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Evaluation of Neuroprotective Effect of *Hibiscus sabdariffa* Linn. Aqueous Extract Against Ischaemic-Reperfusion Insult by Bilateral Common Carotid Artery Occlusion in Adult Male Rats

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Summary: Ischaemic brain injury continues to be devastating, causing social, medical and relationship disruption. Oxidative damage has been reported to be one of the mechanisms for brain damage following ischaemic stroke. The antioxidant activity of *Hibiscus sabdariffa* L. was investigated for a possible protective effect against ischaemia-induced brain damage in rats. Adult male Wistar rats (n=35) were divided into five groups of 7 rats per group. Group 1 served as control was given tap water*;* Group 2: 500 mg/kg daily of *Hibiscus sabdariffa* L. extract (HSE); Group 3: bilateral common carotid artery occlusion (BCCAO) for 30 minutes followed by reperfusion for 24 hours; Group 4: 500 mg/kg (HSE) before BCCAO; Group 5: 500 mg/kg vitamin E before BCCAO. All administrations were oral and lasted 3 weeks. Behavioural studies namely: transitions, rearings, groomings and forelimb grip strength were carried out. Rats were thereafter euthanized and biochemical [malondialdehyde (MDA), reduced glutathione (GSH), superoxide dismutase (SOD) and catalase (CAT)], histological and morphological investigations were carried out on rat whole brain. Animals pretreated with HSE showed a significant ($p<0.05$) reduction in their body weight compared to the control group. BCCAO produced a significant ($p<0.05$) reduction in GSH, SOD and CAT while elevating MDA non-significantly. The HSE and Vitamin E pretreatment ameliorated these biochemical alterations and also attenuated reactive changes in cortical neurons. BCCAO treatment increased grooming and forelimb strength which both HSE and vitamin E pretreatment reversed. The results suggest that *H. sabdariffa* L and vitamin E were protective in acute cerebral ischaemia induced by bilateral common carotid artery occlusion in adult male rats.

Keywords*: Hibiscus sabdariffa*, Ischaemia, Reperfusion, Stroke, Rat brain

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INTRODUCTION

About fifteen million people worldwide suffer a stroke and nearly six million died, while yet another five million are left permanently disabled and this disability include confusion, paralysis, loss of vision and/or speech according to the World Heart Federation (WHF, 2014). Ischaemic brain injury continues to be devastating, causing social, medical and relationship disruption. Ischaemic injury may result from hypoperfusion, thrombus, embolus, arterial stenosis, haemorrhage or small vessel disease, all of which leads to brain injury with attendant neurological deficiency (Kumar and Clark, 2012). Since clinical therapy of this debilitating disorder is not yet perfected, prophylactic steps will be a good measure for neuroprotection (Woranan *et al*., 2013). Treatment modalities depending on synthetic drugs have been the main mode of management. However, the interest of the scientists to explore the pharmacological actions or confirm the veracity of claims made about herbs in various official books of herbal medicine is increasing (Pujari *et al*., 2014). Expectedly, the low income populations turn to the use of plant products for treating various ailments and diseases especially in rural areas majorly because of their availability and low cost. While the quest for plants with medicinal properties having wide range of biological activities continue to receive attention, scientists need to evaluate the constituents, pharmacological properties, and perform detailed screening of bioactive substances for chemotherapeutic purposes (Farombi, 2003; Barku *et al*., 2013).

Oxidative damage has been reported to be one of the mechanisms for brain damage following ischaemic stroke. Antioxidants are agents that neutralize reactive oxygen species (ROS) and reduce the molecular destruction that ROS mediate (Farombi et al., 2008). Previous studies have shown the neuroprotective effects of plant extracts with antioxidant activity in different animal models with induced brain damage using *Ginkgo biloba* extract in transient focal ischaemia (Wayne *et al*., 2001), *Hibiscus sabdariffa* calyx and *Camellia sinensis* tea on some pro-oxidants induced oxidative stress in brain (Ganiyu, 2009),

Hibiscus sabdariffa (calyx) against potassium bromate induced brain damage (Josiah *et al*., 2010), *Hibiscus sabdariffa* anthocyanins on 2, 4-dinitrophenylhydrazine-induced tissue damage (Ologundudu *et al*., 2010) and *Phoenix dactylifera* on focal cerebral ischemia and (Kalantaripour *et al*., 2012).

Hibiscus sabdariffa L. a shrub of family Malvaceae is averagely 3.5m tall and has a penetrating tap root. It is cultivated in Central and West Africa, South East Asia, West Indies, Jamaica and Central America. *Hibiscus sabdariffa* has served as a therapeutic resource and has also been utilized for preparing candies, jellies and hot and cold beverages (Herera-Arellano, 2004). The plant is reported to be rich in phenol, flavonoids and Vitamin C with a free radical scavenging capacity (Oboh and Okhai, 2012). This antioxidant activity was investigated in this study to ascertain its potential neuroptotection on induced ischaemia brain injury. Alpha-tocopherol (vitaminE), a lipid peroxyl radical-scavenging antioxidant (Yoshikawa and Naito, 2002) was used as a standard antioxidant.

The regulation of cognition, motor activities and primary sensory functions are under the control of the cerebral cortex while the hippocampus is responsible for learning, coding and storage of memory (Afifi and Bergman, 2005). Ischaemic injury to these parts of the brain will certainly compromise their integrity and adversely altering their anatomy and physiology.

This study therefore was aimed at determining the effect of *Hibiscus sabdariffa* L. extract on ischaemia-induced brain damage, in adult male Wistar rats using vitamin E as a standard antioxidant with a view to answering the research question of whether *Hibiscus sabdariffa* L. extract (HSE) can protect the brain from ischaemic injury induced by bilateral common carotid artery occlusion.

MATERIALS AND METHODS

Preparation and administration of plant extract

Dried calyces of *Hibiscus sabdariffa* L. were purchased from Bodija market in Ibadan, Nigeria. Authentication was done at the Botany department, University of Ibadan, Nigeria where a voucher specimen (UIH-22429) was deposited at the herbarium. Extraction of the plant material was carried out using Ologundudu and Obi (2005)'s method. Briefly, 50 g of the dried *Hibiscus sabdariffa* L calyx was boiled in 250 mL of distilled water in a cooking pot on a stove for 15 minutes. The boiled material was allowed to cool and then sieved from the decoction. The dark red decoction was filtered in four layers of sieve cloth. A known volume (one mL) of the aqueous filtrate was evaporated at room temperature and the concentration of the filtrate was determined from the weight changes to be 132 mg per ml. The filtrate termed *Hibiscus sabdariffa* extract (HSE) was stored in a refrigerator until required for administration via oral gavage. Rats were administered HSE 500 mg/kg body weight according to Mordi *et al*. (2011) orally for 3 weeks. The toxicity study of aqueous extract of HSE reported by Sireeratawong *et al.* (2013) showed that it was not toxic even at 5000 mg/kg body weight for rat. Mordi *et al.*, (2011) utilized 500 mg/kg which was reported influence metabolic parameters

Administration of drugs

Vitamin E (Gujarat LiquiPharmacaps, Gujarat, India) was purchased from Diadem Pharmacy and Supermarket, Oyo road, Ibadan, Nigeria. Each soft gelatin capsule contains 400 mg tocopheryl acetate BP and a single dose of 500 mg/kg daily was administered orally using an oral gavage.

Ethical approval

Before the commencement of the study, the research protocol was approved by the Animal Care and Use Research Ethics Committee (UI-ACUREC) of the University of Ibadan, Ibadan Oyo state, Nigeria, with reference number UI-ACUREC/App/2014/003.

Experimental animals

Thirty-five adult male Wistar rats weighing 140-240g were purchased from the Central Animal House of the College of Medicine, University of Ibadan, Nigeria. The rats were randomized into five experimental groups of seven rats per group. Members of each group were housed in transparent plastic cages measuring 39 × 29 × 27 cm and soft wood shavings as beddings and allowed to acclimatize to the animal house condition at 12h/12h light-dark cycle for one week prior to commencing the experiment. Rats were given free access to feed (Vital Feeds, Jos, Nigeria) and water *ad libitum.* Animals were grouped and treated as shown in Table 1.

Table 1: Grouping and treatment of experimental animals.

S/N	Group	Treatment
1	Control	Tap water
2	HSE	500 mg/kg of HSE daily for 24 days
3	BCCAO	BCCAO only on day 25 of experiment
4	BCCAO+HSE	500 mg/kg (single daily dose) of HSE for 24 days before BCCAO
5	BCCAO+VIT E	500 mg/kg (single daily dose) of Vitamin E for 24 days before BCCAO

BCCAO = Bilateral common carotid artery occlusion, HSE = *Hibiscus sabdariffa* extract.

Procedure for inducing ischaemic brain injury in animals

Ischaemic brain injury was induced in rats according to the method of Iwasaki *et al*. (1989) with slight modification. Briefly, rats were anaesthesized with ketamine (100 mg/kg) intra peritoneal and

supplemented as needed. Animals were fixed on a clean dissecting board with pins and the heads of the rats were stabilized on the dissecting board with the aid of plaster. The ventral surface of the rat's neck was cleaned with cotton wool and methylated spirit and a midline incision was made on the neck of the animal from below the mandible to the manubro-sternal junction. Blunt dissection was used to separate the skin from the fascia and salivary glands. The salivary glands were lifted to expose the sternomastoid and sternohyoid muscles. After retracting the sternomastoid laterally, the common carotid artery (CCA) was isolated from the surrounding sternohyoid and vagus nerve by careful blunt dissection. The CCA was then lifted up and a silk suture (4/0) passed under it. The procedure was repeated on the other sides of the neck. Occlusion time was 30 minutes according to the method of Sindhura and Eswaraiah (2014). Skin was closed by interrupted sutures and a single dose of 100 mg/kg body weight of ampiclox injection then given intra-peritoneally to prevent bacterial infection. Reperfusion was effected by releasing the knot of the ligature and maintained for 24 hours. Animals were thereafter returned to their cages with fresh beddings and monitored periodically till the animals were clinically stable.

Behavioural studies

Open Field analysis: The method of Brown *et al*., (1999) was employed with slight modification. The apparatus consisted of a square arena (56 x 56 x 20 cm) made of white wood with its floor divided by lines into 16 squares that allowed the definition of central and peripheral parts. At the beginning of the session, each rat was individually placed into the centre of the arena and its activity was recorded in 5 minutes. After the 5 minutes test, rats were returned to their home cages and the open field was cleaned with methylated spirit and permitted to dry between tests. Behavioural test scored according to Brown *et al*., (1999) included: Transition (Frequency with which the rats crossed one of the grid lines with all four paws), Rearing (Frequency with which the rats stood on their hind legs in the maze) and Grooming (Duration of time the animal spent licking or scratching itself while stationary).

Forelimb Grip Test: This test involves the forepaws of the rats being placed on a horizontally suspended metal wire (measuring 7 mm in diameter and 1m in length) placed about 1m above a landing area. The length of time each rat was able to stay suspended before falling off the wire or before supporting the forepaws with the hind paws is recorded. This test reflects the muscular strength in the animals (Olopade *et al.,* 2012).

Sacrifice of animals

The animals were sacrificed by cervical dislocation after the 24 hours of reperfusion. The whole brain was dissected out, rinsed, weighed and brain weights recorded. Employing the method of Owoeye *et al.* (2015), one-half of the brain of all rats was fixed in 10% neutral buffered formalin for histology while the other half was stored in cold phosphate buffered saline (PBS) at pH 7.4 for biochemical analysis. Biochemical parameters analyzed included: lipid peroxidation, superoxide dismutase, catalase and glutathione.

Biochemical Assays

Brain samples were homogenized in 50 mMTris–HCl buffer (pH 7.4) containing 1.15% potassium chloride, and the homogenate was then centrifuged at 10,000 g for 15 minutes at 4 °C. Protein concentration was determined by the method of Lowry et al. (1951). Reduced glutathione (GSH) was determined at 412 nm in a colorimeter using the method described by Beutler *et al.,* (1963). Lipid peroxidation was quantified as malondialdehyde (MDA) according to the method described by Ohkawa *et al*. (1979). The supernatant was collected for the estimation of catalase (CAT) activity and was determined in erythrocyte lysate using Aebi's method (Aebi, 1984). Superoxide dismutase (SOD) was assayed by the method of McCord and Fridovich (1969).

Histology

The brain tissues were processed through the stages of fixation, dehydration, clearing, infiltration, wax embedding, sectioning and staining using haematoxylin and eosin. Slides were viewed using Olympus light microscope (CH model) and images were captured using Olympus light microscope (CH model, Japan).

Histomorphometric studies

Histomorphometric analyses were done using computerized image analyzer (TSView CX image software file version 6.2.4.3) and Image motic 2000 (China). Capillary and pyramidal cell densities were done by counting in ten areas of frontal cerebral cortex and CA1 area randomly at high magnification (H&E x 40 objective) and then calculating the density.

Statistical analysis

Results were expressed as mean ± SD. The difference between means for different groups was analyzed using One-way Analysis of Variance (ANOVA) followed by LSD post-test using computer based fitting program (SPSS). The diagrammatic representation of the data (graphs) was performed by using Graph Pad prism 5.04 (2010) software. A p-value of <0.05 was considered statistically significant.

RESULTS

General observation

In the course of the experiment, there was no mortality during the surgical procedure performed on the animals, however, four rats died in less than 24 hours during reperfusion; one each from BCCAO,

BCCAO+HSE and two from BCCAO+VIT E group. Rats that received the plant extract also showed a significant weight loss compared to other animal groups.

Phytochemical analysis of *Hibiscus sabdariffa* Linn

The phytochemical analysis conducted showed the presence of cardenolides, anthraquinones, saponins, tannins and flavonoids, but absence of alkaloids.

Gross morphology

The brain-to-body weight ratio showed a higher ratio for the BCCAO group compared with all other groups which was insignificant and was therefore not displayed. The weight differences (final weight minus initial weight) in the animals analyzed showed weight loss in the HSE group BCCAO+HSE groups (Fig. 1).

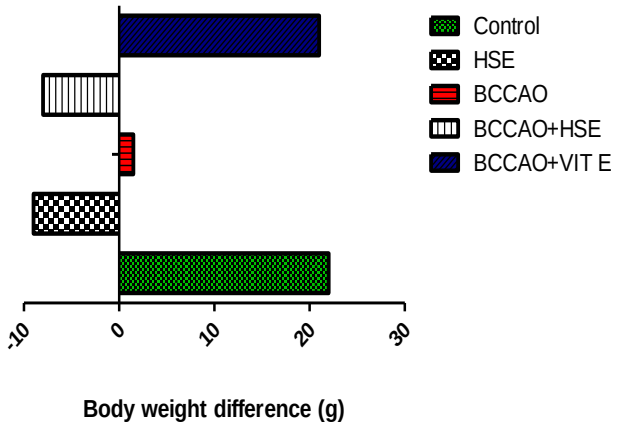

Figure 1**:** Effect of Hibiscus sabdariffa and BCCAO body weight difference in grams. Values are presented as mean±SD (n=5) HSE- Hibiscus sabdariffa Linn. extract, BCCAO- bilateral common carotid artery occlusion, VIT E- vitamin E.

Behavioural and forelimb grip tests

Figure 2 shows a non-significant reduction in the number of transitions of rats in the BCCAO group relative to control group whereas the BCCAO+VIT E significantly ($p<0.05$) elevated it relative to BCCAO only group. The same figure shows a significant reduction ($p<0.05$) in the number of rearing of rats in the BCCAO group relative to control group while the BCCAO+VIT E significantly elevated it relative to BCCAO whereas there was a further reduction in the HSE group. However, BCCAO treatment significantly ($p<0.05$) increased the grooming numbers and forelimb grip strength compared with the control, while these parameters were significantly reduced by both BCCAO+HSE and BCCAO+VIT E treatments compared with BCCAO.

Biochemical parameters.

Figure 3 shows a significant reduction ($p<0.05$) in the level of GSH and activity of SOD in the BCCAO group relative to the control while BCCAO+HSE and BCCAO+VIT E treatments significantly elevated these parameters in comparison with the BCCAO group. MDA levels were significantly reduced in the BCCAO+HSE and BCCAO+VIT E groups when compared with BCCAO group. CAT shows a significant reduction ($p<0.05$) in the activity of CAT in the BCCAO group relative to the control while BCCAO+HSE and BCCAO+VIT E treatments significantly elevated it when compared with the BCCAO group.

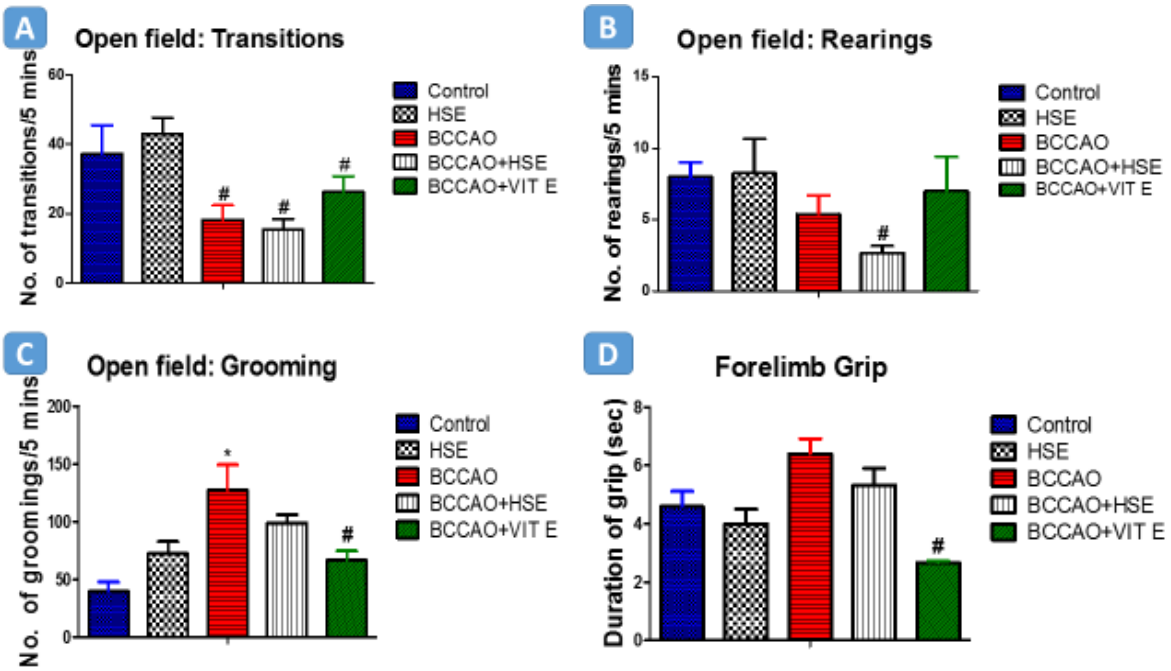

Figure 2: Effect of Hibiscus sabdariffa and bilateral common carotid artery occlusion on behavioural parameters. Values are presented as mean±SD (n=5). HSE- Hibiscus sabdariffa Linn. extract, BCCAO- bilateral common carotid artery occlusion, VIT E- vitamin E. *significantly different from control ($p<0.05$). #significantly different from BCCAO ($p<0.05$).

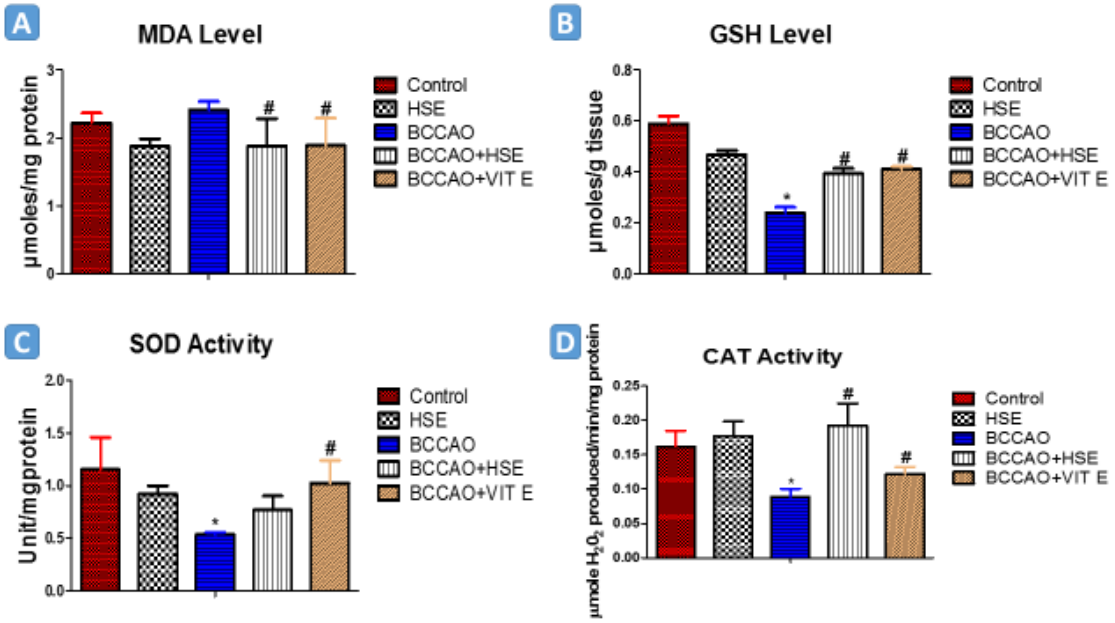

Figure 3: Effect of Hibiscus sabdariffa and bilateral common carotid artery occlusion on biochemical parameters. Data are presented as mean±SD (n=5). MDA- Malondialdehyde, GSH- reduced glutathione, SOD- superoxide dismutase, CAT- catalase. HSE- Hibiscus sabdariffa Linn. extract, BCCAO- bilateral common carotid artery occlusion, VIT E- vitamin E. *significantly different from control ($p<0.05$). #significantly different from BCCAO ($p<0.05$).

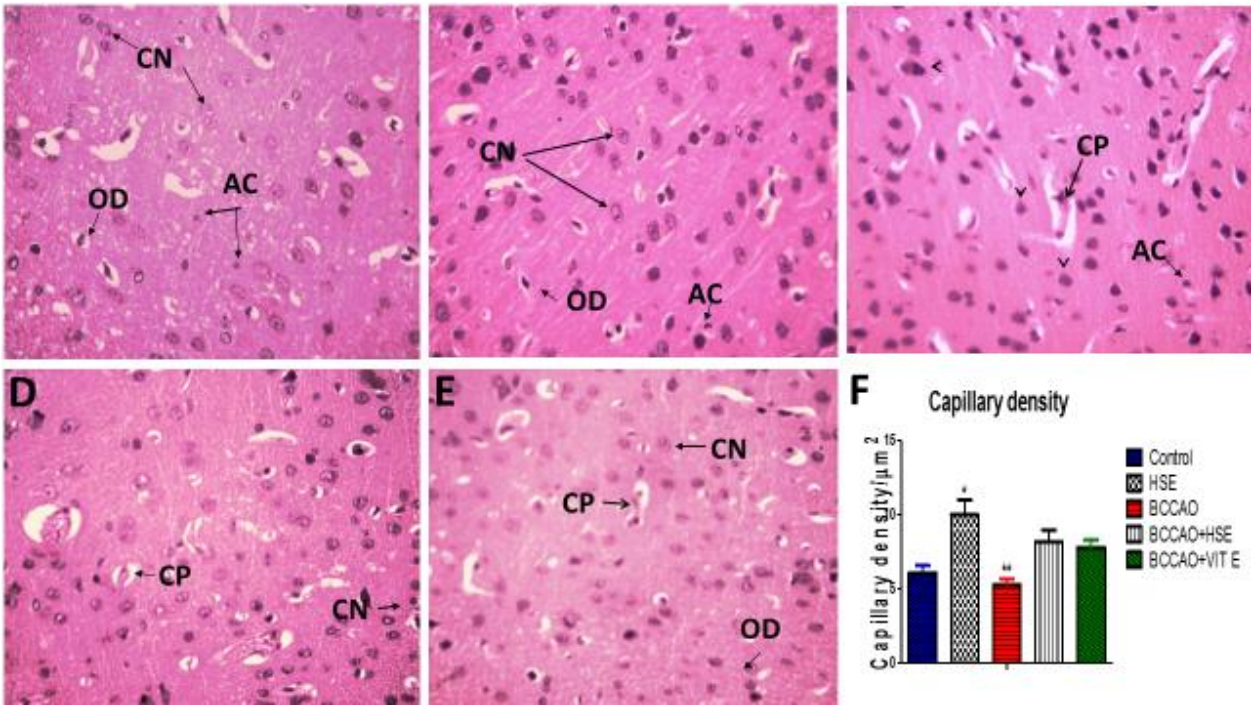

Figure 4: Photomicrographs showing the effects of Hibiscus sabdariffa and bilateral common carotid artery occlusion on the forebrain cortices of rats: A- Control group; B: HSE; C: BCCAO; D: BCCAO+HSE; E: BCCAO+VIT E. CN - cortical neuron, OD - oligodendrocyte, AC - astrocyte, CP - capillary. *significantly different from control ($p<0.05$). #significantly different from BCCAO ($p<0.05$). H & E X 400.

Histological evaluation of the cerebellar cortex and cornu ammonis1 (CA1) of hippocampal formation. The histology of the cerebral cortex in the Control, BCCAO+HSE and BCCAO+VIT E groups in addition to normal cortical cerebral neurons with some exhibiting open chromatin pattern with glia cells scattered within the parenchyma (Fig. 4**)**. However, representative cortical neurons in the BCCAO group showed dark cortical neurons with evidence of pyknosis signaling cellular death. Similarly, pyramidal neurons of the CA1 of Control, BCCAO+HSE and BCCAO+VIT E groups showed large neurons with open chromatin nuclei compared with the BCCAO group (Fig. 5) whose neurons are scattered and pyknotic.

Histomorphometry

The density of the capillaries in the cerebral cortex and the pyramidal cell density in the CA1 of rat brain were

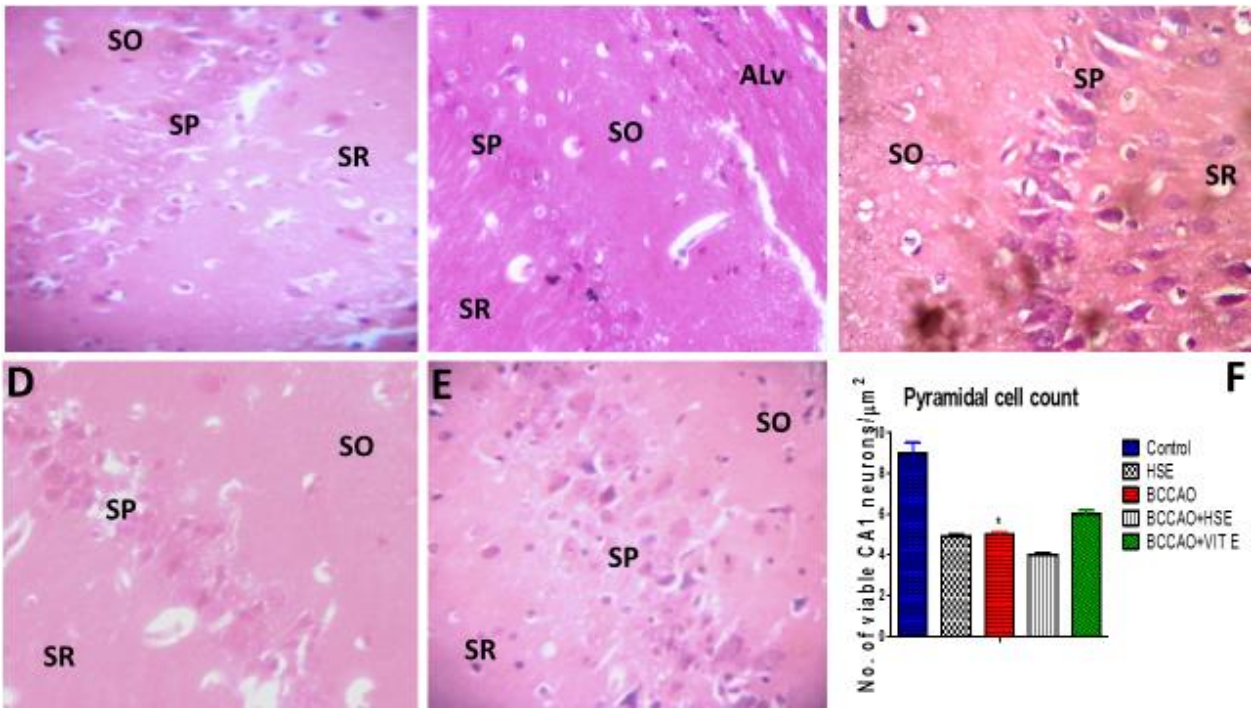

Figure 5: Photomicrographs showing the effects of *Hibiscus sabdariffa* and bilateral common carotid artery occlusion on the cornu ammonis1 (CA1) hippocampus of rats: A- Control group; B: HSE; C: BCCAO; D: BCCAO+HSE; E: BCCAO+VIT E. SO- stratum oriens, SR-stratum radiatum, SP- stratum pyramidal of cornu ammonis1 (CA1). *significantly different from control ($p<0.05$). #significantly different from BCCAO ($p<0.05$). H & E X 400.

counted and displayed in Fig. 4F and Fig. 5F respectively. A significant reduction of the capillary density in BCCAO group compared with HSE group while BCCAO+HSE and BCCAO+VIT E groups showed a non-significant elevation compared with the BCCAO group is shown in Fig. 4F. A significant reduction of the pyramidal density in BCCAO group compared with control and a non-significant elevation in the BCCAO+VIT E group compared with the BCCAO group is shown in Fig. 5F.

DISCUSSION

The present study showed that BCCAO and reperfusion for twenty-four hours altered the oxidative parameters of rat brain and histology of the Cornu ammonis1 (CA1) of hippocampus and the cerebral cortical neurons which pretreatment with HSE and vitamin E ameliorated.

Our findings of gross weight loss in the two groups treated with HSE at 500 mg/kg body weight for three weeks was supported by the report of Sireeratawong *et al*. (2013) that chronic administration of the water extract of *H. sabdariffa* L. produced a decrease in weight of organs (kidney and liver) which they attributed to the size of the organ and weight variation in the animals. However, acute toxicity studies of Sireeratawong *et al*. (2013) of the water extract of *H. sabdariffa* L. calyces did not cause toxicity in animals with a dose of up to 5,000 mg/kg body weight.

The reduction of GSH and elevation of MDA levels in the BCCAO group suggested enhanced lipid peroxidation and increased oxidative state in agreement with earlier reports (Vekaria et al., 2012). Animals pretreated with HSE and vitamin E showed a reduction of lipid peroxidation as indicated by lower levels of MDA and increased GSH levels as shown in animals in groups BCCAO+VIT E and BCCAO+HSE. Hence, this suggested that the mechanism of protection of the brain by HSE and vitamin E might be due to their antioxidant property. Glutathione (GSH) is one of the primary endogeneous antioxidant defense systems in brain, which removes hydrogen peroxides, thus a decline in GSH levels in the BCCAO group could exercabate the oxidative state (Vekaria, 2012). It has been shown that depletion in GSH levels in ischemic reperfusion injury can be attributed to several factors such as cleavage of GSH levels to cysteine, decrease in synthesis of GSH and formation of mixed disulfides, causing their cellular stores to be depleted (Kosower and Seligman, 1978; Nagini, 2003). Superoxide dismutase is an important endogenous antioxidant which prevents production of free radicals as well as decomposing superoxide radicals to produce hydrogen peroxide whereas catalase decomposes the hydrogen peroxide and converts it to water and diatomic oxygen (Chaudhary *et al*., 2003). The reduction of SOD and CAT enzymatic activities by BCCAO compares well with the earlier reports (Vekaria et al., 2012), however, in the pretreated groups (BCCAO+HSE and BCCAO+VIT E) the activities of both SOD and CAT were significantly higher compared with the BCCAO group, a reflection of the potency of the antioxidant capabilities of both HSE and vitamin E. The increased production of SOD was matched by a subsequent elevation of CAT which therefore, prevented the accumulation of hydrogen peroxide, which on conversion to hydroxyl radicals might produce deleterious effect on the brain (Pigeolet *et al.*, 1990).

Furthermore, the reduction in locomotion and rearing observed in the BCCAO group was an indication of

reduction of both horizontal and vertical motion suggesting weakness of the muscle groups. But this did not tally with the increased grooming activity which is an index of displacement response (Espejo, 1997) and forelimb grip strength an index of its skeletal muscle power in the forelimbs. However, pretreatment with HSE and vitamin E did not ameliorate the behavioural alterations induced by BCCAO for reasons not very clear.

Histopathological observations in the present study revealed that acute BCCAO induced ischaemic neuronal alterations in the CA1 of hippocampus which is consistent with previous reports (Zhen and Doré, 2007; Nandagopa *et al*., 2010; Vekaria et al., 2012) that the CA1 subfield of the hippocampus is one of the brain regions most vulnerable to ischaemic insult. The pyramidal neuronal degeneration observed in the BCCAO group was not mitigated by pretreatment as shown in the histology of the BCCAO+HSE and BCCAO+VIT E groups, which was further evidenced in the morphometric test where the density of the viable pyramidal CA1 neurons were not ameliorated in both groups. However, evidence of neuronal changes in the forebrain cortex observed in the rats of the BCCAO group supported the findings of Vekaria et al. (2012). Morphological changes of the neurons as well as staining pattern of the neurons in the BCCAO group were evidences of neuronal structural alterations when compared with control. Degenerating cortical neurons observed in the BCCAO group was mitigated in pretreated groups confirming that it was able to preserve the integrity of forebrain cortical neuron since BCCAO+HSE group showed fewer degenerating cortical neurons compared with the BCCAO group. In addition, increase in brain capillary density in animals exposed to vascular occlusion after pretreatment indicated increase in survival of brain capillaries, as a consequence of which there will be an increased blood flow and stabilization of cerebral energy state (Zechariah *et al.*, 2013) possibly due to the metabolites the vessels will transport.

The implications of the oxidative stress and injury to the cerebral cortical by BCCAO and reperfusion is a possible reduction in the cortical activities that govern cognition and motor activities due to possible reduction in the projection fibres that should have emanated from the degenerated cortical neurons as corticobulbar and corticospinal tracts whose major function is to regulate movements (Afifi and Bergman, 2005). Similarly, the effect on CA1 pyramidal neurons might lead to ineffective memory coding and recording of different forms of memory i.e semantic, episodic and spatial due to interruption of the trisynaptic pathway of the perforant path which the CA1 neurons of the hippocampus participates in. The CA1 neurons receive Schaffers collaterals, a projection from the cornu ammonis3 for onward projection to the subiculum and entorhinal cortex, a neural process that will be disrupted by the death of CA1 neurons (Scharfman, 2007). The ability of HSE and Vitamin E to ameliorate these effects of BCCAO suggests a diminution of the possible consequences on the pretreated groups thus protecting the cortical and pyramidal neurons.

In conclusion, the aqueous extract of *H. sabdariffa* L. demonstrated protection against oxidative stress and cortical neuronal damage induced by BCCAO and reperfusion injury in adult male rats possibly via its antioxidant activity, whereas it partially protected the CA1 pyramidal neurons. The protective role of HSE may be attributed to the polyphenolic compounds present in the plant.

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