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Antioxidant Effect of Co-Administration of Aqueous Extract of *Hibiscus Sabdariffa Linn (Malvaceae)* Calyx and Vitamin E on Carbamazepine-Induced Testicular Changes in Adult Wistar Rats

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Abstract: **Antioxidants are molecules capable of slowing or preventing the oxidation of other damaging oxidants. The antioxidant potential of many flavonoid containing plants is increasingly being exploited in therapy. *Hibiscus sabdariffa* (HS) is one of the most widely used nutraceuticals, used traditionally to combat various illnesses due to its high flavonoid contents. The present study was aimed at evaluating the ameliorative effect of co-administration of HS and vitamin E on sub-chronic carbamazepine (CBZ)-induced oxidative stress and alterations in semen antioxidants characteristics. Thirty young male Wistar rats were divided at random into five groups containing 6 rats per groups. Group I and II were administered normal saline and carbamazepine (20 mg /kg), respectively, while group III was treated with CBZ and aqueous extract of *Hibiscus Sabdariffa* (200 mg/kg); group IV was treated with CBZ and vitamin E (VE) 30 minutes after treatment with CBZ. The regimens were administered orally by gavage once daily for a period of 8 weeks. At the end of the treatment, four animals from each group were randomly selected and sacrificed by jugular fenestration.**

Keywords: **Carbamazepine, catalase, glutathione peroxidase, *Hibiscus sabdariffa*, osmotic fragility, superoxide dismutase and vitamin E.**

1. INTRODUCTION

Carbamazepine (CBZ) is an anticonvulsant and mood-stabilizing drug used primarily in the treatment of epilepsy and bipolar disorder, as well as trigeminal neuralgia. In men with epilepsy, long-term use of liver enzyme inducing antiepileptic drugs (AEDs), is associated with increased serum concentration of sex hormone-binding globulin and reduced bioactive serum testosterone, which may affect reproductive function accompanying oxidative stress from carbamazepine administration [1]. In addition, the use of certain antiepileptic drugs may reduce sperm motility, induce sperm abnormalities and decrease testicular volume. Change in sperm quality can obviously have a direct effect on fertility in men [2]. *Hibiscus sabdariffa L.* is used in folk medicine against many complaints that include high blood pressure, liver diseases and fever [6][7]. In Nigeria, a decoction of the seeds is given to augment or induce lactation in poor milk let-down [8]. *Hibiscus sabdariffa L.* is taken as a common local drink, popularly known as "*zobo*" in Nigeria. It is cultivated for leaf, fleshy calyx, seed or fibre according to the respective properties of the two major varieties variety.

ruber (red) and variety intermedius (green). The thick red and fleshy cup-shapes calyces of the flower are consumed worldwide as a cold beverage and as a hot drink (sour tea). It is known as roselle or red sorrel (English), *karkade* (Arabic), *yakuwa* (Hausa), *amukan* (Yoruba) and *okworo ozo* (Ibo). *Hisbiscus sabdariffa Linn* is a herb belonging to the Malvaceae family and it is grown in Central and West Africa, South- East Asia, and elsewhere in parts of West Indies, Jamaica and Central America. In Africa, roselle is frequently cooked as a side dish, eaten with pulverized peanuts. For stewing as sauce or filling for tarts or pies, the products were indistinguishable from cranberry sauce in taste and appearance [10]. *Hibiscus sabdariffa L.* is an important source of vitamins, minerals, and bioactive compounds, such as organic acids, phytosterols, and polyphenols, some of them with antioxidant properties. The phenolic content in the plant consists mainly of anthocyanins like delphinidin-3-glucoside, sambubioside, and cyanidin-3- sambubioside; other flavonoids like gossypetin, hibiscetin, and their respective glycosides; protocatechuic acid, eugenol, and sterols like -sitoesterol and ergoesterol [11]. *Hibiscus sabdariffa L.* is used as a source of food and beverages in local communities in Africa and other parts of the world. *Hibiscus sabdariffa L.*is reported to have antioxidant effect on some haematological indices and in alleviating the toxicity induced by chronic administration of sodium nitrate in Wistar rats [12]. In India, Africa and Mexico, roselle plants are valued in native medicine. Infusions of the leaves or calyces are regarded as diuretic, cholerectic, febrifugal and hypotensive, decreasing the viscosity of the blood and stimulating intestinal peristalsis. Pharmacognosists in Senegal recommend roselle extract for lowering blood pressure. Sharaf (1962) [13], confirmed the hypotensive activity of the calyces and found them antispasmodic, anthelmintic and antibacterial as well. In 1964, the aqueous extract was found effective against *Ascaris gallinarum* in poultry. Sharaf (1962) [13] showed that both the aqueous extract and the colouring matter of the calyces are lethal to *Mycobacterium tuberculosis.* In experiments with domestic fowl, roselle extract decreased the rate of absorption of alcohol and so lessened its effect on the system. In Guatemala, roselle "ade" is a favorite remedy for the after-effects of drunkenness. The heated leaves are applied to cracks in the feet and on boils and ulcers to speed maturation of new cells. A lotion made from the leaves is used on sores and wounds [14]. The brownish-yellow seed oil is claimed to heal sores on camels. In India, a decoction of the seeds is given to relieve dysuria, strangury and mild cases of dyspepsia and debility.Vitamin E can protect critical cellular structures against damage both from free radicals such as peroxyl radical, hydroxyl radical, and superoxide, and from oxidation products such as malondialdehyde (MDA) and hydroxynonenal [15]. Vitamin E, as an important antioxidant, playing a role in inhibition of mutagen formation and repair of membranes and DNA. Therefore, it has been suggested that vitamin E may be useful in cancer prevention [16]. Vitamin E supplementation in cancer patients showed that vitamin E has an important neuroprotective effect [17]. Vitamin E has the ability to protect neuronal tissue in several neurodegenerative disorders, including Alzheimer's disease [18]. Oxidative stress is thought to contribute to the development of a wide range of diseases, such as Alzheimer's disease [19][20], Parkinson's disease [21], diabetes [22][23], rheumatoid arthritis [24] and neurodegeneration in motor neurone diseases [25]. In many of these cases, it is unclear if oxidants trigger the disease, or if they are produced as a secondary consequence of the disease and from general tissue damage. One case in which this link is particularly well-understood is the role of oxidative stress in cardiovascular disease. Here, low-density lipoprotein (LDL) oxidation appears to trigger the process of atherogenesis, which results in atherosclerosis, and finally cardiovascular disease [26].

2. MATERIALS AND METHODS

2.1experimental Design and Treatment of Animals:

2.1.1 Acute toxicity studies of Hibiscus sabdariffa:

Acute toxicity (LD50) of *Hibiscus sabdariffa l.* was determined using the method of Lorke [27]. The study of *Hibiscus sabdariffa l.* on Wistar rat toxicity was divided into two phases. Nine Wistar rats were used in the first phase in 3 divided groups of 3 each named group A, B and C. Group A received extract doses of 10 mg/kg body weight orally, while group B and C received extract doses of 100 and 1000 mg/kg body weight by oral route respectively. The treatment animals were observed for 24 hours for sign of discomfort or death. In the second phase, four Wistar rats were divided into four groups, named group D, E, F and G. Group D received the extract at a dose of 600 mg/kg body weight orally, group E, F and G received the extract at a dose of 1000, 1600 and 2900 mg/kg body weight, respectively. The animals were also observed for 24 hours for sign of discomfort or death. No sign of discomfort or death was observed during the period of observation. Therefore, the extract was considered relatively non-toxic even at doses higher than 5000 mg/kg [27].

2.1.2 Experimental animals:

Thirty adult male Wistar rats between the ages of 8-12 weeks, weighing from 120-180 g were used for this research. The animals were obtained from the Department of Pharmacology and Therapeutics, Ahmadu Bello University, Zaria. The rats were housed in the animal house of the Department of Human Physiology, Faculty of Medicine, Ahmadu Bello University Zaria, Nigeria. The animals were kept in cages under normal environmental temperature (20 – 22 ^{o}C) and fed with standard pellet diet and water given *ad libitum*.

2.1.3 Identification of plant and extract preparation:

The *Hibiscus sabdariffa l.* plant was authenticated and given herbarium/voucher number 1056. The calyces were then shaded-dried and pounded into powder using pestle and mortar and extraction was done using the method of cold maceration [28]. Five hundred gram (500 g) of the powder was poured into a conical flask and one litre of distilled water was added to it and was shaken for 1 hour, and then allowed to stand overnight. It was then filtered using a filter paper and the filtrate was poured into an evaporating dish, and concentrated with water bath (70-75 ^{o}C) to granules. The granules/extract was then scrape off and preserved in a sample bottle.

2.2 Experimental procedure:

A total of 30 Wistar rats were used for the experiment. They were divided into five groups of six animals each. Group I served as control and given 5 ml/kg of 0.9% saline, while groups II, III, IV and V were treated with 20 mg/kg of carbamazepine, carbamazepine (20 mg/kg) + 200 mg/kg/day of *H. sabdariffa* 30 minutes after administration of carbamazepine, carbamazepine (20 mg/kg) + vitamin E (50 IU/kg body weight) 30 minutes after administration of carbamazepine and carbamazepine (20 mg/kg) + *H. sabdariffa* (20 mg/kg) + vitamin E (50 IU/kg body weight) orally for 8 weeks, respectively. After 24 hours from the last treatment, the animals were used for the experiment. Each rat was sacrificed by jugular fenestration. After fenestration the rat was dissected and the epididymis was exposed by scrotal incisions and transferred into petri dish. The weight of the epididymis was recorded for each rat.

2.3 Preparation of testicular homogenate:

A testis of each animal labeled according to the groups was removed from storage (freezer) and allowed to defreeze. The testes was homogenized in 10 ml phosphate buffer solution (pH 7.4) using a pestle and mortar. The homogenate formed was poured into test tubes labeled according to the group and number of each animal. The resultant homogenates were centrifuged at 2,000 revolutions per minute for 10 minutes at 4°C. The supernatant was collected and used for the experiments within 24 hours.

2.4 Assay of superoxide dismutase activity:

Activity of SOD in the rat serum was determined using NWLSS SOD assay kit (Product NWK-SOD02, Specificity: Cu/Zn, Mn and Fe Superoxide Dismutase, Sensitivity: 5 U/mL). The assay kit is based on the principle of superoxide inhibition of auto-oxidation of haematoxylin as described by Martin *et al*. (1987) [29].

2.5 Assay of glutathione peroxidase activity:

The activity of glutathione peroxidase was assessed using NWLSSTM cGPx (GPx1) ELISA assay kit (Product NWKGPX02, Specificity: Glutathione peroxidase, Sensitivity: 12.5 pg/ml). The NWLSS™ cGPx Assay was based on a sandwich enzyme-linked immunosorbent assay (ELISA), where sample GPx concentration was determined by comparing the 450 nm absorbance of sample wells to the absorbance of known standards [30].

2.6 Assay of catalase (CAT) activity:

The CAT activity was assessed using NWLSS CAT activity assay kit (Product NWK-CAT01, Specificity: Catalase (sensitivity: 6.0 U Catalase/mL). Catalase enzyme activity was measured based on the principle of catalase consumption of H_2O_2 substrate at 240 nm [31].

2.7 Assessment of lipid peroxidation:

Malondialdehyde (MDA) level was estimated in the testicular homogenate according to the method of Burge and Aust (1987) [32]. Thiobarbituric acid (TBA) reacted with malondialdehyde (MDA) in acidic medium at temperature 95^{0}C for

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10 min to form thiobarbituric acid reactive substance (TBARs). In brief, 0.1 ml of tissue homogenate (Tris-HCl buffer, pH 7.5) was treated with 2 ml of TBA-TCA-HCl (1:1:1 ratio) reagent (thiobarbituric acid 0.37%, 0.25 N HCl and 15% TCA), and placed in water bath for 15 min and then cooled. The absorbance of clear supernatant was measured against reference blank at 535 nm. Concentration was calculated using the molar absorptivity of MDA which was 1.56 x105 M^{-1} cm^{-1} and expressed as nmol/mg protein.

2.8 Erythrocyte osmotic fragility determination:

Sodium chloride (NaCl) solution was prepared according to Faulkner and King (1970) [33] in volume of 500 ml for each of the samples and in concentrations, ranging from 0.0 to 0.9% at pH 7.4. A set of 6 test tubes, each containing 10 ml of NaCl solution of concentrations, ranging from 0.0 to 0.9% was arranged serially in a test tube rack. One set was used to analyse each sample. The test tubes were labeled with corresponding 0.9% NaCl concentration. One ml pipette was used to transfer 0.02 ml of blood sample into each of the six test tubes. Mixing was done by gently inverting the test tubes about 5 times. The test tubes were allowed to stand at room temperature (27 ^{o}C) for 30 minutes. The contents of the test tubes were maintained at pH 7.4. Thereafter, the contents of the test tubes were re-mixed and centrifuged at 1,500 x g for 15 minutes. The supernatant of each test tube was transferred into a cuvette. The concentration of haemoglobin in the supernatant solution was measured using a spectrophotometer at 540 nm by reading the absorbance. The same procedure was repeated for every blood sample of each rat used for the study. The percent haemolysis was calculated using the formula [33]:

$$\text{Percentage haemolysis} = \frac{\text{Optical density of test} \times 100}{\text{Optical density of distilled water}}$$

2.9 Statistical analysis:

The results obtained were presented as mean ± SEM. The data were analyzed using ANOVA and Tukey's post hoc test to determine the level of significance between the control and experimental groups. Values of $P < 0.05$ were considered to be of statistically significance.

3. RESULTS

The result showed that mean MDA in the CBZ-treated alone group was significantly higher when compared to control group and groups co-treated with HS, Vitamin E, and HS and vitamin E ($P < 0.05$) (Figure 1).

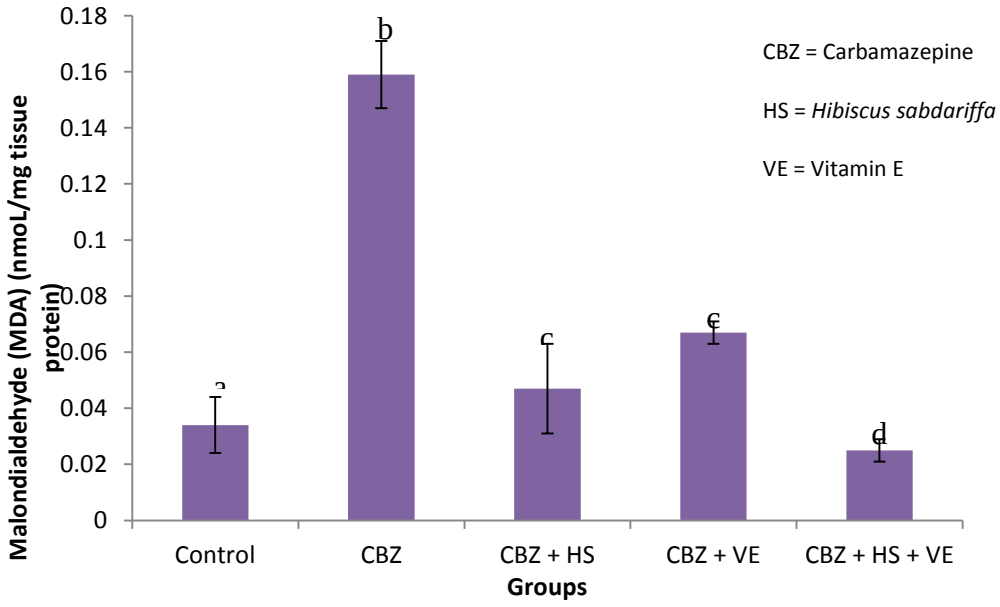

Figure 1: Changes in testicular Malondialdehyde levels in control and treatment groups of adult male Wistar rats

$^{a, b, c}$ = Means with different superscript letters are significantly ($P < 0.05$) different

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Figure 2 shows decrease in mean value testicular SOD of the CBZ-treated group as compare to the control group ($P < 0.05$). While there were significant increases in mean in the groups treated with CBZ in combination with vitamin E, and HS and vitamin E as when compared to the CBZ-treated alone and control groups ($P < 0.05$) but no different in seen in CBZ + HS group.

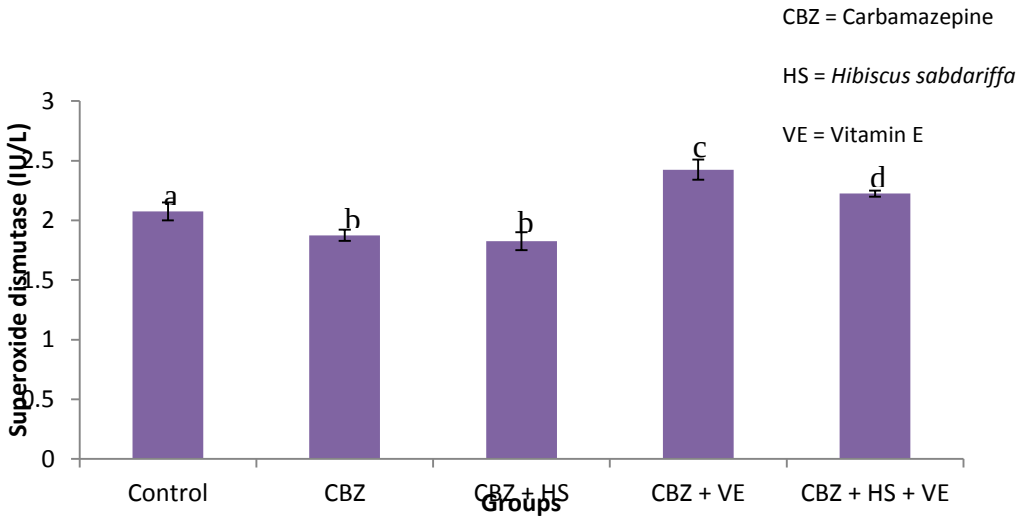

Figure 2: Testicular superoxide dismutase activity in control and treatments groups of adult male Wistar rats

[a, b, c, d] = Means with different superscript letters are significantly ($P < 0.05$) different

There was statistically significant decrease in mean value of GPx in CBZ-treated group as compared to control group ($P < 0.05$). Also, increments in mean of GPx were seen in treatment groups (except for CBZ + HS group) ($P < 0.05$) when compared to the CBZ-treated group and control group (Figure 3).

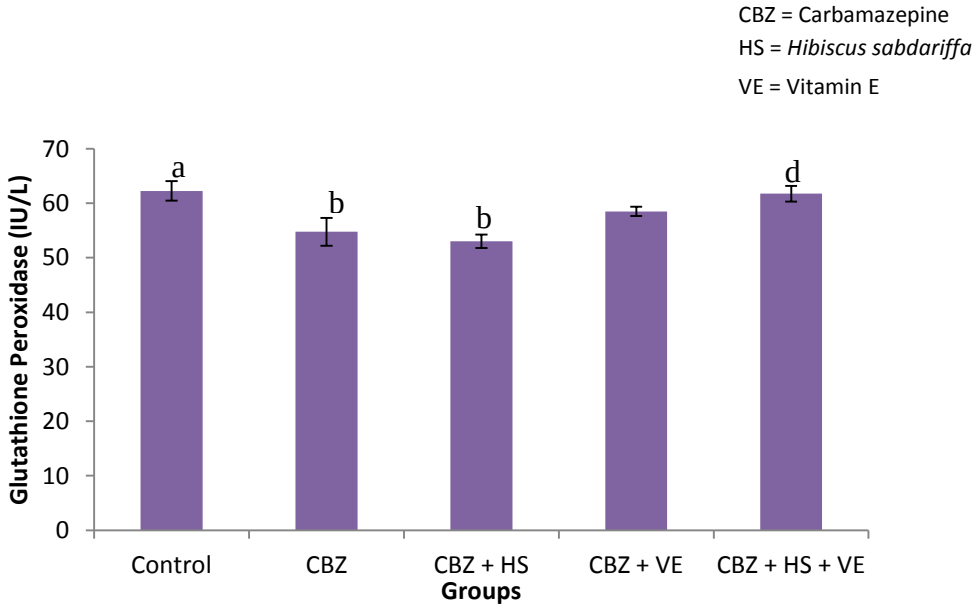

Figure 3: Changes in testicular glutathione peroxidase activity in control and treatment groups of adult male Wistar rats

[a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different

Figure 4 shows relatively decrease in mean testicular catalase level in CBZ-treated group as compared to control group but significant decrease in the groups treated with CBZ in combination with HS and vitamin E only ($P < 0.05$) and increase in mean level of catalase in group treated with CBZ in combination with HS +VE.

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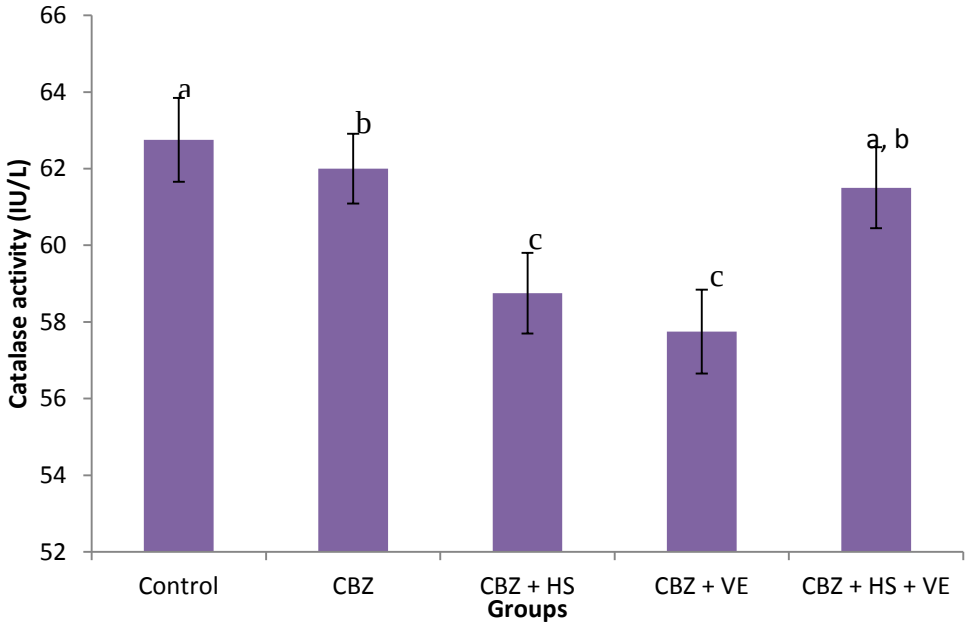

Figure 4: Changes in testicular catalase activity in control and treated groups of adult male Wistar rats

[a, b, c] = Means with different superscript letters are significantly ($P < 0.05$) different

Figure 5 shows high percentage haemolysis in CBZ-treated group as compared to control group, but lower percentage haemolysis in groups treated with CBZ in combination with HS, VE and HS + VE but the results were statistically not significant ($P > 0.05$).

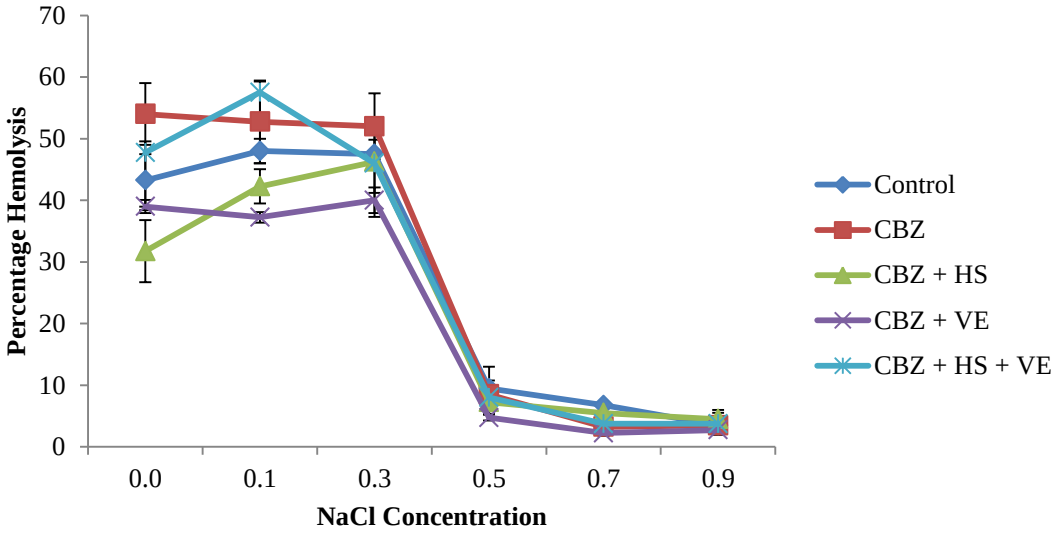

Figure 5: Erythrocyte osmotic fragility of rats administered orally with Normal saline (NS), Carbamazepine (CBZ), and /or aqueous extract of *H. sabdariffa* and /or Vitamin E in adult male Wistar rats

4. DISCUSSION

Flavonoids which are part of the phytochemical constituents of *Hibiscus sabdariffa* exhibit a wide range of biological activities, one of which is their ability to scavenge for hydroxyl radicals, and superoxide anion radicals, and thus health-promoting action [34].

Serum MDA level is an index of free radical generation, which increases in conditions of oxidative stress [35]. The result obtained in this present study indicates significantly higher MDA level in CBZ-treated as compared to the control rats. This is in conformity with previous findings by Tarun *et al*. (2010) [36]. Malondialdehyde levels in the groups treated with CBZ + HS, and CBZ + VE were significantly lower than in the CBZ treated group. This is suggestive of a compensatory mechanism by the testicular antioxidant enzymes, induced by the synergic effect of HS and VE to reduce the generation of free radicals; thus, ameliorating oxidative stress in the testes.

Antioxidant status of the testes in this study shows significant increase in antioxidant enzymes (SOD and GPx) activities in the groups treated with CBZ + VE and CBZ + HS + VE. These findings were consisted with the report of Arm and Alaa-Eldin (2006) [37]. The antioxidant enzymes play a crucial role in the cellular defence mechanism against ROS [38]. Superoxide dismutase serves as the first line of defense against the deleterious effect of ROS. The function of intracellular SOD is to scavenge superoxide (O_2-), produced by cellular metabolism, by catalyzing dismutation of superoxide to oxygen (O_2) and hydrogen peroxide (H_2O_2) [38][39]. The increased dismutation of O_2- by SOD leads to increase in production of H_2O_2, which is further detoxified to H_2O and O_2 by CAT and GPx. The potent antioxidant activity of *Hibiscus sabdariffa* obtained in the present study is in agreement with the finding of Kabele *et al*. (2013) [40], who reported the plant possess a potent intrinsic ability to scavenge free radicals [40].

The erythrocyte possesses a membrane rich in sulpydryl groups, polyunsaturated fatty acid content. Therefore, continuous exposure to high concentration of oxygen species radicals of cellular oxidation makes RBC particularly susceptible and sensitive to redox imbalance that can alter its mechanical properties. Besides, the alteration in mechanical properties may start as a result physiological and pathological oxidative stress [38]. Therefore, it serves as a marker of oxidative stress. Parameters of erythrocytes have been demonstrated to be negatively affected by oxidative stress caused by CBZ, resulting in changes of membrane fluidity, inactivation of membrane receptors, increase in lipid peroxidation and activation of proteolysis [42]. Exposure to oral treatment with CBZ such as occurred in the present study has also been demonstrated to increase oxidant stress markers of erythrocyte [43]. Although, the results of erythrocyte osmotic fragility were not significant in this study between control group and CBZ treated-groups and compared to the CBZ + HS, CBZ + VE and CBZ + HS + VE, a slight higher percentage haemolysis was observed in CBZ group. This perhaps apparently indicating an increased in oxidative stress in this group, but the effect was no pronounced in other groups treated with CBZ. This fact may be due to activation of compensatory processes as evidenced in the increased in enzymatic antioxidant in the CBZ + HS, CBZ + VE and CBZ + HS + VE.

5. CONCLUSION

Following oral administration of carbamazepine for 8 weeks in adult male Wistar rats, a significant testicular increase in MDA and decrease in SOD, GPx and CAT, and relatively increase in percentage haemolysis of red blood cells were observed, but these effects were reduced in the groups treated with CBZ in combination with oral administration of aqueous root extract of *H. sabdariffa* and vitamin E. In conclusion, the results obtained from the present study demonstrated that CBZ treatment induced oxidative stress in the testes of Wistar rats. *Hibiscus sabdariffa* and vitamin E or their combination ameliorated carbamazepine-induced oxidative stress responses in the testes of Wistar rats. Therefore, *Hibiscus sabdariffa* has an anti-oxidant protective effect on fertility.

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