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ORIGINAL ARTICLE

Neuroprotective Potential of Methanolic Extract of *Saraca asoca* Bark against Doxorubicin-Induced Neurotoxicity

Sri Pragnya Cheruku, Mallikarjuna Rao Chamallamudi, Grandhi Venkata Ramalingayya, Subhankar Biswas, Karthik Gourishetti, Krishnadas Nandakumar, Raviraj Devkar[1], Sanchari Basu Mallik, Madhavan Nampoothiri, Nitesh Kumar

Departments of Pharmacology and [1]Pharmacognosy, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal, Karnataka, India

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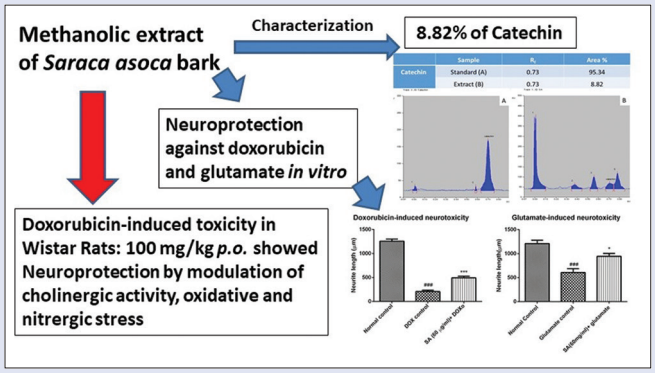
ABSTRACT

Background: Doxorubicin (DOX) is a widely used anthracycline-based anticancer agent, which causes a gradual decline in cognition on long-term usage in breast cancer patients that affect their quality of life negatively. **Aim:** The present study was designed to assess the neuroprotective potential of *Saraca asoca* bark against Dox-induced neurotoxicity, considering its previously reported anticlastogenic effect. **Materials and Methods:** Methanolic extract of bark was prepared by Soxhlet extraction and characterized by high-performance thin-layer chromatography (catechin-8.82%). *In vitro* studies were performed in IMR-32 neuroblastoma cells using cell viability and neurite growth assay to determine the neuroprotective potential of the extract against DOX-induced neurotoxicity. *In vivo* neuroprotection study of extract (100 and 200 mg/kg p. o) was performed by assessing episodic memory through novel object recognition test (NORT) in DOX-induced (10 cycles, 2.5 mg/kg, 5 days apart) memory deficit model. In addition, antioxidant markers and acetylcholinesterase activity in hippocampus and frontal cortex were evaluated. **Results and Conclusion:** The extract showed significant *in vitro* neuroprotection toward differentiated cells in morphological features and cell viability assessment against DOX (IC_{50}-63.35 and 45.17 µg/ml against 1 µg/ml and 2 µg/ml DOX, respectively, and 0.46 µg/ml for DOX alone in 3-[4,5 dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide assay). DOX-treated animals showed depletion in episodic memory while extract at 100 mg/kg significantly prevented it. Similar changes were observed in antioxidant markers levels in the brain. DOX treatment elevated acetylcholinesterase activity which was significantly reversed by the treatment with extract. Thus, it can be stated that the neuroprotective and antioxidant potentials of extract possibly underlie the efficacy against the DOX-induced neurotoxicity.

Key words: Chemoprotection, doxorubicin, episodic memory, novel object recognition test, *Saraca asoca*

SUMMARY

- Saraca asoca at 100 mg/kg p. o showed effective protection against doxorubicin (DOX)
- Neuroprotection by modulation of cholinergic activity, oxidative, and nitrergic stress
- *In vitro* protection in IMR-32 against DOX and glutamate-induced toxicity.



Abbreviations Used: DOX: Doxorubicin; *S. asoca: Saraca asoca*; DMEM: Dulbecco's Modified Eagle's Medium (DMEM); MTT: 3-[4,5 dimethyl thiazol-2-yl]-2,5-diphenyl tetrazolium bromide; FBS: Fetal bovine serum; RA: Retinoic acid; CMC: Carboxymethyl cellulose; SOD: Superoxide dismutase; GSH: Reduced glutathione; HPTLC: High-performance thin-layer chromatography; NORT: Novel object recognition task; ITI: Intertrial interval; MPO: Myeloperoxidase; MDA: Malondialdehyde; RI: Recognition index; DI: Discrimination index.

Correspondence:

Dr. Nitesh Kumar,
Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal University, Manipal - 576 104, Karnataka, India.
E-mail: niteshkumar43@gmail.com
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INTRODUCTION

Doxorubicin (DOX) is widely used to treat a wide range of malignancies by multiple mechanisms such as interfering with DNA replication causing DNA damage, by inhibiting topoisomerase enzyme and also by free radical generation causing oxidative stress, thereby producing a cytotoxic effect[1-3] In contrast to its therapeutic effect against cancer cells, the normal cells are also affected leading to various toxicities such as cumulative cardiotoxicity, hepatotoxicity, potent myelosuppression[2,3] and also cognitive decline.[1,4] Impaired cognition due to chemotherapy is the biggest challenge for cancer survivors and is commonly known as "chemobrain" or "chemofog" which attributes to the disorganized mental state during or after chemotherapy in patients with cancer. It is manifested by a decrease in learning and memory, executive functioning, and information processing speed.[1,5,6] Asher and Myers reported that chemobrain is prevalent in about 75% of patients with cancer during treatment while about 15%–35% cancer survivors experienced subtle

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cognitive decline months and years after treatment impacting their quality of life (QOL).[7] Since the early 1990s, empirical research is being conducted on chemotherapy-induced cognitive impairment caused by antineoplastic agents.[1,8,9] Over the past decade, authors reported that the patients diagnosed with breast cancer were profoundly affected with cognitive decline during and after chemotherapy.[1,7,10,11] Previous studies suggested that chronic administration of DOX has resulted in a cognitive decline in patients with breast cancer.[1,6] Preclinically, DOX demonstrated a deteriorating effect in the cognitive domain.[12]

Treatment-related toxicities due to chemotherapy are inevitable. Neurostimulants such as modafinil and methylphenidate, herbal supplements such as *Gingko biloba*, natural antioxidants such as Vitamin E are regarded as effective interventions against cognitive impairment. Although no sufficient evidence is available on the efficacy of these interventions in combating chemobrain condition.[5,7,13] Preclinical studies purported that antioxidants have proved to prevent oxidative stress and implied to slow the rate of cognitive decline in aging and disease.[5]

Saraca asoca (SA), commonly known as *Saraca indica* or Sita-Ashok or Ashoka, is native to India.[5,7] It is purported to have various biological activities such as aphrodisiac,[14] antibacterial, anti-oxytocic, and anti-menorrhagic activity.[15] Apart from this, the bark of SA was demonstrated for hemoprotective and anticlastogenic effect against cisplatin,[16] cardioprotective effect against cyclophosphamide-induced toxicity.[17] There are no clear reports till now indicating its neuroprotective effect against antineoplastic agents especially DOX.

DOX has demonstrated to cause cognitive impairment in healthy rodents by the mechanism of neuroinflammation, DNA damage and oxidative stress mediated by central and peripheral activation of inflammatory cytokines[4,9,12] which makes it paramount to circumvent the toxic effect of chemotherapy by antioxidant supplementation. The bark of SA has been studied extensively for the accumulation of various metabolic components and is rich in catechin components.[18] The present study is designed to standardize the methanolic bark extract of SA by quantifying the catechin components by high-performance thin layer chromatography (HPTLC) compared against a standard (+) catechin hydrate and to investigate the chemoprotective effect of the bark extract against DOX-induced neurotoxicity along with its protective effect against other major organ toxicities.

MATERIALS AND METHODS

Materials

DOX (Fresenius Kabi Oncology Ltd., Solan, India), Trypsin - ethylenediaminetetraacetic acid (EDTA), dipotassium EDTA, 3-(4,5 dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), fetal bovine serum (FBS), retinoic acid (RA), gallic acid, and methanol were acquired from Himedia chemicals, India. (+) Catechin hydrate (catechin), Dulbecco's Modified Eagle's Medium (DMEM), Ellman's reagent or 5,5'-Dithiobis-(2-nitrobenzoic acid), Acetylthiocholine iodide was procured from Sigma Aldrich, USA. Sterile 96 and 6 well plates, 25 and 75 cm^2 culture flasks were procured from Tarsons Product Pvt Ltd., USA.

Preparation of methanolic extract of *Saraca asoca* bark by Soxhlet extraction method

The bark of SA was acquired from a local vendor in Udupi, Karnataka and authenticated by a taxonomist. The bark was desiccated at room temperature for 1 week until completely dry and ground in an electric grinder. The powder obtained was macerated in 100% methanol for 24 h followed by extraction at 50°C–55°C using hot percolation method by Soxhlet extraction. After 2 to 3 cycles, the methanolic extract obtained was evaporated to dryness under reduced pressure using rotavapor at 50°C–55°C. This process was repeated 4 to 5 times until all the components are extracted. The obtained extract is concentrated and dried at 37˚C. Traces of methanol were evaporated by lyophilization at −48°C. The extract was stored in the desiccator until use.[19,20]

Quantitative analysis of catechin

Catechin was estimated in the bark extract of SA by densitometry using HPTLC using CAMAG HPTLC instrumentation system from Switzerland. A solution of (+)-Catechin hydrate (standard, from Sigma Aldrich, USA.) (100 µg/ml) and SA extract (1000 µg/ml) were prepared in methanol. Ten microliter of standard (100 µg/ml) and extract (1 mg/ml) samples were spotted in duplicates on the HPLC plate (size 20 cm × 10 cm, precoated with silica gel GF254) using automated Linomat 5 applicator under nitrogen gas flow with a band of 6 mm and the track distance of 8 mm. The chromatographic conditions were mobile phase: Toluene: Ethyl acetate: Formic acid: methanol (3:6:1.6:0.4),[21] detection wavelength: 254 nm. The chamber (CAMAG Twin Trough) was saturated for 20 min after which, the plate was run up to 8 cm and air dried. CAMAG TLC scanner-3 with slit dimensions 6 mm × 0.45 mm and CATS evaluation software for densitometry was used for the evaluation. The percentage of catechin in the extract was calculated by the software.

In vitro cytoprotection studies

IMR-32 cell line is derived from 13-month old Caucasian male diagnosed with neuroblastoma. The cell line as purchased from NCCS, Pune, maintained and subcultured in T-25 flasks containing proliferation medium supplemented with DMEM, 10% FBS, 1% gentamicin at 37 ˚C in 5% humidified atmospheric CO_2 incubator with 95% air. After 70%–80%, confluency was obtained, the cells were trypsinized by the addition of proliferation medium containing 0.2% Trypsin – 0.02% disodium EDTA and the viable cells counted by trypan blue exclusion were used to study the neuroprotective effect of the compounds.

3-(4,5 dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide cell viability assay in undifferentiated and differentiated IMR-32 cell line

Five-thousand cells/well were seeded in microplate consisting of 50 µl of proliferation medium and incubated overnight. After 24 h, from the stock solutions of catechin hydrate (standard) and SA (50 mg/ml), 50 µl of concentrations ranging from 31.23 to 250 µg/ml was added to the wells, and the cells were pretreated for an hour followed by the addition of 50 µl of DOX (1 and 2 µg/ml). After a 24 h treatment, 50 µl of MTT was added and incubated for 3 h, and then, the medium was discarded. Formed blue formazan crystals observed under the microscope was dissolved in 100 µl of dimethyl sulfoxide, and the absorbance was read at 540 nm. The protective effect of pretreated compounds against DOX toxicity was evaluated. Along with this, cytoprotective effect of standard catechin hydrate, plant extract SA, and DOX at different concentrations were evaluated individually by the same method. IC_{50} of the percentage cell viability of the compounds were obtained.

Evaluation of neuroprotection against Doxorubicin and Glutamate-induced neurotoxicity in differentiated IMR-32 cell line

Two million cells/well were seeded in 6-well plates supplemented with proliferation medium overnight, after which the media was replaced by differentiation medium containing DMEM, 5% FBS for first 3 days, 15 µM alltrans RA (ATRA), 1% gentamicin, thereby arresting

proliferation and promoting neuronal differentiation. Depending on the extent of differentiation, the complete media was replaced by serum-free differentiation media. The cells were used for experimentation after 12 days of differentiation into long neurites extensions observed under an inverted microscope. The cells were supplemented with fresh media every 3 days. Glutamate (0.5 mM) and DOX (1.5 mg/ml) were used to induce neurotoxicity in the neuronal cells and investigated for the neuroprotective effect of SA (60 µg/ml) based on the IC_{50} values of the compounds obtained in MTT assay. L-glutamate has shown its neurotoxicity at 0.5 mM concentration.[22] The cells were treated for 48 h, after which, the medium was removed, and the cells were washed and replaced with phosphate-buffered saline.

The cells were observed for the effect of compounds on the neurite length which was viewed under an inverted microscope at × 40 objective, and the images were taken. The neurite length defined as a straight-line distance from the tip of the neurite to the junction between the cell body and the neurite base expressed in µm. It was calculated for the treatment groups and evaluated the neuroprotective effect.

In vivo studies

Animals

The study was conducted after obtaining Ethical Approval from the Institutional Animal Ethics Committee, Kasturba Medical College, Manipal University (No: IAEC/KMC/80/2014). Six months old healthy male Wistar rats, weighing 200–250 g were procured from the Central Animal Research Facility of Manipal University, Manipal as per CPCSCEA guidelines. The animals could acclimatize for 1 week before the initiation of the experiment and maintained under controlled conditions of temperature and humidity with a 12 h day and night cycle with free access to rat pellet diet and water *ad libitum*. The animals were randomized according to their body weight.

Doxorubicin-induced memory deficit model

The animals were divided into five treatment groups (n = 6 each), Group I served as vehicle control, administered with 0.25% carboxymethylcellulose (CMC) p. o. and saline intraperitoneally Groups II, III, and IV were induced with DOX as per protocol. Group II served as disease control and was administered with 0.25% (CMC) p. o. Groups III and IV were pretreated with plant extract 100 and 200 mg/kg p. o. prepared in 0.25% CMC. The animals were pretreated 45 min before the administration of DOX (2.5 mg/kg i.p.). Ten cycles of DOX (Adriamycin) 2.5 mg/kg, freshly prepared in distilled water was administered i.p. every 5th day for 57 days at a dosing volume of 2 ml/kg body weight.

The animals pretreated with SA bark extract 1 week before the first cycle of DOX induction. On day 57, at the last cycle of DOX, the animal behavior toward the novel and familiar objects in an open field arena of size 49 cm × 49 cm × 49 cm was observed at fixed time intervals by novel object recognition task (NORT). The behavior was monitored with the help of a camera (model: QuickCam Pro9000, Logitech International S. A., Lausanne, Switzerland) mounted 160 cm above the observation arenas, two stopwatches, and a timer alarm. The study was carried out between 9 am and 4 pm. The animals could habituate to the observation arena during the habituation phase. The familiarization and choice trials with an intertrial interval (ITI) of 4 h was performed. During the familiarization, trial animals could explore two similar objects (a1 and a2). After an ITI, a choice trial was conducted during which a familiar and a novel object was placed (a3 and b). The exploration time, recognition and discrimination indices (DIs) of an animals' on the familiar and novel objects were calculated.[23] Immediately after the study, animals were anesthetized using ketamine. The brain was surgically removed, and hippocampus (H) and frontal cortex (FC) areas were isolated for further biochemical estimations.

Assessment of oxidative and nitrosative stress markers

Oxidative stress and nitrosative markers which include catalase, superoxide dismutase (SOD), reduced glutathione (GSH) and lipid peroxidation, nitrite levels, and myeloperoxidase (MPO) levels were estimated in the H and frontal cortex.[23]

Estimation of ex vivo and in vivo acetylcholinesterase activity

The brain tissue homogenates were estimated for the protective effect of standard Catechin hydrate and SA bark extract against DOX-induced toxicity by the UV-spectrophotometric method. A volume of 0.02 ml of acetylthiocholine iodide, 0.1 ml of DTNB, 2.6 ml of phosphate buffer (pH 8), and 0.4 ml of tissue supernatant was added directly to the cuvette. The change in the absorbance was recorded at 412 nm at a time interval of the 60 s for 4 min by the UV-kinetic method.

Estimation of *ex vivo* acetylcholinesterase activity was performed in untreated rat brain, which was isolated and homogenized at specified conditions by ice-cold 0.1 M phosphate buffer. The brain supernatant collected was incubated at different concentrations with standards donepezil and catechin hydrate ranging from 1000 µM to 31.25 µM, and extract SA ranging from 1000 to 31.25 µg/ml for 45 min with the brain tissue supernatant after which acetylcholinesterase activity was estimated. The result was expressed as micromoles of acetylthiocholine iodide hydrolyzed per min per mg of tissue.

Estimation of *in vivo* acetylcholinesterase activity was performed in the treated tissue samples. The result was expressed as micromoles of acetylthiocholine iodide hydrolyzed per min per mg of protein.[23]

Statistical analysis

Data were statistically analyzed using Graph Pad Prism 5.0 software. Results of the *in vitro* and *in vivo* studies were expressed as mean ± SEM. Statistical comparisons for novel object recognition task results were analyzed by nonparametric test and paired Student's *t*-test, two-tailed, Kruskal–Wallis test followed by *post hoc* Dunnett's multiple comparison tests. All the other results were analyzed by one-way ANOVA followed by *post hoc* Dunnett's Multiple Comparison tests. The DOX-treated group was compared with normal control group. $P < 0.05$ was considered statistically significant at 95% confidence interval.

RESULTS

Densitometric evaluation of (+) catechin by high-performance thin-layer chromatography

Methanolic extract of SA bark showed 8.82% of catechin by (HPTLC) [Figure 1].

In vitro studies

Effect of methanolic bark extract of SA on cell viability in IMR-32 cells: SA bark extract has shown an IC_{50} of 494 µg/ml on the undifferentiated IMR-32 cell line. Dox showed IC_{50} value 0.48 µg/ml. Bark extract of SA defended the cell death. The IC_{50} of bark extract along with 1 and 2 µg/ml DOX was 63.35 and 45.17 µg/ml, respectively, on the differentiated IMR-32 cell line.

Effect of treatment on DOX and glutamate-induced neurotoxicity in differentiated IMR-32 cells The differentiated cells were treated with bark extract SA along with DOX (1.5 µg/ml) and glutamate (0.5 mM) and incubated for 24 h after which the cells were observed under an inverted microscope for the morphological alterations. There was significant toxicity in IMR-32 cells after treatment with glutamate and DOX alone. The cells

were found degenerated, rounded, and detached. This degeneration caused due to the toxicants were significantly protected by treatment with extract at 60 µg/ml. Microscopically, images showed a very few numbers of cell bodies in DOX alone treatment group and DOX and *S. acoca* combination treatment group and a wide number of intact cells [Figure 2].

In vivo pharmacology (doxorubicin-induced memory deficit model)

In DOX-induced memory deficit model, chronic DOX treatment for 50 days induced episodic memory deficits which were demonstrated by the significant reduction in the exploration time of the novel object compared to saline and CMC treated groups ($P < 0.001$) which was also evident by recognition index (RI) and DI ($P < 0.01$ RI; $P < 0.001$; DI). Animals pretreated with bark extract at 100 mg/kg ($P < 0.001$) showed a significant increase exploration time of novel object ($P < 0.001$) and significant RI ($P < 0.05$) and DI ($P < 0.01$) comparable to DOX and saline-treated rats. However, animals treated at a higher dose of bark extract SA 200 mg/kg p. o. explored both novel and familiar objects almost equally [Table 1].

Effect of treatment on *ex vivo* and *in vivo* acetylcholinesterase activity

Ex vivo treatment of bark extract of SA demonstrated potent acetylcholinesterase inhibition potential at an IC_{50} of 36.91 µg/ml compared to standards donepezil and catechin hydrate with an IC_{50} of 190.18 µM (79 µg/ml) and 72.85 µM (21.46 µg/ml), respectively [Table 2].

	Sample	R_f	Area %
Catechin	Standard (A)	0.73	95.34
	Extract (B)	0.73	8.82

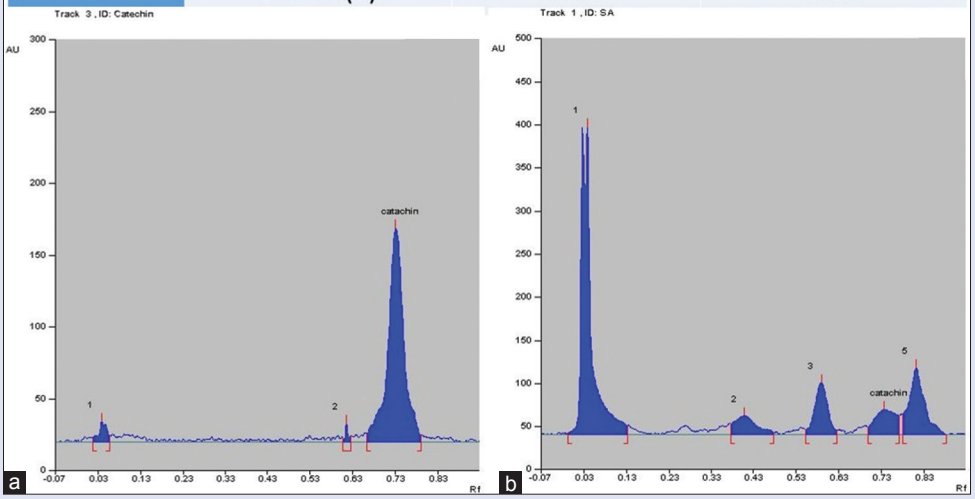

Figure 1: Characterization of extract by high-performance thin-layer chromatography. Densitometric evaluation of standard catechin (a), catechin in methanolic extract of *Saraca asoca* bark (b)

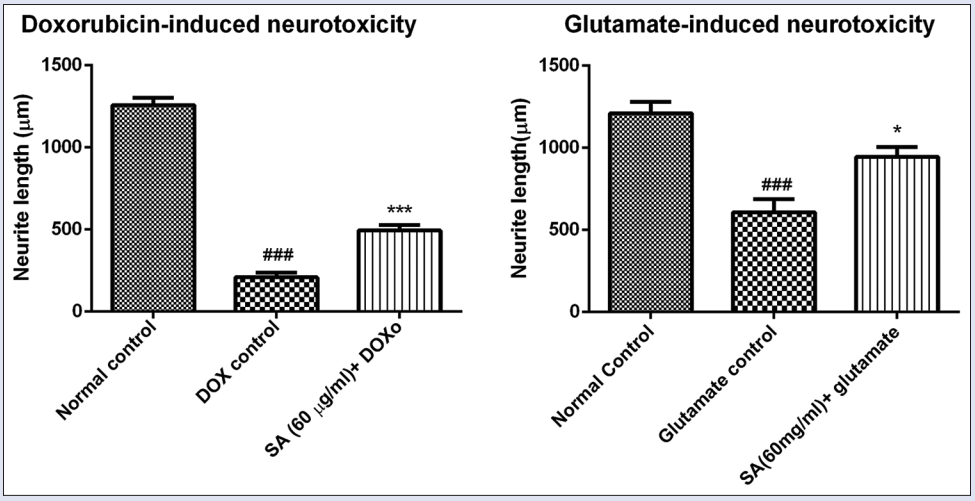

Figure 2: Effect of the extract against doxorubicin and Glutamate-induced morphological changes on IMR-32. Effect of doxorubicin alone and glutamate alone; doxorubicin + treatment and glutamate + treatment on the length of the neurites. ###$P < 0.001$ versus normal control; ***$P < 0.001$ versus doxorubicin control; *$P < 0.05$ versus glutamate control

Table 1: Novel object recognition test in doxorubicin-induced memory deficit model

Treatment	Mean±SEM					
	Familiarization trial		Choice/recognition trial		Recognition and discrimination index	
	Exploration time(s)		Exploration time(s)		RI	DI
	a_1	a_2	a_3	b	-	-
Vehicle control	11.83±1.94	16.10±1.89	7.2±1.2	19.93±2.11***	0.74±0.03	12.72±1.92
DOX control	8.36±1.63	7.13±0.75	8.28±1.04	5.73±0.90	0.41±0.05##	−2.55±0.05###
DOX + SA (100 mg/kg)	12.40±2.34	15.42±2.6	5.95±1.14	18.23±2.34***	0.76±0.03**	12.28±1.73**
DOX + SA (200 mg/kg)	12.19±1.77	12.35±1.62	11.28±2.14	9.83±1.87	0.46±0.08	−1.45±2.99

Exploration time of two familiar objects (a_1 and a_2) in familiarization trial and of one familiar object and one novel object (a_3 and b) in a choice trial with an ITI of 4 h by the animals' in each treatment group. Vehicle control was administered saline *i.p.* and CMC (2 ml/kg) *p.o.* DOX control was administered with DOX *i.p.* and CMC orally.[23] ***P<0.001compared to familiar object in choice/recognition trial. Recognition and discrimination indices of a novel object (b) in each treatment group. **P<0.01 compared to the DOX group. ##P<0.01 and ###P<0.001 compared to the vehicle group, *P<0.05 versus DOX group, *P<0.01 versus DOX group. DOX: Doxorubicin; SA: Saraca asoca; RI: Recognition Index; DI: Discriminative index; ITI: Inter-trial interval; CMC: Carboxymethyl cellulose; SEM: Standard error of mean; *i.p*: Intraperitoneally

Table 2: Effect on drug treatment on acetylcholinesterase activity

Ex vivo study		
Treatment (*ex vivo*)	IC50 (acetylcholinesterase inhibition)	
Donepezil	190.18 µM	
SA	36.91 µg/ml	
***In vivo* study**		
Treatment (*in vivo*)	Hippocampus	Frontal cortex
Vehicle control	0.013±0.001	0.012.±0.001
DOX control	0.020±0.002###	0.014±0.001
DOX + SA (100 mg/kg)	0.014±0.001**	0.012±0.001
DOX + SA (200 mg/kg)	0.016±0.001*	0.013±0.001

Ex vivo study: IC50 values of percentage reduction of acetylcholinesterase activity in the brain homogenate treated with different concentrations of Donepezil and SA bark extract ranging from 500 µM (µg/ml) to 31.25 µM (µg/ml).
In vivo study: Percentage reduction in the acetylcholinesterase activity in the brain after treatment with DOX, DOX+SA (100 mg/kg) and DOX+SA (200 mg/kg)

In vivo chronic administration of DOX increased the AchE activity in hippocampal samples (P < 0.001) compared to the vehicle group. Pre-treatment with bark extract significantly prevented the toxic effect of DOX. t. A similar trend was evident in FC samples; however, the result was not significant [Table 2].

Effect of treatment on oxidative stress markers in brain

Chronic DOX administration significantly increased the malondialdehyde (MDA) levels compared to the vehicle control in H (P < 0.01) and FC (P < 0.001) samples which was significantly reversed by bark extract at 100 mg/kg (P < 0.01 [H]; P < 0.001 [FC]) and 200 mg/kg p. o.(P < 0.001, H; P < 0.01, FC). A trend in the decrease in CAT and GSH antioxidant levels due to DOX administration was observed which was improved by treatment with bark extract at doses 100 and 200 mg/kg. SOD levels significantly decreased in FC samples due to DOX. However, there was no effect on SOD levels of H due to DOX and treatment [Table 3].

Effect of treatment on myeloperoxidase and nitrite in brain

There was a notable increase in nitrite and MPO levels by DOX treatment in the H and FC which was significantly reversed by treatment with bark extract at 100 and 200 mg/kg [Table 3].

DISCUSSION

There are no clinically proven treatments to prevent cognitive decline due to chemotherapy. Antioxidants supplements have known to prevent oxidative stress peripherally and delay the process of degeneration in the brain.[5] SA bark extract is known for its antioxidant and anticancer activity.[15] This may be one such alternative for enhancing the quality of life in the patients undergoing DOX chemotherapy. As in the earlier studies, it has shown the myeloprotective effect against cisplatin[16] and cardioprotective effect against cyclophosphamide[17] in rodents. With this evidence, we further evaluated the extract for its potential anti-chemo brain activity in an established DOX-induced chemobrain model for episodic memory.

Earlier studies have confirmed that the bark of SA is composed of chemical constituents such as catechin, (-)-epicatechin, epi afzelechin-(4β-8)-epicatechin, procyanidin B2, tannins, lignin glycosides, steroids, saponins, leukocyanidin, leukopelargonidin, and β-sitosterol.[15] Previous research shows that flavan-3-ols (catechins), a subclass of flavonoids is one type of phenolic components known to have medium polarity. For the extraction of phenolic components, most widely used solvents are water, methanol, ethanol, and acetone. Among them, methanol is considered as the best suitable solvent for extraction of phenolic compounds, since, it is medium polar in nature, and mediates considerable extraction efficiency of antioxidant compounds. In light of the previous research, methanol was proved to be the suitable solvent for extraction of catechin components from *Saraca asoca* bark.[24-26] Our study standardized the SA extract based on the presence of catechin by HPTLC analysis. Catechin was detected in the methanolic bark extract comparable to standard (+) catechin hydrate at R_f and peak area of 0.73 and 8.82%. Polyphenolic components, such as catechin, epicatechin, and other polyphenols, are known to be potent natural antioxidants. In an earlier study, we found that catechin ameliorated DOX-induced neurotoxicity and prevents the episodic memory loss.[23] Geetha *et al.* reported antimutagenic properties of epicatechin.[27] In addition, it showed profound improvement in learning and spatial memory in mice[28] and also improved cognition in older adults.[29] Thus, the potent antioxidant property of the extract strengthens the hypothesis that the extract may be a potent chemoprotective agent against DOX.

The *in vitro* study was conducted in IMR-32 human neuroblastoma cell line to evaluate the protective effect against DOX-induced neurotoxicity. Previous literature indicates that the IMR-32 neuroblastoma cell line has been studied for neuroprotective evaluation.[30,31] The cell viability assay conducted on undifferentiated IMR-32 cell line was to estimate the toxicity profile of the bark extract. An IC_{50} of 494 µg/ml concentration implies that the bark extract may not be cytotoxic in nature at such high concentration; therefore, it may not interfere with antitumor properties of DOX. Cassano *et al.* and Silvis *et al.* demonstrated that the cell line showed normal neuronal

Table 3: Effect on antioxidant status in hippocampus and frontal cortex

Treatment	Catalase		SOD		GSH	
	HC	FC	HC	FC	HC	FC
Vehicle control	4.37±1.05	3.43±0.57	27.62±0.41	39.16±1.54	0.0123±0.0004	0.0125±0.0005
DOX control	0.92±0.18$^{\#}$	1.86±0.28	37.25±2.34	27.99±1.05#	0.102±0.0002	0.0092±0.0002$^{\#\#}$
DOX + SA (100 mg/kg)	0.15±0.97	6.31±1.36*	41.30±7.02	31.56±0.75	0.0150±0.0013	0.0105±0.0002
DOX + SA (200 mg/kg)	7.17±2.03***	4.73±2.15	45.39±5.70	29.09±2.88	00.0168±0.0020*	0.0090±0.0032
	TBARS		**Nitrite**		**Percentage of MPO**	
	HC	FC	HC	FC	HC	FC
	842.63±83.8	691.8±87.8	9.6±1.2	7.2±1.45	-	-
	1410.55±107.44$^{\#\#\#}$	1115.8±132.7$^{\#\#}$	25.84±6.36$^{\#\#\#}$	14.01±1.48$^{\#}$	62.09±0.59	55.48±1.52
	958.32±45.61***	719.3±23.9**	9.57±1.29**	5.88±2.10**	36.38±2.43*	32.49±3.65**
	1001.05±48.35**	606.2±41.2***	14.52±1.95*	2.79±1.14***	28.95±8.85*	31.70±4.86**

Effect of DOX (2.5 mg/kg) and DOX + SA treatments (SA 100, 200 mg/kg) on antioxidant systems. Catalase activity (μM of hydrogen peroxide decomposed/min/mg of protein), SOD activity (units of SOD (1 unit=50% adrenochrome formed)/min/mg of protein), GSH levels (μmol of GSH/mg of protein), TBARS (MDA formed nM/mg of protein), nitrite (nM/mg of protein), percentage increase in the MPO levels. All the values are mean±SEM of six animals. $^{\#}P<0.05$ versus vehicle group, $^{\#\#}P<0.001$ versus vehicle group, $^{***}P<0.001$ versus DOX group, $^{**}P<0.01$ versus DOX group $^{*}P<0.05$ versus DOX group. HC: Hippocampus; FC: Frontal cortex; TBARS: Thiobarbituric acid reacting substances; DOX: Doxorubicin; SA: Saraca asoca; SOD: Superoxide dismutase; GSH: Reduced glutathione; MDA: Malondialdehyde; MPO: Myeloperoxidase; SEM; Standard error of mean

phenotypic differentiation and formation of neurite extensions when treated with ATRA.[32,33] Induction with ATRA manifested formation of neurite extensions. SA bark extract at 60 μg/ml along with DOX at 1.5 μg/ml showed comparative retention in neurite length in comparison to the DOX alone treated well. The presence of apoptotic cell bodies in treatment wells, however, less when compared to DOX alone, could be due to DOX treatment. A similar course was observed in studies conducted to evaluate neuroprotective potential against DOX.[30] Comparatively fewer cell bodies and presence of more neurite growth extensions purport the extract's protective effect ($P < 0.001$). In addition, the treatment showed significant reversal ($P < 0.01$) against glutamate-induced neurotoxicity. DOX mediates TNF-alpha induced upregulation of the excess release of glutamate from hemichannels leading to glutamate-induced excitotoxicity causing neurodegeneration.[4] The conducted *in vitro* study confirmed its neuroprotective effect of the extract against glutamate, demonstrating its possible role in preventing glutamate-induced excitotoxicity. Furthermore, this can be proposed for molecular evaluation studies to identify the possible protective mechanism.

An *in vivo* study was conducted to evaluate the effect of extract in counteracting effect DOX systemic toxicity mainly by cognitive decline mediated by oxidative and nitrergic stress. The study was conducted using 50 days treatment including 10 cycles, given every 5 days at a dose of 2.5 mg/kg. The chronic exposure of DOX for 57 days produced a reduction in body weight, comparable to the vehicle although it was not significant. This was significantly reversed by treatment with the SA (100 mg/kg) treatment.

Episodic memory is mainly associated with the H and FC. Most of the conscious learning is performed by the declarative memory function in the H and FC, which is said to be temporally dated conscious recollection of past experiences and events. The animals' ability to recollect past experiences (episodic memory) is evaluated by NORT in which the animals were allowed to explore two familiar objects in the familiarization trial followed by choice trial after anITI. In the choice trial, one object was replaced with a novel object and the ability to explore and discriminate the objects was evaluated.[34] The animals irrespective of the treatment tend to explore the two similar objects for almost equal time during the familiarization phase. However, the animals which were able to remember the familiar object were able to discriminate the novel object and spent significantly more time exploring a novel object. In DOX study, animals were treated for 57 days, after which the episodic memory was assessed. An ITIof 4 h was given which was standardized in our laboratory. DOX-treated animals showed insignificant difference in exploration time of the novel or familiar object. This signifies that the animal failed to recognize the previously encountered familiar objects. Vehicle-treated animals were able to discriminate the novel object from the familiar one. Treatment with extract at 100 mg/kg p. o. showed significantly more exploration time toward the novel object ($P < 0.001$) compared to familiar one and reversed the impairment caused due to DOX. This suggests the beneficial effect of SA against DOX-induced cognitive impairment. However, the higher dose of extract, DOX + SA 200 mg/kg did not reverse the impairment caused due to DOX as the animals failed to recognize the novel object over the familiar object.

Cholinergic system in the brain is mainly responsible for learning and memory function, which contributes to the cognitive ability. Acetylcholinesterase is an enzyme responsible for the degradation of acetylcholine terminating the cholinergic transmission. DOX causes neurodegeneration leading to impairment in the cognitive domain[1] which makes it necessary to evaluate the acetylcholinesterase activity in the brain. Chronic exposure of the toxicant may attribute to the

decreased acetylcholine levels in the body due to a decrease in the choline uptake due to neurodegeneration in the H and FC. According to Agamy *et al*. suggested a possible mechanistic role of DOX-induced oxidative and nitrosative stress which might abnormally increase AchE levels implying an increased degradation of acetylcholine which could be one of the pathways of DOX-induced neurotoxicity.[35] *Ex vivo* acetylcholinesterase inhibition activity study signified potential degradation of substrate acetylthiocholine iodide when treated with SA, determined by intense yellow-colored formed byproduct, thionitrobenzoic acid. This entails a possible acetylcholinesterase inhibition potential of SA. After chronic exposure of DOX, a similar activity was shown. *S. acosa* had significantly reduced the AchE activity in the H which could be due to its potent anti-oxidant activity indicating the restoration of cognitive function.

Oxidative stress is one of the major pathways for the neurodegenerative disorders such as Alzheimer's disease. Most of the cancer chemotherapeutic drugs including DOX produce cognitive decline by producing oxidative stress and nitrergic stress. DOX will undergo redox cycling and absorbed into the body not only affects the cancer cells but also the normal cells. This effect in the brain leads to neuroinflammation and mitochondrial dysfunction leading to apoptosis and neurodegeneration. In the present study, oxidative stress markers such as catalase, SOD, GSH, MDA and inflammatory markers, nitrite, and MPO levels were evaluated. DOX increased the oxidative and nitrosative stress which was significantly ameliorated by SA.

CONCLUSION

SA bark extract showed an improvement in episodic memory which was impaired by DOX treatment. The neuroprotective and antioxidant potential of the test extract possibly underlies the efficacy to reverse cognitive deficits associated with the DOX-induced chemobrain like condition. SA extract at 100 mg/kg p.o. showed better chemoprotective activity against extract at 200 mg/kg, p.o.

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Conflicts of interest

There are no conflicts of interest.

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