



Original article

A new cerebral ischemic injury model in rats, preventive effect of gallic acid and *in silico* approaches

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ARTICLE INFO

Article history:

Received 2 April 2021

Revised 17 May 2021

Accepted 18 May 2021

Available online 24 May 2021

Keywords:

GA (Gallic acid)

Multiple occlusion-reperfusion of bilateral common carotid arteries (MO/RCA)

In silico

Antioxidants

Inflammatory mediators

ABSTRACT

Current study was designed multiple occlusions and reperfusion of bilateral carotid arteries induced cerebral injury model and evaluated the protective effect of gallic acid on it. *In silico* study was involved to study gallic acid binding affinity on cerebrotonic proteins compared with standard drugs using *Autodoc vina tool*. Cerebral ischemia was induced by occlusion of bilateral common carotid arteries for 10 mins followed by 10 reperfusions (1 cycle), cycle was continued to 3 cycles (MO/RCA), then pathological changes were observed by estimation of brain antioxidants as superoxide dismutase, glutathione, catalase, oxidants like malonaldehyde, cerebral infarction area, histopathology, and study gallic acid treatment against cerebral injury. Gallic acid exhibited a strong binding affinity on targeted cerebrotoxic proteins. MO/RCA rat brain antioxidant levels were significantly decreased and increased MDA levels ($p < 0.0001$), Infarction size compared to sham rats. Gallic acid treatment rat brain MDA levels significantly decreased ($p < 0.4476$) and increased SOD ($p < 0.0001$), CAT ($p < 0.0001$), GSH ($p < 0.0001$), cerebral infarction area when compared to MO/RCA group. Developed model showed significant cerebral ischemic injury in rats, injury was ameliorated by Gallic acid treatment and *in silico* approaches also inhibit the cerebrotoxic protein function by targeting on active sites.

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1. Introduction

Throughout the world, Currently Ischaemic stroke (IS) is a cerebrovascular disease with high range of severity in morbidity and mortality (Feigin et al., 2016). The occurrence of this disease is of 2 varieties i.e. focal & global. When the amount of blood flow is less or decrease in flow of blood to specific regions of brain-embolic middle cerebral artery occlusion (MCAO) is known as Focal ischemia. global ischemia happens when cerebral blood flow (CBF) is reduced throughout the parts of brain which in turn leads to car-

diac arrest (Smith, 2004; Traystman, 2003). The tissue damage occurs when there is a back flow of blood to the tissues, Cerebral ischemia-reperfusion injury (CIRI) which is very common in ischemic stroke. CIRI persuades oxidative stress which in turn triggers neuronal loss and cognitive impairment, circulation regeneration results in inflammation, and harmful oxidation effects (Ritzel et al., 2015), hence inflammatory agents play a key role in damage of ischemic brain tissue (Liu et al., 2018) along with antioxidant enzymes to modulate and provoke neuronal cell defence against toxic reactive oxygen species (ROS) (Ding et al., 2015; Farrell-Dillon et al., 2017) with this justification antioxidants have been advised *in vitro* and *in vivo* for a desirable pursuit for CIRI therapy, as the crucial role was played by oxidative stress. Globally 11% of total deaths are happened due to stroke when compared to all life-threatening diseases, especially in India the main cause for death is stroke according to the epidemiological statistics (Banerjee and Das, 2016; Kamalakannan et al., 2017) and cerebrovascular diseases are considered the second most common cause of expected deaths in 2020 (Huang and McNamara, 2004).

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Peer review under responsibility of King Saud University.



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In the current scenario we must pay attention to the management of ischemic brain damage with relevant research findings (O'Rourke, 2004). Among the phytoactives, Gallic acid (GA), 3,4,5-trihydroxybenzoic acid, was more focussed by researchers, with its unique and potential constituents present in various parts of plants and different fruits like grapes (Cedó et al., 2014; Scoccia et al., 2016; Yang et al., 2020), gallnuts (Liu et al., 2019; Wang and Li, 2017), pomegranates (Singh et al., 2014; Zhang et al., 2018), and tea leaves (Jiang et al., 2019). To be specific, GA and derivatives are used as flavouring agents and preservatives in food industry (Alfei et al., 2019; Fereidoonfar et al., 2019) also it has many pharmaceutical applications as antioxidants (Phonsatta et al., 2017; Wang et al., 2019), antimicrobial (Wang et al., 2019) (Rosman et al., 2018), anticancer (T. Zhang et al., 2019), anti-inflammatory, gastroprotective (Pandurangan et al., 2015), cardio-protective (El-Hussainy et al., 2016), neuroprotective (Maya et al., 2018b), in addition to the usage in prevention of metabolic diseases (Bak et al., 2013; Huang et al., 2016). The current study is more focussed antioxidant/oxidant status and infarction size in MO/RCA and evaluate the protective effect of Gallic acid on injury in rats.

2. Materials and methods

2.1. Drugs and chemicals

Gallic acid, 2,3,5-Triphenyl tetrazolium chloride (TTC), 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), NADH, Phenazine methosulphate, were purchased from Sisco research laboratories Pvt. Ltd. Sodium lauryl sulphate, Glacial acetic acid, dipotassium hydrogen phosphate, Tris buffer were purchased from Fisher scientific. Trichloro acetic acid, Sodium pyro phosphate, H₂O₂ were purchased from S D Fine-Chem Limited. N-butanol, Pottassium dihydrogen phosphate were purchased from Merck Life Science Pvt Ltd. All other chemicals were of the highest purity commercially available.

2.2. Docking studies

2.2.1. Experimental procedure of binding energy between Gallic acid and neurotoxic proteins by using PyRx tool

Tumor necrosis factor α (Dong et al., 2017), Caspases 3 and Bax (Chaitanya and Babu, 2008), Nitric oxide synthase (Chen et al., 2017), Interleukin 6 (Aref et al., 2020), Glutamate receptors (Sun et al., 2019) and Acid sensing ion channel (Dibas et al., 2018) activation involved in disease progression. The virtual screening was performed by using PyRx virtual screening tool between Gallic acid and above cerebrotoxic proteins which are involved in cerebral stroke they are like Bax (PDB ID: 5 W60), Tumor necrosis factor α (PDB ID : 5UUI), Caspases 3 (PDB ID : 2DKO), Interleukin (PDB ID : 1ALU), Nitric oxide synthase (PDB ID : 6CIC) compared to standard drugs such as Minocycline (Pub Chem Id : 54675783), Quercetin (Pub Chem Id:5280343) with Acid sensing ion channel (PDB ID : 3S3X); Memantine (Pub Chem Id: 4054) with Glutamate receptor (Pub Chem Id:5ZG2). Minocycline (Naderi et al., 2020), Quercetin (Wang et al., 2020), Memantine (Tanaka et al., 2018) reported significant neuroprotective effect on targeting reported cerebrotoxic proteins. Hence current study was using these compounds as standard. Discovery studio2017 R2 tool for visualizing protein and drug interaction and Autodoc vina tool used for binding affinity toward neurotoxicity proteins. Here we downloaded proteins 3D structure from the PDB database using PDB codes. Then deleted ligand groups, water molecules, and heteroatom and applied force field in protein molecule using BIOVIA Discovery studio. The protein was later saved for virtual screening. Minimized protein and

ligand converted to PDBQT, selected maximized GRID parameter then performed docking study (Trott and Olson, 2009). Highest negative binding energy indicates good stability at binding site. The model with highest negative binding energy compound and protein complex was selected and visualized protein and ligand type of interaction and distance using BIOVIA Discovery studio2017 R2 tool.

2.2.2. Prediction of protein active site

Active sites of Caspases 3, identified as HIS A: 121, ARG B: 207 CYS A: 163, SER A: 58, TYR B: 204. Active sites of Interleukin 6, identified as GLN A: 156, ARG A: 40, LYS A: 171 GLN: 175. Active sites of Nitric oxide synthase, identified as PHE B: 709, ALA B: 417, ARG B: 419, ALA A: 417, CYS A: 420. Active sites of Glutamate receptor, identified as THR A: 501, PRO A: 499, GLU A: 726, TYR A: 471, PRO A: 515, ARG B: 506. All active sites were predicted by using *Bio via Drug discovery studio visualizer* 2017. After loading the protein structure to DSV, it reads the protein and highlights the probable active site, i.e., residue information in yellow colour (Design LI, 2014).

2.3. Animals

Adult wistar rats (220–250 g) either sex was procured from Sainath agency Pvt. Ltd., Hyderabad, Telangana, India and maintained under a 12/12-h light/dark cycle, in an ambient room temperature (24 ± 1 °C). Animals were provided with an adequate supply of food and water. Study protocol approved by IAEC/CESCOP/2019-OCT-02.

2.4. Experimental protocol for cerebral ischemia

Rats were divided into five groups; each group contain six rats. Group I Normal rats
Group II Sham rats
Group III Disease rats (MO/RCA)
Group IV Pre-treatment of GA (25 mg/kg B.Wt. i.p) for 10 days followed by MO/RCA surgery.
Group V Pre-treatment of GA (50 mg/kg B.Wt. i.p) for 10 days followed by MO/RCA surgery.

2.5. Induction of cerebral ischemic rats by MO/RCA model

Ketamine (60 mg/kg.), xylazine (10 mg/kg) was used to anesthetize rats. Both common carotid arteries left, and right were carefully separated and maintained from all muscles, ligaments, their adventitial sheath, and vagus nerve. Both common carotid arteries occluded 10 mins, 10 reperfusion (1 cycle), cycle was continued to 3 cycles (MO/RCA). By using waxed silk suture, the skin was closed. Dilated pupils, absence of cornea reflex on light exposure and rectal temperature maintenance at 37 ± 0.5 °C observed after completion of 3rd cycles. Hypothermia development been prevented using a heating lamp on the surgical table.

After completion of 3 cycles, rats were euthanized, isolated the brain, washed in cooled 0.9% saline, then estimated cerebral infarction size using TTC staining method (Bederson et al., 1986). Brain was homogenized as 10% (w/v) in cold phosphate buffer (0.05 M, pH 7.4). The homogenates were centrifuged at 1000 × g for 10 min at 4°C. Supernatant were subjected for estimation of glutathione (GSH) (Ellman, 1959) superoxidase dismutase (SOD) (Kono, 1978) and malondialdehyde (MDA) (M Stefan and Gudrun, 1974) and Catalase (A Claibome, 1985).

2.6. Histological studies

Sagittal brain sections were fixed in freshly prepared 10% neutral buffered formalin and embedded in paraffin. In addition, 4- μm -thick paraffin sections were prepared and stained with hematoxylin and eosin (H&E) for histopathological examination. Sections were examined using a light microscope (Tabassum et al., 2013).

2.7. Statistical method

The mean \pm SEM were calculated for all data significant different between means where evaluated by *t*-test and one-way Analysis Variance (ANOVA followed by Dunnet comparison test $p < 0.05$ considered as statistically significant).

3. Results

3.1. Docking analysis

3.1.1. Binding affinity on Bax

Gallic acid was seen to form hydrogen bonds with ASP A: 53, TRP A: 158, PRO A: 13 having interaction distance 1.62 Å, 1.92 Å, 2.62 Å respectively and exhibited binding energy -5.6 K Cal/mol (Fig. 1) (Table 1).

Minocycline was exhibited hydrogen bonds with ASP A: 98, ASP A: 102 having interacting distance of 1.90 Å, 2.03 Å respectively. Amide pi Stacked with GLY A: 179 having interacting distance of 3.89 Å, Pi alkyl with VAL A: 180 having interacting distance of 4.05 Å and exhibited binding energy -6.2 Kcal/mol (Fig. 1; Table 2).

3.1.2. Binding affinity on Tumor necrosis factor α protein

Gallic acid was seen to form hydrogen bonds with GLN A: 149, VAL A: 150, ALA A: 18, GLY A: 150 having interacting distance 1.74 Å, 1.85 Å, 2.38 Å, 2.18 Å respectively. Pi sigma with VAL A: 17 having interacting distance 3.05 Å, Pi alkyl with ARG A: 32 having interacting distance 4.35 Å and exhibited binding energy -5.6 Kcal/mol (Fig. 2; Table 1).

Minocycline was showed hydrogen bonds with ASP A: 140, LYS A: 65, PHE A: 144, PRO A: 20 having interacting distance of 1.93 Å, 1.95 Å, 2.19 Å, 2.35 Å and exhibited binding energy -7.1 Kcal/mol (Fig. 2; Table 2).

3.1.3. Binding affinity on Caspases 3

Gallic acid was observed to form hydrogen bonds with ASP B: 228, THR A: 77, LYS B: 224 having interaction distance 1.32 Å, 1.23 Å, 1.62 Å respectively. Pi alkyl with LEU A: 81, ALA B: 227 having interacting distance of 3.22 Å, 4.23 Å respectively and exhibited binding energy -5.5 Kcal/mol (Fig. 3; Table 1).

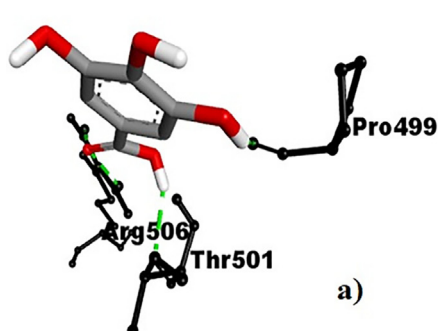


Table 1
Type of interaction and interaction distance between Gallic acid on Neurotoxic proteins.

S. No	Protein Name	PyRx Kcal/mol	Type of interaction with residue (Interaction Distance)
1	Bax	-5.6	Hydrogen bonding ASPA: 53 (2.25 Å), SER A: 55 (2.49 Å), THR A :56 (2.68 Å), TRP A: 158 (2.73 Å), PRO A: 13 (2.93 Å)
2	Tumor necrosis factor α	-5.6	Hydrogen bonding GLU A: 149 (1.85 Å), VAL A: 150 (1.52 Å), GLY A: 148 (1.62 Å), ALA A: 18 (2.63 Å) Pi alkyl bonding ARG A: 32 (3.62 Å) Pi sigma bonding VAL A: 17 (4.26 Å)
3	Caspases 3	-5.5	Hydrogen bonding THR A: 77 (1.91 Å), ASP B: 228 (1.86 Å), LYS B: 224 (2.64 Å) Pi Alkyl bonding ALA B: 227 (3.36 Å), LEU A: 81 (4.50 Å)
4	Nitric oxide synthase	-6.7	Hydrogen bonding PHE B: 709 (1.88 Å), ARG B: 704 (1.90 Å), SER B :418 (2.23 Å) Pi sigma bonding ALA B: 417 (2.67 Å) Pi alkyl bonding ARG B: 419 (4.38 Å) Pi Pi T Shaped HIS B: 346 (4.05 Å)
5	Interleukin 6	-5.9	Hydrogen bonding SER A: 107 (1.89 Å), GLU A: 42 (2.90 Å), ARG A: 104 (2.45 Å), ASP A: 160 (2.23 Å) Pi alkyl bonding PHE A: 105 (4.89 Å)
6	Glutamate receptors	-5.8	Hydrogen bonding ARG A: 506 (1.80 Å), THR A: 501 (2.91 Å), PRO A :499 (2.29 Å) Pi Anion bonding GLU A :726 (3.16 Å) Pi Pi Stacked TYR A : 471 (4.15 Å)
7	Acid sensing ion channel	-6.7	Hydrogen bonding GLN C: 279 (2.03 Å), ARG C: 370 (2.15 Å), ARG B: 370 (2.23 Å), GLY N A: 277 (2.62 Å), GLU A: 417 (2.764 Å), GLU B: 47 (2.95 Å)

Minocycline was seen to form hydrogen bonds with HIS A: 121, ARG B: 207, CYS A: 163 having interacting distance of 1.55Å, 2.22 Å 2.52Å respectively and exhibited binding energy -7.4 Kcal/mol (Fig. 3; Table 2).

3.1.4. Binding affinity on Nitric oxide synthase

Gallic acid was exhibited hydrogen bonds with PHE B: 709, ARG B: 706, SER B: 418 having interacting distance of 1.53 Å, 2.02 Å, 2.57 Å respectively. Pi sigma ALA B: 417 having interacting distance of 3.62 Å. Pi Pi T Shaped with HIS B: 346 having interacting distance of 3.65 Å. Pi alkyl with ARG A: 419 having interacting distance of 4.62 Å and exhibited binding energy -6.7 Kcal/mol (Fig. 4; Table 1).

Minocyclin was seen to form hydrogen bonds with TRP B: 592, GLU B: 597 having interacting distance of 1.85 Å, 2.85 Å respectively. Pi alkyl with VAL B: 572, MET B: 575 having interacting distance of 3.98 Å, 4.25 Å respectively. Alkyl with PHE B: 589 having interacting distance of 4.32 Å and exhibited binding energy -8.8 Kcal/mol (Fig. 4; Table 2).

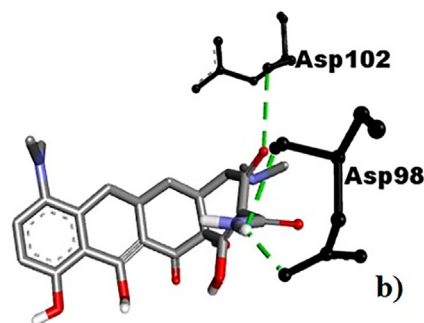


Fig. 1. Binding affinity on Bax Protein.

Table 2
Type of interaction and interaction distance between Minocycline and neurotoxicity proteins.

S. No	Protein Name	PyRx Kcal/mol	Type of interaction with residue (Interaction Distance)
1	Bax	−6.2	Hydrogen Bonding ASP A: 98(1.90 Å), ASP A: 102(2.03 Å) Amide pi Stacked GLY A: 179(3.89 Å) Pi alkyl VAL A: 180(4.05 Å)
2	Tumour necrosis factor α	−7.1	Hydrogen Bonding ASP A: 140 (1.93 Å), LYS A: 65 (1.95 Å), PHE A: 144 (2.19 Å), PRO A: 20 (2.35 Å)
3	Caspases 3	−7.4	Hydrogen Bonding HIS A: 121 (1.55 Å), ARG B: 207 (2.22 Å), CYS A: 163 (2.52 Å)
4	Nitric oxide synthase	−6.6	Hydrogen Bonding TRP B: 592 (1.85 Å), GLU B: 597 (2.85 Å) Pi alkyl bonding VAL B: 572 3.98 Å), MET B: 575 4.25 Å) Alkyl bonding PHE B: 589 (4.32Å), CYS B: 420 (4.52 Å)
5	Interleukin 6	−8.8	Hydrogen Bonding ARG A: 104 (1.95 Å), GLN A: 156 (2.55 Å), GLU A: 106 (3.98Å) Pi sigma bonding PHE A: 105 (4.58 Å)

3.1.5. Binding affinity on Interleukin 6

Gallic acid was showed to form hydrogen bonds with SER A:107, GLUA: 42, ARG A: 104, ASP A: 160 having interaction distance 1.80 Å, 1.12 Å, 2.23 Å, 2.12 Å respectively. Pi-Pi shaped with PHE A :105 having interacting distance of 3.12 Å and exhibited binding energy −5.9Kcal/mol (Fig. 5; Table 1). Minocycline was observed to form hydrogen bonds with ARG A: 104, GLN A: 156, GLU A: 106 having interacting distance of 1.95Å, 2.55 Å, 3.98 Å respectively. Pi sigma with PHE A: 105 having interacting distance of 4.58 Å and exhibited binding energy −6.6Kcal/mol (Fig. 6; Table 2).

3.1.6. Binding affinity on acid sensing ion channel

Gallic acid was shown to form hydrogen bonds with GLN C: 279, ARG C: 370, GLU B: 417, GLN A: 277, GLU A :417 having interaction distance 1.50 Å, 1.56 Å, 1.62 Å, 2.52 Å, 2.72 Å respectively and exhibited binding energy −8.0 Kcal/mol (Fig. 6; Table 1). Quercetin was observed to form hydrogen bonds with GLU C: 98, ARG C: 191, GLU C: 154, ARG F: 28, SER C: 241, GLU C: 243 having interacting distance of 1.62 Å, 1.85 Å, 2.32 Å, 2.62 Å, 2.74 Å, 2.82 Å respectively and exhibited binding energy −9.3Kcal/mol (Fig. 6; Table 3).

3.1.7. Binding affinity on Glutamate

Gallic acid was seen to form hydrogen bonds with PRO A: 499, THR A: 501, ARG A: 506 having interaction distance 1.95 Å, 1.99 Å, 2.62 Å respectively. Pi Pi Stacked with TYR A: 471 having interac-

tion distance 2.52 Å, Pi anion with GLU A: 726 having interaction distance 2.95 Å and exhibited binding energy 5.8Kcal/mol (Fig. 7; Table 1). Memantine observed to form hydrogen bonds with GLN B: 663, SER B: 661 having interacting distance of 2.25 Å, 2.56 Å respectively. Pi alkyl with TYR B: 694, LYS B: 690 with having interacting distance of 3.62 Å, 4.22 Å respectively and exhibited binding energy −5.6Kcal/mol (Fig. 7; Table 4).

3.2. Cerebral infarction area

Gallic acid (25 mg/kg, 50 mg/kg, B.wt) treated rats the percent cerebral infarct volumes were found to be reduced. In this study, there was a significant increase in percent cerebral infarction in MO/RCA group compared to normal control group. Treatment with Gallic acid significantly reduced in percent cerebral infarction compared to MO/RCA group, indicating the cerebroprotective action of Gallic acid (Figs. 8, 9; Table 5).

3.3. Effect of Gallic acid on biochemical parameters in MO/RCA rats

Results were revealed that potential neuroprotective activity of GA. MDA levels exhibited significant increase and all other enzymatic and non-enzymatic parameters (SOD, CAT, and GSH) showed a significant decrease in the MO/RCA group. The animals from GA treated groups had shown a significant protection by reducing the elevated levels of MDA (P < 0.4476) and marked increase in SOD (P < 0.001), CAT (P < 0.01), and GSH (P < 0.001) as compared to MO/RCA treated group. The levels of all enzymatic and non-enzymatic parameters were normal in normal and sham group (Fig. 10; Table 5).

3.4. Histopathology

In this study we unveil that during cerebral ischemia cells can lose their cell permeability barrier properties, leading to edema formation and degradation, and the hippocampus region of the brain showed decreased thickness of the pyramidal layer, with increased apoptotic neurons with dystrophic changes in the form of shrunken and irregular of MO/RCA group when compared to normal, sham and treated groups. However, MO/RCA surgery induced severe ischemia and neuronal damage, manifested as decreased in the intact of neurons when compared with normal rats and sham rats. Moreover, Gallic acid 25 mg and 50 mg/kg treatment showed restore neuronal cells contact when compared with MO/RCA rats. In this neuronal intact more in Gallic acid 50 mg/kg when compared with Gallic acid 25 mg/kg treatment groups (Fig. 11).

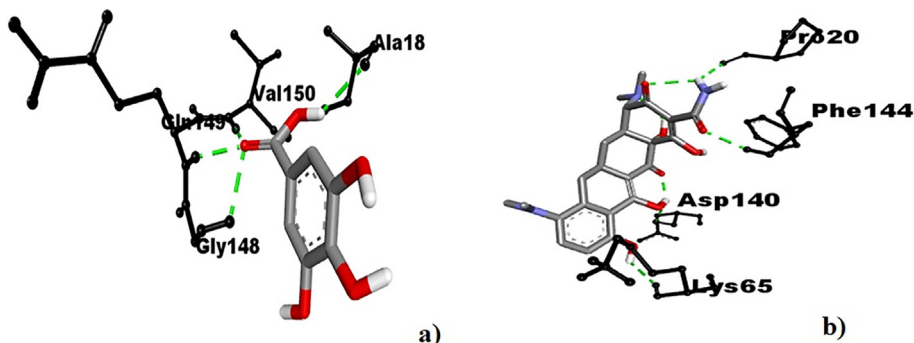


Fig. 2. Binding affinity on Tumour necrosis factor α.

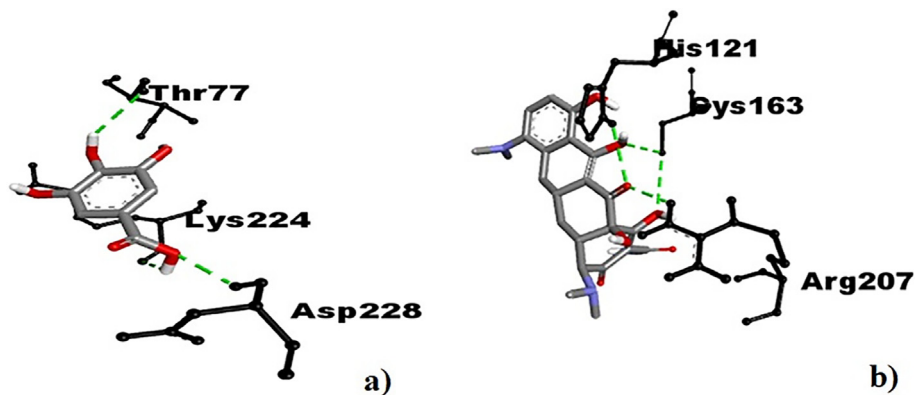


Fig. 3. Binding affinity on Caspases 3.

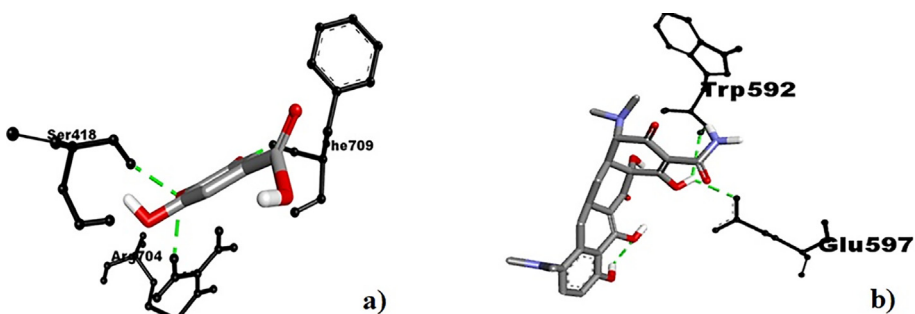


Fig. 4. Binding affinity on Nitric oxide synthase.

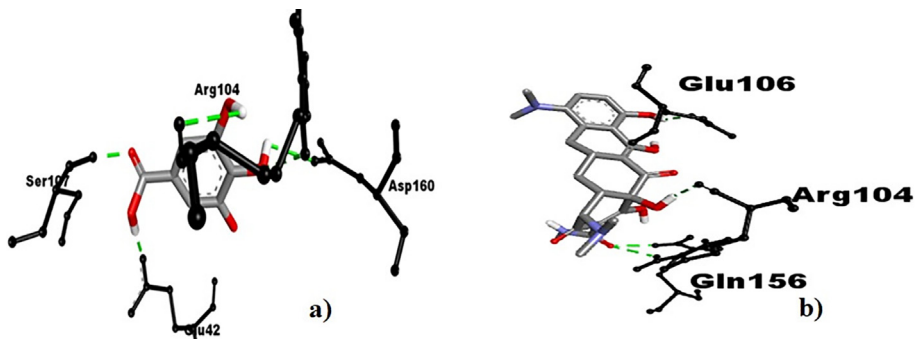


Fig. 5. Binding affinity on Interleukin 6.

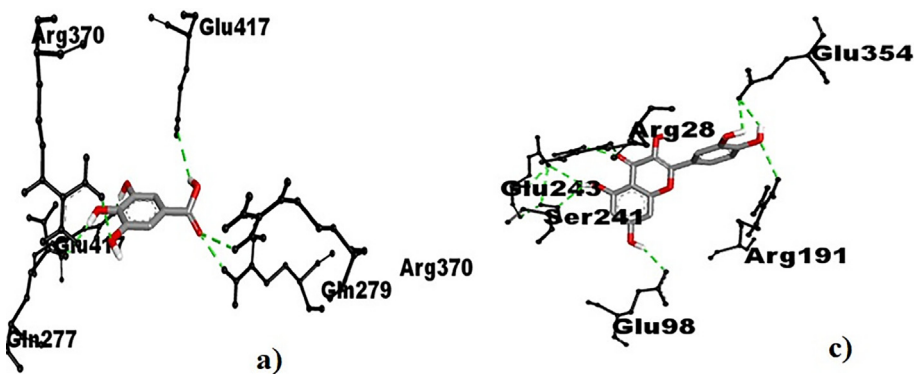


Fig. 6. Binding affinity on Acid sensing Channels.

Table 3

Type of interaction and interaction distance between Quercetin on Acid sensing ion channel.

S. No	Protein Name	PyRx Kcal/mol	Type of interaction with residue (Interaction Distance)
1	Acid sensing ion channel	−9.3	Hydrogen Bonding GLU C: 98 (1.62 Å), ARG C: 191 (1.85 Å), GLU C: 154 (2.32 Å), ARG F: 28 (2.62 Å), SER C: 241 (2.74 Å), GLU C: 243 (2.82 Å)

3.5. Histopathological studies

Fig. 11.

4. Discussion

Cerebral ischemia and reperfusion both lead to neuronal damage (Jiang et al., 2010). In this study, we evaluated, for the first time, the brain antioxidant/stress parameters and infarction size in multiple occlusion and reperfusion of bilateral common carotid arteries, evaluated protective effect of known neuroprotective effective agent GA against cerebral injury. Our study demonstrated multiple occlusion reperfusion of bilateral common carotid arteries rats showed significantly decreased antioxidant enzymes, increased infarct size and cerebral histological damage. Furthermore, we found that GA treatment markedly reduced MDA, as well as oppose the decreases in SOD, catalase and GSH induced by multiple occlusion/reperfusion of bilateral common carotid arteries induced cerebral ischemia injury rats. *In silico* studies was included to assessed binding affinity on cerebrototoxic proteins in cerebral ischemia. Cerebral damage was evaluated by the infarct size and the histopathological changes of brain were assessed by hematoxylineosin (H–E) staining in the brain slices. Our results revealed that "MO/RCA rats showed significant increase infarct size and histological damage in ischemia/reperfusion mice compared to the sham rats and protective effects of GA in a dose dependent manner. The protective effects of GA at 50 mg/kg were more apparent than those at 25 mg/kg, probably due to much higher brain concentrations of GA at a high dose.

It is well understood that oxidative metabolism is necessary for brain survival, but it is also correlated with the production of reactive oxygen species (Madamanchi et al., 2005; Schreiber et al., 2007; Zhao et al., 2018). GSH, an endogenous antioxidant, is a key element of cellular antioxidant defences since it functions both to directly detoxify reactive oxygen species and as a substrate for various peroxidases. Glutathione system dysfunction has been linked to a variety of neurodegenerative diseases (Chandra Jagetia et al., 2003; X. Zhang et al., 2019) and it can contribute to

oxidative damage following transient ischemia. Catalase is an enzyme that scavenges harmful oxygen by products such as hydrogen peroxide, and a rise in catalase levels suggests antioxidant activity. Catalase is an enzyme that converts toxic hydrogen peroxide to water and oxygen. Catalase activity is mainly identified in peroxisomes, which are subcellular organelles (Weydert CJ, 2010). Inflammatory cells contain a significant amount of hydrogen peroxide in order to destroy pathogens. Its high concentration is cytotoxic, and its deposition causes cellular targets such as DNA, proteins, and lipids to oxidize, resulting in mutagenesis and cell death. Catalase eliminates cell H₂O₂, thereby protecting it from oxidative damage. SOD, another defensive system to work against reactive oxygen species-induced tissue damage, catalyzes the dismutation of superoxide anion to hydrogen peroxide and prevents the formation of the hydroxyl radical (Cheng et al., 2019; Li et al., 2008). MDA is a widely used oxidative stress biomarker in several diseases, including stroke. It is a by-product of lipid peroxidation induced by the deterioration of cellular membrane phospholipids. Since MDA enters the blood after being released into the extracellular space, it has been used as an effective diagnostic tool of lipid oxidation (Menon et al., 2020).

Sudden bursts of reactive oxygen species cannot be managed by endogenous antioxidant systems during ischemia/reperfusion, and the accumulation of reactive oxygen species causes oxidative damage to cellular membranes, proteins, and DNA (Thiyagarajan and Sharma, 2004; Zheng et al., 2007). As a result, antioxidants have been recognized as a potential therapy for ischemic stroke (J Murdoch, 1990; Powers and Jackson, 2008). In this study we found that the MO/RCA rats brain antioxidant enzymes GSH, catalase and SOD were significantly decreased, MDA levels was increased compared to sham rats, GA treatment markedly increased GSH, catalase and SOD levels and decreased MDA levels in MO/RCA rats.

Astrogliosis is most common in neurological disorders that cause apoptosis, neuroinflammation, and neuronal death. TNF, IL-6, and apoptotic proteins such as caspase 8, Bax are examples of neuroinflammatory factors formed by Glia cell activity (Jayaraj et al., 2019; Sun et al., 2014). Neuronal death in a stroke is a complicated event involving, among other things, metabolic dysfunction, excitotoxicity, loss of calcium homeostasis, and oxidative stress (Alexi, 2000). During ischemic stroke, increased glutamate release leads to increased Ca²⁺ level. The massive Ca²⁺ entry activates enzymes such as proteases, oxidases, phospholipases and endonucleases that can hydrolyze the DNA molecule and destroy the cytoskeleton (Nicotera and Lipton, 1999; Park et al., 2020; Welch KM, 1997). Few studies have indicated that acid sensing ion channel 1a knockout or pharmacological inhibition of acid sensing ion channel 1a decreased infarct volume significantly (Xiong et al., 2006). The histopathology studies revealed that in MCAO/R rats hippocampus region of the brain showed decreased

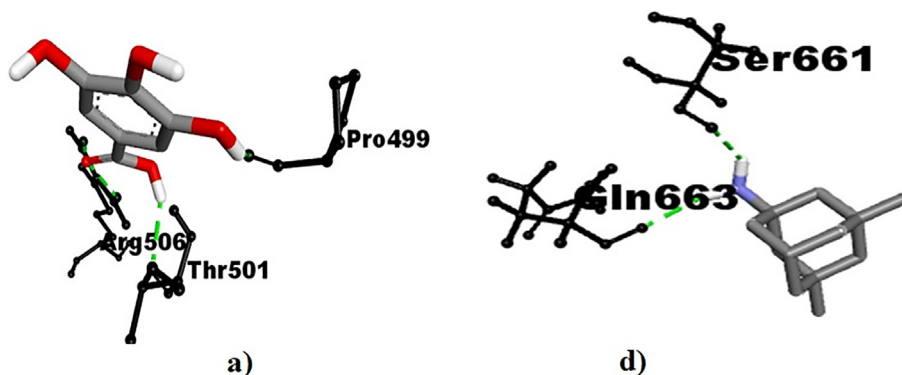


Fig. 7. Binding affinity on Glutamate receptors Gallic acid b) Minocycline c) Quercetin d) Memantine.

Table 4
Type of interaction and interaction distance between Memantine on Glutamate receptor.

S No	Protein Name	PyRx Kcal/mol	Type of interaction with residue (Interaction Distance)
1	Glutamate receptors	-5.6	Hydrogen Bonding GLN B: 663 (2.25 Å), SER B: 661 (2.56 Å) Pi alkyl TYR B: 694 (3.62 Å), LYS B: 690 (4.22 Å)

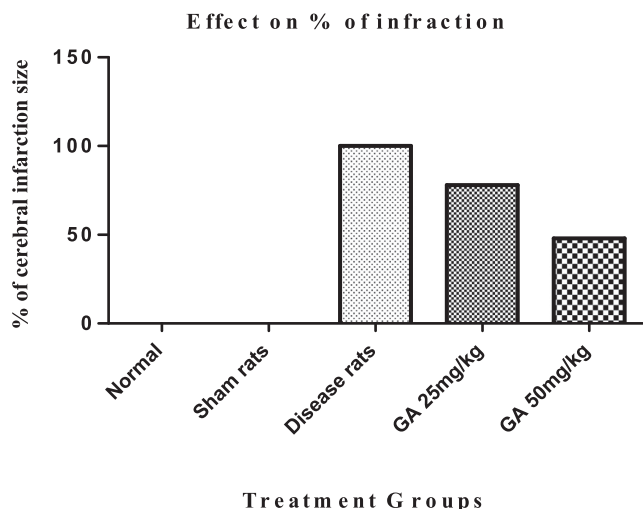


Fig. 8. Effect of Gallic acid on % of cerebral infarction Size in MO/RCA rats.

thickness of the pyramidal layer, with increased apoptotic neurons, decreased in the intact of neurons and irregular when compared to sham group. Moreover, Gallic acid treatment restore neuronal cells contact when compared with MO/RCA rats. Similarly, Cerebral

infraction area was increased in MO/RCA rats as compared to control groups, it was attenuated by gallic acid treatment.

GA exhibits cerebroprotection by antioxidant and inflammation pathways in animal models of neurodegenerative diseases due to its susceptibility to NMDA receptors and excitotoxicity caused by glutamate after cerebral ischemia accompanied by Ca⁺² influx and thus intracellular Ca⁺² accumulation induced neuronal apoptosis (Mansouri et al., 2013). Antioxidative effect of GA may oppose the activation NMDA receptors and thereby has protective effect on neurotoxicity and excitotoxicity following brain injury (Korani et al., 2014). *In silico* study reported that GA form good binding affinity with active sites of neurotoxic proteins, hydrogen bonding with amino acid residues HIS A: 121, ARG B : 207 CYS A : 163, SER A : 58, TYR B : 204 of caspase 3 ,GLN A: 156, ARG A: 40 , LYS A : 171 GLN : 175 of Interleukin 6, PHE B : 709, ALA B : 417, ARG B : 419, ALA A : 417 , CYS A : 420 of Nitric oxide synthase, THR A: 501, PRO A : 499, GLU A: 726, TYR A : 471, PRO A : 515, ARG B : 506 Glutamate receptor. *In silico* studies showed good binding of gallic acid with the caspase 3, Interleukin 6, Nitric oxide synthase and Glutamate receptor and also exhibited good binding energy, it was observed with marketed compounds such as Minocycline, Quercetin and Memantine, it is indicating it as a possible therapeutic strategy for treatment of cerebral ischemic injury. GA inhibited the expression of mitochondrial apoptotic signalling molecules reported in various *in vivo* studies. Cerebral ischemia stimulates both the intrinsic pathway, which results from mitochondrial crash-associated stimulation of caspase-9, and the extrinsic pathway, which results from death receptor activation and subsequent stimulation of caspase-8.(Sacco et al., 2009). Several studies revealed that protective effect of GA against oxidative stress, apoptosis, and inflammation (Abdel-Moneim et al., 2017; Maya et al., 2018a, 2018b). GA has been reported to possess antioxidant, neuroprotective activities, in the treatment of depression (Nagpal et al., 2012) by acting on intracellular antioxidant enzymes such as superoxide dismutase (SOD) and catalase, by lowering hydroperoxide through the glutathione peroxidase process (Nordberg and Arnér, 2001). In summary, our results demonstrate

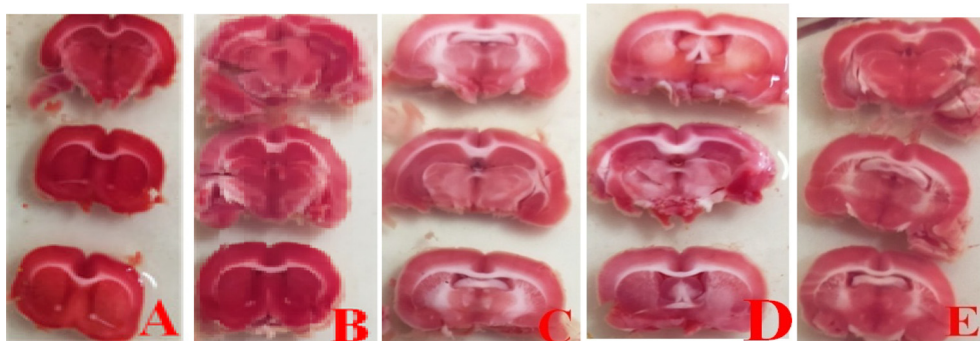


Fig. 9. GA inhibiting MO/RCA increased Percent of Cerebral infarct volume A) Normal rats B) Sham rats C) MO/RCA rats D) MO/RCA + GA 25 mg/kg rats E) MO/RCA + GA 50 mg/kg rats.

Table 5
Effect of Gallic acid on brain hippocampus biochemical parameters in MO/RCA injury rats.

Treatment	Infarction size (%)	GSH (nmol/mg)	MDA (nmol/g)	SOD (U/mg)	Catalase(nmol/mg)
Normal rats	0	0.19 ± 0.01	6.43 ± 0.17	16.7 ± 0.18	0.0043 ± 0.00025
Sham rats	0	0.13 ± 0.01**	7.41 ± 0.15***	13.5 ± 0.96**	0.0020 ± 0.00068**
MO/RCA rats	100%	0.10 ± 0.01***	10.77 ± 0.99***	9.8 ± 0.11***	0.00022 ± 0.00005***
Gallic acid 25 mg/kg	22.03%	0.13 ± 0.01*	10.28 ± 0.18 ^{ns}	15.7 ± 0.42***	0.00106 ± 0.00005***
Gallic acid 50 mg/kg	52.12%	0.18 ± 0.01***	9.90 ± 0.55 ^{ns}	15.7 ± 0.42***	0.0038 ± 0.00067***

Mean ± SEM were calculated for all data significant different between means where evaluated by One way Analysis Variance (ANOVA followed by *t* test comparison test p < 0.05*, p < 0.01**, p < 0.001*** considered as statistical significant.

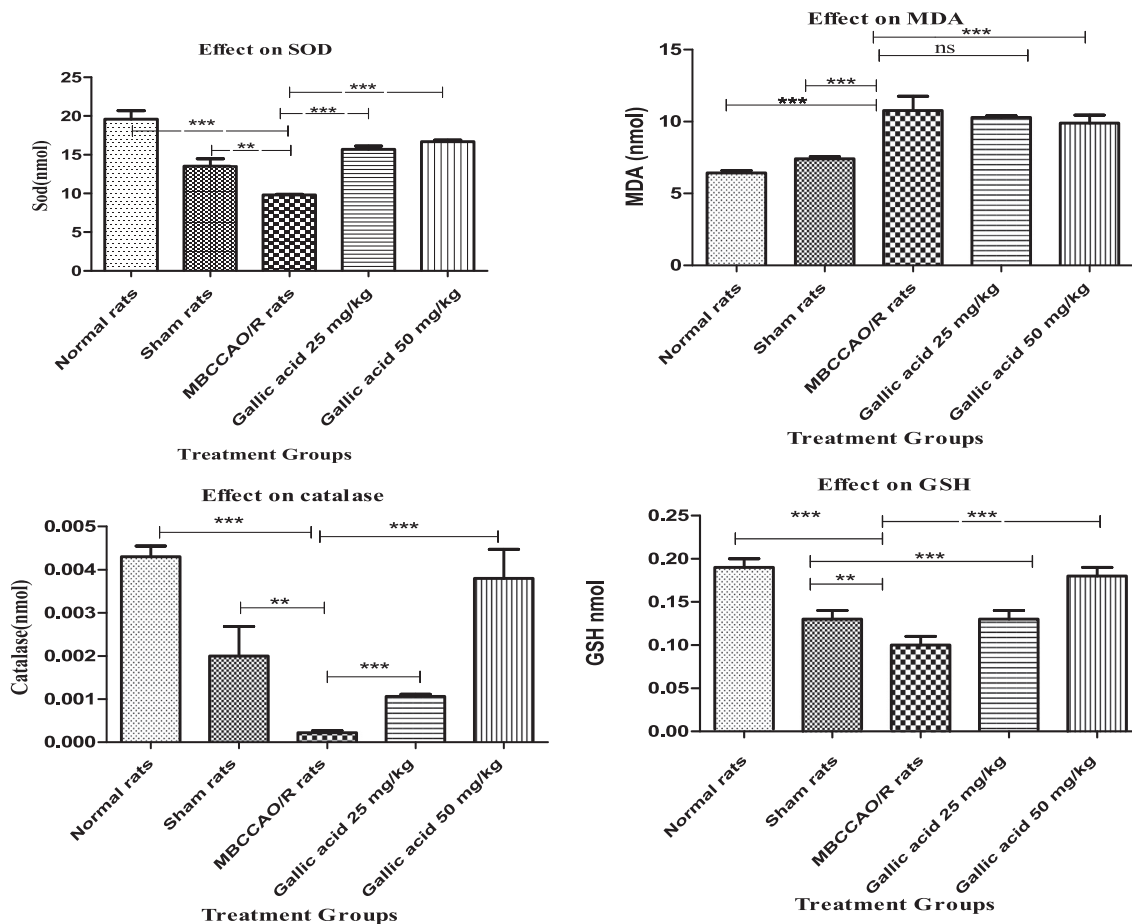


Fig. 10. Effect of Gallic acid on brain biochemical parameters in MO/RCA rats.

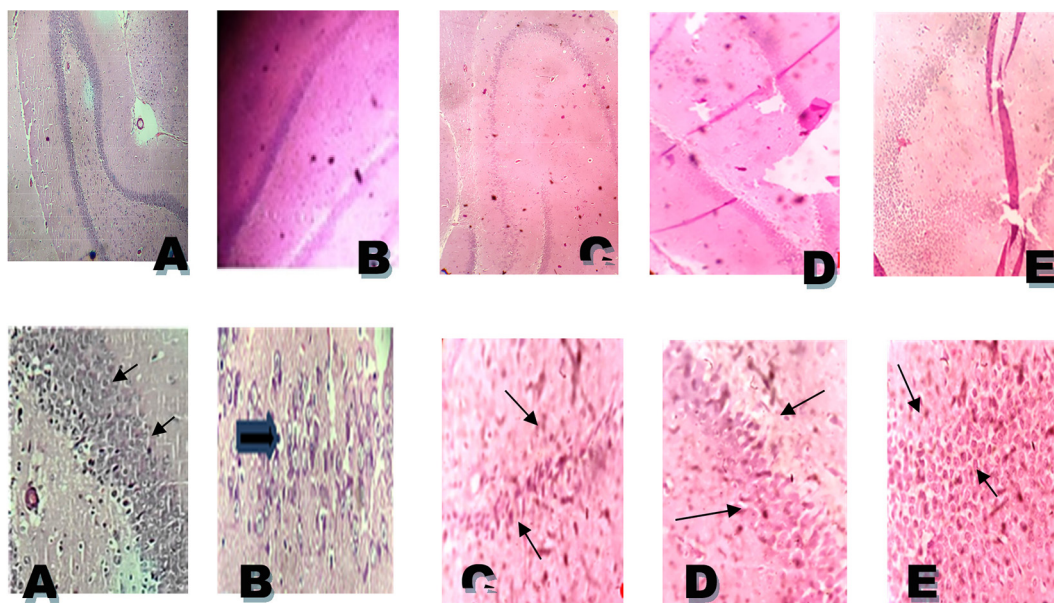


Fig. 11. Effect of MO/RCA on brain hippocampus region of rats: The brain hippocampus region (x40) after induction of Cerebral stroke by MO/RCA. (A) Normal rats (B) Sham rats (C) MO/RCA (D) MO/RCA + GA 25 mg/kg rats. (E) MO/RCA + GA 50 mg/kg rats. In treated rats hippocampus region neuronal cells are less scattered, more neuronal cells in contact (black arrow). Treated rats showing increased thickness of pyramidal cell layer in the hippocampus region, with decreased apoptotic neurons with dystrophic changes in the form of shrunken and irregular (black arrows).

that designed multiple occlusion and perfusion of carotid artery model showed significant decreased rat brain antioxidant and increased MDA in rat, it was clinically resembled to cerebral stroke, attenuated by known antioxidant gallic acid treatment.

Compliance with Ethical Standards

All the experimental protocol was carried out with the approval of Institutional animal ethical committee (IAEC), protocol number (IAEC/CESCOP/2019-OCT-02).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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