IAA Journal of Biological Sciences 10(1):68-77, 2023. ISSN: 2636-7254

Gallic Acid Reversed NeuN and Mbp Reactivity in Hippocampal CA3 Doxorubicin Challenge: Enhancement of Neuronal Integrity

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ABSTRACT

Doxorubicin is a chemotherapeutic agent that adversely affects the brain, causes deficits in learning, memory, attention, motor activity, and executive function. The impairment of cognition such as learning and memory directly affect the hippocampus. Gallic acid has antioxidant and neuroprotective activities. This study was designed to investigate the protectiveeffect of gallic acid on doxorubicin-induced neuron damage in hippocampal CA3. Thirty-two (32) albino Wistar rats were randomized into four (4) sub-groups of 8 rats each. Group 1 rats received 0.1mL of normal saline intraperitoneal for two weeks. Group 2 rats received 15mg/kg of Doxorubicin weekly intraperitoneally for two weeks. Group 3 rats received 15mg/kg of Doxorubicin weekly intraperitoneally and orally treated with gallic acid at dose of 60mg/kg daily for two weeks. Group 4 rats received 15mg/kg of Doxorubicin weekly and orally treated with gallic acid at dose of 120mg/kg daily for two weeks. Thereafter, tissue antioxidant marker enzymes (SOD,GSH, MDA), H and E, and immunohistochemistry of NeuN and Myelin Basic Protein were carried out to ascertain the effects of Gallic acid on Doxorubicin challenge. DOX altered the antioxidant parameters by decreasing the GSH, SOD and increased MDA. The findings revealed the presence of cytoplasm vacuolations, degenerating neurons and pyknotic nuclei prominent in doxorubicin group upon haematoxylin and eosin staining, positive immunoreactivity with few neurons upon NeuN staining and decreased MBP expression. Gallic acid reversed the effects on the doxorubicin. Gallic acid dose-dependent its neuroprotective effect and would be beneficial in the doxorubicin chemotherapy when administered for longer duration.

Keywords: NeuN, Myelin Basic Protein, Doxorubicin, Gallic acid, Neurotoxicity

INTRODUCTION

Chemotherapy is a widely recognised treatment for patients with cancer [1]. It adversely affects brain structure and functions, these causes loss of neurons, deficits in learning, memory, attention, motor activity, and executive function [2,3]. Seventy percent (70%) of cancer patients receiving chemotherapy develop cognitive impairment at some point during or after treatment [4]. Doxorubicin is a potent chemotherapeutic drug, a broad spectrum anthracyline [1]. It is associated with cognitive impairment and neurotoxicity that affects treatment outcome [4,5]. Doxorubicin has access to the blood-brain-barrier (BBB) and, influences the release of inflammatory factors, neurotransmitters, neurogenesis and survival pathways to initiate neurotoxic events [1]. Doxorubicin damages normal and non-cancerous cells [6]. Doxorubicin acts via DNA cross-linking which results in the disruption of the cell cycle and subsequent death of cancer cells and rapidly dividing healthy non-cancerous cells [7,8,9]. In the central nervous system (CNS), doxorubicin enhances neuronal cell death in the early and late days of administration [10]. The death of neurons impairs cognitive functions such as learning and memory loss in the hippocampus. Due to the therapeutic benefits of doxorubicin in cancer treatment, it has become increasingly essential to find remedies with protective effects against doxorubicin induced adverse effects such as chemobrain. Bioactive plant compounds are found in fruits, vegetables and grains which can that provide health benefits in reducing the risk of diseases [11]. For instance, phenolic compounds are a main class of secondary metabolites in plants with diverse benefits. Gallic acid (GA) is a

polyphenol compound found in many vegetables and fruits such as bananas, strawberries, sumac, green tea, and oak bark [12]. It exhibits antioxidant, anti-inflammatory, anticancer and antiviral activities [12,13,14,15]. Plant derived compounds with antioxidant properties are useful in mitigating the therapeutic adverse effect of drugs including doxorubicin. The hippocampus is associated with learning, memory and emotion. The hippocampus proper is defined by the dentate gyrus and Cornu Ammonis (CA). While the dentate gyrus contains the fascia dentate and the hilus while the CA is anatomically and functionally differentiated into distinct sub-fields named CA1, CA2, CA3, and CA4. The CA3 is the largest in the hippocampus and receives fibers from the dentate granule cells on their proximal dendrites [16].The CA3 plays specific role in memory processes, and linked to cognitive deficit. Seizure susceptibility and neurodegeneration [17].The integrity of neurons in health and disease can be accessed in order to ascertain the functional status at a given period. For example, the NeuN is expressed only by neurons and allows the neuron to be demonstrated in health and disease [18]. The neuronal nuclear antigen (NeuN) reactivity could depict the functional status of neuron and detects matured post-mitotic neurons. Neuronal damage is reported in doxorubicin chemotherapy [4]. There exists a need to develop interventions to combat the neuronal damage accompanying chemobrain condition so as to improve the treatment outcome. Gallic acid and its derivatives have demonstrated broad range of beneficial effects in the prevention and/or management of several disorders including cancers. It has acceptable safety profiles, which make it an option as dietary supplements. Therefore, our concern in this present study is to evaluate the effect of Gallic acid on Doxorubicin-induced neuronal damage in hippocampal CA3 myelination and functional state of neurons.

MATERIALS AND METHODS

Gallic acid was purchased from Zigma, Aldrich USA. Doxorubicin was procured from Sterling Biotech. India. Analytical and Standard graded chemicals were obtained from registered chemical stores in Enugu State, Nigeria. Thirty-two (32) Wistar rats weighing between 160g -190g of age 6-8 weeks were purchased and housed at the animal house of the Enugu State University of College of Medicine, Nigeria. The rats were housed in well aerated laboratory cages with soft wood shavings as bedding. They were allowed two weeks to acclimatize, fed with pelleted animal feed. The animals were maintained under laboratory conditions (temperature 26-28^{o}C with relative humidity of 60-70 percent, and a 12- hours- light-dark cycle). The rats were weighed and randomized into groups. The protocol for the conduct of the study was reviewed and approved by the Faculty Research Ethic Committee (FREC) of Enugu State University of College of Medicine, Nigeria with certificate number.

Experimental Design

The rats were randomized into four (4) sub-groups of 8 rats each. Group 1 rats received 0.1mL of normal saline intraperitoneal for two weeks. Group 2 rats received 15mg/kg of Doxorubicin weekly (I.p)/ 2 dose Group 3 rats received 15mg/kg of Doxorubicin weekly (I.p)/ 2 dose and orally treated with daily doses of gallic acid at dose of 60mg/kg daily for two weeks. Group 4 rats received 15mg/kg of Doxorubicin weekly (I.p)/ 2 dose and orally treated with a daily dose of gallic acid at dose of 120mg /kg for two weeks.

Termination of Experiment

Collection of Tissue for Evaluation of Oxidative Markers

After the blood collection all the rats was sacrificed under light ester anesthesia. The brain was quickly harvested, half of the brain harvested was homogenised for evaluation of oxidative markers: Total brain Superoxide dismutase (SOD), Malondialdehyde (MDA) and glutathione (GSH). The remaining brain tissue were fixed in 10% formal saline for H&E, NeuN and MBP immunohistochemical staining following the standard protocols for each marker.

Determination of Antioxidant Biomarkers

The activities of total SOD, MDA, and glutathione peroxidase in the tissue were determined using commercially available kits from BioVision Research Products

(Linda Vista Avenue, USA) according to the instruction of the manufacturer. Thereafter, data obtained from the oxidative markers were recorded and analysed using the statistical package for Social Science (SPSS) version 25(Chicago, USA). All values were presented as mean ± standard error of the mean. Comparison of multiple groups was analysed by one-way analysis of variance. $p < 0.05$, $p < 0.01$ considered to be significant.

Histological Techniques

The hippocampi were fixed in 10% formal saline 72 hrs and dehydrated through ascending grades of alcohols (50 % , 70 %, 90% and absolute alcohol) for 45 minutes each. The tissues were cleared in three changes of xylene for 45mins each and impregnated in three changed of molten paraffin wax for 30mins each. The impregnated tissues were then embedded in paraffin and to solidify. The embedded paraffin block were trimmed and prepared for sectioning. The tissues were serially sectioned to form ribbons of 5µm thick on the rotary microtome. Sections were routinely stained for H&E following the standard procedure and micrographed.

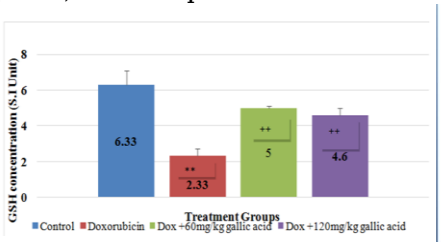
Immunohistochemistry of NeuN and MBP

The Avidin-Biotin Complex (ABC) Immunoperoxidase method to demonstrated NeuN and MBP. The processed tissue was sectioned at 5 microns on the rotary microtome and placed on the hot plate at 70 degrees for at least 1 hour. Sections were brought down to water by passing them on 2 changes of xylene, then 3 changes of descending grades (100%, 90%, 70% & 50%) of alcohol and finally water. Antigen retrieval was performed on the section by heating them on a citric acid solution of pH 6.0 using the microwave at power 100 for 15 minutes. The sections were equilibrated gradually with cool water to displace the hot citric acid for at-least 5 mins for the sections to cool. Peroxidase blocking was carried out on the sections by simply covering sections with 3% hydrogen peroxide ($H_2O_{2)}$ for 15 minutes. Sections were washed with phosphate buffered saline (PBS) and protein blocking was performed using Avidin for 15 minutes. Sections were washed with Phosphate buffered saline (PBS) and endogenous biotin in tissues was blocked using biotin for 15 minutes. After washing with phosphate buffered saline (PBS) sections were incubated with the diluted NeuN or MBP primary antibody (manufactured by Novocastra, LEICA Germany) was diluted 1:100 for 60 minutes. Excess antibodies were washed off with PBS and a secondary antibody (LINK) was applied on section for 15 minutes. Sections were washed and the (LABEL) which is the (Hpp) were applied on the section for 15 minutes. A working DAB solution was constituted by mixing 1 drop (20microns) of the DAB chromogen to 1ml of the DAB substrate. This working solution was applied on sections after washing off the HPP with PBS for at least 5 minutes. The brown reactions begin to appear at this moment especially for a positive target. Excess DAB solution and precipitate were washed off with water. Sections were counter-stained with haematoxylin solutions for at least 2 minutes. Sections were dehydrated in alcohol, cleared in xylene and mounted in DPX. The slides were interpreted and photographed.

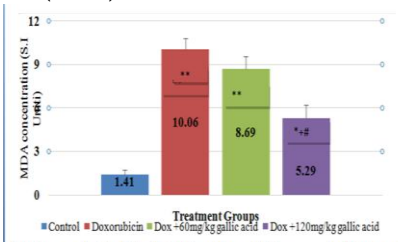
RESULTS

Assessment of Antioxidant Markers

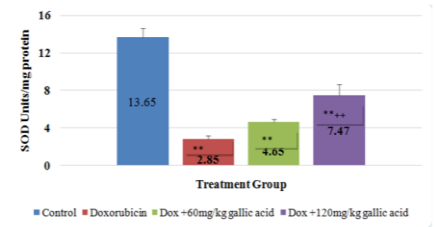
The effect of Gallic acid on the tissue levels of glutathione (GSH), Malondialdehyde (MDA) and superoxide dismutase (SOD) of wistar rats treated with Doxorubicin is shown below.



Showing result of effect of Gallic acid on Glutathione conc. in Wistar rat hippocampal tissue, following Doxorubicin treatment
** Represents comparison with group 1(**p<0.01 and * p<0.05)*
+Represents comparison with group 2 (++p<0.01 and + p<0.05)



Showing result of effect of Gallic acid on MDA conc. in Wistar rat hippocampal tissue, following Doxorubicin treatment
** Represents comparison with group 1(**p<0.01 and * p<0.05)*
+Represents comparison with group 2 (++p<0.01 and + p<0.05)
 # Represents comparison with group 3(##p<0.01 and # p<0.05)



Showing result of effect of Gallic acid on SOD conc. in Wistar rat hippocampal tissue, following Doxorubicin treatment
** Represents comparison with group 1(**p<0.01 and * p<0.05)*
+Represents comparison with group 2 (++p<0.01 and + p<0.05)

Histological Assessment

The hippocampal section of the normal saline revealed normal neurons in the granular (Black arrow) and pyramidal layer (white arrow). Doxorubicin revealed cytoplasmic vacuolations (Black arrow) with and degenerating neurons that had pyknotic nuclei prominent in the granular layer (white arrow). The low dose of gallic acid treated group showing less neurons with cytoplasmic vacuolations (Black arrow) and few regenerating neurons with centric nuclei (white arrow) prominent in the granular layer. The high dose of gallic acid treated group showed few neurons with cytoplasmic vacuolations (Black arrow) and few regenerating neurons with centric nuclei (white arrow) prominent in the granular layer. (Figure 2:A to D). H & E, X400. In the immunohistochemistry of NeuN to assess neuron integrity all the hippocampal sections depict positive immunoreactivity normal expression in the normal saline. Only few neuron expressed NeuN in the doxorubicintreated while the expression of NeuN increased in the low dose and high gallic acid (Figure 2:E to H). NeuN, x400. Myelin Basic Protein (MBP) expression of hippocampal sections also revealed positive immunoreactivity in all the groups. Hippocampal section of doxorubicin group showed positive prominent myelin expression. The low dose and high dose gallic acid treated relatively normal myelin expression compared to the normal saline and doxorubicin. (Figure 2 to L)Mbp, x400.

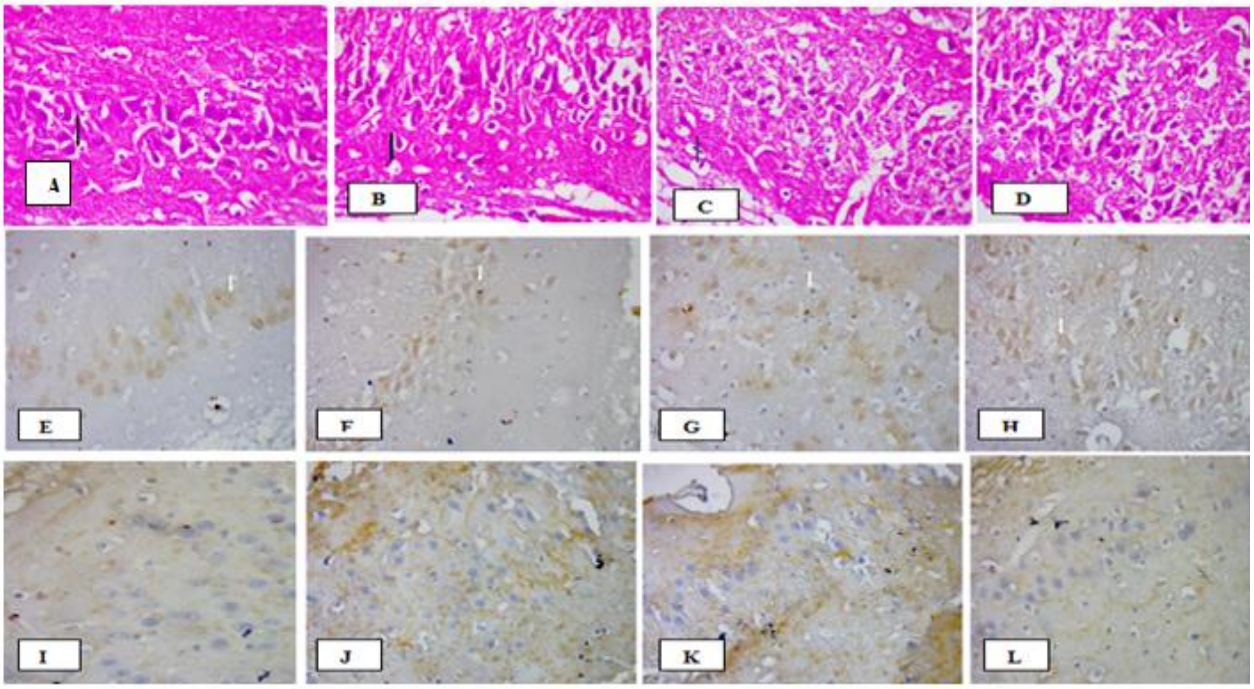


Figure 2: Hippocampal section (A) Normal saline (B) Doxorubicin only (C) DOX +Low dose Gallic acid (D) DOX + high dose of gallic acid. H & E. x400. (E)Normal saline (F) Doxorubicin only (G) DOX +Low dose Gallic acid (H) DOX + high dose of gallic acid NeuN, x400. (I) Normal saline (J) Doxorubicin only (K) DOX +Low dose Gallic acid (L) DOX + high dose of gallic acid.MBP. X400.

DISCUSSION

Oxidative stress is a key factor in the etiology of various diseases including the central nervous system, it is associated with haematological and behavioural changes such as in depression and anxiety [19]. Superoxide dismutase (SOD) and glutathione peroxidase (GSH) are some of the constituents of this defense system [20] DOX altered oxidative parameters by decreasing the GSH, and SOD while increasing MDA in this study. This result is aligning with previous studies [21, 22, 23]. Oxidative stress occurs when the production of reactive oxygen species exceeds the antioxidant defense capacity [24]. During oxidative stress, the productionthe body's antioxidant is depleted, such as vitamins C and E, and

glutathione [25]. Consequently, elicits oxidation of proteins, membrane lipids, DNA damage, and cell apoptosis [26] and causes overproduction of MDA [27].

MDA is a small but stable product of lipid peroxidation that is produced by degradation of the unstable peroxides of unsaturated fatty acids. Excessive lipid peroxidation in nerve cells causes cell death [28]. In order to fight free radicals, antioxidant defense mechanisms prevent the adverse effects of oxidant. Diminished GSH levels elevate cellular vulnerability toward oxidative stress which is characterized by heighten levels of reactive oxygen species that contribute to oxidative stress mechanism [29]. In the CNS, GSH deficiency plays a key role in the pathogenesis of many CNS diseases including Alzheimer's with cognitive impairment [30]. but, gallic acid reversed the above effect by exhibiting antioxidant property,it increased SOD, GSH and decreased MDA.This signifies both reversing oxidation and lipid peroxidation in the treated groups. Its antioxidant effect was better appreciated in the high dosage thus agreeing with previous studies [31, 32, 33]. Doxorubicin adversely affects brain structure and functions, like loss of neurons, deficits in learning, memory, attention, motor activity, and executive function [2,3]. Our findings revealed the presence of cytoplasm vacuolations and pyknotic nuclei in doxorubicin. These are features of cellular adaptive changes in cells response to a harmful agent. Cytoplasmic vacuolation could occur in cells as a result of exposure to pathogens resulting in cell death [34]. Pyknosisis the irreversible condensation of chromatin in the nucleus of a cell undergoing necrosis or apoptosis and fragmentation of the nucleus as in degenerating neurons [35, 36]. Such neuronal degeneration might ultimately involve cell death. It is evident that oxidative stress is one of major contributing factors to neurodegeneration besides inflammation [37, 38, 39]. This also collaborate the induction of oxidative stress by Doxorubicin in the alteration of the marker enzymes. It was found that doxorubicin damaged mitochondrial function in the hippocampus, resulting in elevated mitochondrial ROS levels and calcium disorder [10, 40, 41]. Oxidative stress and neuron degeneration caused by abnormal mitochondria are one of the important causes of cognitive impairment [10]. Gallic acid was able to reverse the doxorubicin-induced neurotoxicity, through the reversal of oxidative stress and probably anti inflammatory activity in the brain. This effect of gallic acid confirm finding of other previous works [42, 43, 44].

NeuN is a neuronal nuclear antigen that is commonly used as a biomarker for neurons. The NeuN protein is associated with neuronal nuclei and the perinuclear cytoplasm and it is exclusive to the nervous tissue [45, 46]. The antibody for NeuN was implored in this study as an indicator to assess the functional state of neurons following administration of Dox and gallic acid. It is suitably applied in morphological diagnosis of cancer and histopathological research in the detection of post mitotic neurons that demonstrate the presence of the neuronal nuclei protein (NeuN) [47, 48]. NeuN also identifies areas of low chromatin density and absence dense packing of DNA [49]. This signifies NeuN functions at the level of the cell nucleus. In this study, positive immunoreactivity with few neurons was observed implying there was loss of neurons in the hippocampus that are actively dividing. It is established that injured neurons have faulty expression of NeuN protein in the cell, for example axonal injury leads to an almost complete loss of NeuN expression. On the contrary, a negative result of the reaction to NeuN might by inferred as: absence of neuron protein expression in a cell, decrease protein synthesis leaving small amount undetectable [18] or Post translational modifications such as protein to protein interaction by phosphorylation [49]. This study affirm the positive reaction of NeuN in the rat brain and the reliability of the application [50]. NeuN immunoreactivity can be affected by prolonging fixation in formalin which reduces the reactivity. In this study, the hippocampus was fixed in 10% neural formal saline only for 72 hrs prior to processing, hence chemical unmasking of the antigen, standard and specific protocol were used with paraffin wax sections. NeuN is also associated with the identification of pathological changed in existing neuronal population [18]. NeuN completely disappearance from damaged or dying pyramidal neurons of the hippocampus [45,46,51,52]. The loss of Neun immunoreactivity in this study was also linked to neuronal death and

temporarily suspension of synthesis of the protein due to neuronal damage by doxorubicin and aligns with other reports [53,54,55]. Gallic acid co-administered with Doxorubicin confers protective effect against the doxorubicin effect. The study also assessed the potential of gallic acid in myelination of neurons of the hippocampal CA3 using the Myelin Basic Protein (MBP). The MBP is a protein believed to be important in the process of myelination of nerves in the nervous system. The MBP maintains the proper structure of myelin interacting with the lipids in the myelin membrane [56]. Research has shown that MBP binds negatively charged lipids on the cytosolic surface of oligodendrocytes membranes and it is responsible for adhesion of these surfaces in the multi-layered myelin sheath [57,58,59].We observed dose-dependent changes in the immunoreactivity and expression levels of MBP. There was increased MBP in the doxorubicin which can be attributed tothe disruption of the Blood brain barrier (BBB) permeability and tight junction expression. The MBP expression decreased in the low dose and high dose gallic acids treatment simultaneously, this further confirmed the potency of Gallic acid in reversing the effect doxorubicin has on the hippocampus. The anti-oxidative, anti-inflammatory and anticancer activities of gallic acid are implicated. It has been suggested that it serves a role as a prognostic indicator of disease progression [59]. This current study shows that Gallic acid co-administered with doxorubicin, in high dosage exhibited ameliorative effect on the hippocampal CA3. In addition, it exhibited dose-dependent effect attributed to the photochemical load contained in each dose.

CONCLUSION

In the present study treatment with gallic acid reversed the effect of oxidative stress via antioxidant effect and the higher dose was more effective. Gallic acid is a promising supplement in chemotherapeutic treatment while using doxorubicin in order to enhance neuronal preservation and treatment outcome.

Conflicts of Interest

The Author declared that research was conducted in the absence of any commercial or financial relationship that could be construed as a potential conflict of interest

Acknowledgement

We thank the staff of the histology laboratory for providing the technical assistance.

Author Contributions

FBE: Conception and design.OYP:Execution of research, OSE and MOE: wrote the draft; assembled and analyzed performed the histology;VOA and AHE: gave conceptual advice, contributed to the discussion, reviewed, and edited the draft. FBE. is the corresponding author. All authors read and approved the manuscript.

REFERENCES

1. Du, J., Zhang, A., Li, J., Liu, X., Wu, S., Wang, B., Wang, Y. & Jia H. (2021) Doxorubicin-Induced Cognitive Impairment: The Mechanistic Insights. Frontier in oncology. 11, 673340
2. Yamada, T. H., Denburg, N. L., Beglinger, L. J., Schultz, S. K. (2010). Neuropsychological Outcomes of Older Breast Cancer Survivors: Cognitive Features Ten or More Years after Chemotherapy. J Neuropsychiatry Clin Neurosci. 22(1):48– 54.
3. Wang, L., Apple, A. C., Schroeder, M. P., Ryals, A. J., Voss, J. L., Gitelman, D., et al. (2016). Reduced Prefrontal Activation During Working and Long-Term Memory Tasks and Impaired Patient-Reported Cognition Among Cancer Survivors Postchemotherapy Compared With Healthy Controls. Cancer 122 (2):258–68.
4. Xu, Z., Luo, F., Wang, Y., Zou, B.S., Man, Y., Liu, J. S., et al. (2020). Cognitive Impairments in Breast Cancer Survivors Treated With Chemotherapy: A Study Based on Event-Related Potentials. Cancer Chemother Pharmacol. 85(1):61–7.
5. Zandbergen, N., de Rooij, B. H., Vos, M. C., Pijnenborg, J. M. A., Boll, D., Kruitwagen, R. F. P. M., van de Poll-Franse, L. V., Ezendam, N. P. M. (2019). Changes in health-related quality of life among gynecologic

cancer survivors during the two years after initial treatment: a longitudinal analysis. Acta Oncol. 58(5):790-800.

6. Dietrich, J., Han, R., Yang, Y., Mayer-Proschel, M., & Noble, M. (2006). CNS progenitor cells and oligodendrocytes are targets of chemotherapeutic agents in vitro and in vivo. Journal of Biological. 5, 22.
7. Pei, Y., Liu, Y., Xie, C., Zhang, X., You, H. (2020). Detecting the Formation Kinetics of Doxorubicin-DNA Interstrand Cross-link At the Single-Molecule Level and Clinically Relevant Concentrations of Doxorubicin. Anal Chem. 92 (6):4504–11.
8. Manchon, J. F., Dabaghian, Y., Uzor, N. E., Kesler, S. R., Wefel, J. S., Tsvetkov, A. S. (2016). Levetiracetam Mitigates Doxorubicin-Induced DNA and Synaptic Damage in Neurons. Sci Rep. 6:25705
9. Dasari, S., & Tchounwou, P. B. (2014). Cisplatin in Cancer Therapy: Molecular Mechanisms of Action. Eur J Pharmacol. 740:364–78.
10. Ramalingayya, G. V., Cheruku, S. P., Nayak, P. G., Kishore, A., Shenoy, R., Rao, C. M., et al. (2017). Rutin Protects Against Neuronal Damage In Vitro and Ameliorates Doxorubicin-Induced Memory Deficits In Vivo in Wistar Rats. Drug Des Devel Ther 11:1011–26.
11. Rui, H. L. (2004). Potential Synergy of Phytochemicals in Cancer Prevention: Mechanism of Action, The Journal of Nutrition, 134(12): 3479S–3485S
12. Zhu, L., Gu, P. Q., Shen, H. (2019) Gallic acid improved inflammation via NF-κB pathway in TNBS-induced ulcerative colitis. Int Immunopharmacol. 67:129–137.
13. Dehghani, M. A., Shakiba, M. N., Moghimipour, E., Khorsandi, L., Atefikhah. M., Mahdavinia, M. (2020) Protective effect of gallic acid and gallic acid-loaded Eudragit-RS 100 nanoparticles on cisplatin-induced mitochondrial dysfunction and inflammation in rat kidney. Biochimica et Biophysica Acta – Molecular Basis of Disease. 1866:165911
14. Shruthi, S., Shenoy, K. B. (2018) Genoprotective effects of gallic acid against cisplatin induced genotoxicity in bone marrow cells of mice. Academic Oup Com. 7:951–958.
15. Kim, S. H., Jun, C. D., Suk, K., Choi, B. J., Lim, H., Park, S., et al. (2006) Gallic acid inhibits histamine release and proinflammatory cytokine production in mast cells. Toxicol Sci.91:123–131
16. Daugherty, A. M., Bender, A.R., Raz, N., Ofen, N. (2016). Age differences in hippocampal subfield volumes from childhood to late adulthood. Hippocampus. 26(2):220-8.
17. Cherubini, E., & Miles, R. (2015). The CA3 region of the hippocampus: how is it? What is it for? How does it do it? Front Cell Neurosci. 9:19
18. Gusel'nikova, V. V., & Korzhevskiy, D. E. (2015). NeuN As a Neuronal Nuclear Antigen and Neuron Differentiation Marker. Acta naturae.7(2), 42–47
19. Fedoce, A. D. G., Ferreira, F., Bota, R. G., Bonet-Costa, V., Sun, P. Y., & Davies, K. J. A. (2018). The role of oxidative stress in anxiety disorder: cause or consequence? Free radical research. 52(7), 737–750.
20. Ighodaro, O. M., & Akinloye, O. A. (2018) First line defence antioxidants-superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX): Their fundamental role in the entire antioxidant defence grid, Alexandria Journal of Medicine, 54:4, 287-293
21. Kitamura, Y., Ushio, S., Sumiyoshi, Y., Wada, Y., Miyazaki, I., Asanuma, M., Sendo, T. (2021). N-Acetylcysteine Attenuates the Anxiety-Like Behavior and Spatial Cognition Impairment Induced by Doxorubicin and Cyclophosphamide Combination Treatment in Rats. Pharmacology. 106(5-6):286-293.
22. Wenjiao, L., Mingzi, O., Xiaomeng, M., Tiantian, H., Dajin, P., Shijun, Q. (2021).Kai-Xin-San Attenuates Doxorubicin-Induced Cognitive Impairment by Reducing Inflammation, Oxidative Stress, and Neural Degeneration in 4T1 Breast Cancer Mice, Evidence-Based

Complementary and Alternative Medicine.
23. Mohit, K., Vikas, K., Ashok, J., Murli, M., Sahabuddin, A., Pinaki, G., Divya, V., Razia, K. (2016) Ameliorative effect of naringin against doxorubicin-induced acute cardiac toxicity in rats, Pharmaceutical Biology. 54:4, 637-647
24. Jorge L., & MarÃ-a, E. G. (2009).The role of antioxidants and antioxidant-related enzymes in protective responses to environmentally induced oxidative stress. Mutation Research/Genetic Toxicology and Environmental Mutagenesis. 674,137-147
25. Pandya, C. D., Howell, K. R., & Pillai, A. (2013). Antioxidants as potential therapeutics for neuropsychiatric disorders. Progress in neuro-psychopharmacology & biological psychiatry, 46, 214–223
26. Lundgren, C. A. K., Sjöstrand, D., Biner O. et al. (2018). "Scavenging of superoxide by a membrane-bound superoxide oxidase," Nature Chemical Biology. 14(8):788–793
27. Gaweł, S., Wardas, M., Niedworok, E., Wardas, P. (2004). Malondialdehyde (MDA) as a lipid peroxidation marker. Wiad Lek. 57(9-10):453-5.
28. Michael, M. G., & Brent, R. S. (2017). Lipid peroxidation in cell death, Biochemical and Biophysical Research Communications. 482(3):419-425
29. Gawryluk, J. W., Wang, J. F., Andreazza, A. C., Shao, L., Young, L. T. (2011). Decreased levels of glutathione, the major brain antioxidant, in post-mortem prefrontal cortex from patients with psychiatric disorders. Int J Neuropsychopharmacol. 14(1):123-30.
30. Guoyao, W. U., Yun, Z. F., Sheng, Y., Joanne, R. and Turner N. D. (2004). Glutathione metabolism and its implications for health. The American Journal of Nutritional Sciences; 134:489-492.
31. Mohammad, T. M., Yaghoub, F., Maryam, J. S., Alireza, S., Bahareh, N., Maryam, R., (2013). Neuroprotective effects of oral gallic acid against oxidative stress induced by 6-hydroxydopamine in rats. Food Chemistry. 138:1028-1033
32. Mehrdad, S. K., Yaghoub, F., Alireza, S., Hadi, F. M., Mohammad, T. M. (2014). Protective effects of gallic acid against chronic cerebral hypoperfusion-induced cognitive deficit and brain oxidative damage in rats, European Journal of Pharmacology. 733:62-67
33. Salehi, A., Rabiei, Z., & Setorki, M. (2018). Effect of gallic acid on chronic restraint stress-induced anxiety and memory loss in male BALB/c mice. Iranian journal of basic medical sciences. 21(12):1232–1237.
34. Andrey, V. S., Ilya, V. D., Alexey, A. K., Lola, M. R., Sergey, V. K. (2016). Cytoplasmic vacuolization in cell death and survival. *Oncotarget*. 7(34): 55863-55889
35. Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E. S., Baehrecke, E. H., et al. (2009). "Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009". Cell Death and Differentiation. 16 (1): 3–11
36. Kumar, V., Abbas, A., Nelson, F., Mitchell, R. (2007). Robbins Basic Pathology (8th ed.). 6, 9–10.
37. Pereira, T. M. C., Côco, L. Z., Ton, A. M. M., Meyrelles, S. S., Campos-Toimil, M., Campagnaro, B. P., Vasquez, E. C. (2021). "The Emerging Scenario of the Gut-Brain Axis: The Therapeutic Actions of the New Actor Kefir against Neurodegenerative Diseases". Antioxidants. 10 (11): 1845
38. Singh, A., Kukreti, R., Saso, L., Kukreti, S. (2019). "Oxidative Stress: A Key Modulator in Neurodegenerative Diseases". Molecules. 24 (8): 1583.
39. Stephenson, J., Nutma, E., van der Valk, P., Amor, S. (2018). "Inflammation in CNS neurodegenerative diseases". Immunology. 154 (2): 204–219.
40. Shokoohinia, Y., Hosseinzadeh, L., Moieni-Arya, M., Mostafaie, A., Mohammadi-Motlagh, H. R. (2014) .Osthole Attenuates Doxorubicin-Induced Apoptosis in PC12 Cells Through Inhibition of Mitochondrial

Dysfunction and ROS Production. BioMed Res Int. 156848.

41. Park, H. S., Kim, C. J., Kwak, H. B., No, M. H., Heo, J. W., Kim, T. W. (2018). Physical Exercise Prevents Cognitive Impairment by Enhancing Hippocampal Neuroplasticity and Mitochondrial Function in Doxorubicin-Induced Chemobrain. Neuropharmacology. 133:451–61.
42. Nagpal, K., Singh, S. K., Mishra, D. N. (2012b). Nanoparticle mediated brain targeted delivery of gallic acid: in vivo behavioral and biochemical studies for improved antioxidant and antidepressant-like activity. Drug Deliv 19:378–91
43. Zhongbing, L., Guangjun, N., Belton, P. S., et al. (2006). Structure–activity relationship analysis of anti-oxidant ability and neuroprotective effect of gallic acid derivatives. Neurochem Int. 48:263–74
44. Krogh, R., Yunes, R. A., Andricopulo, A. D. (2000). Structure–activity relationships for the analgesic activity of gallic acid derivatives. Farmaco 55:730–5
45. Kirik, O.V., Sukhorukova, E.G., Vlasov, T.D., Korzhevskii, D.E. (2009). Selective death of the striatum neurons in rats after the transient occlusion of the middle cerebral artery. Morfologiia. 135(2):80–82.
46. Korzhevskii, D.E., Kirik, O.V., Baisa, A.E., Vlasov, T.D. (2009). Simulation of Unilateral Ischemic injury to the Striated Neurons Inflicted by Short-Term Occlusion of the Middle Cerebral Artery. Bull Exp Biol Med. 147(2):255–256.
47. Petrova, E. S., Isaeva, E. N., Korzhevskii, D. E. (2014). Effect of allotransplants containing dissociated cells of rat embryonic spinal cord on nerve fiber regeneration in a recipient. Bull Exp Biol Med.158(1):123-6
48. Chowdhury, T. G., Jimenez, J. C., Bomar, J. M., Cruz-Martin, A., Cantle, J. P., Portera-Cailliau, C. (2010) Fate of cajal-retzius neurons in the postnatal mouse neocortex. Front Neuroanat 4:10.
49. Lind, D., Franken, S., Kappler, J., Jankowski, J., Schilling, K. (2005). Characterization of the neuronal marker NeuN as a multiply phosphorylated antigen with discrete subcellular localization. J Neurosci Res. 79(3):295-302.
50. Darlington, P. J., Goldman, J. S., Cui, Q. L., Antel, J. P., Kennedy, T. E. (2008). Widespread immunoreactivity for neuronal nuclei in cultured human and rodent astrocytes. J Neurochem 104:1201–1209.
51. Tippett, L.J., Waldvogel, H.J., Thomas, S.J., Hogg, V.M., van Roon-Mom, W., Synek ,B.J., Graybiel, A.M., Faull, R.L. (2007). Brain. Striosomes and mood dysfunction in Huntington's disease. 130(1):206–221.
52. Korzhevskii, D. E., Khozhai, L.I., Gilerovich, E. G., Grigoriev, I. P., Gilyarov, A. V., Otellin, V.A. (2006). In the conference abstract-book ≪Structural, functional and neurochemical regularities of the brain asymmetry and plasticity-2006≫. Proceedings of the All-Russian conference with international participation, Moscow. 139–142.
53. Igarashi, T., Huang, T.T., Noble, L.J. (2001). Regional vulnerability after traumatic brain injury: gender differences in mice that overexpress human copper, zinc superoxide dismutase. Exp Neurol. 172(2):332–341.
54. Davoli, M.A., Fourtounis J., Tam J., Xanthoudakis S., Nicholson D., Robertson G.S., Ng G.Y., Xu D. (2002). Immunohistochemical and biochemical assessment of caspase-3-activation and DNA fragmentation following transient focal ischemia in rat. Neuroscience. 115:125–136.
55. Unal-Cevik, I., Kilinc, M., Gursoy-Ozdemir, Y., Gurer, G., Dalkara, T. (2004). Loss of NeuN immunoreactivity after cerebral ischemia does not indicate neuronal cell loss: a cautionary note. Brain Res. 1015(1-2):169–174.
56. Sakamoto, Y., Kitamura, K., Yoshimura, K., Nishijima, T., Uyemura, K .(March 1987). "Complete amino acid sequence of PO protein in bovine peripheral nerve myelin". The Journal of Biological Chemistry. 262 (9): 4208–4214.

57. Harauz, G.,& Musse, A.A.(2007). Structural and functional aspects of myelin basic protein determination in health and disease. Neurochemical research 32 (2):137-158.
58. Tikka, S., Monogioudi, E., Gotsopoulos, A. *et al.* (2016). Proteomic profiling in the brain of CLN1 Disease Model reveals affected functional modules. Neuromol Med. 18: 109-133
59. Berger, T., Rubner, P., Schautzer, F., Egg, R., Ulmer, H., Mayringer, I. (2003). Antimyelin antibodies as a predictor of clinically definite multiple sclerosis after a first demyelinating event. The New England Journal of Medicine. 349(2): 139–145.