Neuroprotective effect of Mulmina™ against chemotherapy-induced cognitive decline in normal mice

MANAS KINRA[1], NIRAJA RANADIVE[1], KARTHIK GOURISHETTI[1], PAWAN GANESH NAYAK[1], RAJESH N. JAGDALE[2], SYED MUSHTAQ AHMED[2], KAGGUNDI V. RAGHAVENDRA[2], JAYESH MUDGAL[1] and KRISHNADAS NANDAKUMAR[1]

[1]Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal, Karnataka 576104; [2]Juggat Pharma, Jagdale Industries Pvt. Ltd., Bengaluru, Karnataka 560078, India

Received February 26, 2020; Accepted September 14, 2020

DOI: 10.3892/br.2020.1377

Abstract. The aim of the present study was to evaluate a marketed formulation against chemotherapy-induced cognitive dysfunction. The formulation, Mulmina™, contains natural compounds which are known to help in improving function as well as in preventing cognitive decline. All of the phytoconstituents in the formulation have been tested individually but this is the first study where such a formulation has been evaluated against chemotherapy-induced cognitive decline (CICD) in a mouse model. CICD was induced by cyclophosphamide (50 mg/kg), methotrexate (5 mg/kg), and 5-fluorouracil (5 mg/kg) (CMF), administered intraperitonially. CMF was administered in three cycles, with one injection per week for three weeks. The decline in cognition of the mice was evaluated by a test of locomotor activity (Open Field Test) followed by a test for spatial memory (Morris Water Maze). Biochemical parameters evaluated include brain cytokine levels and BDNF levels via ELISA. Hematological counts were also performed to evaluate any changes in blood profile using a veterinary blood cell counter. Levels of oxidative stress markers with respect to catalase activity and lipid peroxidation were also evaluated in the brain using UV-spectrophotometric analysis. Mulmina™ was able to show significant improvement in cognitive function post chemotherapy when compared to the untreated animals. Apart from improvement in spatial memory, there was also an improvement in biochemical parameters. The particular combination of phytochemicals in Mulmina™ proved themselves successful in alleviating the CICD in this preliminary study and pave a path for future studies which can establish the solid grounds with respect to molecular and pharmacological basis for the mechanism of action of Mulmina™.

Correspondence to: Professor Krishnadas Nandakumar or Professor Jayesh Mudgal, Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Madhav Nagar, Manipal, Karnataka 576104, India
E-mail: nandakumar.k@manipal.edu
E-mail: jayesh.mudgal@manipal.edu

Key words: 'chemobrain', chemotherapy, cognition, neuroinflammation, cytokines

Introduction

Cancer incidence is increasing globally (1), and this has caused an increase in the search for anticancer therapies (2). Among the adverse effects exerted by anticancer drugs (3), only a few are manageable, and the majority of these agents significantly affect the quality of life of the patients even after the completion of the chemotherapy regime. One such adverse effect is 'chemobrain', where a severe decline in cognitive functions are manifested. This manifestation adversely affects the memory, learning ability, ability to process information and day-to-day activities. The main underlying reasons for this condition are reported to be oxidative stress due to chemotherapy, apoptosis, chemotherapy-induced inhibition of neuronal proliferation and differentiation and activation of microglia (4,5). These are associated with chromatin remodelling which causes aberrant expression of neurotrophic proteins in the brain (6). Although the incidence and actuality of 'chemobrain' have been a subject of deliberation, recent studies have confirmed that it is not only applicable to a majority of patients but also measurable in terms of decrease in quality of life and outcomes of the adverse effects. As many as 70% of patients who have undergone cancer chemotherapy have reported that these effects persist long after the termination of treatment, especially in patients suffering from breast (7), ovarian (8) and prostate cancer (9).

Natural products have attracted the significant attention of the scientific community for protection against 'chemobrain'. A few such promising products include mangiferin from mango pulp (10), curcumin (11), and brahmi (12). In the present study, a combination of such natural products, which are commercially available as an ayurvedic proprietary balya/poshak Mulmina™ Mango was assessed. The combination of *Mangifera indica* fruit pulp, *Centella asiatica* whole plant extract, *Curcuma longa* rhizome extract along with various vitamins and minerals makes Mulmina™ Mango a potential candidate for preventive care in the case of 'chemobrain'.

Materials and methods

Animals. Forty male Swiss Albino mice (8-10 weeks, 20-30 g initial weight) were obtained from the inbred strains of the Central Animal Research Facility (CARF), Manipal Academy of Higher Education, Manipal, India. All of the experiments were performed after approval from the Institutional Animal Ethics Committee of Manipal Academy of Higher Education (approval no. IAEC/KMC/115/2018) in accordance with the guidelines set out by them, following the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines and in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (https://grants.nih.gov/grants/olaw/guide-for-the-care-and-use-of-laboratory-animals.pdf). The animals were housed in groups of 8, under controlled laboratory conditions, maintained on a 12-h day and night cycle. Food and water were available *ad libitum*.

Chemicals and reagents. All the reagents used for the study were of analytical grade. Thiobarbituric acid, trichloroacetic acid, disodium hydrogen phosphate and, sodium dihydrogen phosphate were obtained from Sigma-Aldrich Co. LLC (Merck KGaA) and the chemotherapeutic drugs were of pharmaceutical grade. Cyclophosphamide (Endoxan-N), methotrexate (Biotrexate) and 5-flurouracil (Fluracil) were obtained from a local registered pharmacy. ELISA kits were obtained from Thermo Fisher Scientific Inc.

Drug treatments. Animals were randomly assigned into 5 groups (n=8): Group I: Normal control; Group II: CMF (cyclophosphamide+methotrexate+5-fluorouracil); Group III: Mulmina™ 40; Group IV: Mulmina™ 80; Group V: donepezil (a medication used to treat Alzheimer's disease). All of the treatments were administered orally (p.o.) whereas chemotherapy was given through an intraperitoneal (i.p.) route. Chemotherapy consisted of cyclophosphamide (50 mg/kg), methotrexate (5 mg/kg) and 5-fluorourcail (5 mg/kg) (CMF). Mulmina™ 40 group received 40 ml/kg/day of Mulmina™ + CMF and Mulmina™ 80 group received 80 ml/kg/day of Mulmina™ + CMF. These doses were converted from human equivalent dose as per FDA HED conversion table. Donepezil was administered at a dose of 2 mg/kg p.o. suspended in the vehicle 0.25% w/v carboxymethylcellulose (CMC) along with CMF. The normal control group received 10 ml/kg CMC p.o. and 0.1 ml of saline i.p. to keep the conditions of animal handling similar across the groups and did not receive CMF. Chemotherapy was given in three cycles over a period of 21 days (once a week for three weeks) and the treatments were given every day for the same 21-day period.

The health and general behaviour of the animals were monitored twice daily, once in the morning and once in the evening. On the days of chemotherapy, animals were closely observed for 3 h post administration. On all other days, animals were observed for 1 h after administration of the standard drug as well as Mulmina™. The animals were administered with the standard drug and Mulmina™ between 8-9 a.m. in the morning and between 4-5 p.m. in the evening.

On completion of the chemotherapy cycle, the open field test was performed to evaluate any loss of locomotor activity following which, the water maze test was performed. After the water maze test, the mice were anesthetised with thiopentone sodium (25 mg/kg, i.v.), and blood was withdrawn via retro-orbital puncture for biochemical estimations. Later, the same animals were sacrificed with carbon dioxide (CO_2) overdose (flow rate of 20% chamber volume per min) as per BU ASC guidelines (13), and brain tissue was collected for biochemical estimations.

Behavioral parameters

Open field test. The open field test (OFT) was used to assess the effect of the treatment groups on exploratory behavior of the animals. Locomotor activity (LMA) was assessed in mice individually placed into a clean, novel glass jar (30x30x60 cm). The open field was divided into nine virtual quadrants (10x10 cm) and LMA was measured by counting the number of line crossings, latency to first line crossing, centre square entries, time spent in the centre square and, time spent in the periphery over a 5-min period. The apparatus was cleaned with 70% ethanol between experiments as previously described (14).

Morris water maze test. The maze was a circular pool with a diameter of 150 cm with an escape platform of 40 cm height. The pool was virtually divided into 4 quadrants: North-west, north-east, south-west and south-east using ANY-maze software (Stoelting Co.). The escape platform was kept 2 cm below the water and was 10 cm in dimension. The pool was filled with fresh water every day before experiments and maintained at the temperature of 27˚C. The parameters observed were escape latency and retention time as previously described (15).

Estimation of antioxidant parameters in brain homogenate

Catalase. Catalase activity was measured in the whole brain homogenate. A total of 0.1 ml of supernatant was added to a cuvette containing 1.9 of 50 mM phosphate buffer (pH 7.0). The reaction was started by the addition of 1 ml freshly prepared 10 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from the changes in absorbance at 240 nm. The activity of catalase was expressed as μmol of H_2O_2 decomposed/min/mg protein as described (16).

Lipid peroxidation. The TBA-TCA-HCl reagent was sonicated and mildly heated to dissolve the components. Thereafter, 0.5 ml of brain homogenate and 1 ml of TBA-TCA reagent was heated at 80˚C for 10 min. The reaction mixture was centrifuged at 4˚C at 2,000 x g for 20 min. The amount of malondialdehyde (MDA) was measured spectrophotometrically at 532 nm as previously described (17).

Estimation of hematological parameters. Estimation of the hematological parameters was performed using veterinary blood cell counter (PCE-210 Fully Automatic Blood Cell Counter, ERMA Inc., Tokyo, Japan). The tested parameters were red blood cell count ($10^6/\mu l$), haemoglobin (g/dl), white blood cell count ($10^3/\mu l$), number of monocytes ($10^3/\mu l$), lymphocytes ($10^3/\mu l$), granulocytes ($10^3/\mu l$) and platelets ($10^3/\mu l$).

Estimation of IL-6, TNF-α and BDNF. ELISAs were performed for these parameters as per the manufacturer's instructions.

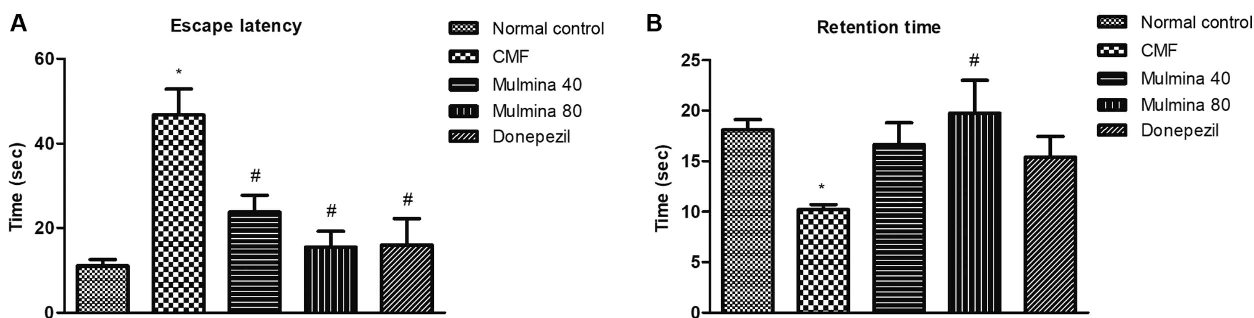

Figure 1. Effect of test drugs (CMF, Mulmina 40, Mulmina 80 and donepezil) on (A) escape latency and (B) retention time as assessed by the Morris water maze. The data were analyzed using one-way analysis of variance (ANOVA) employing Bonferroni as the post-hoc test. *P<0.05 compared to the normal control; #P<0.05 compared to the CMF group. Treatment: CMF group received cyclophosphamide + methotrexate + 5-fluorouracil; Mulmina™ 40 group received 40 ml/kg/day of Mulmina™+ CMF and Mulmina™ 80 group received 80 ml/kg/day of Mulmina™ + CMF.

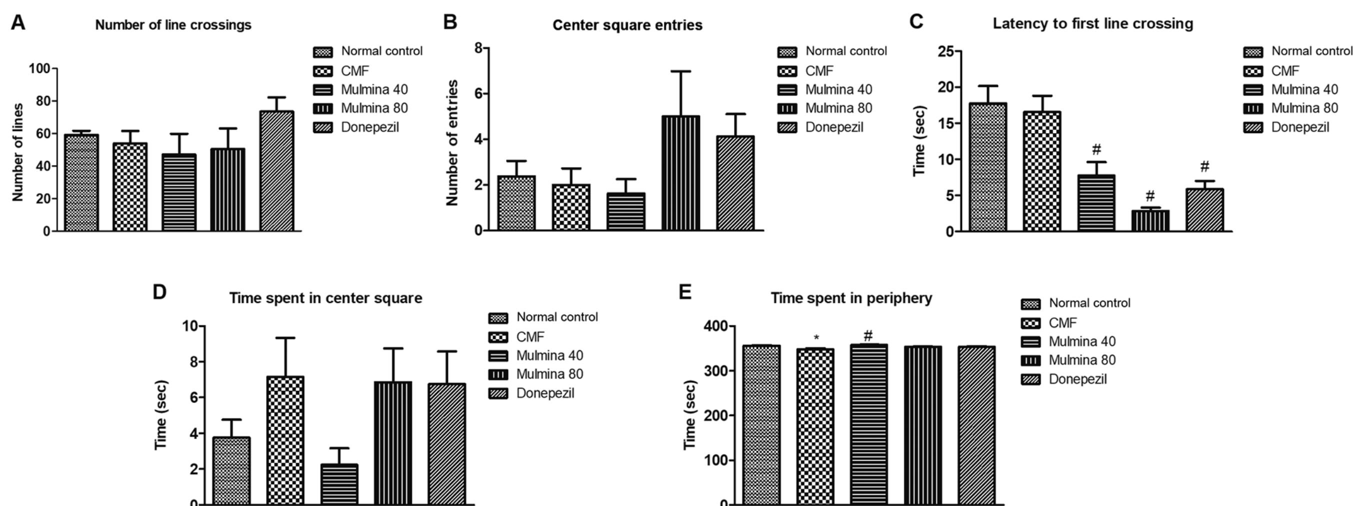

Figure 2. Effect of test drugs (CMF, Mulmina 40, Mulmina 80 and donepezil) on (A) number of line crossings, (B) center square entries, (C) latency to first line crossing, (D) time spent in center square and (E) time spent in periphery in the open field test. The data were analyzed using one-way analysis of variance (ANOVA) employing Bonferroni as the post-hoc test. *P<0.05 compared to the normal control; #P<0.05 compared to the CMF group. Treatment: CMF group received cyclophosphamide + methotrexate + 5-fluorouracil; Mulmina™ 40 group received 40 ml/kg/day of Mulmina™ + CMF and Mulmina™ 80 group received 80 ml/kg/day of Mulmina™ + CMF.

ELISA kit for interleukin (IL)-6 (E-EL-M0044), tumor necrosis factor (TNF)-α (E-EL-M0049), and brain-derived neurotrophic factor (BDNF) (E-EL-M0203) were obtained from Elabscience Inc.

Statistical analysis. Data are expressed as mean ± SEM (n=8) and were analyzed using GraphPad Prism version 8.4 (GraphPad Software, Inc.). All the parameters in the study were analyzed by one-way analysis of variance (ANOVA) followed by Bonferroni as the post-hoc test. The difference between the two groups was considered statistically significant at P<0.05.

Results

Memory and locomotor activity

Assessment of memory by escape latency (ELT) and retention time (RT) using the Morris water maze. ELT is the amount of time each animal takes to reach the island zone for the first time during the retention trial. The difference between the normal control (11±1.4 sec) and CMF (47±6.1 sec) groups was found to be significant (P<0.05). Low dose [40 ml/kg/day (24±4.0 sec)] as well as high dose [80 ml/kg/day (16±3.7 sec)] of Mulmina™ significantly reduced the escape latency compared to the CMF group. Similarly, donepezil (16±6.3 sec) was also found to be significant in reducing the escape latency (P<0.05) compared to the CMF group (Fig. 1A). RT is the amount of time the animal spends in D-quadrant during the duration of the retention trial which was 60 sec. The CMF (10±0.49 sec) group was found to be significantly different from the normal control (18±1.1 sec) (P<0.05). Although the low dose of Mulmina™ (17±2.2 sec) and donepezil (15±2.0 sec) did not show a significant difference, a high dose of Mulmina™ (20±3.3 sec) was found to be significant at P<0.05 compared to the CMF group (Fig. 1B).

Assessment of locomotor activity by open field test. The number of line crossings (LC) is the number of lines the animal crosses while exploring the arena which is divided into nine equal squares and reflects upon the locomotor activity of the animal directly. No significant difference was observed between the groups in regards to LC (Fig. 2A). Center square entry (CS) is the number of times an animal enters into the

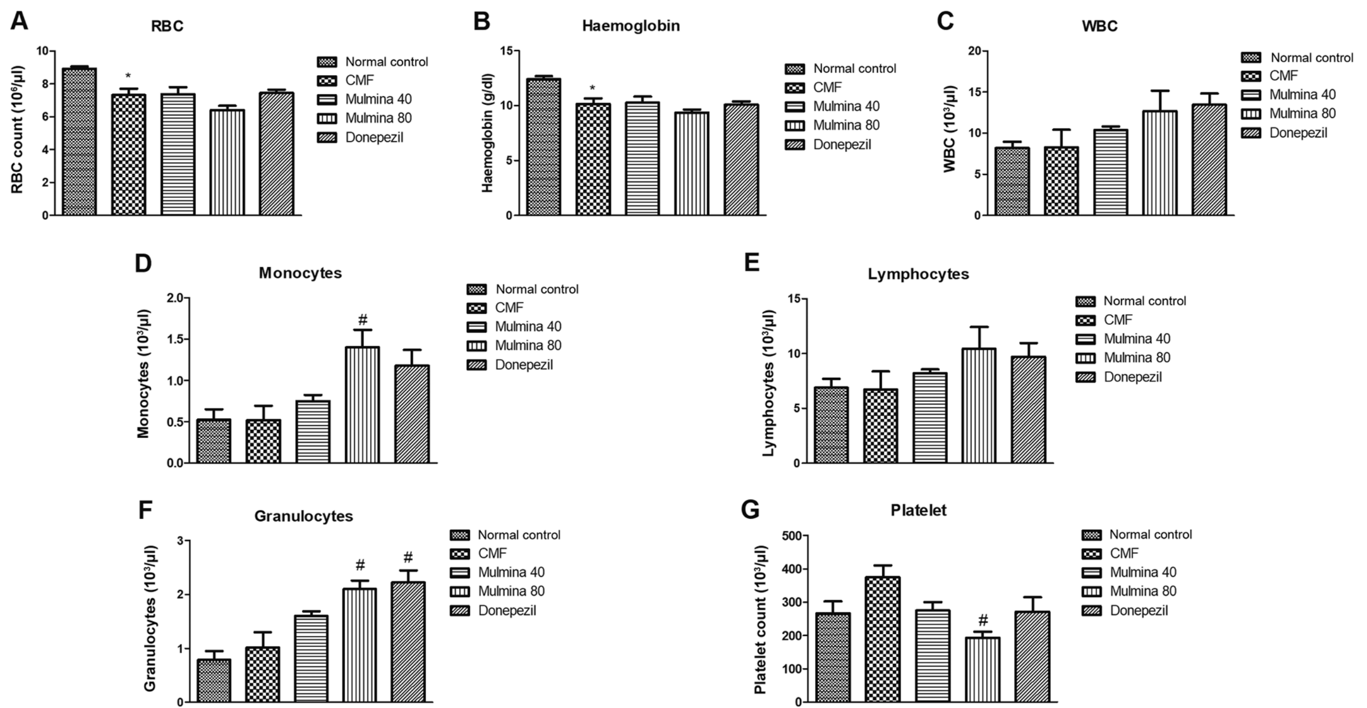

Figure 3. Effect of test drugs (CMF, Mulmina 40, Mulmina 80 and donepezil) on (A) red blood cell count, (B) hemoglobin levels, (C) white blood cell count, (D) monocyte count, (E) lymphocyte count, (F) granulocyte count and (G) platelet count. The data were analyzed using one-way analysis of variance (ANOVA) employing Bonferroni as the post-hoc test. *P<0.05 compared to the normal control; #P<0.05 compared to the CMF group. Treatment: CMF group received cyclophosphamide + methotrexate + 5-fluorouracil; Mulmina™ 40 group received 40 ml/kg/day of Mulmina™+ CMF and Mulmina™ 80 group received 80 ml/kg/day of Mulmina™ + CMF.

center square in the arena while exploring it. No statistically significant difference was observed in terms of CS (Fig. 2B). Latency to first line crossing (FLC) is the measure of the time taken by an animal to cross the first line after being introduced into the arena and is measured in sec. The low (7.8±1.9 sec) as well as the high dose of Mulmina™ (2.9±0.46 sec) and donepezil (5.9±1.2 sec) significantly reduced the FLC when compared to the CMF group (17±2.3 sec) (P<0.05) (Fig. 2C). Time spent in the center square (CST) is the amount of time an animal spends in the center square while exploring the open field arena and is measured in sec. No significant difference was observed between the groups in regards to CST (Fig. 2D). Time spent in the periphery (PT) is the amount of time an animal spends in the periphery/along the walls of the open field arena and is measured in sec. The CMF (347±2.9 sec) group spent significantly more amount of time in the periphery as compared to the normal group (356±1.0 sec) (P<0.05) while Mulmina™ 40 (358±0.92 sec) treated animals spent significantly lesser time in the periphery as compared to the CMF group (347±2.9 sec) (Fig. 2E).

Hematological parameters

Effect of test drugs on the complete blood cell count. The trend observed in red blood cell (RBC) count and hemoglobin content was almost similar whereas the RBC count and hemoglobin were found to be significantly reduced in the CMF group (7.31±0.39 $10^6/\mu$l; 12.38±0.30 g/dl) compared to the normal control (8.92±0.14 $10^6/\mu$l; 6.84±0.63 g/dl) but changes in any other group were statistically insignificant (Fig. 3A and B). CMF treatment increased the total WBC count in all the groups compared to the normal control group. This difference, although evident, was not found to be significant (Fig. 3C). The mice showed an elevation in monocyte count ($10^3/\mu$l) in all the groups upon treatment with CMF. This elevation was statistically significant in the high dose Mulmina™ group (1.40±0.81) when compared to the normal control (0.52±0.12) (P<0.05) (Fig. 3D). No statistical significance in lymphocyte count was observed among the treatment groups and CMF when compared to the normal control (Fig. 3E). CMF administration increased the granulocyte count ($10^3/\mu$l) among all the test groups but only the high dose of Mulmina™ (2.10±0.15) and donepezil (2.22±0.22) showed a statistically significant increase in the count when compared to the normal control (0.78±0.16) (P<0.05) (Fig. 3F). Following administration of CMF, the platelet count ($10^3/\mu$l) was observed to be elevated in the CMF group, but it was not significant statistically. Among the test groups, the high dose of Mulmina™ group showed a significant decrease in platelet count when compared to the normal control (P<0.05) (Fig. 3G).

Biochemical parameters

Effect of test drugs on the levels of cytokines in the mouse brain. Interleukin (IL)-6 is predominantly a pro-inflammatory cytokine whose expression as well as released levels have been reported to increase in acute as well as chronic inflammatory conditions and is a well-known player in neuroinflammatory conditions. For brain IL-6, the difference between the normal control (14±1.8) and CMF group (66±7.0) was found to be significant (P<0.05). Although the low dose of Mulmina™ (54±2.7) did reduce the level of IL-6, the difference was not statistically significant. The high dose group (35±4.4) showed a significant reduction (P<0.0.05) as well as the donepezil group (44±3.5)

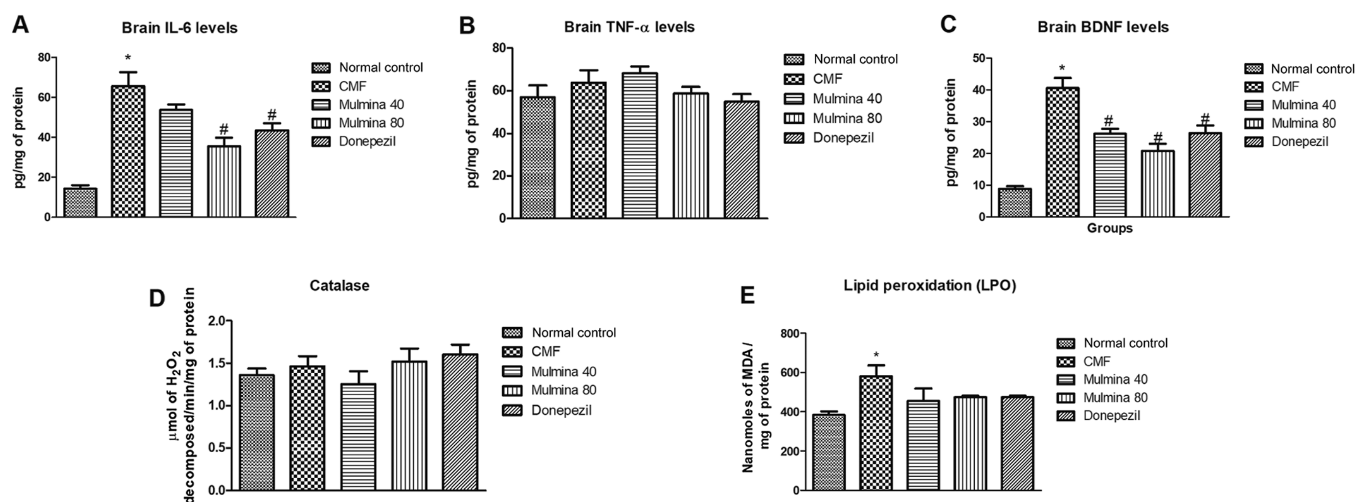

Figure 4. Effect of test drugs (CMF, Mulmina 40, Mulmina 80 and donepezil) on (A) brain IL-6 levels, (B) brain TNF-α levels, (C) brain BDNF levels, (D) brain catalase levels and (E) lipid peroxidation in the brain. The data were analyzed using one-way analysis of variance (ANOVA) employing Bonferroni as the post-hoc test. *P<0.05 compared to the normal control; #P<0.05 compared to the CMF group. Treatment: CMF group received cyclophosphamide + methotrexate + 5-fluorouracil; Mulmina™ 40 group received 40 ml/kg/day of Mulmina™ + CMF and Mulmina™ 80 group received 80 ml/kg/day of Mulmina™ + CMF. IL, interleukin; BDNF, brain derived neurotrophic factor; TNF, tumor necrosis factor.

(P<0.05) (Fig. 4A). Tumor necrosis factor (TNF)-α is a cytokine involved in systemic inflammation and a key mediator in acute phase reaction of inflammation. We did not find any significant difference between the groups with respect to brain TNF-α levels (Fig. 4B).

Effect of test drugs on BDNF in the brain. Brain derived neurotrophic factor (BDNF) is a neuronal growth factor which regulates the survival of existing neurons as well as growth and differentiation of new neurons and synapses. We observed a significant (P<0.05) increase in BDNF in the CMF group (41±3.2) when compared to the normal control (8.9±0.81). This increase was in line with the earlier published report which showed that activation of microglial cells for an extended time triggers a release of BDNF (18). This long-term activation of microglia was seen in the CMF model. All the treatment groups were able to decrease this elevation in BDNF showing a reduction in inflammation indirectly. Low (26±1.6) as well as high (21±2.3) dose of Mulmina™ (26±1.6) and donepezil (26±2.4) were significantly able to reduce the BDNF levels as compared to CMF group (P<0.05) (Fig. 4C).

Effect of test drugs on the level of oxidative stress in the brain. Catalase is an endogenous antioxidant enzyme and its levels can be estimated spectrophotometrically by assessing the rate of degradation of an externally added known quantity of hydrogen peroxide to tissue supernatants. No significant difference was observed in regards to the catalase levels among the groups (Fig. 4D). Lipid peroxidation (LPO) is the process by which free radicals scavenge the electrons from lipids in cell membranes causing a destructive damage to tissue. The CMF (581±55) group showed a significant increase in lipid peroxidation confirming the injury, compared to the normal control (383±19) group. There was a decrease in the levels of LPO among the treatment groups as well, but the differences were not statistically significant (Fig. 4E).

Discussion

In the present study, mice were challenged with cyclophosphamide, methotrexate and 5-fluorouracil (CMF) chemotherapy. The effect of proprietary ayurvedic balya/poshak Mulmina™ Mango against chemotherapy-induced cognitive decline (CICD) was investigated. In the majority of cases, 'chemobrain' presents itself with cognitive dysfunction along with elevated pro-inflammatory cytokines in the brain, increased neuronal cell death and a severe decline in neurogenesis. Three cycles, over a period of 21 days of CMF chemotherapy were able to elicit these effects thus establishing the validity of the model. Two doses of Mulmina™ i.e. 40 and 80 mg/kg/day were chosen for this study which were directly converted from the human dose and administered to mice through an oral route every day in equally divided doses. When put through the Morris water maze, the mice treated with both doses of Mulmina™ showed significant improvement in spatial memory of the animals.

The Morris water maze results can be affected positively as well as negatively if the locomotion of animals is disturbed. To assure that the results obtained from the Morris water maze were dependant on the effect of test drugs only, we accessed the locomotion of animals using the Open Field Test which concluded that all the animals were equal in cognitive abilities before the beginning of the water maze test.

To correlate the behavioral data with biochemical and hematologic parameters, a battery of assays and estimations were performed. In regards to the hematological parameters, the CMF group showed a decline in red blood count (RBC) count which was not increased by any of the treatments. With respect to total and differential white blood cell (WBC) count, our results indicated an increase in total white blood cell count as well as in monocytes, lymphocytes, and granulocytes throughout the test groups, except for the normal control, which is a clear indicator of exacerbated inflammatory reaction in the body. On estimation of some common cytokines involved

in the condition, the levels of IL-6 were highly elevated in the brain in the CMF group and the high dose of Mulmina™ significantly restored the levels of pro-inflammatory cytokines. We also evaluated basic markers of oxidative stress in the brain. Lipid peroxidation was significantly increased in the CMF group. However, Mulmina™ did not restore the brain lipid peroxidation levels induced by CMF treatment.

We employed donepezil as our standard drug in the present study, and it was observed that there were no significant differences between the effects shown by Mulmina™ and donepezil in the study. Mulmina™ contains fruit pulp of *Mangifera indica*, whole plant extract of *Centella asiatica* and, rhizome powder of *Curcuma longa*. Mangiferin, the main constituent of *Mangifera indica*, has been reported to possess antioxidant, anti-inflammatory, immunomodulation and anti-apoptosis activities. Since mangiferin can cross the blood-brain barrier, it can potentially modulate the damage caused by chemotherapy in brain as well as the periphery (19). *Centella asiatica* contains a triterpenoid called asiatic acid. Asiatic acid has demonstrated the ability to reduce oxidative stress and inflammation in rat brain (20). Curcumins in *Curcuma longa* are also known to reduce the inflammation as well as oxidative stress through inhibition of apoptosis, TNF-α, iNOS, RNS, COX-2, and LOX (21).

Although it was confirm in the present study that Mulmina™ has some effect against CMF-mediated CICD, further research is warranted, especially at the molecular level to come to a definitive conclusion with respect to the alterations in proteins in apoptotic/pyroptotic pathways, potency and the possible long-term curative/prophylactic beneficial effects of Mulmina™.

Acknowledgements

The authors would like to acknowledge Manipal Academy of Higher Education for providing the infrastructure required to carry out the work. We also acknowledge the collaboration between Manipal Academy of Higher Education and Griffith University, Gold Coast, Australia, from which the animal model was developed and validated by Dr Devinder Arora, Senior Lecturer, School of Pharmacy and Pharmacology, Griffith University, QLD 4222, Australia.

Funding

The project was supported by Juggat Pharma, Jagdale Industries Pvt. Ltd., Bengaluru.

Availability of data and materials

The datasets used during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

Conception and design of the study was carried out by JM, KN along with RNJ and SMA. Data acquisition was conducted by MK, NR and KG. Data analysis and interpretation was carried out by MK, KG, PGN, JM and KN. Critical intellectual input during the study was accomplished by KN and KVR. Draft preparation was carried out by MK, NR, PGN, JM and KN. All authors read and approved the manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All guidelines dictated by the Institutional Animal Ethics Committee of Manipal Academy of Higher Education (MAHE) (Manipal, Karnataka, India) (approval no. IAEC/KMC/115/2018) and Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) were followed while conducting the animal experiments.

Patient consent for publication

Not applicable.

Competing interests

The authors do declare a competing interest. This research was a collaborative project between the Department of Pharmacology, Manipal College of Pharmaceutical Sciences, Manipal Academy of Higher Education, Manipal and Juggat Pharma, Jagdale Industries Pvt. Ltd., Bengaluru. Jagdale Industries sponsored the study and were involved in the design of the study for the purposes of assessing their marketable product. However, the work was carried out at the Department of Pharmacology, Manipal College of Pharmaceutical Sciences where the study was independently performed in an unbiased manner and data were reported based on the experimental observations.

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