Anti-Apoptotic and Anti-Inflammatory Protective Mechanisms of *Gmelina Arborea* Stem Bark Extract on Ischemic Reperfusion Injury in Albino Wistar Rats

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Abstract

Ethnopharmacological relevance

*Gemlina arborea* Roxb. ex.'s entire plant is utilized in medicine to treat several diseases.

Objective and design

Cerebral infarction occurs through multiple mechanisms, and herbs are natural multi-component with numerous protective mechanisms. This research investigates the neuroprotective effect of *Gemlina arborea* stem bark extract in preventing cerebral infarction.

Methodology

Male albino Wistar rats were inducted with ischemic injury using the BCCAO method. Neurological effects were examined by motor defects, locomotor activity, and forced swim test, while biochemical activities of the brain oxidative stress biomarkers studied include; (lipid peroxidation, superoxide dismutase, catalase levels, glutathione levels, total calcium levels, and sodium-potassium-ATPase) and histopathological examination. Western blotting analysis was used to study the anti-apoptotic and anti-inflammatory mechanisms.

Results

The elevated oxidative stress biomarkers in the treated group showed extract-inhibited cerebral infarction. Histological examination shows inhibited neutrophil migration and cell damage. The western blot assay depicts reductions in the inflammatory indicators (p38 MAPK, TNF-α) and attenuation of apoptotic mediators (Bcl2/Bax ratio), down-regulation of caspase-3 expression and a significantly raised anti-inflammatory mediators (IL-10 and actin) in a dose-dependent manner, suggested potential mechanisms by which the extract prevented ischemic reperfusion injury.
Conclusion

Overall, the estimation of inflammatory and apoptotic mediators revealed the involvement of the anti-inflammatory mechanisms of *Gmelina arborea* stem bark extract in preventing cerebral infarction.

**Keywords**: Anti-Apoptotic, Anti-Inflammatory, Gmelina Arborea, Cerebral-infarction, Ischemic-Reperfusion, Western-blot-analysis, Albino-Wistar-Rats
Introduction

Brain disorders and stroke are the leading causes of mortality and morbidity in most patients. They often result in permanent or temporary disabilities, if not death, in many cases. The majority of stroke cases end up suffering from physical and mental disabilities [1]. Brain problems and their associated neurodegenerative diseases are the world's leading causes of death [2], contributing to about 5 million annually. Stroke is also quite prevalent and a cause of disability, which is common in the elderly population [3] and predicted to rise to 1.4 billion by 2050 [1]. With advances in science and technology in medical fields, it has become easy to diagnose and treat many diseases, but strokes with multiple etiologies and mechanisms still pose challenges during treatment [4]. Even with modern medicine, stroke is usually caused by multi-faceted mechanisms and pathways that are still difficult to identify and target.

Removing damaged cells and preserving tissue homeostasis depends on the tightly controlled, intrinsic cell death mechanism known as apoptosis [5, 6]. The caspases, a family of cysteine proteases that cleave a variety of cellular substrates and cause cell deconstruction, are what control the process [7]. The FasL/FasR interaction, which recruits the FADD and procaspase-8 to create the death-inducing signalling complex (DISC), is one example of a death ligand that binds to a death receptor to start the extrinsic route [8]. By controlling the release of cytochrome c from mitochondria and subsequent activation of caspase-9 through the creation of the apoptosome, Bcl-2 family proteins govern the intrinsic route, also referred to as the mitochondrial pathway [9]. When the caspases-3, -6, and -7 are activated, these two pathways combine to produce the morphological characteristics of apoptosis, such as DNA fragmentation and membrane blebbing [10]. Herbal drugs act by various mechanisms and minimal side effects, so they can be considered alternatives to synthetic drugs in stroke treatment [12]. According to reports, alternative medicine holds promise
for the future and true bioactive components from medicinal plants may be used in an alternative medication or treatment plan [13–15].

*Gmelina arborea* Roxab., belongs to the family *Verbaneaceae*, it is widely distributed in the South East Asia and India [16, 17]. The whole plant has been reported to be used as medicine in various ailments such as wound-healing, antidiarrheal, antiulcer, and antidiabetic [17–19]. It is rich in antioxidants, and a wild folkloric plant with proven efficacy against amnesia and inflammation [17, 20]. Among the phytochemical components isolate reported are luteolin, indole alkaloids, hentriacontanol and lignans (arboreol, isoarboreol, methyl arboreol, arborone, gmelanone, gummadiol, and 7-oxodihydrogmelinol) [16, 17]. Also, coumarin glycosides and iridoid glycosides [14] are presence the in roots and leaves of *Gmelina arborea*. The stem bark is used to treat abdominal complications and inflammations, and various chemical constituents like flavonoids and polyphenols have been isolated from the plant [21]. Here, we investigate the anti-apoptotic and anti-inflammatory protective mechanisms of *gmelina arborea* stem bark extract on ischemic reperfusion injury in albino Wistar rats.

**Materials and Methods**

**Plant material processing**

The *Gmelina arborea* plant was collected from the Tirumala hills, Tirupathi, India, and authenticated by Prof. Jayaraman, PARC, Chennai, and a reference number PARC/2022/264-11 remained deposited in the herbarium. The plant's stem barks were carefully separated and shade-dried at 30±5°C. The dried bark was ground in a rotary mill and 50g of powder was packed in a muslin cloth and extracted with 70%v/v ethanol in distilled water using ultra-sound assisted extraction. Afterwards, filtered using a Whatman filter paper 42mesh, and evaporated to dryness
using a rotary evaporator. The crude extract (yield-21.65% w/w) was stored in a dry and cap-sealed container until use.

**Experimental Treatment Regimens and Induction of Ischemic Injury**

Male Albino Wistar rats (n=36, 8-10 weeks old, weighing 200-250g) were acclimatize to laboratory conditions with a temperature of 24±3°C, 50% relative humidity, and a 12:12 light and dark schedule. They were housed in polypropylene cages with free access to standard pellet food, fresh water, and labitum. Rats were randomly divided into four groups (n=9). The first group was the sham-operated group (SG), and the second group, the Ischemic reperfusion group (IRG), received saline (10ml/kg/day via gavage). The remaining two groups, [extract treated groups (GE-250 & GE-500)], received Gmelina extract (2.5% and 5% w/v in saline at 10ml/kg/day via gavage, respectively), for 14 days, and the rats were subjected to a surgical process to investigate the effect of the extract. The bilateral common carotid artery occlusion (BCCAO) method was used to induce cerebral ischemia and reperfusion (CIR) to investigate the neuroprotective effect of Gmelina extract in preventing brain injury [22]. Rats of all the groups were anaesthetized using a solution of 100mg/kg ketamine and 10mg/kg xylazine via injection (i.p.). A Midline incision was made on the neck above the sternum, parallel to the trachea, to expose bilateral common carotid arteries lateral to the sternocleidomastoid. Small artery clips were used to clamp and occlude the carotid arteries to cause ischemia in the brain. After 30 min, ischemic reperfusion was caused by removing the clips to allow the blood to flow through carotid arteries. The surgical wound was sutured correctly, and betadine ointment (10%w/w) was applied. A thermostat-controlled IR lamp was used to control the rats' body temperature (37±0.5°C) throughout the operation. The sham group rats were also operated
similarly without causing occlusion or reperfusion. Animals were placed in a warm area (30-32°C) for observation for 24 hrs to stabilize after surgery.

**Neuromotor Activity Examination**

**Estimating Neuromotor Score**

The operated rats were allowed to reperfusion for 24 hrs and were subjected to neurological examination to evaluate for motor defects. The evaluation was conducted in 3 stages as described by [23].

**Evaluation of Locomotor Activity:**

The movement of rats, signifying the locomotor activity, was evaluated using an act photometer, after allowing the animal to acclimatize for 2 min. The investigation was conducted as describe by [24, 25].

**Forced Swim Test:**

A forced swim test after 24 hr of reperfusion was performed for 5min, and the total duration of immobility was noted follow the method of [26].

**Measurement of Infarct Area:**

Rats of all the groups were anaesthetized using a solution of 100mg/kg ketamine and 10mg/kg xylazine via injection (IP). They were gracefully sacrificed by decapitation, and their brains were collected carefully. The tissues were washed using normal saline (4°C, 0.9%w/v) for 2 mins and sliced into 2mm thick sections using an acrylic template. The slices were stained using 2,3,5-triphenyl tetrazolium chloride (TTC) and incubated at 37°C for 15 min [27]. The stained slices were
stored in 10% buffered formalin (pH-7.2) for 24 hr, and the area of infarction was measured using Image J 1.42 image processing software.

**Estimation of Biochemical Parameters in Brain Tissue**

**Preparation of Tissue Homogenate**

After sacrificing the rats, the brain was collected and washed with normal saline (4°C, 0.9% w/v) and homogenized in 0.05M Phosphate buffer saline (PBS) (10% w/v, pH 7.4) and 0.1M EDTA. This was centrifuged at 4500 rpm for 15 min to yield a supernatant liquid collected and stored at 4°C until use.

**Estimation of Lipid peroxidation levels (LPO)**

Supernatant liquid (200µl) was added to 50µl sodium dodecyl sulphate (SDS) solution (8.1% w/v) and allowed to react for 10 min. 375µl Thiobarbituric acid (0.6% w/v) was added to the above solution and incubated in a water bath at 37°C for 1 hr. 1.25ml of solution of butanol and pyridine (3:2) was added and centrifuged at 2000 rpm for 5 min. The absorbance was measured at 532 nm against a blank reference, and results were expressed in terms of nM of MDA formed from gram protein in a minute [28].

**Estimation of Superoxide Dismutase Levels (SOD)**

Supernatant liquid (100 µl) was mixed with 880 µl carbonate buffer (0.05M) and 20µl epinephrine (30mM), and the pH was adjusted to 7.8 with acetic acid (0.05% v/v) and 0.1mM EDTA solution. The mixture was allowed to stand and react for 5 min, and absorbance was measured at 480 nm.
**Estimation of Catalase levels (CAT)**

Supernatant liquid (100µl) was added to 10µl ethanol (90%v/v) at 4°C, and the resultant mixture was allowed to stand to attain room temperature (24°C). 50µl of the solution was mixed with 10µl of triton and 250µl phosphate buffered hydrogen peroxide (0.66M, pH-7.8) and allowed to react for 10min. The absorbance was measured at 240nm [29].

**Estimation of Reduced Glutathione Levels (GSH)**

The supernatant liquid was mixed with sulfosalicylic acid (4%w/v, 1:1 volume) and centrifuged at 1200rpm for 5min. Supernatant liquid (0.5ml) was collected and mixed with 4.5ml of 5-5'-dithiobis [2-nitrobenzoic acid] (DTNB, 0.01M). The mixture was allowed to react for 5min, and absorbance was measured at 412nm [30].

**Estimation of Total Calcium Levels (TC)**

The serum's calcium level was estimated using a calcium estimation kit (Merck; spectroquant#10004).

**Estimation of Sodium-Potassium-ATPase (Na+K+ATPase) Activity:**

The reaction mixture was prepared using 5 mM Magnesium chloride, 20 mM potassium chloride, 80 mM sodium chloride, and 40 mM Tris-HCl buffer (pH-7.4) to a final volume of 2ml [31]. The reaction was initiated using 0.2ml of 25mM ATP solution in a water bath at 37°C and allowed to react for 10 min. Then the reaction was terminated using 0.1ml trichloroacetic acid (10%w/v) at 4°C. This was centrifuged at 1000 rpm for 5min, and the supernatant was collected to estimate the released inorganic phosphate [31]. The control readings were drawn by adding one mM ouabain to the reaction mixture.
Histopathological Evaluation of the Rat's Brain Tissues

The brain tissues were removed and stored in a formalin solution (10% v/v). They were dehydrated with a series of ethanol (30%, 50%, 70%, 90% v/v) and sectioned in paraffin using a rotary microtome of 5µ thickness. The sections were rehydrated and stained using hematoxylin and eosin and visualized under a microscope (40X magnification) for histological changes.

Western blot analysis:

The brain tissue homogenate was subjected to SDS-PAGE (4% gel) electrophoresis after precipitating the proteins using radio immune precipitation (RIPA) buffer (50 mM Tris-HCl; pH-8.0, 150 mM sodium chloride, 0.1% w/v triton X-100, 0.5% w/v sodium deoxycholate, 0.1% w/v sodium dodecyl sulphate (SDS), 1mM sodium orthovanadate, 1mM sodium fluoride, 10µl/ml protease inhibitor enzyme). 15μL samples were loaded into the cells along with 6 μL of Tris gel buffer. The solutions were left to run for 60 min from 60V to 140V using Bio-Rad-Trans-Blot.

Protein electrotransfer was performed onto polyvinylidene fluoride (PVF) membrane using 25 mM Tris in 20% v/v methanol and 190 mM glycine. The membrane was washed with PBS before and after staining using ponceau (0.2%w/v in 5% glacial acetic acid). The membrane with proteins was blocked using bovine serum albumin (BSA) (3%w/v), Tris (20 mM Tris+150 mM 0.9% saline), and 0.1% w/v tween 20 in 1x PBS for 1hr. The membrane was washed twice with cold PBS solution and incubated overnight using standard primary antibodies (TNF-α, 1:1000; Bax, 1:500; Bcl2, 1:1000; Caspase and Actin, 1:1000; IL-10, 1:1000). The membrane was washed with PBS and incubated for 2hr with standard secondary antibody (p38MAPK, 1:2000). Visualization was carried out using chromomeric substrate (10ml 3,3’-diaminobenzidine (DAB), 0.05%w/v in 10µl hydrogen peroxide).
Statistical analysis

All the values were polled and presented as mean ± standard deviation (SD). The data were analyzed using one-way ANOVA in Graph pad Prism version 5.04. The significance of the data between the groups was estimated by performing Dunnet's test, and P<0.001 were considered significant.

Ethics approval

The study was approved by the Institutional Animal Ethical Committee of QIS College of Pharmacy, Ongole, Andhra Pradesh, India on 27th July 2022. With the certified reference number 1921/PO/Re/S/17/CPCSEA. All the experiments were conducted according to the CPCSEA guidelines.

Results

Effect of Gmelina arborea Stem Bark on Neurological Function

The ischemia and reperfusion were performed in the rats using the BCCAO method. Rats were observed during 24 hrs of reperfusion of blood flow to the brain and analyzed for neuromotor skills. Figure 1 showed a significant variation in the overall performance in the neurological examination. The Sham group (SG) scored above eight on an overall 9-point scale, significantly similar to the gmelina extract-treated group at a dose of 500mg/kg (GE-500) with an average score above 8. The Ischemia/reperfused (IRG) demonstrated poor neuromotor ability with less than a 2-point score. Gmelina treated group at a dose of 250mg/kg (GE-250) had a moderate score of around 5 points, as depicted in Table 1.

The locomotors activity test and forced swim test showed significant improvement in the rats' brain function in Figures 2 a & b, showing the high movement of over 200 counts in SG rats. IRG rats
showed a minor movement of about 50% of the SG group. GE-250 and GE-500 showed a higher and more significant movement than the IRG group.

**Effect of *Gmelina arborea* Stem Bark on the Infarct area of BCCAO Rat Brain**

The percentage area of infarction was significantly lowered with the pretreatment with *Gmelina arborea* stem bark extract at two doses (GE-250, GE-500). GE-250 showed a significantly less pale area, and GE-500 showed the highest activity inhibiting the infarction. The measured area of infarction in the brain was significantly higher in the IRG group (72.36 %) compared to GE-250 (24.48 %) and GE-500 (5.36%) in a dose dependent effect. SG showed no infarction or discolouration in the tissue. Individual infarction percentages were compared with the stained brain tissue in Figures 3a & b. In Figure 3a, line a is the triphenyl tetrazolium chloride (TTC) stained brain slices of all the groups of rats, while the line b is the percentage of brain infarction.

**Effect of *Gmelina arborea* Stem Bark on the Infarct area of BCCAO Rat Brain**

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Effect of *Gmelina Arborea* Stem Bark Extract on the Brain Enzyme Levels

The results showed a significant infarction and brain damage by estimating the enzymes in the brain tissue homogenate. Lipid peroxidation (LPO), superoxide dismutase (SOD), reduced glutathione (GSH), and catalases (CAT) were estimated using standard procedures. Table 2 and Figure 4(A-F) show the individual enzyme levels in the brain. The injury caused due to ischemic reperfusion caused the lowering of SOD, GSH, and CAT in IRG compared to the SG. GE-500 showed a marked elevation of the enzymes compared to GE-250, indicating that the *Gmelina* extract showed a dose-dependent activity around a 100% reduction in the antioxidant enzymes in the IRG group compared with SG. All the levels were normalized in GE-500 when compared to SG. A spike in the LPO levels was noticed in IRG compared to SG and extract-treated groups. LPO values hiked almost 100% in IRG compared to SG, indicating severe infarction due to BCCAO, but significantly lowered with the treatment with *Gmelina arborea* stem bark extract in both doses 250 and 500mg/kg.

Effect of *Gmelina Arborea* Stem Bark on Total Calcium and Na+/K+ATPase Activity

The results in Table 3 clearly show the elevation of total calcium levels in IRG due to injury in the brain. The damage caused a 3-fold calcium elevation compared to the expected values in the SG group. GE-500 showed a normalization of values indicating significant neuroprotective activity, while the SG group recorded a 2-fold decrease in Na+/K+ ATPase activity in the IRG, possibly due to severe injury in the brain. GE-250 and GE-500 activity significantly prevented the infarction caused by the ischemia. GE-500 similarly elevated the Na+/K+ ATPase activity in the SG group, as evident from Figure 4(A-F).
Histopathological findings

The microscopic sections of brain tissue of various groups are illustrated in Figure 5. Section of IRG shows an apparent neutrophil infiltration in the intracellular spaces of the tissue and increased vacuole size. The neutrophil activity was considerably low in GE-250 and GE-500 groups, suggesting that there were preventive or inhibitory mechanisms involved in the neuroprotective activity of gmelina in BCCAO rats.

Effect of *Gmelina Arborea* Stem Bark Extract on the Inflammatory and Apoptotic Mediators in the Rat Brain

The brain's inflammatory mediators and cell apoptosis factors were identified using western blot analysis. A significant presence of mediators like TNF-α, p38MAPK, and Barware was detected in the IRG group rats, and the amount of those factors detected in the SG group and the GE-500 were significantly less, as evident from the statistical analysis, too. The results have been illustrated in Figures 6 and 7. Cell survival mediator actin was detected in all the groups but with an insignificant variation. Cell apoptotic mediator Caspsase-3 was distinctive in IRG but negligible in SG and GE-500 groups. A significant amount of Bcl2 was detected in the SG group compared to IRG and GE-250. The highest amount of IL-10 in GE-500 was not detected in SG and IRG. Overall, the results were similar between SG and GE-500, except that the detection of TNF, P38, and IL-10 was more significant in GE-500 than in the SG group, and Bcl2 was highest in the SG group.
Discussion

Cerebral infarction or stroke is one of the significant causes of death and disability in patients worldwide [32]. Various stroke etiologies include ischemia/reperfusion injuries in the brain, cell and tissue necrosis, depolarization around the infarcted area, various inflammations, and oxidative stress [32, 33]. It is desirable to address all the etiologies of brain damage by limiting the number of drugs used and treating the stroke effectively without causing notable side effects, but achieving this is a real challenge [33]. As the herbal drugs are promising concerning the diversified mechanisms of action and lack of side effects, an extract of *Gmelina arborea* was selected based on the ethnomedicinal claims to investigate the neuroprotective activity on ischemia/reperfusion injury induced via BCCAO in rats.

Ischemic reperfusion injury resulted in a significant reduction in the neurological performance of the rats, as evident from the lowered neurological score and locomotor activity. Rats in IRG performed very poorly in all the tests, and the rats of GE-250 and GE-500 showed better performance. GE-500 almost normalized the neurological score compared to SG. A study has previously reported that brain damage caused a drop in neural activity [34], and our findings indicated a significant inhibition of the brain damage caused by BCCAO in *gmelina* extract-treated groups compared to IRG. TTC is a sensitive stain that converts the intracellular NAD and NADPH into a red pigment. The depth of the colour indicates the amount of regular cellular activity in any tissue and vice versa. The stain remains non-reactive and colourless with no NAD or NADPH, which indicates cell death or unviable cells. In our research, IRG showed pale and discoloured areas in the brain sections after TTC treatment, suggestive of marked cerebral infarction as a result of ischemia due to BCCAO. The cerebral cortex, sub-cortex, and hippocampus regions were affected due to reperfusion.
It is known that brain damage occurring through various mechanisms, such as damage due to oxidative free radicals, inflammations and cell apoptosis, ion imbalance, and necrosis, are most common and significant [35]. In this study, we elucidate the involvement of oxidation mechanisms of infarction. Results obtained suggest that brain damage due to ischemic reperfusion involves oxidative mechanisms. The elevation of LPO and fall in the levels of antioxidant mediators, SOD, CAT, and GSH indicate oxidative damage at the cellular level of brain tissue. A rise in the levels of LPO in IRG indicates the damage of neural cell membranes caused by lipid peroxidation [33]. Normalizing the levels in the extract-treated groups suggests that the extract effectively inhibited lipid peroxidation, thereby protecting the nerve cell integrity in brain tissue. The reduction in the SOD, CAT, and GSH in the IRG group suggests that their levels were exhausted to fight oxidative free radicals to protect the brain cells [36]. Catalase dismutase inhibits the OH free radical formation, and the low levels of SOD and CAT in the IRG group indicate a significant oxidative activity involved, which was prevented with the treatment of *Gmelina* extract. Decreased GSH level indicates neurodegenerative diseases caused by oxidative stress in the brain. The elevation of the antioxidant enzymes and lowering of the LPO by the extract suggests that *Gmelina* exhibited antioxidant mechanisms to protect the brain from infarction due to ischemia and reperfusion. Furthermore, GSH combats oxidative stress in a non-enzymatic manner by directly scavenging ROS [37], while SOD is a crucial component of the antioxidant metalloenzymes that provide protection [38]. Our findings are consistent with earlier reports (Kou et al., 2020; Morsy et al., 2022; Xu et al., 2019), where modulation of antioxidant GSH levels and SOD activity demonstrated a significant role of oxidative stress in the process of ischemia-reperfusion injury.

Due to BCCAO, there was a significant loss of blood flow to the hippocampus, cortical areas, and striatum of the brain. This might have caused a reversible loss of sodium and potassium ATPase...
pump, thus explaining the lack of proper nerve impulse transmission and energy balance in the neuron, and the alteration of neuromotor functions observed. This may also sometimes result in injury with a lowered ATP generation in the nerve cell, consequently lowering the glucose level and causing the intracellular accumulation of Na+ [40]. In addition, sodium (Na+) accumulation inside the cell also causes membrane depolarization, releasing neurotransmitters and producing glutamate, leading to the rise in the intracellular levels of calcium. All the above factors initiate the ageing of cells and, eventually, premature cell death [41]. The extract-treated groups showed a low Na+/K+ ATPase activity and low intracellular calcium level, inhibiting cellular apoptosis and necrosis. GE-500 effectively lowered the levels compared to GE-250, indicating that the extract showed a dose-based neuroprotective activity. Furthermore, our findings revealed the effect of *gmelina* extract to be dose-dependent. The GE-500 shown to be more effective by neurological score, locomotor activity, effect on the % infarction reducing antioxidant enzymes and induction of apoptotic mediators. The similar dose-dependent manner have been observed in the standardized GA stem bark aqueous extract protected against ADR-induced nephrotoxicity in animals in a dose-dependent manner [42], suggesting *gmelina* extract to be a useful chemotherapeutic agent.

p38 MAPK is responsible for cell apoptosis and triggers the release of cytokines responsible for the inflammation caused by neutrophils and macrophages [43]. The presence of p38 MAPK in the IRG showed an inflammatory activity that resulted in cell necrosis and damage, as evident from the histopathology section. Interestingly, very low IL-10 was seen in SG and IRG, indicating no anti-inflammatory mediators at the cellular level. Interleukins generally aid in preventing or lowering inflammation due to neutrophil infiltration of the tissue and maintain homeostasis [43]. IL-10 was seen prominently in the groups treated with the extract (GE-250, GE-500), indicating that the anti-inflammatory activity of *Gmelina* assisted in preventing infarction due to ischemia by stimulating
the release of IL-10. The absence of IL-10 in the SG group indicates no inflammatory activity in the tissue. However, IL-10 is a neuroprotective anti-inflammatory cytokine primarily produced by microglia, macrophages, and astrocytes in ischemia injury. It functions by inhibiting pro-inflammatory cytokines (IL-1β, TNF-α, and IFN-γ) and suppresses cytokine receptors’ expression and activation. Studies have shown that an increase or over-expression of IL-10 reduces brain injury and infarct size and enhances neurological outcomes in experimental stroke [44, 45].

The tumour necrosis factor (TNF-α) is a well-reported mediator during sterile inflammation and substantially exacerbates liver injury in ischemic reperfusion injury. Also, it is involved in the recruitment of neutrophils by increasing the expression of adhesion molecules and chemokines, which then release ROS and proteases to increase damage [6, 37]. The GE-500 showed a significantly lower TNF-α level than the SG group, indicating that Gmelina exhibited a higher protective effect at a high dose, demonstrating strong anti-inflammatory mechanisms in preventing the ischemic reperfusion injury caused by BCCAO. However, our findings on the effect of Gmelina on p38 MAPK and TNF-α agree with the studies of [40, 46, 47] where extract specific inhibitor of p38 MAPK offers protection against ischemia-reperfusion injury.

Caspase-3 is also one of those apoptotic mediators that lead to necrosis and death of cells. As the mechanism stated by earlier researchers, the release of caspase indicates the loss of cell wall integrity, protein destruction, and DNA fragmentation [48]. Similarly, Bax also causes cell death by destroying the mitochondrial matrix and depleting the energy resources in the cell. Higher levels of caspase and Bax were detected in IRG, which infers nerve cell damage due to the direct cellular destruction and denaturation of DNA and mitochondria. The extract-treated group did not detect the significant presence of caspase and Bax, suggesting the caspase inhibitory effect of Gmelina extract. Bcl2 is a neuroprotective mediator that inhibits the Bax and other apoptotic mediators and
protects the mitochondrial membranes from destruction by Bax. GE-500 showed a significant level of Bcl2 compared to the SG group, indicating the inhibition of apoptotic mediators, thereby preventing nerve cell damage. Our findings are consistent with earlier investigations on medicinal plants implication on members of apoptotic genes such as inhibiting Bax, caspases 3, 8, 9, and increasing Bcl-2 [6, 37, 49]. Therefore, the plausible Gmelina extract effect could be an apoptosis stimulation mechanism via elevation of ROS levels resulting in a change in the expression levels of Bcl-2 family genes that reduces cell viability in the ischemic reperfusion animal model. Consequently, the results of the present investigation showed that Gmelina could play a protective function in ischemic injury (BCCAO) induction by increasing mitochondrial activity, decreasing ROS generation, and preventing apoptosis and inflammation.

Conclusion
Our study evaluates the mechanisms of neuroprotective activity of Gmelina arborea on the BCCAO infarcted rats. The findings suggest significant neuroprotection via increasing the antioxidant enzymes in the brain tissues and a reduction in lipid peroxidation, as well as potential mechanisms for stroke prevention. Furthermore, the western blotting assay underscores Gmelina's involvement in anti-apoptotic and anti-inflammatory mechanisms in preventing infarction, which is clinically significance. Overall, the extract of Gmelina arborea inhibited inflammatory mediators and apoptosis factors, in addition to activating antioxidant mechanisms in a dose-dependent manner. Further studies will be needed to establish its potential role in stroke prevention and management.
**List of Abbreviations**


**Declarations**

**Ethics approval**

The study was approved by IAEC (IAEC/SVCOP/007/2020-21) and conducted according to the CPCSEA guidelines.

**Consent to Participate**

Not applicable

**Consent to Publication**

All the authors have read and agreed to the final copy of the finding as contained in the manuscript.

**Availability of data and materials**

The datasets/information used for this study are available on reasonable request.

**Conflicting interest**

All authors report that there was no conflict of interest in this work.

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Figure 1. Effect of *Gmelina Arborea* Stem Bark on the Neurological Score of Ischemia/Reperfused Rats

SG-Sham control group; IRG-ischemia reperfused group; GE-250-*Gmelina* extract 250mg/kg/day; GE-500-*Gmelina* extract 500mg/kg/day; All values were expressed as mean ± SD (n=9) *p<0.001 significant compared to SG group, +p<0.001 significant compared to IRG group.
Figure 2. Effect of *Gmelina Arborea* Stem Bark on the Locomotor Activity of Ischemia/Reperfused Rats
SG-Sham control group; IRG-ischemia reperfused group; GE-250-\textit{Gmelina} extract 250mg/kg/day; 
GE-500-\textit{Gmelina} extract 500mg/kg/day; All values were expressed as mean ± SD (n=9) *p< 0.001 significant compared to SG group, +p<0.001 significant compared to IRG group.
while the line b is the percentage of brain infarction.
Figure 3a & b. Effect of Gmelina Arborea Stem Bark on the Percentage Infarction of Ischemia/Reperfused Rats

A-Sham control group; B/IRG-ischemia reperfused group; C/GE-250-Gmelina extract 250mg/kg/day; D/GE-500-Gmelina extract 500mg/kg/day; All values were expressed as mean ± SD (n=9) *p< 0.001 significant compared to SG group, +p<0.001 significant compared to IRG group.
Figure 4. Effect of *Gmelina Arborea* Stem Back Extract on Antioxidant Enzymes, Excitotoxicity Mediator, and Na\(^+\)/K\(^+\)ATPase Activity in Brain Tissue Homogenate
A. Comparison of lipid peroxidation (LPO); B. Comparison of superoxide dismutase (SOD) level in; C. Comparison of reduced glutathione (GSH) level; D. Comparison of Catalase level; E. Comparison of total calcium; F. Comparison of Na+/K+ATPase activity.

SG-Sham control group; IRG-ischemia reperfused group; GE-250- *Gmelina* extract 250mg/kg/day; GE-500- *Gmelina* extract 500mg/kg/day; All values were expressed as mean±SD (n=9). ***p< 0.001 more significant, **p<0.001 significant, *p< 0.001 less significant compared to SG group, +++p<0.001 significant compared to IRG group.
Figure 5. Photographs of Histopathological Sections of the Brain of Various Groups of Ischemic/Reperfused Rats

SG-Sham control group; IRG-ischemia reperfused group; GE-250-*Gmelina* extract 250mg/kg/day; GE-500-*Gmelina* extract 500mg/kg/day.
Figure 6. Western Blot Analysis of Cell Apoptotic Mediators in the Brain Tissue Homogenate

A. Western blot analysis of various mediators in brain tissue homogenate; B. Representation of caspase-3 levels in various groups of rats; C. Representation of Bax level in various groups of rats; D. Representation of Bcl2 level in various groups of rats.

SG-Sham control group; IRG-ischemia reperfused group; GE-250-Gmelina extract 250mg/kg/day; GE-500-Gmelina extract 500mg/kg/day; All values were expressed as mean±SD (n=9) ***p< 0.001 more significant, *p<0.001 less significant compared to SG group, +++p<0.001 significant compared to IRG group.
Figure 7. Western Blot Analysis of Inflammatory and Anti-Inflammatory Mediators in the Brain Tissue Homogenate

A. Representation of TNF level in various groups of rats; B. Representation of p38MAPK level in various groups of rats; C. Representation of Actin level in various groups of rats; D. Representation of IL-10 level in various groups of rats

SG-Sham control group; IRG-ischemia reperfused group; GE-250-Gmelina extract 250mg/kg/day; GE-500-Gmelina extract 500mg/kg/day; All values were expressed as mean ± SD (n=9) ***p<0.001 significant compared to SG group, +++p<0.001 more significant ++p<0.001 less significant compared to IRG group.
Table 1. Effect of *Gmelina arborea* Stem Bark Extraction the BCCAO Ischemia/Reperfused Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Movement (count)</th>
<th>Swim test immobility (sec)</th>
<th>% infarction</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>204.55±3.283</td>
<td>43.55±3.005</td>
<td>--</td>
</tr>
<tr>
<td>IRG</td>
<td>95.00±3.240*</td>
<td>96.55±3.909*</td>
<td>72.36±3.240</td>
</tr>
<tr>
<td>GE-250</td>
<td>153.88±3.180**</td>
<td>64.77±3.492**</td>
<td>24.48±2.283*</td>
</tr>
<tr>
<td>GE-500</td>
<td>192.55±3.046**</td>
<td>42.88±3.408*</td>
<td>5.36±1.080*</td>
</tr>
</tbody>
</table>

SG-Sham control group; IRG-ischemia reperfused group; GE-250-*Gmelina* extract 250mg/kg/day; GE-500-*Gmelina* extract 500mg/kg/day; All values were expressed as mean ± SD (n=9) *p< 0.001 significant compared to SG group, +p<0.001 significant compared to IRG group.
Table 2. Effect of *Gmelina Arborea* Stem Bark Extract on the Antioxidant Enzyme Levels in the Rat Brain Tissue Homogenate

<table>
<thead>
<tr>
<th>GROUP</th>
<th>SOD (U/mg protein)</th>
<th>CAT (µM/min/mg)</th>
<th>GSH (µM of GSH/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>44.733±2.583</td>
<td>32.182±1.558</td>
<td>18.978±0.971</td>
</tr>
<tr>
<td>GE-250</td>
<td>25.748±2.615**</td>
<td>23.627±1.311**</td>
<td>12.408±1.514**</td>
</tr>
<tr>
<td>GE-500</td>
<td>41.102±2.292³+</td>
<td>29.167±0.622³+</td>
<td>18.023±0.850³+</td>
</tr>
</tbody>
</table>

SG-Sham control group; IRG-ischemia reperfused group; GE-250-*Gmelina* extract 250mg/kg/day; GE-500-*Gmelina* extract 500mg/kg/day; All values were expressed as mean ± SD (n=9) *p< 0.001 most significant, a significant, b less significant compared to SG group, +p<0.001 significant compared to IRG group.
Table 3. Effect of *Gmelina Arborea* Stem Back Extract on the Peroxidation, Excitotoxicity Mediator, and Na⁺/K⁺ATPase in Rat Brain Tissue Homogenate

<table>
<thead>
<tr>
<th>GROUP</th>
<th>LPO (nM/mg protein)</th>
<th>Total Calcium (µg/mg protein)</th>
<th>Na⁺/K⁺ATPase</th>
</tr>
</thead>
<tbody>
<tr>
<td>SG</td>
<td>10.080±1.097</td>
<td>8.885±0.843</td>
<td>8.485±0.785</td>
</tr>
<tr>
<td>IRG</td>
<td>32.890±0.842*</td>
<td>34.707±1.557*</td>
<td>3.122±0.460*</td>
</tr>
<tr>
<td>GE-250</td>
<td>23.452±1.178**</td>
<td>29.287±1.319**</td>
<td>4.885±0.716**</td>
</tr>
<tr>
<td>GE-500</td>
<td>12.737±1.126**</td>
<td>17.225±0.921**</td>
<td>7.212±0.878**</td>
</tr>
</tbody>
</table>

SG-Sham control group; IRG-ischemia reperfused group; GE-250-*Gmelina* extract 250mg/kg/day; GE-500-*Gmelina* extract 500mg/kg/day. All values were expressed as mean±SD(n=9) *p< 0.001 most significant, a*significant, b*less significant compared to SG group, p<0.001 significant compared to IRG group.