the tissue homogenate (Kumar et al. 2014). Nitrite level (Bredt and Snyder 1994) and myeloperoxidase (MPO) (Graff et al. 1998) activity were also estimated in brain homogenate. Percentage increase in MPO levels were Total protein was estimated in all the tissue samples by using Pierce® BCA Protein Assay Kit as per the experimental protocol of Thermo Scientific (Waltham, MA, USA).

Statistical analysis

Data were statistically analyzed using Graph Pad Prism (trial version) software. Results of the in vitro and in vivo studies were expressed as mean ± SEM. Novel object recognition test results, exploration time and memory indices (RI and DI) were analyzed by paired Student's *t* test and a non-parametric test (Kruskal–Wallis test followed by Dunnett's post hoc test) respectively. All the other results were analyzed by One-way ANOVA followed by Dunnett's multiple comparison tests.

Table 1 Effect of different concentrations of catechin against doxorubicin at two concentrations of 1 and 2 μg/ml

Conc (μg/ml) of catechin	Percentage cell viability/proliferation rate (mean ± SEM)		
	Catechin	Dox (1 μg/ml)	Dox (2 μg/ml)
0	100 ± 0.046	24.2 ± 3.65[a]	20.8 ± 1.1[a]
31.25	99.91 ± 0.010	47.9 ± 0.019	43.7 ± 0.01
62.5	96.54 ± 0.054	59.9 ± 0.017	53.9 ± 0.006
125	93.17 ± 0.002	93.2 ± 0.027	89.33 ± 0.023
250	68.82 ± 0.003	129.4 ± 0.057	122.64 ± 0.012

Data are mean ± SEM of three readings in triplicate. [a] Dox only treatment, Catechin column represents catechin only treatment. Control showed 100 ± 0.046% viability. DOX alone treatment showed percentage viability 24.2 ± 3.65 and 20.8 ± 1.1 and 1 and 2 μg/ml concentration, respectively. Catechin at 31.25, 62.5, 125, 250, 500 μg/ml concentration showed percentage viability 99.91 ± 0.010, 96.54 ± 0.054, 93.17 ± 0.002, 90.63 ± 0.018, 68.82 ± 0.003 respectively. IC_{50} values for different treatments were: Dox—0.46 μg/ml, catechin—821.1 μg/ml, catechin + Dox (1 μg/ml)—37.61 μg/ml, catechin + Dox (2 μg/ml)—53.47 μg/ml

Results

In vitro neuroprotection studies

Effect of catechin and DOX per se on cell viability/proliferation in IMR-32 cells

Catechin was found to be safe in IMR-32 cells (IC_{50}—821.1 μg/ml). DOX showed toxicity on IMR-32 cells (IC_{50}—0.46 μg/ml). Catechin treatment showed significant protective action in ameliorating DOX-induced death. The IC_{50} value for catechin along with DOX at 1 μg/ml was 37.61 μg/ml and along with DOX at 2 μg/ml was 42.87 μg/ml, while percentage viability after treatment with DOX alone was 24.2 ± 3.65 at 1 μg/ml and 20.8 ± 1.1 at 2 μg/ml (Table 1).

Effect of catechin on doxorubicin-induced neurotoxicity in differentiated IMR-32 cells

There was significant toxicity in the IMR-32 cells after treatment with DOX alone. The cells clearly appeared to be degenerated, rounded and detached. This degeneration caused by the toxicant was reversed by treatment with catechin. The treatment with catechin (40 μg/ml) had shown very few cells prone to toxicity while most of the cells were protected and appeared as normal cells (Fig. 1). DOX showed a significant decrease in the length of neurite as they caused damage to the cellular processes. Pre-treatment with catechin significantly increased the length of the neurite compared to DOX group (Fig. 2).

Effect on cell cycle

Doxorubicin at 1.5 μg/ml showed arrest of cell cycle in both S-phase and G_2-M phase. Catechin pretreatment at 40 μg/ml showed comparative prevention in cell cycle arrest of DOX (Fig. 3).

In vivo pharmacology

Time-induced memory deficit model

In time-induced memory deficit model, it was noted that vehicle-treated rats were not able to discriminate the novel object as they spent almost equal time exploring both novel and familiar objects after an ITI of 24 h. However, treatment with catechin has resulted in significant increase of novel object exploration time as compared to familiar one in a dose-dependent manner. This is also evident from the discriminative and recognition indices among the treatment groups. Animals treated with catechin at the lowest tested dose, i.e., 50 mg/kg, *p.o.* had spent comparatively more time in exploring the novel object in the choice trial, however, the difference was not statistically significant. Catechin prevented the time-induced episodic memory deficits in a dose-dependent manner, the most effective being 200 mg/kg, *p.o.* (Figs. 4, 5). Interestingly catechin at a dose of 100 mg/kg showed a lesser exploration time compared to 50 and 100 mg/kg.

Doxorubicin-induced memory deficits model

Rats treated with saline and CMC spent significantly more time exploring the novel object compared to the

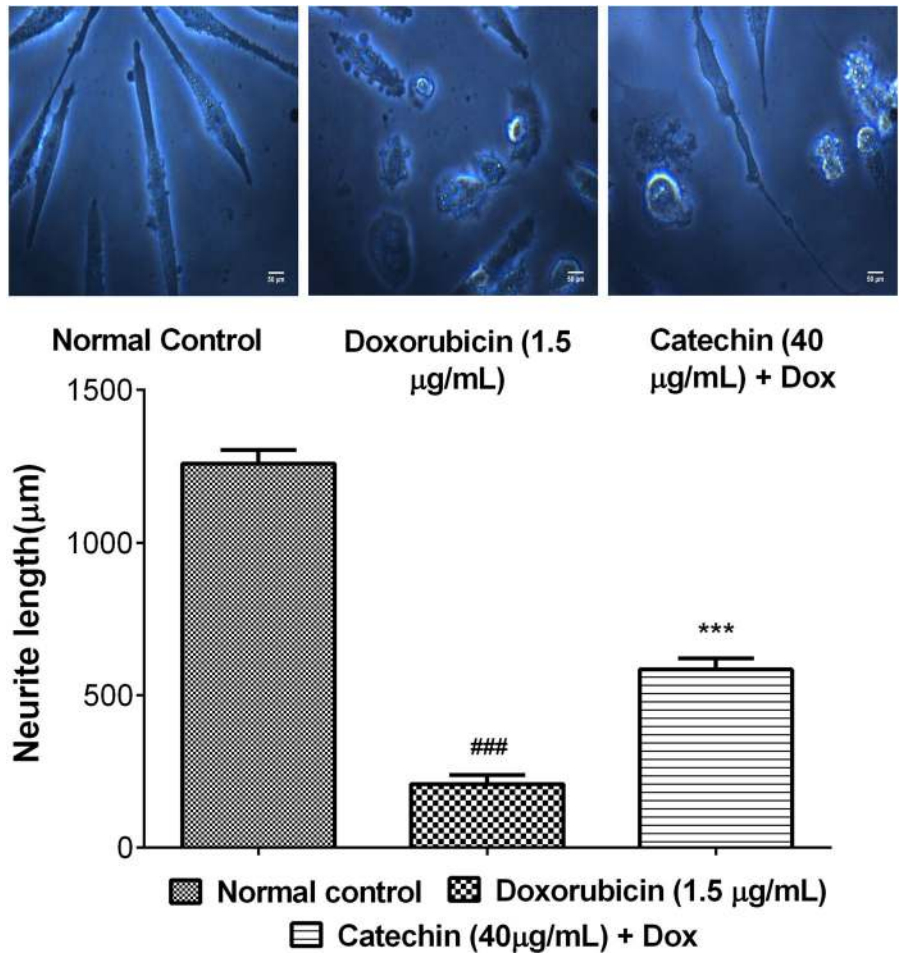

Fig. 2 Effect of treatment on catechin on doxorubicin-induced neurotoxicity in differentiated IMR-32 cells. Scale bar: 50 μm. Data are presented as mean ± SEM of triplicates performed thrice where ### $p < 0.001$ versus normal control; *** $p < 0.001$ versus doxorubicin control

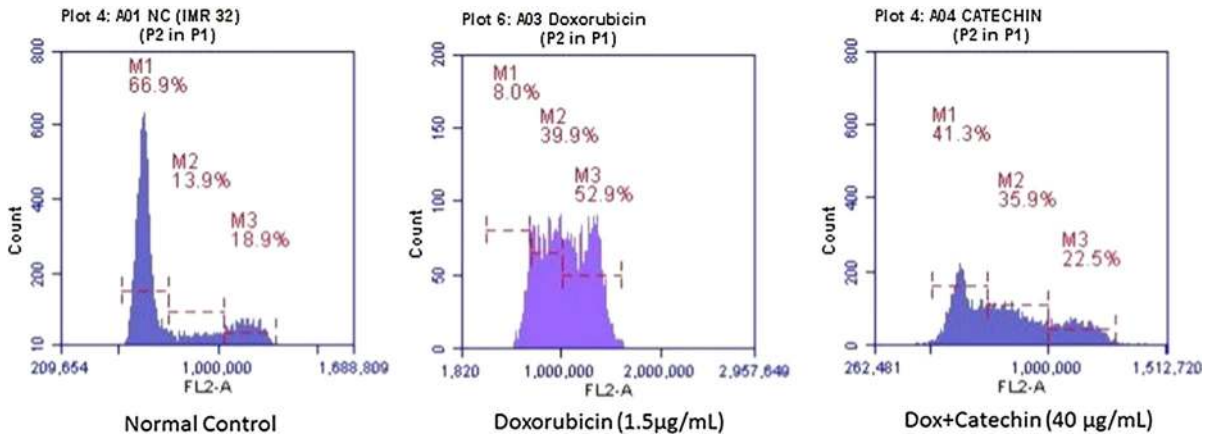

Fig. 3 Effect of catechin on the altered cell cycle of doxorubicin-induced toxicity

familiar object, hence it is clear that they were able to recognize the novel object and showed discrimination. Chronic treatment with ten cycles of DOX has resulted in episodic memory deficits which were evident from the significant reduction in discriminative and recognition indices as compared to saline control. DOX-

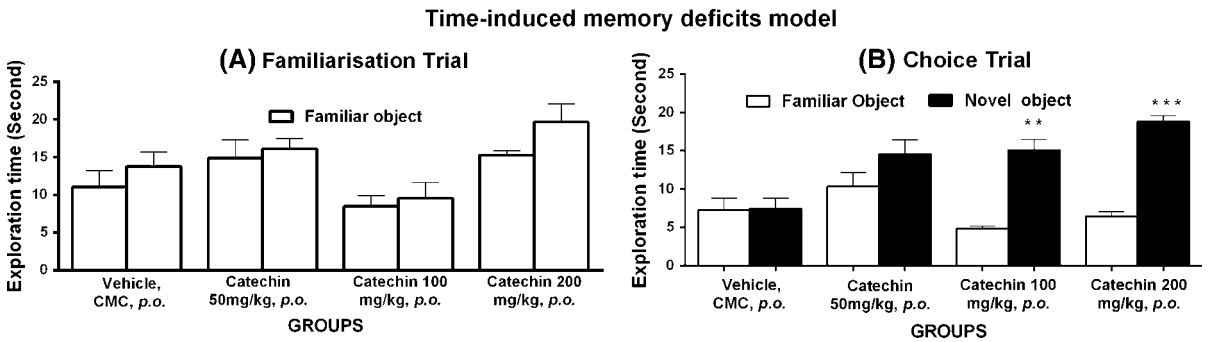

Fig. 4 Effect of catechin in exploration time in either familiarization or choice trial in NORT test in Time-induced memory deficit model. Illustration represents mean ± SEM of exploration time in either familiarization (**a**) or choice trial (**b**) in NORT test, n = 6–8, $^{**}p < 0.01$, $^{***}p < 0.001$ versus familiar object. In (**a**—familiarization trial) left and right bars explain two similar objects

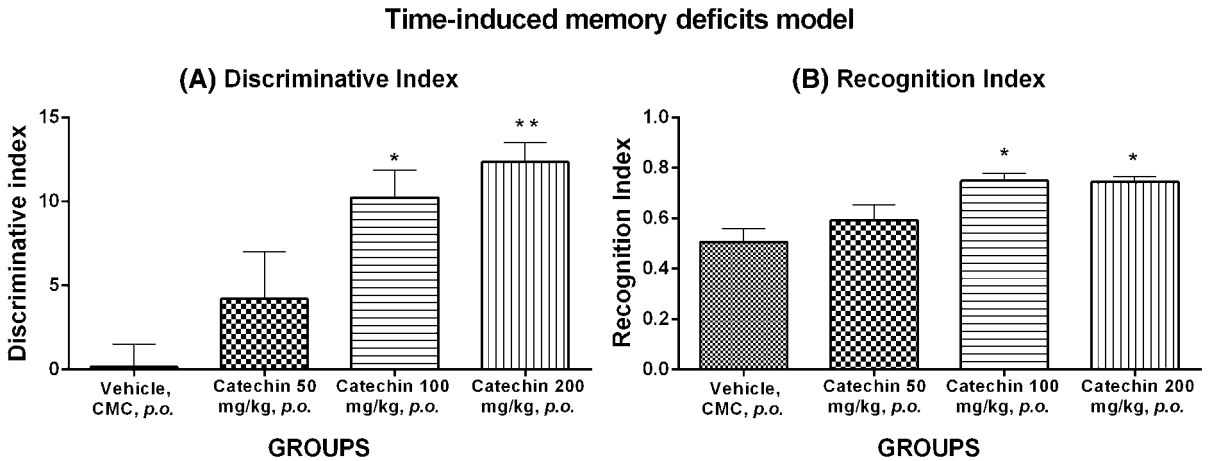

Fig. 5 Effect of catechin in discriminative and recognition indices in NORT test in time-induced memory deficit model. Illustration represents mean ± SEM of discriminative (**a**) and recognition (**b**) indices in NORT test, n = 6–8, $^{*}p < 0.05$, $^{**}p < 0.01$ versus vehicle

treated rats have spent almost equal time exploring either novel or familiar objects. However, the daily treatment with catechin at 100 mg/kg, *p.o.* prevented DOX-induced episodic memory deficits which are evident from the significant increase in RI and DI as compared to DOX control group and was comparable to that of saline control (Figs. 6, 7).

Effect of doxorubicin on body weight of animal

Ten cycles of DOX (2.5 mg/kg) given every 5 days resulted in significant reduction in the body weight of the animals. DOX significantly produced a reduction in the body weight of the animals compared to normal control group. Catechin 100 mg/kg did not show any significant decrease in the body weight compared to DOX group (Table 2).

Evaluation of acetylcholinesterase activity

Catechin was found to be more potent in ex vivo evaluation of acetylcholinesterase activity compared to donepezil in inhibiting the activity of acetylcholinesterase with IC_{50} 21.15 µg/ml and 79.11 µg/ml, respectively (Table 3A).

In in vivo evaluation, DOX showed a significant increase in acetylcholinesterase activity in hippocampus and a moderate increase in cerebral cortex compared to vehicle control which was prevented by simultaneous treatment of catechin (Table 3B).

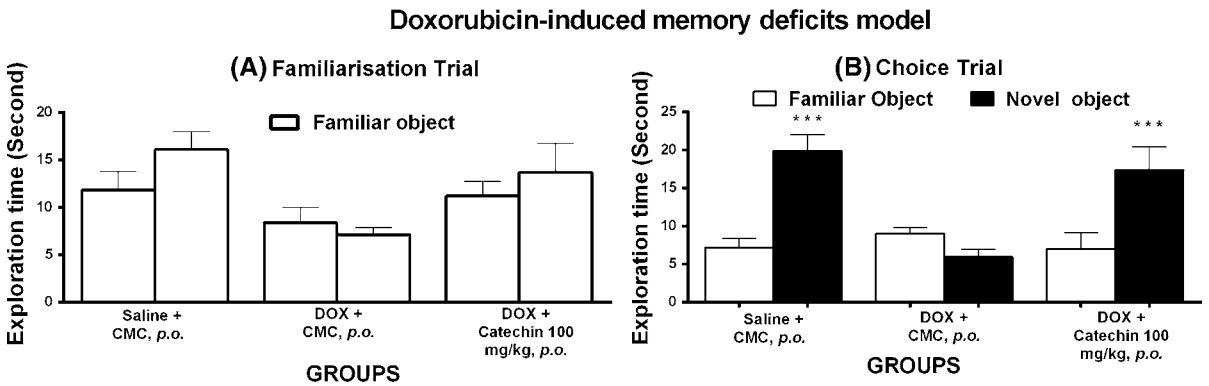

Fig. 6 Effect on familiarization or choice trial in NORT for DOX-induced memory deficits. Illustration represents mean ± SEM of exploration time in either familiarization (**a**) or choice trial (**b**) in NORT for DOX-induced memory deficits, n = 7–9, ***$p < 0.001$ versus familiar object. In (**a**—familiarization trial) left and right bars explain two similar objects

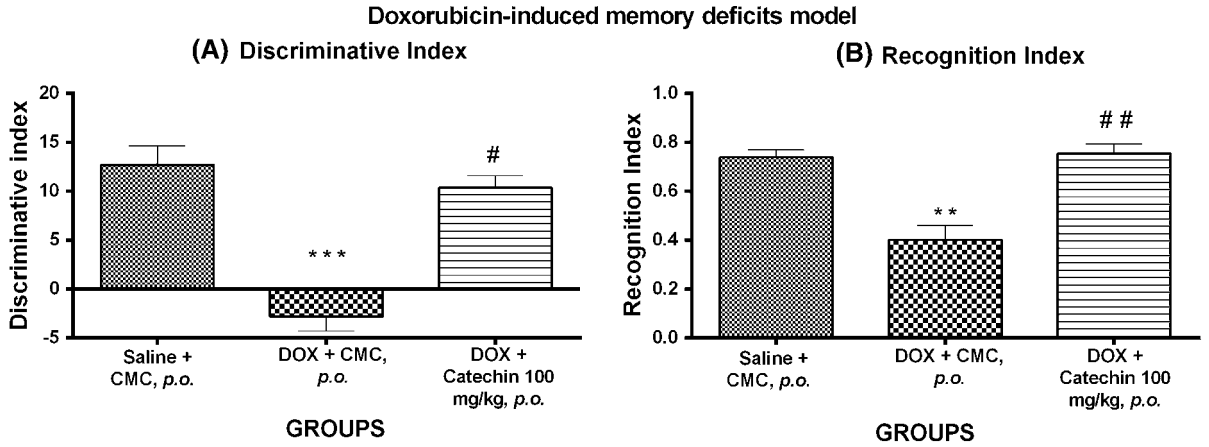

Fig. 7 Effect on discriminative and recognition indices in DOX-induced memory deficit. Illustration represents mean ± SEM of discriminative (**a**) and recognition (**b**) indices in NORT for DOX-induced memory deficits, n = 7–9, **$p < 0.01$, ***$p < 0.001$ versus saline control and $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ versus DOX control

Table 2 Effect of on body weight of animals before and after the treatments

Treatments	Body weight (g) (mean ± SEM)	
	Day 1	Day 50
Vehicle	185.17 ± 4.53	247.83 ± 5.23***
Dox	183 ± 5.0	205.5 ± 8.35$^{\#\#}$
Dox + catechin (100 mg/kg)	172.5 ± 7.35	194.38 ± 7.55$^{\#\#\#}$

Data are presented in mean ± SEM of six animals where *** $p < 0.001$ compared to day 1 of normal control, $^{\#\#}$ $p < 0.01$ compared to day 50 of normal control, $^{\#\#\#}$ $p < 0.001$ compared to day 50 of normal control

Table 3 Acetylcholinesterase activity

Treatment	IC_{50} (50% acetylcholinesterase inhibition)	
(A) Ex vivo acetylcholinesterase activity		
Donepezil	79.11 μg/ml	
(+) Catechin hydrate	21.15 μg/ml	
Treatment	Hippocampus	Frontal cortex
(B) In vivo AchE activity (μmol of acetylthiocholine iodide hydrolyzed/min/mg of protein)		
Vehicle (CMC)	0.013 ± 0.001	0.012. ± 0.001
Dox + CMC	0.020 ± 0.002$^{###}$	0.014 ± 0.001
Dox + catechin 100 mg/kg	0.016 ± 0.001*	0.011 ± 0.0004

(A) IC_{50} values of percentage reduction of acetylcholinesterase activity in the brain homogenate treated with different concentrations of Donepezil, catechin ranging from 500 to 1.25 μg/ml; (B) Data are presented in mean ± SEM, where, $^{###}$ $p < 0.001$ compared to vehicle group, * $p < 0.05$ compared to doxorubicin group

Antioxidant parameters in Frontal cortex and hippocampus

DOX significantly ($p < 0.01$) increased levels of MDA compared to vehicle control in both hippocampus and frontal cortex which was reversed significantly ($p < 0.05$) by catechin at a dose of 100 mg/kg, *p.o.* The improvement in antioxidant profile by catechin treatment compared to DOX only treatment were observed in hippocampus and frontal cortex on the catalase and SOD activity and GSH level (Table 4).

Effect on inflammatory markers

Nitrite level was significantly ($p < 0.05$) increased by DOX treatment compared to vehicle control. Treatment with catechin significantly ($p < 0.05$) prevented a rise in nitrite level compared to DOX alone treatment group (Table 5).

Percentage increase in MPO level was calculated from the mean MPO value of vehicle control. DOX treatment showed more than 50% increase in MPO levels in both hippocampus and frontal cortex compared to vehicle control group (62.09 ± 0.59 and 55.48 ± 1.52% in the hippocampus and frontal cortex, respectively). Treatment with catechin prevented the rise in MPO level compared to DOX alone treatment group (21.98 ± 9.44 and 36.76 ± 4.39% in the hippocampus and the frontal cortex respectively) (Table 5).

Discussion

Flavonoids are a group of compounds known for chemo-preventive potential due to their antioxidant properties. Earlier reports have proven that various plant-derived phenolic products such as curcumin, garlic, alpha-lipoic acid, epigallocatechin gallate, catechin hydrate etc. show a similar effect against various toxic agents (Walle 2007; Yazawa et al. 2006). Hence, the present study was designed to explore the neuroprotective potential of catechin against a well-known anticancer agent, DOX.

There are various reports available for evaluation of in vitro neuroprotective action of tested compounds on undifferentiated and differentiated neuroblastoma cell lines such as on SH-SY5Y (Nampoothiri et al. 2014), IMR32 (Kim et al. 2002), PC-12 (Kim et al. 2002; Tabakman et al. 2002) etc. Starvation and alpha retinoic acid had been used for differentiation of neuroblastoma cells to cholinergic phenotype, thereby leading to growth in the form of neurite length. These studies mostly involved few important parameters namely percentage viability, neurite length, cell cycle etc. The present study was also designed in a similar way, where percentage viability was assessed in undifferentiated IMR-32 cells and assessment of neurite length in differentiated IMR-32 cells. In undifferentiated IMR-32 cells, catechin pretreatment led to higher percentage viability against DOX-induced toxicity and indicated that catechin pretreatment protected the cells from DOX-induced toxicities. The lowest effective dose of catechin on

Table 4 Effect of treatments on antioxidant parameters in rats

Gr.	CAT		SOD		GSH		Lipid peroxidation	
	HC	FC	HC	FC	HC	FC	HC	FC
Antioxidant profile of hippocampus (HC) and frontal cortex (FC)								
I	5.07 ± 1.16	4.19 ± 0.48	27.62 ± 0.41	39.16 ± 1.54	0.012 ± 0.0004	0.013 ± 0.001	842.63 ± 83.8	691.8 ± 87.8
II	1.0 ± 0.2$^{\#\#}$	1.77 ± 0.31	37.25 ± 2.3$^{\#}$	27.99 ± 1.05$^{\#}$	0.0102 ± 0.0002	0.009 ± 0.0002$^{\#\#}$	1410.55 ± 107.44$^{\#\#\#}$	1115.8 ± 132.7$^{\#\#}$
III	1.76 ± 0.18	4.58 ± 0.67	45.7 ± 7.88*	37.5 ± 3.53*	0.017 ± 0.003*	0.013 ± 0.001**	1058.94 ± 53.26**	808.3 ± 74.2*

CAT catalase activity (unit/min/mg of protein), *SOD* SOD activity (units/min/mg of protein), *GSH* reduced Glutathione levels (μM/mg of protein), *Lipid peroxidation* malondialdehyde (MDA) formed (nM/mg of protein), *HC* hippocampus, *FC* frontal cortex, *Gr.* group. Animal groups represented as group I—vehicle (0.25% CMC, *p.o.*), group II—doxorubicin *i.p.* + vehicle *p.o.*, group III—Dox *i.p.* + catechin 100 mg/kg *p.o.*

All values are mean ± SEM of six samples where $^{\#}$ $p < 0.05$ versus vehicle group, $^{\#\#}$ $p < 0.01$ versus vehicle group, $^{\#\#\#}$ $p < 0.001$ versus vehicle group, * $p < 0.05$ versus doxorubicin group, ** $p < 0.01$ versus doxorubicin group

the undifferentiated cell line was selected for further study on differentiated cells. Based on this, the concentration of catechin selected on differentiated cells was 40 μg/ml, which was between 31.25 and 62.5 μg/ml, the lowest effective concentrations on undifferentiated cells. In differentiated cells, DOX treatment produced a significant decrease in neurite length and cell cycle arrest in S and G2/M phase compared to normal control. Catechin pretreatment showed a significant increase in neurite length and prevention of cell cycle arrest compared to DOX alone treatment. This finding strengthened the earlier result of neuroprotection on undifferentiated IMR32 cells. Based on these in vitro results, it could be stated that catechin showed a neuroprotective effect against DOX-induced toxicity. The possibility of catechin to inhibit the anti-cancer potential of DOX is minimal. A recent finding has been reported for (−)-epigallocatechin-3-o-gallate (EGCG) which is a similar flavonoid. Together with DOX EGCG increases the anti-cancer action against multi-drug resistant breast cancer (Cheng et al. 2016). The report also indicated the beneficial role of the combination by minimizing the cardiotoxicity of DOX.

Hence, the in vivo study was conducted to evaluate the effect of catechin in counteracting the toxic effect of DOX i.e., cognitive decline and underlying mechanism. In vivo study is more significant for DOX, as it is reported to cross blood brain barrier to a lesser extent, however, clinical reports showed a cognitive decline associated with DOX. A recent report by Grandhi et al. (2016), states that DOX induces cognitive decline thereby altering episodic memory. To induce cognitive deficit, they reported DOX for a course of 50 days including ten cycles, given every 5 days at a dose of 2.5 mg/kg. The same model was used in the present study. The dose of catechin for DOX -induced cognitive decline model was selected based on time-induced memory deficit model.

In time-induced memory deficit model, episodic memory was explored by increasing the time delay between familiarization and choice trials. This study was conducted using three doses of catechin (50, 100, 200 mg/kg). A dose-dependent increase in RI and DI were observed. However, treatment with catechin at 100 and 200 mg/kg treatment showed a significant increase in RI and DI. Based on the efficacy of catechin in this study and considering long-term

Table 5 Effect of treatments on inflammatory parameters in the hippocampus and frontal cortex in rats

Treatment	Nitrite (nM/mg of protein)		Percentage increase in the myeloperoxidase (MPO) levels	
	Hippocampus	Frontal cortex	Hippocampus	Frontal cortex
Vehicle	9.6 ± 1.2	7.2 ± 1.45	–	–
DOX	25.84 ± 6.36$^{\#\#\#}$	14.01 ± 1.48$^{\#}$	62.09 ± 0.59	55.48 ± 1.52
DOX + Catechin 100 mg/kg	12.94 ± 1.28*	7.12 ± 0.38*	21.98 ± 9.44	36.76 ± 4.39

All values are mean ± SEM of six samples, where $^{\#}$ $p < 0.05$ versus vehicle group, $^{\#\#\#}$ $p < 0.01$ versus vehicle group and * $p < 0.05$ versus doxorubicin group. Percentage increase in myeloperoxidase level was calculated compared to vehicle control

dosing, 100 mg/kg dose was selected in DOX neurotoxicity model.

The chronic exposure of DOX for 50 days produced a minor reduction in body weight which was not significant compared to vehicle control. Thus, it can be stated that change in behavior is mainly attributed due to cognitive decline and not due to peripheral toxicities. Vehicle treated animals could discriminate the novel object from the familiar one, evident by high RI and DI compared to other groups. Animals with DOX treatment showed an insignificant difference in exploration time of the novel or familiar object, evidenced by a significant decrease in RI and DI compared to the vehicle treated group. This signified that the animals did not remember the previously encountered familiar objects. The treatment groups DOX + catechin 100 mg/kg showed significantly ($p < 0.05$) more exploration time towards the novel object compared to the familiar one and reversed the impairment caused due to DOX. This suggested the beneficial effect of catechin against DOX-induced cognitive impairment.

To further elucidate the molecular mechanisms of catechin, we examined its effect on cholinergic neurotransmission, oxidative stress and neuroinflammation in centers of learning and memory viz., hippocampus and frontal cortex. Cholinergic neurotransmission in these centers plays a vital role in developing a neural circuit responsible, which contributes to the cognitive ability. Acetylcholinesterase is an enzyme responsible for the degradation of acetylcholine into acetate and choline and terminating the cholinergic transmission. DOX causes cognitive impairment (Aluise et al. 2010a) which makes it necessary to evaluate the acetylcholinesterase activity in hippocampus and frontal cortex. It has been found that chronic exposure to the toxicants leads to cognitive decline due to a decrease in acetylcholine levels in the hippocampus and frontal cortex, which is either due to a decrease in the choline uptake or increased expression of acetylcholine esterase level (John et al. 2015; Nampoothiri et al. 2015). The toxicant DOX had shown a similar significant increase in the acetylcholinesterase activity which was reversed by catechin treatment. Ex vivo study also showed potent acetylcholine esterase inhibitory effect of catechin. Hence, it can be stated that catechin might have prevented cognitive decline due to a reduction in acetylcholine esterase activity.

Reports have highlighted the role of oxidative stress in altering the cholinergic transmission by inhibiting muscarinic cholinergic receptor in the brain (Fawcett et al. 2002) and considered to be one of the major pathways for the neurodegenerative disorders like AD (Christen 2000). Most of the cancer chemotherapeutic drugs including DOX produce cognitive decline by producing oxidative stress and nitrergic stress (McIntosh and Sapolsky 1996). Doxorubicin causes the overproduction of ROS acting on mitochondrial NADPH oxidase activity. Furthermore, DOX promotes the production of superoxide and leads to enzyme uncoupling adding to oxidative stress (Asensio-López et al. 2016, 2017).

It does not only affect the cancer cells, but also the normal cells. This effect in the brain leads to neuroinflammation and mitochondrial dysfunction leading to apoptosis and neurodegeneration (Mohamed et al. 2011). In this study, the brain areas associated with cognition (learning and memory), hippocampus and frontal cortex were used for the assessment of oxidative stress markers such as catalase, SOD, GSH, and lipid peroxidation. Additionally, a nitrergic stress marker viz., nitrite and MPO, an inflammatory marker, were assessed. Rise in oxidative stress in DOX alone treatment was

marked by significant increase in the lipid peroxidation (MDA levels in hippocampus and cortex) levels in hippocampus and cortex which were significantly reversed by the DOX + catechin treatment. The rise in oxidative stress in cortex and hippocampus was accompanied by depletion of antioxidant defense mechanism exhibited by a decrease in the catalase, SOD and GSH levels. The DOX + catechin treatment groups showed a reversal in antioxidant defense mechanism compared to DOX only control. Nitrergic stress is another free radical associated burden to the neuronal cells, which was evaluated by nitrite levels in frontal cortex and hippocampus. A significant increase in nitrite levels by DOX treatment was observed which was significantly decreased by catechin pretreatment in comparison to DOX only treatment.

MPO level is one of the markers of oxidative stress and mediators of inflammation in the brain. MPO is reported to be present in both neuronal cells and microglia cells. It is found to be overexpressed in neurons of frontal cortex and hippocampus in Alzheimer's disease brain where immunological materials are localized. In the present study, similar results were obtained after DOX treatment. MPO levels increased both in frontal cortex and hippocampus compared to vehicle control by DOX alone treatment while catechin treatment prevented this increase. Previous studies have shown catechin has antioxidant and anti-inflammatory effect against DOX-mediated cardiotoxicity (Abd El-Aziz et al. 2012). Thus, present study justifies the earlier report on the anti-inflammatory and antioxidant role of catechin as one of the mechanisms underlying neuroprotection against DOX-induced toxicity.

Catechin showed in vitro and in vivo neuroprotective potential, which merits it to be a potential adjuvant therapeutic agent to ameliorate cognitive impairment complications associated with DOX chemotherapy. The neuroprotective, antioxidant potential and anti-inflammatory effect of catechin is possibly related to the mechanism to reverse cognitive deficits associated with the DOX-induced chemobrain like condition (assessed by episodic memory deficits).

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