

Medhya Rasayana Restores Memory Function against Doxorubicin-induced Cognitive Decline: Possibly by its Neuroprotective Effect

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ABSTRACT

Background: Medhya Rasayana (MR) is an ayurvedic formulation used to rejuvenate the intellectual functions. It was shown to improve the cognitive function in rats. Hence, the present study was designed to study the neuroprotective effect of MR against Doxorubicin (DOX)-induced cognitive deficits which usually occurs in long-term breast cancer survivors. **Methods:** The neuroprotective effect of MR was studied *in-vitro* using IMR-32 cell lines against DOX-induced toxicity while the protective effect of MR against DOX-induced cognitive dysfunction was evaluated in Novel object recognition task (NORT) assay in rats. **Results:** In IMR-32 cell line, DOX (100 nM) treatment reduced the cell viability and arrested the cell cycle at G₂/M phase. Pre-treatment with MR significantly prevented the DOX-induced cytotoxicity and reduced the G₂/M phase arrest indicating that MR exhibits protection against DOX-induced toxicity. In NORT, DOX treatment significantly decreased the Recognition and discriminative indices (RI and DI) indicating DOX impairs episodic memory. Treatment with MR (1.26 and 1.52 g/kg) significantly protected against DOX-induced cognitive dysfunctions. DOX treatment also increased the brain oxidative stress and produced hepato, cardio, nephrotoxicity and myelosuppression. MR treatment ameliorated the DOX-induced toxicity. **Conclusion:** These results showed that MR pre-treatment could protect against post chemotherapy-induced cognitive dysfunction along with an overall reduction in chemotherapy-induced toxicity.

Key word: Doxorubicin, Medhya Rasayana, Chemobrain, Cognitive dysfunction, Chemotherapy, Novel object recognition task.

Submission Date: 29-09-2018;

Revision Date: 01-12-2018;

Accepted Date: 18-01-2019

DOI: 10.5530/ijper.53.2s.54

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INTRODUCTION

Excellent intellectual ability, high mental capacity and a successful career are the objectives of every person in this competitive world. The oldest traditional Indian system of medicine, Ayurveda recognizes the importance of sexual and brain functions and has dedicated several chapters in Charka Samhita. Medhya Rasayanas (MR) described in Ayurveda are a collection of plants that improve Medhya meaning intellect/retention and Rasayana meaning pro-

cedures or preparation that increase the longevity.¹ Hence, these plants promote cognition and improve brain function. The four main medicinal plants that are used in MR are *Mandukaparni* (*Centella asiatica* Linn.), *Yastimadhu* (*Glycyrrhiza glabra* Linn.), *Guduchi* (*Tinospora cordifolia* (Wild) Miers) and *Shankhapushpi* (*Convolvulus pleuricaulis* Choisy). Apart from these other medicinal plants like *Aindri* (*Bacopa monniera*), *Jyotishmati* (*Celastrus panniculata*), *Kushmanda* (*Benincasa his-*



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pida), *Vacha* (*Acorus calamus*) and *Jatamamsi* (*Nardostachys jatamamsi*) are also used to improve efficacy.

Polyherbal formulations of MR are marketed in India to improve memory in children and aged individuals. Scientific studies have been reported which describes the efficacy of MR in short term memory and also improved efficacy when combined with yoga practices.² The individual plants has been reported to possess nootropic properties and an overall improvement in mental functions in various animal models.³

Cognitive decline is one of the important post chemotherapeutic complication of breast cancer survivors. Doxorubicin (DOX) is widely used along with other chemotherapeutic drugs for the management of breast cancer and has been observed to be responsible for cognitive decline in more than 60% of breast cancer patients.⁴ Many possible mechanisms are postulated related to DOX-induced cognitive deficits. These include neuroinflammation, increased oxidative stress, elevated TNF- α levels in the circulatory system crossing into the brain, blood brain barrier disruption, decreased blood flow and glucose uptake to various parts of the brain, decreased brain derived neurotrophic factor (BDNF) expression, defective hippocampal neurogenesis and glial cell stimulation.^{5,6}

MR is reported to reduce the oxidative stress, improve overall brain functions and maintain good flow to the brain while maintaining brain glucose levels.³ Based on this, we hypothesized that MR could potentially prevent doxorubicin-induced cognitive dysfunction. To verify this claim, we designed experiments to study the neuroprotective activity *in vivo* (By global ischemia) and *in vitro* (IMR-32 cell lines). Further, protective efficacy of MR was assessed against doxorubicin-induced episodic memory deficits in normal healthy rats.

MATERIALS AND METHODS

Chemicals

MR (SriSri Ayurveda, Bengaluru, India), DOX (Fresenius Kabi Oncology Ltd., Solan, India), dyes, media and its supplements used for cell culture study were purchased from HiMedia Laboratories, Mumbai, India. Dulbecco's Modified Eagle's Medium (DMEM) was procured from Sigma-Aldrich Co. LLC, St Louis, MO, USA. Plastic wares used in the cell culture study, were from Tarsons Products Pvt. Ltd., Kolkata, India.

Cell Lines and Animals

Human neuroblastoma (IMR-32) cells were procured from National Centre for Cell Science, Pune, India and cultured using DMEM containing 10% FBS in CO₂

incubator. Female Sprague Dawley rats (8–10 weeks, 150–170 g) were obtained from the inbred strains of Central Animal Research Facility (CARF), Manipal Academy of Higher Education (MAHE), Manipal. All the experiments were performed in accordance with the CPCSEA guidelines followed by Institutional Animal Ethics Committee, Kasturba Medical College, MAHE, Manipal (IAEC/KMC/69/2016). The animals were randomized into different group of 8 with 10 animals each in group. All the procedures were followed under controlled laboratory conditions, maintained on 12 h day and night cycle as described earlier.⁷

Neuroprotective Effect of MR against Doxorubicin in IMR 32 Cell Lines⁸

Neuroprotective effect of MR was determined by performing Sulforhodamine B (SRB) assay. Briefly, 5×10^5 cells were seeded in 96-well plate and incubated for a period of 24 h. Next, cells were incubated with graded concentrations of MR (7.5 – 60 mg/mL) for 48 h. Later, cells were fixed with 10% TCA at 4°C for 1 h followed by washing. Cells were then incubated with 0.057 %w/v SRB (Prepared in 1 % acetic acid) for 30 min in dark at room temperature. The unbound dye was removed by washing with 1 % acetic acid solution while the bound dye was dissolved using 10 mM tris base. Absorbance was read at 540 nm using microplate reader (ELx800, Bio-Tek Instruments Inc., Winooski, VT, USA).

After the above study, neuroprotective potential of MR was evaluated in the presence of DOX (100 nM). Cells were pre-incubated with MR (7.5 – 60 mg/mL) for 24 h. Next, cells were exposed to DOX (100 nM) for 48 h. Cell viability was assessed by SRB assay as detailed previously.⁹

Cell Cycle Analysis

5×10^5 cells were seeded, after 24 h seeding, cells were incubated with MR for 24 h at different concentrations, then exposed to DOX (100 nM). Then cells were fixed in 70 %v/v ethanol for 4 h and centrifuged, suspended in PBS containing propidium iodide with RNase and kept in the dark for 20 min. Samples were subjected to analysis in a flow cytometer (BD Accuri™ C6; BD Biosciences, San Jose, CA, USA). The influence of MR was assessed against DOX-induced cell cycle arrest.

Dox Induced Toxicities and Assessment of Protective Effect of MR *in vivo*^{10,11}

Cognitive decline in healthy rats were induced as described earlier. Grouping was treatment schedules are depicted in Figure 1. MR doses were selected based on the human dose levels prescribed for nootropic

activity. The ability of MR to protect against Dox induced cognitive decline (Novel object recognition task), myelosuppression (Hematological changes) and oxidative stress in brain (Levels of SOD, catalase GSH and LPO in frontal and hippocampal regions of brain) were evaluated as described earlier.⁷

Acute Toxicity Study

Acute toxicity was conducted in healthy adult female rats (Nulliparous and non-pregnant). Limit test was performed for MR at a dose volume of 20 ml/kg. Animal husbandry condition, gross behavioural changes, physical signs of toxicity and mortality rate were observed for short- and long-term outcomes as described in OECD 425.¹²

Statistical Analysis

Prism Version 6.05 (Fully functional demo; GraphPad Software Inc., La Jolla, CA, USA) software was used for statistical analysis. Data are represented as mean \pm SEM. Recognition and discriminative indices were analyzed by Kruskal–Wallis test, followed by Dunn's post hoc test. Other parameters were carried out by one-way analysis of variance, followed by Dunnett's post hoc test. A value of $p < 0.05$ was considered as statistically significant.

RESULTS

Neuroprotection of Medhya Rasayana in Higher Concentration against DOX in Differentiated IMR-32 Cells

MR was evaluated for their neuroprotective effect against DOX treatment in differentiated IMR-32 cells. MR of desired concentrations (7.5-60 mg/mL) were added and incubated for 24h. Further, the cell culture media was aspirated, 100 nM DOX was added and incu-

bated for 24h. A significant reduction in cell viability was observed with DOX treatment (100 nM). The pre-treatment with MR with these concentrations showed a significant increase in cell viability (Figure 2).

Cell Cycle Analysis for 2 h Pre-Treatment of MR by Flowcytometry

Cell cycle analysis was carried out to identify the phase of cell cycle arrested by DOX and MR. It was observed that 100 nM DOX arrested the cell cycle at G₂/M Phase (Figure 3) and pre-treatment with MR at 60 mg/ml was able to protect the toxicity produced by DOX (Table 1).

In vivo Studies

Acute Toxicity Study

MR did not show any signs of toxicity at a dose level of 20ml/kg (25.3 g/kg). Based on these studies and average daily human consumption of syrup we fixed the dose of MR for *in vivo* studies to be 2.53 g/kg.

Novel Object Recognition Test

Recognition Index

Recognition index was used to determine the ratio of time spent between exploring the novel object over the total time spent exploring both familiar and novel objects. Exposure of DOX to the animals for 10 cycles had reduced the recognition index significantly ($*p < 0.05$) when compared to that of normal control. The effect of doxorubicin induced memory deficits was significantly reversed by Standard Donepezil as well as both the doses of MR (Figure 4A).

Discriminative Index

Alternatively, discriminative index was used to determine the animal's ability to differentiate between the novel and familiar object. Administration of DOX for 10 cycles significantly reduced ($*p < 0.05$) the discrimi-

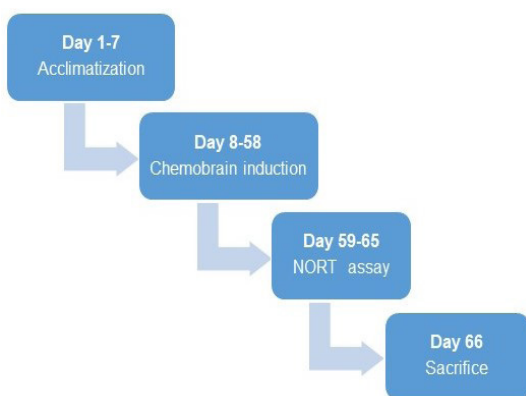


Figure 1: Treatment Protocol for the *in-vivo* Model to Evaluate MR Against DOX-Induced Cognitive Dysfunction.

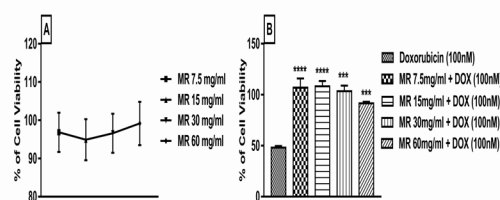


Figure 2: Cell Viability Assay. A. Effect of MR Alone on Cell Viability and B. Protective effect of MR Against DOX-Induced Cytotoxicity.

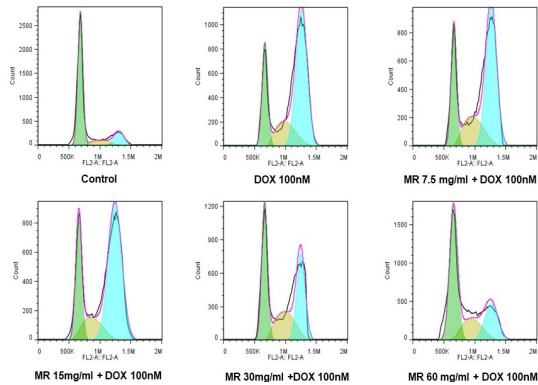


Figure 3: Cell Cycle Analysis by Flow Cytometry Indicating DOX (100 nM) caused G₂/M Phase Arrest and MR at Different Concentrations Prevented this Arrest.

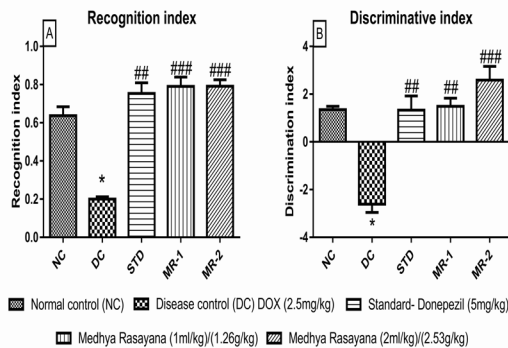


Figure 4: Effect of MR on Episodic Memory in NORT Assay Conducted in DOX Treated Animals- A. Recognition Index and B. Discriminative Index. Statistical analysis was performed using Kruskal-Wallis test followed by Dunn's post-hoc test. * $p < 0.05$ significant compared to NC; ## $p < 0.01$ significant compared to DC; ### $p < 0.001$ significant compared to DC.

native index compared to normal control. The effect induced by DOX was reversed significantly by treatment with both Donepezil and MR (Figure 4B).

Serum Biochemical Estimations

Effect of Treatment and DOX on Serum AST and ALT Levels

DOX administration for 10 cycles during the study resulted in significant ($**p < 0.01$) increase in AST and ALT levels. MR treatment at both the doses prevented these elevations induced by DOX (Figure 5A and B) indication it protected against hepatic toxicity induced by DOX.

Effect of Treatment and DOX on Blood Urea Nitrogen (BUN) Levels

Nephrotoxicity was observed with DOX administration represented by elevated serum BUN levels (Figure 5C).

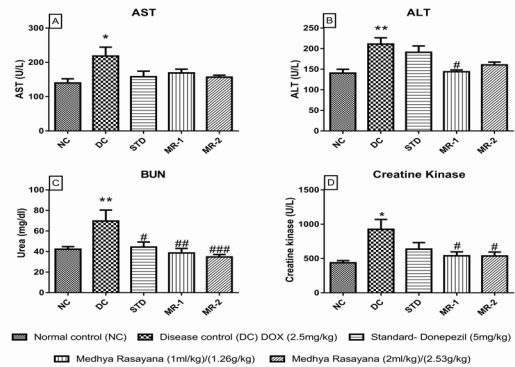


Figure 5: Effect of MR on Serum Biochemical Parameters in DOX treated Animals Where A. Aspartate Aminotransferase (AST); B. Alanine Aminotransferase (ALT); C. Blood Urea Nitrogen (BUN) and D. Creatine Kinase. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test. * $p < 0.05$ significant compared to NC; ** $p < 0.01$ significant compared to NC; # $p < 0.05$ significant compared to DC; ## $p < 0.01$ significant compared to DC; ### $p < 0.001$ significant compared to DC.

The effect of the DOX was significantly reduced by both the doses of MR. However, the higher dose of MR (2.53 g/kg) had shown better nephroprotective action.

Effect of Treatment and DOX on Creatine Kinase Levels

The cardio toxic effect of DOX administration was assessed by estimating creatine kinase levels. It as seen that the DOX group has significantly increased the creatine kinase levels. However, the effect of DOX-induced cardio toxicity was reversed by both the doses of MR (Figure 5D).

Effect of Treatment and DOX on Hematology

No significant difference was found between any of the groups for WBC count. However significant reduction in the number of RBC was found in DOX control group compared to normal control. However, both the doses of MR did not reverse the effect of DOX. Significant reduction in Hb ($***p < 0.001$) and platelet count ($***p < 0.001$) was seen when the animals when exposed to DOX as compared to normal control indicating myelosuppression. The toxicity of DOX was significantly reduced by the higher dose of MR (2.53g/kg) (Figure 6)

Brain Oxidative Stress Parameters

Administration of DOX resulted in decreased levels of catalase, SOD and GSH both in hippocampal and frontal cortex. Further, DOX treatment elevated LPO levels in hippocampal region suggesting that DOX caused oxidative stress in brains of rat. MR treatment protected against these changes induced by DOX (Figure 7 and 8) resulting in improved cognitive functions.

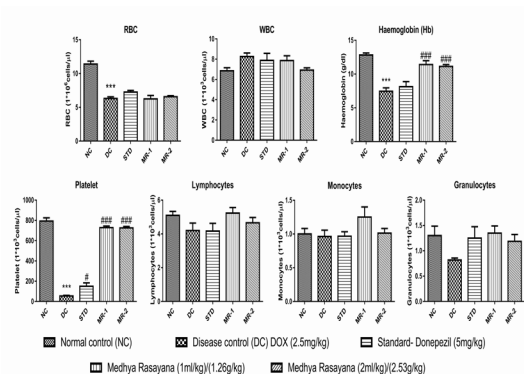


Figure 6: Effect of MR on Hematological Parameters in DOX Treated Animals. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test. * $p < 0.05$ significant compared to NC; * $p < 0.001$ significant compared to NC; # $p < 0.05$ significant compared to DC; ### $p < 0.001$ significant compared to DC.**

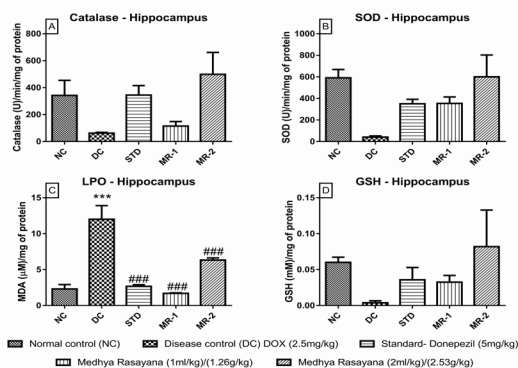


Figure 8: Effect of MR on Antioxidant Levels in Hippocampus in DOX Treated Animals. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test. * $p < 0.05$ significant compared to NC; * $p < 0.001$ significant compared to NC; ### $p < 0.001$ significant compared to DC.**

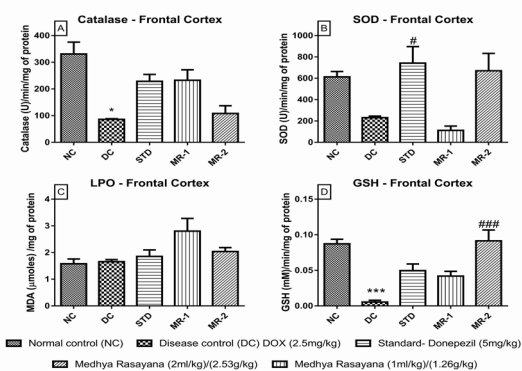


Figure 7: Effect of MR on antioxidant Levels in Frontal Cortex in DOX Treated Animals. Statistical analysis was performed using one-way ANOVA followed by Dunnett's post-hoc test. * $p < 0.05$ significant compared to NC; * $p < 0.001$ significant compared to NC; # $p < 0.05$ significant compared to DC; ### $p < 0.001$ significant compared to DC.**

Table 1: Percentage Cells Present in Different Phases of Cell Cycle Treated with MR and DOX.			
% of cells present in each phase			
Treatment	G ₀ /G ₁ Phase	S Phase	G ₂ /M Phase
Control	69.57	15.87	14.55
Dox (100 nM)	23.57	17.96	58.6
MR 7.5 mg/ml + Dox (100 nM)	24.35	21.41	54.31
MR 15 mg/ml + Dox (100 nM)	23.98	15.04	61.19
MR 30 mg/ml + Dox (100 nM)	38.83	26.3	34.82
MR 60 mg/ml + Dox (100 nM)	55.69	20.97	23.37

DISCUSSION

Chemo brain is a condition where decline in memory is observed mostly after or during the chemotherapy treatment. It is most prevalent in breast cancer survivors administered with DOX regimen. Acute and chronic treatment of DOX affects the grey matter and white matter present in the brain which ultimately affects the cognitive performance.^{13,14} Although, DOX itself cannot cross BBB as it is not lipophilic, it mainly mediates its action by release of proinflammatory mediators, increased oxidative stress and by mitochondrial dysfunction.¹⁵ Currently no approved medication is available to treat chemo brain. Reports have suggested that various constituents of MR is responsible for its neuroprotective activity. Clinical data provide us the basis that MR has neurotropic effect which act on intellect

and retention memory.³ Therefore, in our study we have attempted to evaluate the chemo brain preventive activity of MR by assessing neuroprotective effect in *in vitro* assays as well as in *in vivo* assays through NORT assay. Along with the assessment of episodic memory by NORT assay, antioxidant assays were also performed to evaluate the pathological and toxicological effect of DOX.

In *in vitro* assays, we tried pre-treatment as well as treatment along with toxicant regimens. We observed that DOX treatment caused a significant reduction in the cell viability of IMR-32 and produced G₂/M phase arrest. While treatment with MR caused a significant increase in cell viability showing its neuroprotective effect. We also observed that 1 μM DOX was not able to produce the cell cycle arrest at G₂/M phase but rather

was causing cell cycle arrest at the S phase. Hence, we also reduced the concentration of the toxicant DOX to 100 nM for cell cycle analysis following the previous pre-treatment schedule. During further assessment, we observed that DOX 100 nM produced a significant reduction in the cell viability and pre-treatment with MR at increased concentrations caused a significant increase in cell viability of IMR-32. In cell cycle analysis, DOX 100 nM produced cell cycle arrest at G₂/M phase and MR pre-treatment was able to protect against the cell cycle arrest by DOX.

In vivo studies were undertaken to determine the neuroprotective effect of MR in DOX induced chemo brain in experimental animals. Acute oral toxicity for the drug was done as per the OECD guidelines 423, in which the drug MR has shown no toxicity up to a dose of 20ml/kg (25.3g/kg) in female Sprague Dawley rats indicating that higher dose for further studies can be taken into consideration so that the efficacy of the MR can be better proven. No death of the animals or histopathological changes in the major organs like kidney, liver and heart were noted in the study, indicating that the drug was safe and a higher dose of drug can be used for further study to prove its efficacy.

Episodic memory is mainly associated with frontal cortex and hippocampus which was assessed by NORT assay.¹⁶ In the NORT assay, significant increase in the recognition index was observed at both the doses of MR when compared to that of DOX control. Significant difference was also seen in discriminative index with MR treatment when compared to that of DOX control. This shows that MR was able to reverse the neuronal damage produced by DOX regimen.

Since, DOX regimen has been previously associated with liver toxicity, in our studies, we have determined the levels of serum AST and ALT as markers of liver damage. From the results it is seen that animals administered with DOX had shown high levels of AST and ALT when compared to that of normal control. This is in accordance with the literature support.¹⁷ However, this was significantly reversed by the low dose of MR, which possibly indicates that MR has liver protective activity.

Additionally, markers for nephrotoxicity and cardio toxicity were ascertained in all DOX treated animals. A significant reduction in the BUN levels were found in both the doses of MR when compared to that of DOX indicating towards neuroprotective activity of MR. Cardio toxicity of DOX was assessed by estimating creatine kinase levels and the result found was in accordance with the literature support.^{18,19} In our study, DOX treated animals showed significant increase in the

creatine kinase levels when compared to that of normal control and creatine kinase levels were significantly decreased by high dose of MR (2.53g/kg) showing cardio protective activity.

Haematological parameters were estimated to assess the myelosuppressive effect of DOX. Significant reduction in the RBC and Hb and platelet levels were found in DOX control group when compared to normal control. Alternatively, the effect of DOX on haemoglobin (Hb) and platelet count was significantly reversed by higher dose of MR. However, no significant effect was found between any of the groups in lymphocyte, granulocyte and monocyte count.

Additionally, oxidative stress is one of the major factors which leads to neurodegeneration resulting in cognitive impairment by decreasing BDNF levels.²⁰ Therefore, in the present study, antioxidant parameters were evaluated in both hippocampus and frontal cortex as hippocampus is mostly associated with learning and memory due to dentate gyrus where BDNF is present. Antioxidant estimation was also done in frontal cortex as DOX generally affects the episodic memory, which is mainly associated with frontal cortex.

Administration of DOX results in increased oxidative stress²¹ in cells thus showing decrease levels of catalase and SOD which could not be reversed by both the doses of medhya rasayana in both frontal cortex as well as in hippocampus indicating that medhya rasayana has no anti oxidative property. However, in lipid peroxidation, significant decrease in the MDA levels were found in both the doses of Medhya Rasayana when compared to that of normal control in hippocampus indicating that Medhya Rasayana has significantly reduced the oxidative stress generated due to DOX. But no significance reversal of the DOX induced oxidative stress was seen in frontal cortex when assessed for MDA levels.

GSH levels in both the hippocampus and frontal cortex was reduced in DOX control group when compared to normal control group. Reduced GSH levels in frontal cortex DOX control was significantly reversed by the higher dose (2.53g/kg) of MR when compared to that of lower dose while in hippocampus no reversal of the activity is seen when compared to that of DOX control. The results from these studies showed that MR can play a beneficial role in DOX induced cognitive impairment.

CONCLUSION

The present study was attempted to investigate the role of MR as a neuroprotective agent in chemobrain. In *in vitro* assays MR showed neuroprotection at higher doses and similarly, afforded neuroprotection against DOX-

induced neuronal damage in animal studies through improved antioxidant defense. MR also afforded protection against cardio toxicity, nephrotoxicity and liver toxicity induced by DOX. Therefore, MR may prove to be a potential treatment option for the management of chemo brain. However, further studies are required to verify MR produces these protective actions without comprising the anti-cancer effect of DOX.

ACKNOWLEDGEMENT

The authors are thankful to All India Council for Technical Education (AICTE) and Department of Sciences and Technology – Fund for improvement of S and T infrastructure in Higher Educational Institutions (DST-FIST) to create infrastructure for this project. We are also thankful to Manipal Academy of Higher Education for the support of this project.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ABBREVIATIONS

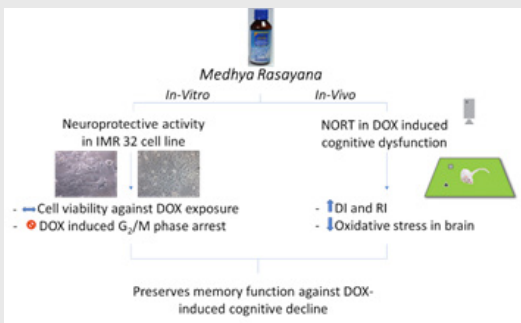
DOX: Doxorubicin; **MR:** Medhya Rasayana; **BDNF:** Brain derived neurotrophic factors; **NORT:** Novel object recognition task; **SOD:** Superoxide dismutase; **MDA:** Malondialdehyde; **GSH:** Glutathione; **AST:** Aspartate aminotransferase; **ALT:** Alanine aminotransferase; **BUN:** Blood urea nitrogen; **DI:** Discriminative index; **RI:** Recognition index; **RBC:** Red blood cell; **Hb:** Hemoglobin; **BBB:** Blood brain barrier; **LPO:** Lipid peroxidation; **TNF – α :** Tumor necrosis factor alpha.

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Cite this article: Gourishetti K, Ghetia S, Jacob LA, Mallik SB, Nayak PG, Kishore A, *et al.* Medhya Rasayana Restores Memory Function against Doxorubicin-induced Cognitive Decline: Possibly by its Neuroprotective Effect. *Indian J of Pharmaceutical Education and Research.* 2019;53(2S):s104-s111.

PICTORIAL ABSTRACT



SUMMARY

- Medhya Rasyana (MR) is a herbal preparation used to improve intellectual ability. We tested this preparation against DOX-induced neurotoxicity and cognitive dysfunction. It was observed that MR protected neurotoxicity against DOX induced cell death in human neuroblastoma cells. Further MR prevented DOX-induced cognitive decline in NORT. These preclinical results suggest that MR may be beneficial in the management of post chemotherapy induced cognitive dysfunction seen in breast cancer survivors.