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Jobelyn® ameliorates neurological deficits in rats with ischemic stroke through inhibition of release of pro-inflammatory cytokines and NF-κB signaling pathway

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ABSTRACT

The effects of Jobelyn® (JB) on neurological deficits and biochemical alterations associated with ischemic stroke induced by bilateral common carotid artery occlusion (BCCAO) in rats were investigated in this study. Male Wistar rats were divided into five groups (n = 8): group 1 served as Sham control; group 2, which served as negative control received normal saline while groups 3–5 were given JB (25, 50 and 100 mg/kg, p.o) daily for 28 days. Then, rats in groups 2–5 were subjected to BCCAO for 30 min and reperfusion afterwards. Neurological deficits were assessed 3 h post-reperfusion using a 9-point neurological scoring scale. The levels of biomarkers of oxidative stress and pro-inflammatory cytokines (tumour necrotic factor-α and interleukin-6), expressions of immunopositive cells of nuclear factor-kappa B (NF-κB) and acetyl-cholinesterase (AChE) activity were determined in brain tissues. Histology of the striatum, prefrontal cortex (PFC) and hippocampus (CA1) was also evaluated. JB improved BCCAO-induced neurological deficits and attenuated increased oxidative stress and AChE activity in rats subjected to BCCAO ($p < 0.05$). Increased brain levels of tumour necrotic factor-α and interleukin-6 as well as expressions of immunopositive cells of NF-κB were decreased by JB. JB reduced brain damage and also increased population of viable neurons in the striatum, PFC and hippocampus of ischemic stroke rats. These findings suggest that the positive effect of JB on neurological function in rats with ischemic stroke may be related to inhibition of oxidative stress, release of pro-inflammatory cytokines and expressions of immunopositive cells of NF-κB.

1. Introduction

Ischemic stroke is a fatal disease caused by sudden obstruction of cerebral blood flow with subsequent neuronal cell death or tissue necrosis [1,2]. Occlusion of the blood vessels (carotid arteries) that supply blood to the brain and subsequent reperfusion are the critical factors involved in the pathology of ischemic stroke [3–5]. Stroke is the second leading cause of death and disability worldwide [6]. According to World Health Organization [WHO], 15 million people suffer stroke worldwide each year, with over 6 million dying and more than half of the survivors being permanently disabled [7]. The death rate due to the disease in most African countries is quite alarming resulting in huge loss of economic manpower and productivity [8,9]. Moreover, stroke is typically associated with neurological deficits and the belief that the disease is incurable even contributes to a wide range of psychiatric disturbances such as anxiety, depression and memory deficits [10].

Over the past few decades, the understanding of the pathophysiology of stroke has increased but little progress has been made in the development of effective drugs for treatment of this debilitating disease [4; 7]. Current approach to the management of stroke focuses on the amelioration of symptoms as cure for the disease still remains elusive [4; 7]. The clinical efficacy of tissue plasminogen activators currently used for stroke has been compromised by serious adverse effects [11,12]. Also, the need to administer them as quick as possible, at least within 3 h after onset of symptoms of the disease makes it almost unrealistic for patients in most African communities to benefit from these drugs [11,12]. Moreover, the

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lack of accessibility to cost effective treatment for stroke is the major reason for the increased fatality and high burden of the disease in developing countries like most African nations [13; 8].

Ischemic stroke accounts for over 85% of all cases of stroke and its pathology is known to be due to activation of oxidative and inflammatory pathways in the brain [7; 14]. Both preclinical and clinical studies have revealed increased biomarkers of oxidative stress and inflammatory cytokines after ischemic stroke [15–16; 7]. Interleukin-6 (IL-6), interleukin-1 (IL-1) and tumor necrosis factor-alpha (TNF-α) are some of the most studied cytokines in stroke pathology [13; 17–19]. In stroke patients, IL-6 has been linked to early neurological deterioration, greater infarct volumes and poorer long-term outcomes [7,16]. High plasma level of TNF-α has also been correlated with infarct volume and neurological deficits in various models of cerebral ischemia [7,14,16]. Also, during reperfusion, there is an increase in serum steroids, which further exacerbates neuronal damage by disrupting glucose homeostasis and increasing oxidative stress in the brain [20,21]. Moreover, increased oxidative stress and leukocyte infiltrations result in the formation of more pro-inflammatory cytokines, which perpetuate neurodegeneration in the brains of animals with ischemic stroke [22–23; 21]. On this basis, the current approach to the treatment of the disease using thrombolytic agents is quite limited in scope as they cannot antagonize the injurious oxidative and inflammatory events that underpin ischemic stroke [7; 24–25]. Thus, oxidative and neuroinflammatory pathways are currently being viewed as promising targets for development of new drugs that could be used to antagonize the multiple mechanisms and mediators involved in ischemic brain injury, which constitute potential goal of neuroprotection [26–27; 24; 7]. Indeed, there are several evidences that have shown that some phytochemicals have the ability to target multiple pathways involved in the pathophysiology of stroke including oxidative stress, inflammation and apoptotic cell death [21]. Moreover, there are various epidemiological data in literature, which show that regular consumption of fruits and vegetables rich in phytochemicals reduce the risk of stroke [21; 28]. Thus, bioactive compounds of plant origin with potent antioxidant and anti-neuroinflammatory activities hold promising strategy for treatment of stroke [13; 21].

Jobelyn® (JB) is a dietary supplement obtained from the polyphenol-rich leaf sheath of *Sorghum bicolor (L) Moench (Poaceae)*. *Sorghum bicolor* is commonly known as millet and has been used in African traditional medicine for treatment of several diseases over hundred years ago [29,30]. JB is manufactured by Health Forever Products Ltd, Lagos, Nigeria and is available as capsules in most pharmaceutical outlets in Nigeria and abroad including United States of America [30]. JB has gained high reputation as a remedy for treatment of moderate to severe anaemia [31]. The manufacturer also claimed that the dietary supplement is beneficial in neurological disorders such as psychosis, stroke and convulsions. It is also known to boost the immune system thereby enhancing the body's defensive mechanisms in response to stress, infections or debilitating diseases [31; 30]. The manufacturer recommends 1 or 2 capsules, 1–3 times per day for the treatment of anaemia [30]. However, the manufacturer also recommends higher doses of JB for chronic diseases like cancer, arthritis and stroke. The major active phytochemical constituents found in JB and their mechanisms of action in neurological disorders are presented in Table 1. Specifically, most of these phytochemicals have been shown to demonstrate antioxidant/neuroprotective and anti-neuroinflammatory effects [33–38]. Luteolin is one of the most studied phytochemicals, which has been reported to protect rat brain against focal ischemia via several mechanisms including inhibition of NF-κB signalling pathway [34,35]. Meanwhile, previous studies carried out in culture cells showed that JB inhibited infiltrations of inflammatory cells, release of inflammatory mediators and formation of free radicals [32]. Recently, JB was reported to attenuate inflammatory responses and neurobehavioural deficits associated with complete Freund-adjuvant-induced arthritis via inhibition of pro-inflammatory cytokines in mouse brain [30]. However, no studies have been carried out to ascertain its potential usefulness in stroke disorder. This study was therefore carried out to evaluate its effect on ischemic stroke using bilateral common carotid artery occlusion model in rats; which closely mimic the clinical and biochemical conditions relevant to the pathology of ischemic stroke in humans.

2. Materials and methods

2.1. Experimental animals

Male Wistar rats (250–300 g) used for this study were obtained from the Central Animal House, College of Medicine, University of Ibadan, Ibadan, Nigeria. The animals were housed in plastic cages (42 × 30 × 27 cm) in standard conditions. They were fed with standard rodent pellet food and water ad libitum throughout the experimental period. However, food but not water, was withdrawn 12 h prior to surgical procedures. They were acclimatized for 1 week prior to commencement of the experiments, thereafter; each animal was randomly distributed according to the experimental design. All procedures in this study were performed in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85-23, revised 1985).

2.2. Drugs and chemicals

JB (Health Forever Products Ltd., Lagos, Nigeria), ketamine hydrochloride (Sigma-Aldrich, St. Louis, MO, USA), thiobarbituric acid (Sigma–Aldrich, St. Louis, MO, USA), trichloroacetic acid (TCA) (Burgoyne Burbidges and Co., Mumbai, India), tris-potassium chloride (Sigma-Aldrich, St. Louis, MO, USA), acetic acid (Sigma-Aldrich, St. Louis, MO, USA), sodium bicarbonate (BDH Chemicals Ltd., Poole, England), sodium carbonate (Fisons, Loughborough Leics, England), adrenaline (Sigma-Aldrich, St. Louis, MO, USA) and 5′, 5′-Dithiobis-(2-nitrobenzoate- DTNB (Sigma-Aldrich, St. Louis, MO, USA) were used in the study.

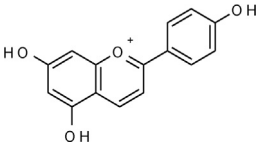
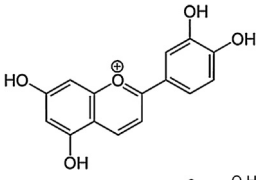
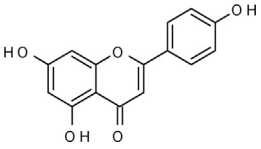
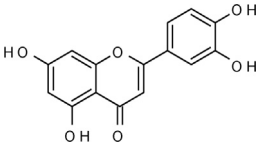
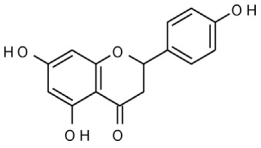
2.3. Drug preparations and treatments

JB and other chemicals used in this study were dissolved in normal saline immediately before use. JB was administered per oral (p.o.) at different doses (25, 50 and 100 mg/kg), which were chosen based on the results obtained from previous investigations [30].

2.4. Induction of ischemic stroke in rats

The male Wistar rats were distributed randomly into five groups (n = 8): group 1 served as Sham control; group 2, which served as negative control received normal saline (10 mL/kg, p.o.) while groups 3–5 received JB (25, 50, 100 mg/kg) daily for 28 days. On day 28 post-treatment, ischemic stroke was induced by bilateral common carotid artery occlusion (BCCAO) as previously described [39]. The rats were anaesthetized with ketamine (50 mg/kg, i.p.) and diazepam (2.5 mg/kg, i.p). Through midline incision of the neck, both common carotid arteries were carefully dissected away from the accompanying vago-sympathetic trunk and ischemia was induced by occluding the common carotid arteries with 4-0 suture for 30 min. Thereafter, the sutures were removed to initiate reperfusion and plasticity of arteries was confirmed by visual inspection. After 5 min reperfusion, the incisions were sutured. Temperature was maintained at 37 ± 0.5 °C throughout the surgical procedure. Upon recovery of the righting reflex, the rats were placed in a

Table 1
Major phytochemical constituents found in Jobelyn(R).

Phytochemicals	Chemical structure	Abundance(μg/g)	Molecular Weight (g)	Mechanism (s) of action
Apigeninidin		39,900	255.24	Antioxidant and anti-inflammatory effect [29,32]
Luteolinidin		450	271.24	Inhibition of NOS and ROS [32,37]
Apigenin		6910	270.24	Enhances adult neurogenesis via generation of neuronal cells, inhibition of oxidative stress and pro-inflammatory cytokines [32,37,38]
Luteolin		570	286.24	Inhibition of oxidative stress, release of interleukin-6, tumor necrosis factor-alpha, cyclooxygenase-2 and nuclear factor-kappa B. Enhances neuroprotection and expression of BDNF [32,34,35,38]
Naringenin		130	272.26	Elevation of brain levels of dopamine, serotonin, noradrenaline and BDNF. Inhibition of oxidative stress and release of pro-inflammatory cytokines [32,33,36]

warm chamber for 3 h before evaluation of neurological functions. However, rats in group 1, which served as the Sham control, were subjected to the same surgical procedures but their arteries were not occluded.

2.5. Assessments of neurological functions

The neurological deficits were assessed 3 h post-reperfusion by a blinder to the identity of treatment groups using a 9-point neurological function scoring scale based on detection of abnormal posture and hemiplegia as previously described [40,41]. Briefly, rat was suspended by the tail 10 cm above the bench top or home cage floor for 5 s and observed for any twisting of the thorax, defined by the ability of the rat to move up towards its tail. In normal rats with no deficit; no twisting will be observed and the rat will reach for the ground (score 0). In rats with damage to their striatum or cortex, there may be a wobble to contralateral side (score 1), some twisting up towards the tail (score 2), or twisting all the way up to touch the tail (score 3). In addition to twisting, forelimb flexion was also evaluated. A normal rat will extend both forepaws towards the ground as if reaching for it (score 0). After damage to the brain as described above [41], the contralateral forepaw may not reach to the ground but flex to the contralateral side. Slight flexion = 1, 45° flexion = 2 and pronounced 90° flexion = 3). The walk beam test was also used to assess the neurological function by placing the animal on the narrow beam (3 cm wide x 70 cm long) raised to about 20 cm above the bench. Limb paralysis was scored by inability to grip and keep all limbs on the beam. Loss of grip and occasional slipping = 1), no grip and limb resting over edge of beam = 2), no grip and limb dangling from beam with an inability to move along the beam = 3. Thereafter, total neurological deficit scores were obtained from the above tests by adding all scores using a 9-point neurological scoring scale [40,41].

2.6. Preparations of brain tissues for biochemical studies

Rats were euthanized under ether anaesthesia immediately after assessment of neurological functions, and the brains were rapidly removed and weighed. Thereafter, the whole brain of each rat was homogenized with 5 mL of 10% w/v phosphate buffer (0.1 M, pH7.4). The brain tissue homogenates were centrifuged at 10,000 rpm for 10 min at 4 °C. The pellets were discarded and the supernatant was immediately separated into different portions for various biochemical studies.

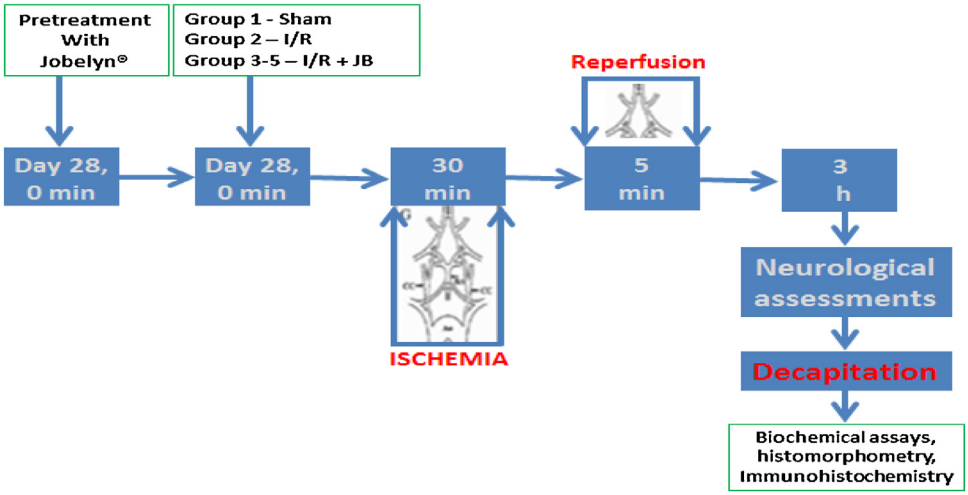


Fig. 1. Procedural induction of ischemic stroke in rats.

2.6.1. Estimation of reduced Glutathione (GSH) concentration

The concentration of GSH in the brain tissue supernatant of rats in each group was determined using the method of Moron et al., [42], which is based upon the development of a relatively stable (yellow) colour when 5′, 5′–dithiobis-(2-nitrobenzoic acid) (DTNB) is added to sulfhydryl compounds. The chromophoric product resulting from the reaction of DTNB with the reduced glutathione, 2-nitro-5-thiobenzoic acid possesses a characteristic absorbance at 412 nm and the amount of reduced glutathione in the sample is proportional to the absorbance at this wavelength. In brief, brain supernatant of 0.4 mL was added to 0.4 mL of 20% trichloroacetic acid (TCA) and mixed by a gentle swirling motion. This was centrifuged at 10,000 rpm for 20 min at 4 °C using cold centrifuge). The supernatant (0.25 mL) was added to 2 mL of 0.6 mM DTNB and the final volume of the solution was made up to 3 mL with 0.75 mL phosphate buffer (0.2 M, pH 8.0). Absorbance was then read at 412 nm against blank reagent [2 mL of 0.6 mM DTNB + 1 mL phosphate buffer (0.2 M, pH 8.0)] using a spectrophotometer. The concentration of reduced GSH in the brain tissues were expressed as micromoles per gram tissue (μmol/g tissue).

2.6.2. Determination of catalase (CAT) activity

Catalase activity in the brain tissue homogenate of rats in each group was determined using the spectrophotometric method described by Sinha [43]. The method is based on measuring the absorbance of the stable yellow complex formed by hydrogen peroxide and ammonium molybdate at 410 nm. Briefly, brain supernatant (0.1 mL) of each treatment group was added to 1 mL substrate (65 μmole/mL hydrogen peroxide in μmol/L sodium potassium phosphate buffer, pH 7.4) and incubated at 37 °C for 30 min. Thereafter, the reaction was stopped by adding 2 mL of ammonium molybdate to the reaction mixture. The absorbance of the yellow complex formed by ammonium molybdate and hydrogen peroxide was measured at 410 nm against the blank using spectrophotometer. The catalase activity was expressed as μmoles of H_2O_2 decomposed per min per mg protein (Unit/mg protein).

2.6.3. Estimation of concentration of malondialdehyde (MDA)

The MDA levels, an index of lipid peroxidation in the brain tissues of rats in each group were determined as previously described [44]. This assay is based on the fact that lipid peroxidation produces polyunsaturated fatty acid peroxides, which generates MDA upon decomposition. The MDA forms a 1:2 adduct with thiobarbituric acid (TBA), which gives rise to a pink colour product when heated in acidic pH, with a maximum absorbance of 532 nm. An aliquot of 0.4 mL of the sample was mixed with 1.6 mL of Tris-potassium chloride (Tris-KCl) buffer to which 0.5 mL of 30% trichloroacetic acid (TCA) was added. Then, 0.5 mL of 0.75% TBA was added and placed in a water bath for 45 min at 80 °C. This was then cooled in ice and centrifuged at 3000 rpm for 15 min. The clear supernatant was collected and absorbance measured against a reference blank of distilled water at 532 nm. The concentration of MDA was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} CM^{-1} and the value was expressed as μmole of MDA per gram tissue.

2.6.4. Estimation of acetyl-cholinesterase (AChE) enzyme activity

Acetyl-cholinesterase enzyme activity, which served as a biomarker of cholinergic function, was measured as previously described [45]. Briefly, aliquots of supernatant (0.4 mL) of the brain tissue was added to 2.6 mL of phosphate buffer (0.1 M, pH 7.4) followed by 0.1 mL of DTNB. Then, 0.1 mL of acetylthiocholine iodide was added to the reaction mixture. The absorbance was then read using a spectrophotometer at a wavelength of 412 nm and the change in absorbance for 10 min at 2 min interval was recorded. The rate of AChE activity was measured following the increase in colour produced from thiocholine when it reacts with DTNB. The change in absorbance per minute was determined and the rate of AChE activity was calculated and expressed as μmol/min/mg protein.

2.6.5. Determination of protein content

The protein content of all treatment groups was determined as described by Gornall et al., [46]. The method is based on the fact that proteins form a colored complex with cupric ions in an alkaline solution like Biuret reagent containing cupper sulphate, potassium iodide and sodium potassium tartarate. The protein and Biuret reagent form complexes with a maximum absorbance at 540 nm. Using the Biuret method, the brain supernatant of 0.4 mL was diluted 10 times with 4 mL of distilled water. This was done to reduce the level of protein in the samples to the sensitivity range of Biuret method. Then, 1 mL of the diluted sample was added to 3 mL of Biuret reagent in triplicate. The mixture was incubated at room temperature for 30 min after which the absorbance was read at 540 nm using distilled water as blank. The protein content of the samples was extrapolated from the standard curve and multiplied by 10 to get the actual amount in the fraction.

2.6.6. Estimation of brain concentrations of tumour necrosis factor-alpha (TNF-α) and interleukin-6 (IL-6)

The brain concentrations of TNF-α and IL-6 were estimated in the supernatant of the brain tissues using ELISA kits according to the

manufacturer's instructions. The absorbance was read at 450 nm using Spectramax M-5 (Molecular Devices, Sunnyvale, CA) multi-functional plate reader equipped with Softmax Pro v 5.4 (SMP 5.4), and a log-log logistic curve-fit was used to determine the unknown sample concentrations in pg/mL.

2.6.7. Preparation of brain tissues for histology

Rats (n = 3) in the respective groups were anaesthetized with ether and perfused transcardially with sterile phosphate buffered saline. The rats were then dissected, flushed with normal saline and perfused with 10% buffered formaldehyde. Thereafter, their brains were harvested, and fixed with 10% phosphate buffered formaldehyde.

2.6.8. Histological studies and Histomorphometry

The brain sections were processed through the stages of fixation, dehydration, clearing, infiltration and embedding in paraffin wax. Coronal sections of the tissue blocks were cut (5–6 μm), fixed on glass slides and subsequently stained using haematoxylin and eosin to demonstrate general histology of the prefrontal cortex and hippocampus (CA1) regions [47]. The slides were then viewed using Leica DM 500 digital light microscope (Germany) and images captured with Leica ICC50 E digital camera (Germany) connected to a computer interface (Magna Fire). Histomorphometric analyses were done using computerized image analyzer (Apache Open Office Tm4 4.0.0. software version). Using an objective lens (x 40) and an ocular lens (x 10), the viable and pyknotic neurons of the prefrontal cerebral cortex, hippocampus (CA1) and striatum of the brains were observed and counted as oviod cytoplasmic membrane-intact cells, without any nuclear condensation or pyknotic spot. Rat brain atlas [48] with an anatomical explanation was used. The density of viable neurons was calculated in ten different areas of the slides of the striatum, PFC and CA1 region of the hippocampus in each experimental group according to the method described by Taveira et al., [49]. Viable neuronal cells were defined as round-shaped, cytoplasmic membrane-intact cells, without any nuclear condensation or distorted aspect.

2.6.9. Imuunohistochemistry

Mice brains in the respective groups for immunohistochemistry were anaesthetized with ether and perfused transcardially with sterile phosphate buffered saline (PBS) and later with 10% buffered formaldehyde. Thereafter, brains were rapidly harvested, and fixed with 10% phosphate buffered formaldehyde at 25 °C for 48 h. The hippocampus [cornus ammonis 1 (CA1)] of the brains was then subjected to the routine method for paraffin wax embedment to get paraffin wax embedded tissue blocks. The expressions of nuclear transcription factor-kappa B (NF-κB) immunopositive cells of the hippocampus of mice brains exposed to BCCAO were determined using immunohistochemistry kit (Santa cruz, Germany) according to the manufacturer's protocol and modified method of Edelstein et al. [50]. Images were revealed using Leica ICC50 E Digital Camera (Germany) that was connected to a computer interface (MagnaFire) and an Olympus BX-51 Binocular research microscope. Expression of immunopositive cells were analyzed using Image *J* software (NIH, Bethesda, MD, USA) [50].

2.7. Statistical analysis

Data were expressed as Mean ± S.E.M. (standard error of mean). The data were analyzed using Graph Pad Prism software version 5. Statistical analysis was done using Kruskal–Wallis test (Non-parametric) and One-way analysis of variance (ANOVA) followed by post–hoc test (Newman-Keul) for multiple comparisons where appropriate. P-value less than 0.05 was considered as statistically significant.

Fig. 2. Effect of Jobelyn® (JB) on neurological score in bilateral common carotid arteries occlusion-induced ischemic stroke in rats. Each column represents the Mean ± SEM (n = 8). #p < 0.05 compared with Sham control group and *p < 0.05 compared with negative control group (ANOVA followed by Newman-Keuls test).

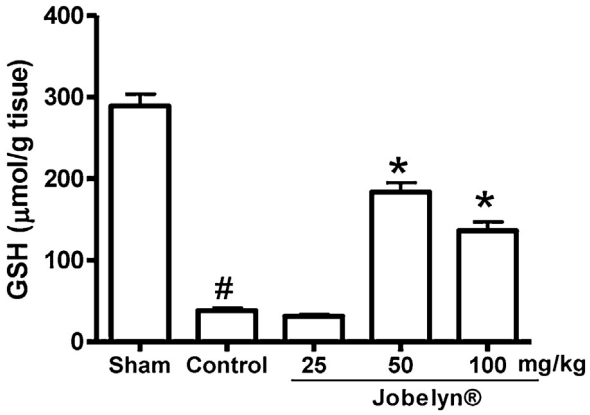


Fig. 3. Effect of Jobelyn® on glutathione (GSH) levels in brain tissue of rats subjected to bilateral common carotid arteries occlusion-induced ischemic stroke in rats. Each column represents the Mean ± SEM (n = 8). #p < 0.05 compared with Sham control group and *p < 0.05 compared with negative control group (ANOVA followed by Newman-Keuls test).

3. Results

3.1. Effect of Jobelyn® on neurological score in BCCAO-induced ischemic stroke

The effect of JB on neurological deficit in BCCAO-induced ischemic stroke in rats is presented in Fig. 1. As shown in Fig. 1, rats subjected to ischemic stroke had increased neurological score deficits when compared with the Sham operated group ($p < 0.05$). However, treatment with JB (25, 50 and 100 mg/kg, p.o.) significantly ($p < 0.05$) improved neurological deficits induced by ischemic stroke in rats (Fig. 2).

3.2. Jobelyn® reduces oxidative stress in brains of rats with ischemic stroke

Figs. 3, 4 and 5 showed the effects of JB on increased oxidative stress in brains of rats subjected to BCCAO-induced ischemic stroke. One-way ANOVA revealed that there were significant differences between treatment groups: GSH [$F(4, 25) = 118.1$, $p < 0.001$] (Fig. 3), catalase [$F(5, 30) = 13.70$, $p < 0.001$] (Fig. 4) and MDA [$F(4, 25) = 29.04$, $p < 0.001$] (Fig. 5). Post-hoc analysis by Newman-keuls test revealed that rats with ischemic stroke had marked increase in

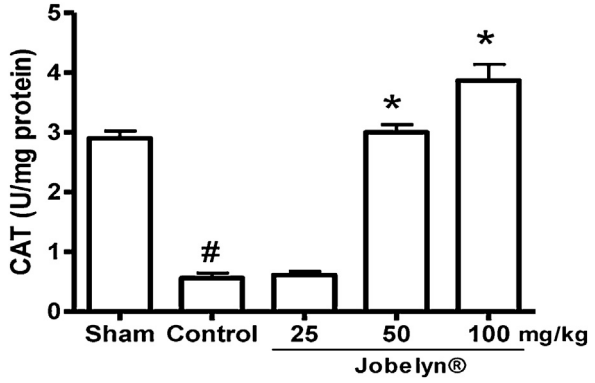


Fig. 4. Effect of Jobelyn® on catalase (CAT) levels in brain tissue of rats subjected to bilateral common carotid arteries occlusion-induced ischemic stroke in rats. Each column represents the Mean ± SEM (n = 8). #p < 0.05 compared with Sham control group and *p < 0.05 compared with negative control group (ANOVA followed by Newman-Keuls test).

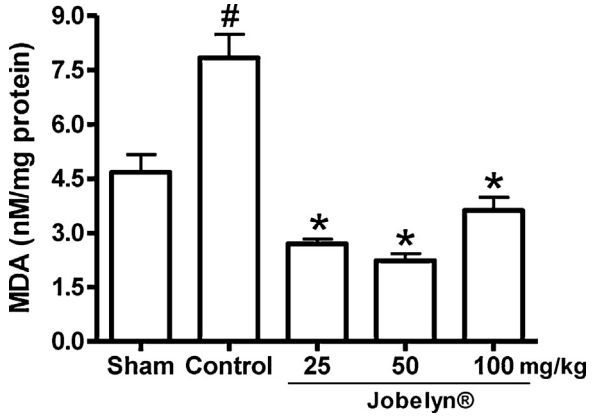


Fig. 5. Effect of Jobelyn® on malondialdehyde (MDA) levels in brain tissue of rats subjected to bilateral common carotid arteries occlusion-induced ischemic stroke in rats. Each column represents the Mean ± SEM (n = 8). #p < 0.05 compared with Sham control group and *p < 0.05 compared with negative control group (ANOVA followed by Newman-Keuls test).

oxidative stress as shown by elevated brain levels of MDA accompanied by reduced GSH content and catalase activity when compared with Sham operated group ($p < 0.05$). As shown in Figs. 3–5, JB (50 and 100 mg/kg, p.o) significantly ($p < 0.05$) reduced MDA and increased antioxidant molecules (GSH and catalase) in the brains of rats with ischemic stroke. However, JB (25 mg/kg, p.o.) did not significantly ($p > 0.05$) alters the level of GSH and activity of catalase in BCCAO-ischemic stroke in rats (Figs. 4 and 5).

3.3. *Jobelyn® inhibits acetyl-cholinesterase activity in brains of rats with ischemic stroke*

Fig. 6 showed the effect of JB on AChE activity in the brains of rats subjected to BCCAO-induced ischemic stroke. One-way ANOVA revealed that there were significant differences between treatment groups [$F(4, 25) = 30.68$, $P < 0.001$]. However, the increased AChE activity in the brains of rats with ischemic stroke was significantly ($p < 0.05$) reduced by JB (25, 50 and 100 mg/kg, p.o.).

3.4. *Jobelyn® decreases the brain levels of interleukin-6 and tumor necrosis factor-α in rats subjected to ischemic stroke*

The effects of JB on the brain levels of interleukin-6 and tumor necrosis factor-α in rat subjected to ischemic stroke are presented in Figs. 7 and 8. One-way ANOVA revealed that there were signifi-

Fig. 6. Effect of Jobelyn® on acetylcholinsterase (AChE) activity in brain tissue of rats subjected to bilateral common carotid arteries occlusion-induced ischemic stroke in rats. Each column represents the Mean ± SEM (n = 8). #p < 0.05 compared with Sham control group and *p < 0.05 compared with negative control group (ANOVA followed by Newman-Keuls test).

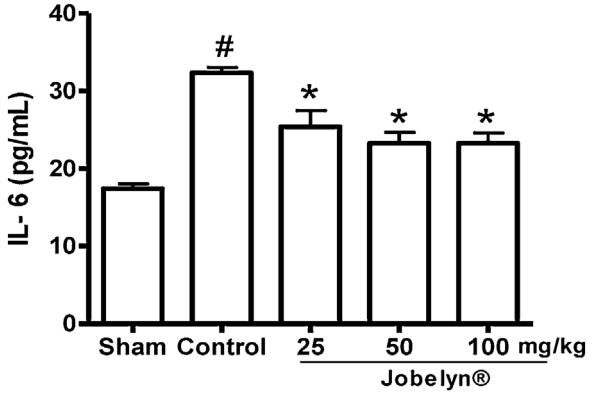


Fig. 7. Effect of Jobelyn® on interleukin-6 (IL-6) concentrations in brain tissue of rats subjected to bilateral common carotid arteries occlusion-induced ischemic stroke in rats. Each column represents the Mean ± SEM (n = 8). #p < 0.05 compared with Sham control group and *p < 0.05 compared with negative control group (ANOVA followed by Newman-Keuls test).

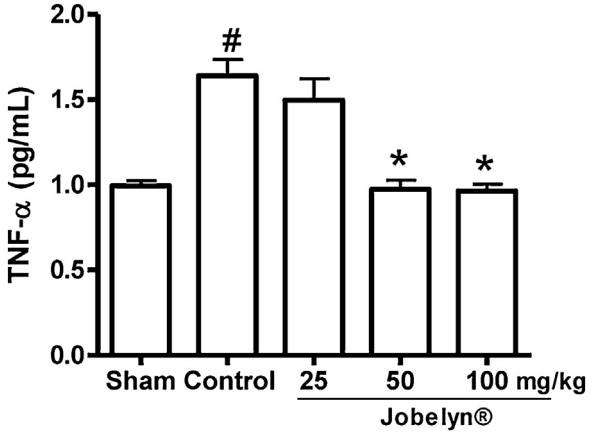


Fig. 8. Effect of Jobelyn® on tumor necrosis factor- α (TNF-α) concentrations in brain tissue of rats subjected to bilateral common carotid arteries occlusion-induced ischemic stroke in rats. Each column represents the Mean ± SEM (n = 8). #p < 0.05 compared with Sham control group and *p < 0.05 compared with negative control group (ANOVA followed by Newman-Keuls test).

cant differences between treatment groups: IL-6 [$F(4, 25) = 16.59$, $P < 0.001$] (Fig. 7) and TNF-α [$F(4, 25) = 17.62$, $p < 0.001$] (Fig. 8). As shown in Figs. 7 and 8, rats with ischemic stroke had significant ($p < 0.05$) increases in the brain concentrations of IL-6 and TNF-α when compared with Sham operated group. However, treatment with JB (50 and 100 mg/kg, p.o.) significantly ($p < 0.05$) attenuated the increases in the brain levels of these cytokines in rats subjected to BCCAO-induced ischemic stroke (Figs. 7 and 8).

3.5. *Effect of Jobelyn® (JB) on BCCAO/reperfusion-induced alteration on striatal neurons of rat brain*

The effects of JB on the cytoarchitecture of the brain tissues of rats subjected to bilateral common carotid artery occlusion-induced ischemic stroke are presented in Fig. 9. H&E staining revealed that there was a significant changes in the cytoarchitectural compositions of the striatum; as revealed by the presence of perivascular oedema and decreased population of viable neuronal cells in the striatum when compared with Sham operated group. However, treatment with JB (25 and50 mg/kg, p.o.) reduces histomorphological alterations induced by ischemic stroke in the striatum. As shown in Fig. 9, JB (50 mg/kg, p.o.) significantly ($p < 0.05$) increased the population of viable neuronal cells in the striatum of rats subjected to BCCAO-induced ischemic stroke.

3.6. *Effect of Jobelyn® (JB) on BCCAO/reperfusion-induced alteration on prefrontal cortex neurons of rat brains*

Fig. 10 showed the effect of JB on the histological and histomorphological changes in the PFC of rats subjected to BCCAO-induced ischemic stroke. H&E staining showed diffused necrosis and loss of pyramidal cells, moderate perivascular oedema and gliosis in the PFC of rats with ischemic stroke. Rats with ischemic stroke also had decreased population of viable neuronal cells in the prefrontal cortex when compared with Sham operated group. However, pretreatments with JB (25, 50 and 100 mg/kg, p.o.) reduced these histomorphological alterations due to exposure to BCCAO/reperfusion in the PFC (Fig. 10). Furthermore, JB (25, 50 and 100 mg/kg, p.o.) significantly ($p < 0.05$) increased the density of viable neuronal cells in a dose-dependent manner when compared with ischemic stroke group (Fig. 10).

3.7. *Effect of Jobelyn® (JB) on BCCAO/reperfusion-induced alteration on hippocampus neurons of rat brains*

The effect of JB on histological and histomorphological changes in the hippocampus of rats subjected to BCCAO-induced ischemia/reperfusion injury is presented in Fig. 11. The H&E staining revealed that BCCAO-induced ischemic stroke caused a significant histological and histomorphological changes in the hippocampus as shown by moderate ischemic necrosis of the neurons, partial loss of nuclei staining and astrocytic response (Fig. 11). There was also a significant decrease in the population of viable neuronal cells in rats with ischemic stroke relative to the Sham operated group. The Sham operated group showed normal hippocampal neurons with predominantly bluish stained nuclei and some blood capillaries containing red cells. As shown in the densitometry count (Fig. 11), the lowest dose of JB (25 mg/kg, p.o.) protected the hippocampal neurons and increased the density of viable neuronal cells in rats subjected to BCCAO/reperfusion.

3.8. *Jobelyn® reduces expression of immunopositive cells of NF-κB*

The photomicrographs of the effect of JB on BCCAO/Reperfusion-induced immunohistochemical changes and expressions of NF-kB immunopositive cells in the hippocampus of rats are presented in Fig. 12. As shown in Fig. 12, rats with ischemic stroke had significant ($p < 0.05$) increased expressions of immunopositive cells of NF-κB [$F(4, 14) = 13.47$, $P < 0.001$] when compared with Sham operated group. However, treatment with JB (25, 50 and 100 mg/kg, p.o.) significantly ($p < 0.05$) attenuated increased expressions of immunopositive cells of NF-κB in the hippocampal region of the brain of rats subjected to ischemic stroke (Fig. 12).

4. Discussion

The results of this study showed that occlusion of the bilateral common carotid artery produced neurological deficits in rats that resemble the clinical features often seen in patients with ischemic stroke [16,39]. However, JB improved the neurological deficits and also reduced oxidative stress as indicated by increases in endogenous antioxidant molecules (GSH and catalase) and decreased MDA levels in the brains of rats subjected to ischemic stroke induced by BCCAO. JB also inhibited the release of pro-inflammatory cytokines (IL-6 and TNF-α) and AChE activity in the brain of rat with ischemic stroke. The marked increased expression of immunopositive cells of NF-κB in rats with ischemic stroke was significantly suppressed by JB. Moreover, oral treatment with JB protected the neurons of the striatum, PFC and hippocampus, and also increased the population of viable neurons in these brain regions of ischemic rats.

Ischemic stroke occurs whenever there is a transient or permanent occlusion of the blood vessels that supply blood to the brain, which often results in tissue necrosis or neuronal cell death [2]. Although blood supply is needed in other parts of the body, the brain in particular is known to be more vulnerable to arterial occlusion because of its high metabolic rate and oxygen demand [51,52]. The brain is one of the first organs to suffer from reduced blood flow, and dies quickly when deprived of adequate oxygen. The functioning of the brain is immediately impaired followed by irreversible damage if the supply of oxygen is interrupted by reduced blood flow. Thus, the function of brain cells depends essentially on adequate cerebral blood flow [51,52]. Specifically, the damages done to the cerebral cortex, the cerebellum and the basal ganglia by ischemic stroke are known to underscore the widespread neurological deficits and long-term disabilities that often characterized the disease [50,53]. Cognitive dysfunction, anxiety, depression, abnormal movements (including tremor, spasticity or rigidity), motor inco-ordination and postural instability are some of the common manifestations of ischemic stroke in clinical settings [26; 16, 54]. The loss of neurological function is the genesis of the poor quality of life of persons recovering from ischemic stroke and reoccurrence of stroke episodes [26,55]. The finding that JB ameliorates neurological deficits in rats subjected to BCCAO-induced ischemic stroke further support its acclaimed usefulness in ethnomedicine for the management of stroke.

Ischemic stroke is one of the leading causes of vascular dementia across the globe and loss of cognition is a major factor that has been shown to affect the quality of life of patients with the disease [26; 16, 54–55]. Thus, the brain activity of AChE, a major biomarker of cholinergic function was measured in this study. Our data showed that animals subjected to ischemic stroke in BCCAO model, had increased brain activity of AChE, the enzyme responsible for the degradation of ACh. Previous studies have established that a decreased synaptic concentration of ACh plays a crucial role in cognitive dysfunctions in stroke [26,55]. The reduced synaptic level of ACh, which exemplify impaired central cholinergic function, has been ascribed majorly to increased AChE activity [55,56]. Moreover, decreased ACh concentrations due to increased AChE enzyme activity have been reported in the cerebral tissues of patients with ischemic stroke [26] and in rats subjected to BCCAO

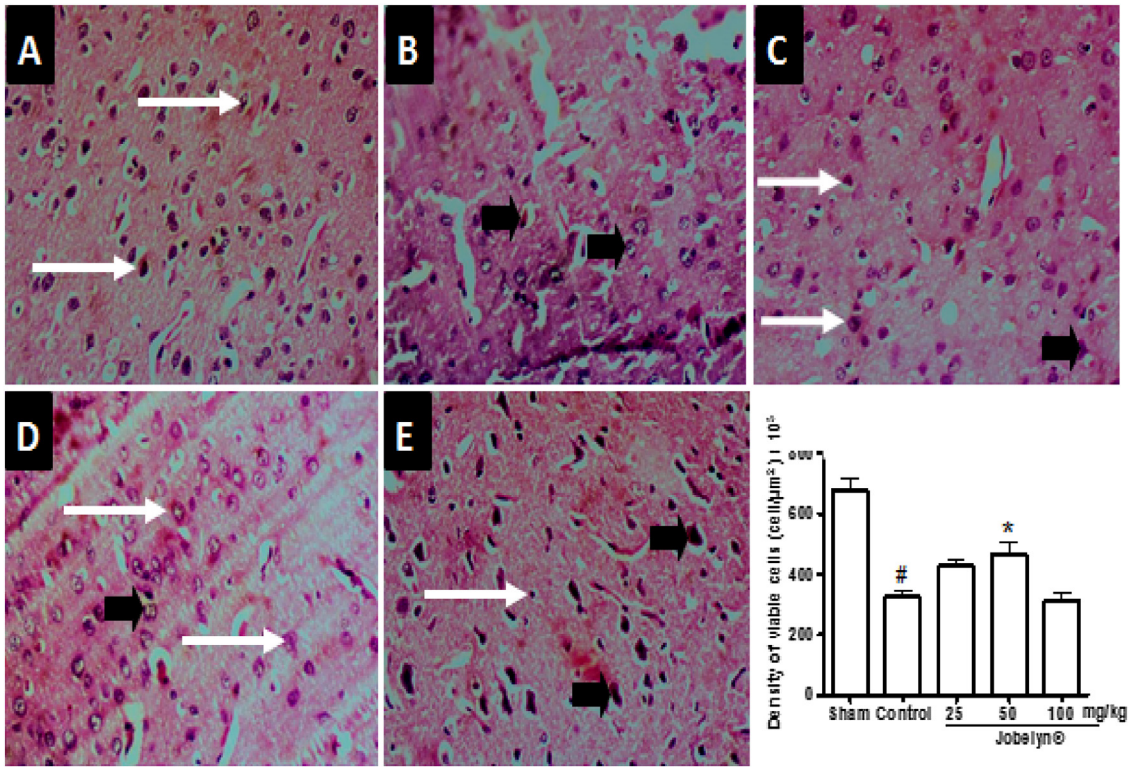


Fig. 9. Representative photomicrographs of stained sections of the striatum of rat brains submitted to ischemia/ reperfusion injury. A = Sham control, B = Negative (BCCAO ischemic stroke) control, C = JB (25 mg/kg, p.o.), D = JB (50 mg/kg, p.o.) and E = JB (100 mg/kg, p.o). Slide A shows normal neuron with no observable changes only consisting of multiple neuronal cells with hyperchromatic nuclei. Slide B revealed neuropil containing mixture of bluish staining and eosinophilic neurons, angulated cells with perivascular oedema. Slide C shows normal neuron with no observable cytoarchitecturalchanges. Slide D reveals few normal neurons with angulated cells and increased population of viable neuronal cells. Slide E showed a few ghost neuronal cells and moderate diffuse pyknotic neurons and decreased population of viable cells. Arrow-normal neuron and Arrow head-dark neurons. H&E eosin stain: x400 for all plates. Calibration bar = 0.01 mm (10 μm) for all figures. Graph represents densitometry count: All values were expressed as mean ± SEM. #$p < 0.05$ compared to sham control group and *$p < 0.05$ compared to negative control group (ANOVA followed by Newman-Keuls test).

[55; 57]. Thus, the ability of JB to reduce brain activity of AChE in rats with ischemic stroke suggests that it might enhance cognitive function in patients with ischemic stroke.

Although ischemic stroke is a complex neuroendocrine disease of multiple pathologies, functional deficits in central microvasculature and neuronal activity have been postulated to be partly due to increased generation of reactive oxygen species (ROS) and release of pro-inflammatory cytokines [26; 7; 14]. This is due to the responses of immune and oxidative defense systems to disruption of cellular homoestasis [58,59]. Ischemic stroke has been reported to disrupt blood flow, limit delivery of oxygen and glucose to neurons causing intracellular ionic imbalance, mitochondrial failure, reduction in ATP and energy depletion, as well as activation of intracellular proteases, lipases and ribonucleases as well as adhesion molecules [26; 59].

Furthermore, the stagnated blood flow and altered rheology induce shear stress on the vascular endothelium and platelets as well as activation of intravascular leukocytes [7]. These actions may initiate several excitotoxic mechanisms that further induce the production of large quantity of neurotoxic substances accompanied by various devastating effects on neuronal integrity and functions [5–59]. The release of reactive oxygen species (ROS) and inflammatory cytokines that cause cell death; have been proposed as the major neuropathophysiological mechanisms that underpin ischemic stroke [14,58]. Specifically, oxidative damage due to increased ROS induces the production of inflammatory cytokines and other cytotoxic products that propagate the ongoing neuronal damage and breakdown of blood brain barrier after ischemic stroke [59; 14]. Moreover, previous studies have reported that inflammatory mediators are increased in the cerebral tissues of patients [26] and rodents [60] with ischemic stroke. Increased brains levels of cytokines have also been reported to cause disruption of blood brain barrier during ischemic stroke [58] accompanied by alterations in neurotransmitters like dopamine and ACh, which have been implicated in movement and cognitive disorders [55]. Moreover, increased concentrations of MDA and low levels of endogenous antioxidants as well as increased levels of inflammatory cytokines have been found to correlate with neurological deficits in ischemic stroke [26; 58]. Hence, inhibition of oxidative and inflammatory pathways is being sought as novel therapeutic targets for the amelioration of neuronal damage caused by ischemic stroke [7,21,26]. In this study, BCCAO was found to decrease brain antioxidant defense systems such as GSH and CAT levels. Moreover, the rats exposed to BCCAO showed increased levels of MDA, suggesting increased lipid peroxidation due to oxidative stress. These findings are in agreement with previous investigations, which showed that alteration in oxidative stress plays a prominent role in stroke-induced neurological dysfunctions [61,62]. Relevant to this, rats with ischemic stroke induced by BCCAO also had increased brain levels of IL-6, TNF-α and increased expression of immnopositive cells of NF-κB, which are also consistent with existing literature [58,6–59]. Meanwhile, the activation of NF-κB has also been found to plays a central role in a variety of chronic inflammatory diseases via regulation of several cellular target genes [58,6–59]. However, oral administration of JB produced a marked decrease in oxidative stress as shown by reduced MDA levels and increases in endogenous antioxidant molecules (GSH and catalase) in the brains of rats with ischemic stroke induced by BCCAO. Moreover, JB also inhibited the release of pro-inflammatory cytokines (IL-6 and TNF-α) and increased expression of immnopositive cells of NF-κB in the

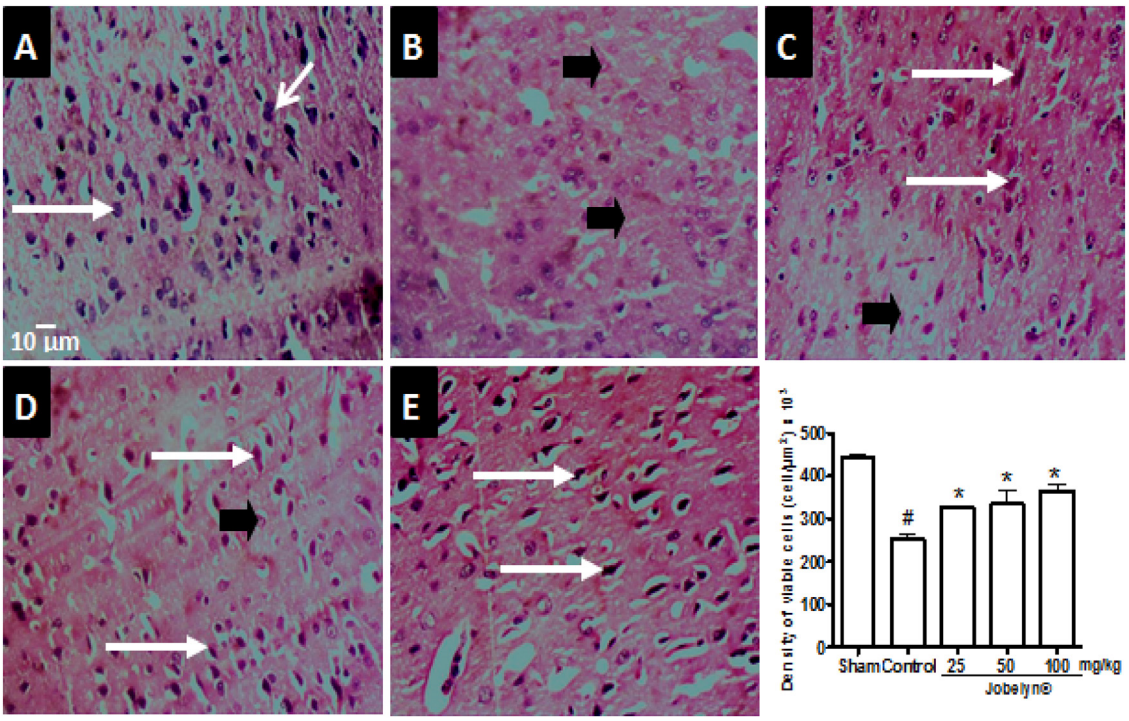


Fig. 10. Representative photomicrographs of stained sections of the prefrontal cortex of rat brains submitted to ischemia/reperfusion injury. A = Sham control, B = Negative (BCCAO ischemic stroke) control, C = JB (25 mg/kg, p.o.), D = JB (50 mg/kg, p.o.), and E = JB (100 mg/kg, p.o). Slide A shows predominantly bluish staining of normal pyramidal neurons with no observable changes. Slide B showed diffused necrosis, loss of pyramidal cells, remnants are shrunken and eosinophilic consisting of perivascular oedema and gliosis. Slide C shows normal neuron with predominantly eosinophilic staining neurons with intact nuclei and increased population of viable neuronal cells. Slide D reveals few normal neurons with no observable lesion and increased population of viable neuronal cells. Slide E showed angulated cells with no other observable changes. Arrow–normal neuron. Arrow head-dark neurons. H & E stain: x400 for all plates. Calibration bar = 0.01 mm (10 µm) for all figures. Graph represents densitometry count: All values were expressed as mean ± SEM. #$p < 0.05$ compared to sham control group and *$p < 0.05$ compared to negative control group (ANOVA followed by Newman-Keuls test).

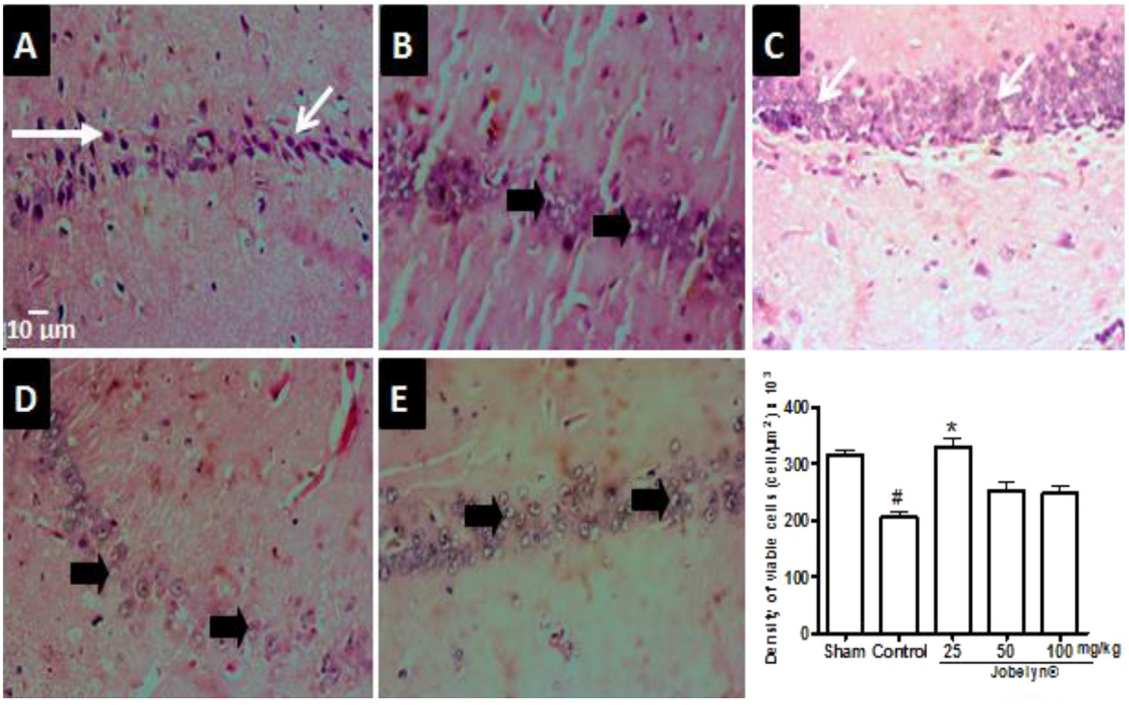


Fig. 11. Representative photomicrographs of stained sections of the hippocampus of rat brains submitted to ischemia/reperfusion injury. A = Sham control 10 mL/kg, B = Negative (BCCAO ischemic stroke) control, C = JB (25 mg/kg, p.o.), D = JB (50 mg/kg, p.o.) and E = JB (100 mg/kg, p.o). Slide A showed normal hippocampal neurons with predominantly bluish stained nuclei and some blood capillaries containing red cells. Slide B showed moderate ischemic necrosis of the neurons, partial loss of nuclei staining and astrocytic death. Slide C showed normal pyramidal neurons with no observable changes. Slide D reveals few normal neurons with distortion of lamina arrangement of neurons. Slide E showed angulated cells with other observable changes. Arrow-normal neuron and Arrow head-dark neurons. H&E stain: x400 for all plates. Calibration bar = 0.01 mm (10 µm) for all figures.Graph represents densitometry count: All values were expressed as mean ± SEM. #$p < 0.05$ compared to sham control group, *$p < 0.05$ compared to negative control group (ANOVA followed by Newman-Keuls test).

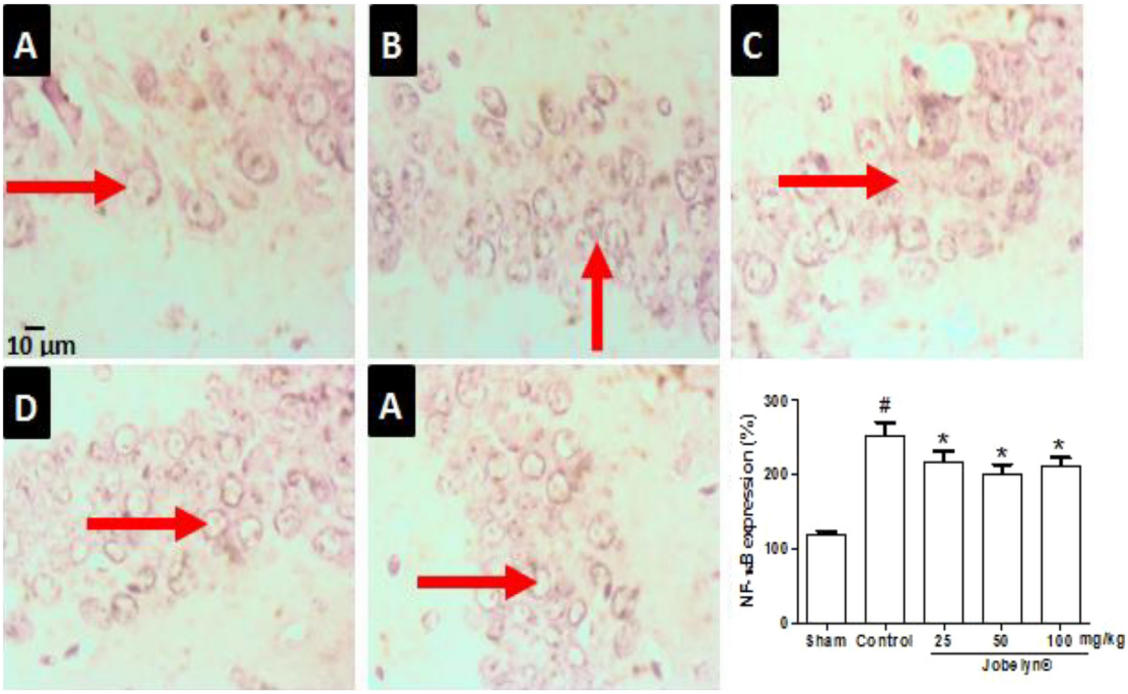


Fig. 12. Representative photomicrographs of the effect of Jobelyn® (JB) on BCCAO/Reperfusion-induced immunohistochemical changes and expressions of NF-kB immunopositive cells in the hippocampus of rat brains. A = Sham control, B = Negative control (BCCAO/reperfusion), C = JB (25 mg/kg, p.o.), D = JB (50 mg/kg, p.o.) and E = JB (100 mg/kg, p.o.). Vertical arrow indicates: High immunopositive cell expression. Horizontal arrow indicates: Low immunopositive cell expression (Immunohistochemical stain: x1000 for all plates). Calibration bar = 0.01 mm (10 µm) for all figures. Immunopositive cell expression bars represent the mean ± SEM (n = 3 animals/group). *$p < 0.05$ compared to sham group, #$p < 0.05$ compared to negative control group (ANOVA followed by Newman-Keuls test).

brain of rat with ischemic stroke. Thus, the ability of JB to attenuate the neurological deficits in rats with ischemic stroke might be related to the suppression of oxidative stress and formation of pro-inflammatory cytokines via inhibition of NF-κB signaling pathway. This suggestion is further reinforced by the data obtained from the histological studies, which showed that JB exhibited neuroprotection as evidenced by reduced extent of neuronal damage and increased population of viable neuronal cells in the striatum, PFC and hippocampus of the brains of rats with ischemic stroke.

Previous studies have shown that different parts of the brain possess different vulnerability factor to ischemic stroke [7,13,63]. Specifically, alterations in the ST, PFC and HC have been linked to the neurological perturbations that closely mimic the behavioral deficits seen in patients suffering from ischemic stroke; as these brain regions play important roles in movement disorders and learning/memory processes [13,64]. Thus, rats exposed to BCCAO showed increased cyto-architectural distortions akin to those seen in the brains of patients with ischemic stroke [7,60]. These include the presence of gliosis, loss of pyramidal cells, ischemic necrosis of neurons as well as perivascular oedema in the ST, PFC and HC, which connotes increased infarct volume probably due to infiltration of intravascular leukocytes [7,64]. Our studies also revealed decreased population of viable neuronal cells in the ST, PFC and HC in ischemic rats, which is in accordance with previous findings [64,65]. Thus, there is increasing interest over the years in the use of compounds with potent antioxidant and anti-inflammatory properties that could protect the neurons in ischemic stroke condition, thereby delaying the onset and progression of the disease [16,21,25,28]. The finding that JB exhibited neuroprotective effect as shown by reduced cyto-architectural distortions and increased population of viable neuronal cells in PFC regions of the brains of rats with ischemic stroke further confirms its potential utility for the management of stroke disorder in traditional medicine. However, more studies involving expression of inflammatory proteins and other behavioral phenotypes are necessary to confirm the anti-stroke effect of JB. Meanwhile, it is important to note that JB has been reported to contain various biologically active compounds like apigeninidin, luteolinidin, apigenin, luteolin and naringenin [32] that are known to demonstrate a wide range of neuropharmacological activities [33–38]. Luteolin for example, has been shown to exhibit positive effects against a wide range of neurological disorders including stroke via inhibition of NF-κB expression [34,35]. Moreover, previous investigations have also established the efficacy of naringenin in attenuation of neuropsychiatric disorders via several mechanisms related to elevation of brain levels of dopamine, serotonin, noradrenaline and BDNF levels [33,36,38]. In fact, the ability of naringenin to boost brain concentrations of dopamine, serotonin, noradrenaline and BDNF may have positive influence on animals with stroke, considering the roles of these molecules in the regulation of neuronal functions. Thus, the presence of these phytochemical constituents might contribute to the ability of JB to improve neurological functions in rats with ischemic stroke, although more studies are necessary to establish this declaration.

5. Conclusion

The results of this investigation showed that Jobelyn® improved neurological deficits induced by bilateral common carotid artery occlusion via inhibition of oxidative stress, neuroinflammation and neurodegeneration. These findings further support the ethnomedicinal application of Jobelyn® as a therapeutic agent for the management of patients with ischemic stroke.

Conflict of interest

The authors declare that they have no conflict of interest.

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