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Hibiscus sabdariffa calyx protect against oxidative stress and aluminium chloride-induced neurotoxicity in the brain of experimental rats

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ABSTRACT

We evaluated the antioxidant and neuroprotective potentials of extracts of *Hibiscus sabdariffa* calyx in Wistar albino male rats injected intraperitoneally with aluminium chloride at a dose of 7 mg/kg/day. Phytochemical screening of *H. sabdariffa* calyx show that coumarin glycosides and steroid were absent after drying at 50 °C. At 30 °C, there were significant ($p < 0.05$) highest amounts of phenols, flavonoids, alkaloids, tannin, and saponin. The extracts showed significantly ($p < 0.05$) high dose-dependent antioxidant activities. MDA significantly ($p < 0.05$) increased, while GSH, GPX, SOD, CAT activities significantly ($p < 0.05$) decreased in the brain of the experimental rats induced with $AlCl_3$, while treatment with the extracts reversed these effects to a relatively normal level. At doses of 500 and 1000 mg/kg body weight, the extracts of the calyx dried at 30 °C exhibited the highest capacity to increase the activities of GSH and GPx. Also, $AlCl_3$ caused significant increases ($p < 0.05$) in the percentage inhibition of acetylcholinesterase and butyrylcholinesterase activities, and a significantly ($p < 0.05$) lower protein levels in the brain of the test rats, while treatment with the extracts, at low and high doses, significantly ($p < 0.05$) reversed these effects in the rat brain to near normal. *H. sabdariffa* exhibited a good potential to protect against oxidative stress and neurotoxicity.

1. Introduction

Neurodegenerative disorders are diseases which often present with clinical signs and symptoms such as decline in cognitive and memory capacity, behavioral changes, impairment in the performance of daily activities, and neuropsychiatric disturbances. The free radical hypothesis postulates that the aging process is associated with multisystem failure which occurs as a result of oxidative damage, which is a consequence of the distortion of the balance between the generation of reactive oxygen species (ROS) and the counter activities of the antioxidant defense system [1]. Apart from the ROS, neurotoxins have also been implicated in the pathogenesis of neurodegenerative disorders by interfering with the antioxidant system of the brain, leading to oxidative stress in the brain, with consequent high rate of degradation of neurotransmitters. A common neurotoxin like aluminium (Al) has been implicated in the pathogenesis of various cognitive disorders. Chronic exposure to Al has also been implicated in the appearance of neurologic signs like progressive neurodegeneration, changes in the neuro-filament of the hippocampus and cerebral cortex, and other biochemical changes of clinical importance. $AlCl_3$ (Aluminium trichloride) has been reported to play an important role in aggravating neurodegenerative disorders like Alzheimer's disease (AD) and Parkinson disease [2]. Findings from basic and clinical experiments have shown that exposure to $AlCl_3$ often leads to deterioration in learning, memory and cognition function [3].

Experiments have also shown that prolonged exposure to Al is capable of inducing oxidative stress and pathological changes in the brain of neonatal rats [4]. $AlCl_3$ reportedly induces the generation of free radical which further triggers the peroxidation of lipids [5]. However, antioxidants are known to function by slowing down or preventing the oxidation of other molecules by these free radicals [6,7]. The major function of antioxidants is the trapping of these free radicals, particularly ROS and reactive nitrogen species (RNS), which are involved in the pathogenesis of several chronic, degenerative, and neurodegenerative diseases [4,8,9]. In healthy individuals, free radicals and other ROS are neutralized by the body's antioxidant defence system, which includes superoxide dismutase (SOD), catalase (CAT) and glutathione (GSH).

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However, in individuals with certain oxidative stress-related diseases, the endogenous antioxidant defence system may not be able to effectively protect against the activities of free radicals, thus, antioxidants from dietary sources or medicinal plants may be an important alternative [10–13]. In this light, several researches which study the natural antioxidant activity of extracts from medicinal plants, as a function of their phytochemical constituents, have been recently conducted [13]. These phytochemical constituents are a group of bioactive compounds that are naturally found in abundance in the root, bark leaves, flowers, and fruits of medicinal plants or herbs [14,15]. Some of the phytochemicals with high antioxidant activities include phenols, flavonoids, phytic acid, and sterols, phenolic acids and their derivatives [16]. Some of these phytochemicals can be easily lost or inactivated during processing, due to their instability and susceptibility to degradation by external factors such as pH, temperature, enzyme activities, chelation by metal ions, light, oxygen, and other factors [17]. Increases in temperature, for example, can result in organoleptic and nutritional losses, as well as changes in the levels of ascorbic acid, phenolic compounds, and carotenoids. These changes could lead to decreases in antioxidant capacity and other effects on bioactivities [18], which consequently lead to reduction in their application; especially as anti-oxidative and anti-neurodegenerative agents.

However, the search for antioxidants from natural sources, like medicinal plants and herbs, has received much attention of recent times. Much efforts have been put into identifying or determining compounds that can act as useful antioxidants, with the intentions of using them to replace the synthetic ones, in the fight against oxidative stress-related diseases like neurodegeneration. The use of the fresh calyx of *Hibiscus sabdariffa,* a medicinal herb, in phytomedicine is increasingly becoming popular. This is adduced to its inherent composition of phytochemicals, with antioxidant and anti-neurodegenerative activities. Interestingly, there has been a growing interest in zobo drink (a Nigeria beverage drink made from the calyx of *H. sabdariffa*) as a nutraceutical because of its possible potential as a neuroprotective agent. Despite the potential medicinal benefits from this plant, there are unverified claims that the calyx of *H. sabdariffa* dried at lower temperatures have better medicinal potentials than those dried at higher temperatures. Equally, there are limited reports describing the effects of different drying temperatures on the bioactive phytochemicals in the extracts of *H. Sabdariffa*. Furthermore, there is a dearth of information which describes the phytochemical constituents, anti-oxidative, and neuroprotective potentials of the calyx (dried at different temperature) of *H. sabdariffa.* Thus, in this study, we determined and evaluated some useful phytochemicals in the extracts of the calyx (dried at different temperature) of *H. sabdariffa,* as well as established their potential to fight against oxidative stress. We also evaluated their potential to protect against aluminium chloride-induced neurotoxicity using experimental rats. In essence, we induced oxidative stress and neurodegenerative conditions in the experimental rats, and treated them with extracts of the calyxes of *H. sabdariffa* calyx (dried at different temperatures).

2. Materials and methods

2.1. Preparation of extracts of H. sabdariffa calyces

Fresh calyx of *H. sabdariffa* were obtained from Uchi market, Auchi, Etsako West Local Government Area of Edo State, Nigeria. The calyx was taxonomically identified at the Department of Botany, Ambrose Alli University, Ekpoma, Edo State, Nigeria. Afterwards, the *H. sabdariffa* calyx were dried at different temperatures (i.e., −58°C, 30 °C, 40 °C and

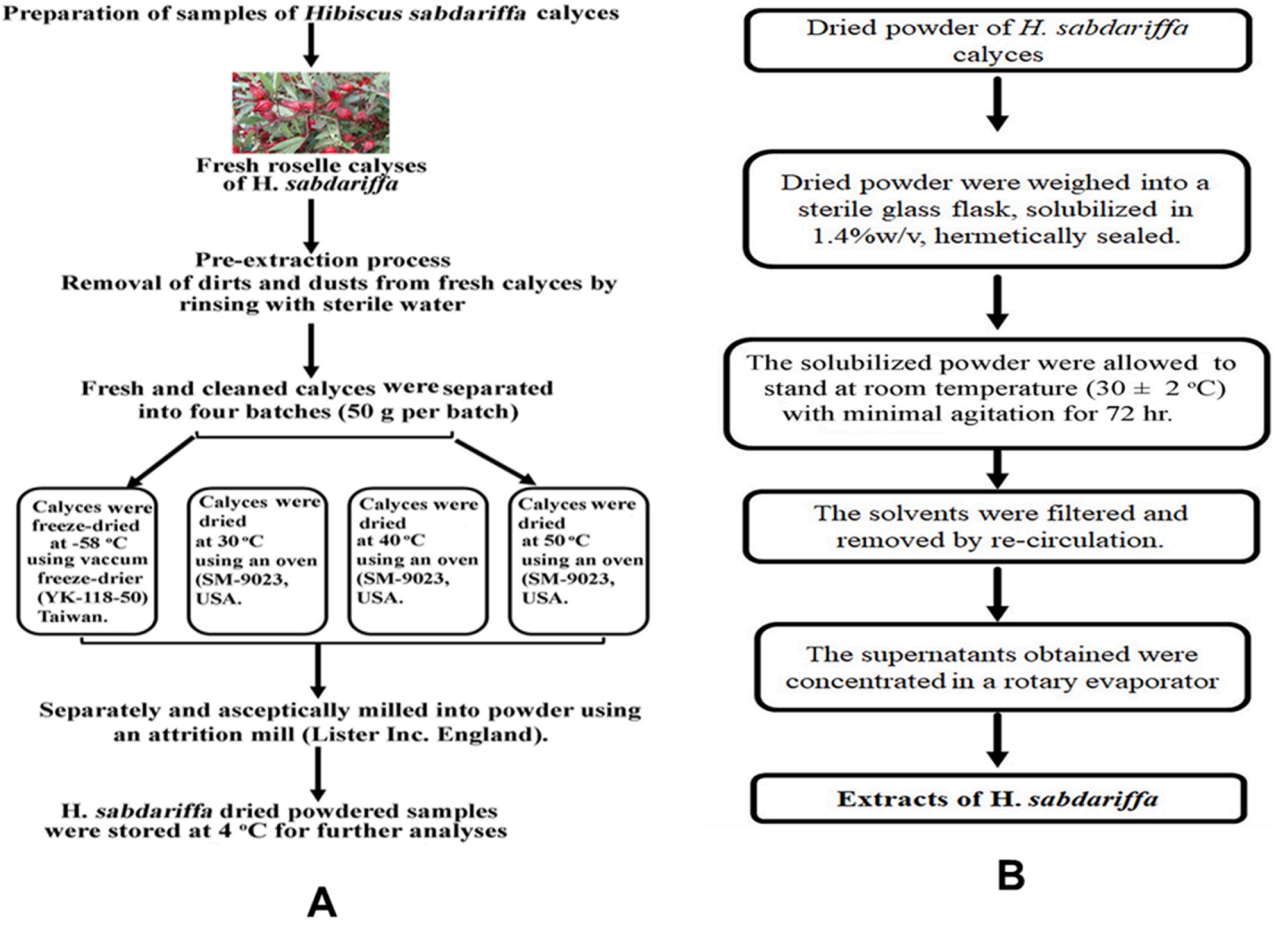

Fig. 1. (A) Flow chart for the preparation of *H. sabdariffa* dried powder. (B) Flow chart for the preparation of extracts of *H. sabdariffa* calyx.

50 °C) and subsequently differentiated based on variations in the drying temperature (Fig. 1A). Extracts of the calyx were prepared according to the method described by Akinnibosun [19]. The differentially dried calyx was weighed and aseptically blended into powdery form. The dried powders were solubilized in 1:4% (w/v) ethanol-water solvent, and the mixtures (solubilized powder) were placed in a sterile glass flask, sealed hermetically and then allowed to stand on a magnetic stirrer for 72 h. The mixtures were filtered and the solvents removed by re-circulation. Concentration of the supernatants was done in a rotary evaporator (model RE201D), under the same temperature and pressure (25 °C and 42 mbar) for all the extracts. This was to ensure that the same condition of evaporation/concentration applies to all the samples/extracts. The concentrated extracts were then stored at − 4 °C until further use (Fig. 1B).

2.2. Qualitative and quantitative phytochemical screening of the extracts of H. sabdariffa calyces

The identification and determination of selected phytochemical constituents (i.e., steroids, phenols, tannin, terpenoids, alkaloids, flavonoids, anthraquinones, saponin, coumarin glycoside, cardiac glycoside, oxalate) of the extracts of the differentially dried calyx of *H. sabdariffa* were done following standard procedures as previously described by our group [6–8,13,20–22].

2.3. Antioxidant study

The in vitro assays used for the assessment of the antioxidant capacity of the extracts of *H. sabdariffa* calyx involved the incubation of the mixtures of the extracts and the relevant reagents in test tubes. The absorbances of the mixtures were measured and the corresponding activities determined.

2.3.1. In vitro assessment of the antioxidant capacity of H. sabdariffa extracts (non-enzymatic assay)

The non-enzymatic antioxidant capacities of the extracts were determined following standard procedures. Specifically, 1,1-Diphenyl-2 picrylhydrazyl (DPPH) radical scavenging activity was evaluated in accordance with the method described by Yamaguchi et al., [23], while the superoxide radical scavenging activity was evaluated in accordance with the method described by Nishikimi et al., [24]. Assessment of reducing power was carried out following the protocol described by Pulido et al., [25], while the ferrous (Fe^{2+}) chelating ability (FRAP) assay was carried out following the protocol described by Benzie and Strain [26].

2.3.2. In vitro assessment of the antioxidant capacity of H. sabdariffa extracts (Enzymatic assay)

The enzymatic antioxidant capacities of the extracts were also determined following standard procedures. Specifically, superoxide dismutase activity was carried out as described by Beauchamp and Fridovich [27], while catalase activity was carried out as described by Chance and Maehly [28]. The activity of glutathione reductase (GR) was evaluated following the method described by Carlberg and Mannervik [29], while the extent of lipid peroxidation was evaluated following the modified method of Ohkawa et al., [30].

2.4. Animal study

2.4.1. Experimental animal and ethic statement

In this study, we used 184 adult male albino experimental rats (8 weeks old, 150 ± 5 g weight). The rats were obtained from the Animal House of the College of Medicine, Ambrose Alli University, Ekpoma, and were hygienically housed in plastic cages placed in a well-ventilated vivarium with natural photoperiod of 12-hr light and dark cycle. They had free access to feed (standard rat chow) and drinking water, and were allowed to adjust to the new environment for a period of 7 days before the commencement of the study. All the rats received proper animal care as indicated in the 'Guide for the Care and Use of Laboratory Animals' prepared by the Animal Use and Care Committee (AUCC). All the protocols used in this study were as approved by the, National Health Research Ethic Committee, Nigeria.

2.4.2. Experimental design

The experimental rats were divided into groups and given different treatments for 28 days as illustrated in Table 1. Rats in group 1 were used as a base line control and given only water and feed, while rats in group 2 (negative control) were administered with aluminium chloride (7 mg/kg/day), intraperitoneally, according to the method of Yuan et al., [4].

2.4.3. Preparation of brain samples

At the end of the experimental protocol, the rats were euthanized with isoflurane inhalation, decapitated and the brains excised. The brains of the rats were dissected, cleared of adhering tissues, weighed, and then homogenized in normal saline 10% w/v 0.1 mmol/L phosphate buffer (pH 7.4). The homogenates were centrifuged at 3000 rpm/g for 10 mins at 4 °C, and the resulting supernatants were used for the respective biochemical assays.

2.5. Biochemical analysis of brain samples

Lipid peroxidation (MDA) were determined by the method of Laskowska-Klita and Szymańska [31]. Glutathione reductase (GPX) activity was assayed using the method of Goldberg and Spooner [32]. Superoxide dismutase (SOD) was estimated using the method of Kakkar et al., [33]. Catalase (CAT) activity was assayed using the method of Aebi [34]. The protein content in the samples were measured according to the method of Markwell et al., [35], using bovine serum albumin as the standard.

2.6. Assay of acetylcholinesterase and butyrylcholinesterase activities in rat brain

The cerebral cortex, striatum, hypothalamus and hippocampus were used for the determination of the activities of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). The assays were performed using the modified Ellman's spectrophotometric method as described by Sullivan and Bonawitz [36]. The activities of AChE and BChE were determined by measuring the formation of the yellow anions obtained from the reaction between Ellman's reagent and the thiocholine generated by the enzymatic hydrolysis of acetylthiocholine iodide (ATCh) and butyrylthiocholine (BCh), respectively (sample 0.1 mL, PBS 0.8 mL, DTNB 0.1 mL, ATCh 0.20 mL and BTCh 0.20 mL). The activities of AChE and BChE in the homogenate of brain samples were expressed as μmol/min/mg protein by using spectrophotometric method (UV 412 nm).

2.7. Statistical analysis

The data obtained from all analyses were reported as Means ± SD, and Means ± SEM, and subjected to one-way analysis of variance (ANOVA), followed by Turkey multiple comparison tests. The probability level at $p < 0.05$ was taken as criterion for statistical significance.

3. Results

3.1. Different drying temperatures affect the amount of the phytochemical constituents of H. sabdariffa

From Table 2, our initial qualitative identification of the phytochemicals in the calyx of *H. sabdariffa*, dried under different

Table 1
Experimental design for neuroprotective study.

Test	Sample group				Control group		
	-58°C	30 °C	40 °C	50 °C	Baseline	Negative	Positive
Baseline control	-	-	-	-	8	-	-
Negative control $AlCl_3$ (7 mg/kg b.wt/day i.p.) + H_2O	-	-	-	-	-	8	-
Positive control Quercetin (50 mg/kg b.wt./day i.p.)	-	-	-	-	-	-	8
Hs extract 1000 mg/kg b.wt. p.o.)	8	8	8	8	-	-	-
Test 1: Hs (250 mg/kg b.wt./day/p.o.) + $AlCl_3$ (7 mg/kg b.wt/day i.p.)	8	8	8	8	-	-	-
Test 2: Hs (500 mg/kg b.wt./day/p.o.) + $AlCl_3$ (7 mg/kg b.wt/day i.p.)	8	8	8	8	-	-	-
Test 3: Hs (1000 mg/kg b.wt./day/p.o.) + $AlCl_3$ (7 mg/kg b.wt/day i.p.)	8	8	8	8	-	-	-
Test 4: Quercetin (50 mg/kg b.wt./day/i.p.) + $AlCl_3$ (7 mg/kg b.wt/day i.p.)	8	8	8	8	-	-	-

Where 8 = number of rats; i.p. = intraperitoneal dose; p.o. = oral dose

Table 2
Phytochemical constituents of aqueous extract of *Hibiscus sabdariffa* calyx.

Phytochemicals	Control	-58°C	30°C	40°C	50°C
Steroid	+	+	+	+	-
Phenol	+	+	+	+	+
Tannin	+	+	+	+	+
Terpenoid	+	+	+	+	+
Alkaloid	+	+	+	+	+
Flavonoid	+	+	+	+	+
Anthraquinone	+	+	+	+	+
Saponin	+	+	+	+	+
Coumarin glycoside	+	-	-	-	-
Cardiac glycoside	+	+	+	+	+

Key: '+ ' indicate the presence of the specified phytochemical, while '−' indicate the absence of the specified phytochemical. The 'Control' indicates extract of fresh calyx of *H. sabdariffa*. The analyses were done in triplicates (n = 3)

temperature, show that coumarin glycosides and steroid were absent after drying at 50 °C. However, other phytochemicals like cardiac glycoside, phenol, tannin, terpenoids, alkaloids, flavonoids, steroid, anthraquinones, and saponins were present after drying at other temperatures.

After the initial identification, we further determined the amount of some of these phytochemicals. Our results, as indicated in Fig. 2a, show that the concentration of the total phenolic content (TPC) was significantly highest ($p < 0.05$) in the extract of *H. sabdariffa* calyx dried at 30 °C (93.50 ± 3.88 mgQAE/g) when compared with that of the extracts dried at 50 °C (78.00 ± 1.39 mgQAE/g), − 58°C (79.00 ± 3.12 mgQAE/g), and − 40°C (78.83 ± 6.35 mgQAE/g). The total flavonoid content (TFC) was significantly highest ($p < 0.05$) in *H. sabdariffa* samples dried at 30 °C (42.22 ± 1.08 mgQE/g) when compared with other samples dried at different temperatures. The lowest flavonoid content was observed in *H. sabdariffa* dried at 40 °C (24.86 ± 4.20mgQAE/g) and − 58°C (25.83 ± 2.45 mgQAE/g), and they were not statistically significant. Also, the total alkaloid content (TAC) of the extract dried at 30 °C (284 ± 1.08 mgQE/g) was significantly higher ($p < 0.05$) than that of the other samples dried at 50 °C and − 58°C, while that dried at 40 °C had the lowest TAC (123 ± 1.64 mgQE/g). In addition, the total tannin content (TTC) of the extract dried at 30 °C (100.77 ± 0.50 mgTAE/g) was significantly higher ($p < 0.05$) than that of other samples dried at 40 °C (73.04 ± 0.67 mgTAE/g), 50 °C (71.07 ± 0.90 mgTAE/g) and 58 °C (63.33 ± 0.35 mgTAE/g). We also show that the total oxalate content of the extract was significantly higher ($p < 0.05$) after drying at − 58 °C (307.50 ± 1.50 mgQAE/g) when compared to that of other samples. The lowest oxalate content was observed in the extract of *H. sabdariffa* calyx dried at 50 °C. However, there was no significant difference ($p > 0.05$) in the total oxalate content in extracts of *H. sabdariffa* calyx dried at 40 °C (212.50 ± 0.52 mgQAE/g) and 30 °C (233.33 ± 0.50 mgQAE/g). And, the total saponin content was significantly higher ($p < 0.05$) in the extract dried at 30 °C (570.44 ± 0.30 mgQE/g) when compared with that of the other samples. The lowest saponin content was observed in the extract dried at 40 °C (388.27 ± 0.10 mgQE/g). The results generally show that the different phytoconstituents of the calyx of *H. sabdariffa* vary in amounts with respect to different drying temperatures. The same is true for the total vitamin C content (TVC) which was retained mostly in the extract of the calyx dried at − 58°C (6.45 ± 0.18 mg/g FW). Interestingly, it was observed that an increased temperature in the drying of *H. sabdariffa* calyx resulted in a reduction in TVC content (Fig. 2b).

3.2. The extracts of the differentially dried Hibiscus sabdariffa calyx show a high non-enzymatic antioxidant potential, which increases with increasing concentration

As indicated in Fig. 3a and b, we assessed the in vitro abilities of the different extracts of *H. sabdariffa* calyx to scavenge DPPH, nitric oxide, FRAP, and ABTS free radicals, and their variations in radical scavenging activity, as well as their minimum inhibitory concentrations (IC_{50}). Our results generally revealed that the extracts from *H. sabdariffa* calyx dried at different temperatures had significantly high antioxidant activities which increases with increasing concentration. However, these antioxidant activities were seen to be lower than those of the controls (Trolox and ascorbic acid) (Fig. 3a), while their minimum inhibitory concentrations (IC_{50}) were significantly higher ($p < 0.05$) than those of the controls (Trolox and ascorbic acid) (Fig. 3b). The IC_{50} values for the DPPH radical scavenging activity of the dried *H. sabdariffa* samples at 58 °C (2283.21 ± 2.28 mg/mL), 30 °C (661.72 ± 0.66, mg/mL), 40 °C (973.90 ± 0.97 mg/mL) and 50 °C (698.57 ± 0.70 mg/mL), were higher than that for Trolox (153.16 ± 0.15 mg/mL). The scavenging of nitric oxide by the extracts, also, significantly increased in a dose-dependent manner. The IC_{50} value of the extracts at − 58°C (1272.18 ± 0.79 μg/mL), 30 °C (1155.65 ± 1.6 μg/mL), 40 °C (1232.11 ± 1.23 μg/mL) and 50 °C (1431.72 ± 1.43 μg/mL) were shown to be significantly higher ($p < 0.05$) than that of the well-known antioxidant, ascorbic acid (778.88 ± 0.79 μg/mL). The FRAP scavenging activity of the extracts show dose-dependent variations, which is similar to that of the control. However, we observed that the extracts of *H. sabdariffa* calyx dried at 40 °C had the highest FRAP scavenging ability at a range of 82.32 ± 9.45 mgFe(II)/g – 319.23 ± 5.46 mgFe(II)/g. But, no significant difference ($p > 0.05$) was observed between the FRAP scavenging capacity of the extract dried at − 58°C (38.60 ± 0.22 mg/mL) and that at 30 °C (43.28 ± 2.21 mg/mL). Overall, all samples showed relatively higher IC_{50} values when compared with the control sample (ascorbic acid). The ABTS radical scavenging activity of all the extracts also increased in a dose-dependent manner. However, among the test samples, the extract of *H. sabdariffa* calyx dried at 30 °C recorded the highest ABTS radical concentration at a range of 3.30 ± 0.68% – 11.11 ± 0.12%.

3.3. The extracts of the differentially dried Hibiscus sabdariffa calyx show a high enzymatic antioxidant potential, which increases in a dose-dependent manner

We also assessed the in vitro effects of the different extracts of

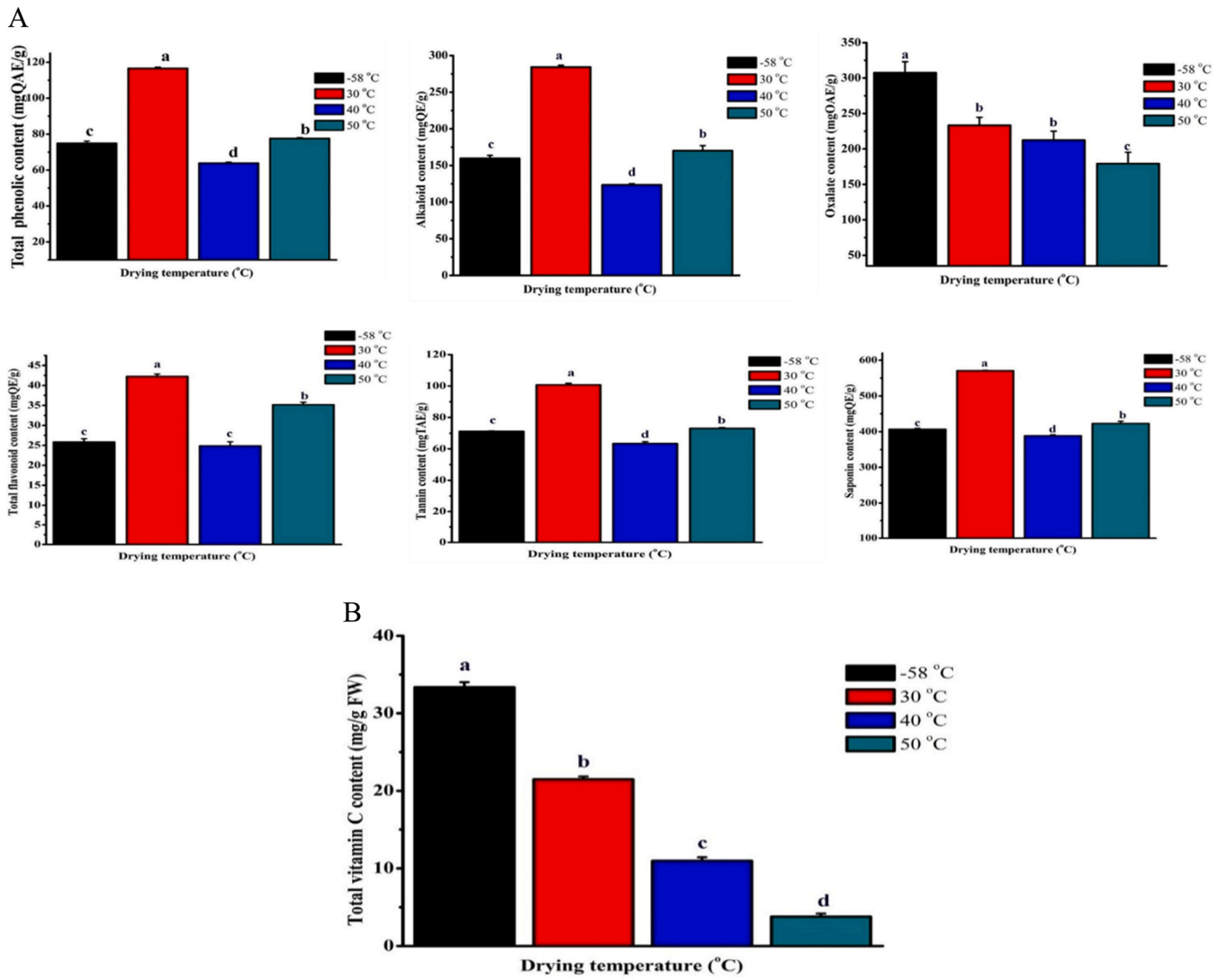

Fig. 2. A: Quantification of total phenolic, total flavonoid, alkaloid, tannin, oxalate, and saponin contents in the extracts of *Hisbiscus sabdariffa* calyx (dried at different temperatures). The bars represent Mean ± SD of triplicate determinations (n = 3). Data were analysed using one-way ANOVA, followed by Turkey's multiple comparisons post hoc test. Bars with different alphabet **(a, b, c, d)** are significantly different at $p < 0.05$. **B**: Quantification of Total Vitamin C contents in extract of *Hibiscus sabdariffa* calyx. The bars represent mean ± SD of triplicate determinations (n = 3). Data were analysed using one-way ANOVA, followed by Turkey's multiple comparisons post hoc test. Bars with different alphabets **(a, b, c, d)** are significantly different at $p < 0.05$.

Hibiscus sabdariffa calyx on glutathione (GSH), catalase (CAT), superoxide dismutase (SOD), and lipid peroxidation activities (Fig. 4a). All the extracts influenced an increase in GSH concentration in a dose-dependent manner. However, among the test samples, the extract of *H. sabdariffa* calyx dried at 30 °C recorded the highest GSH concentration at a range of 7.17 ± 0.52 μg/mL- 142.08 ± 4.90 μg/mL. The CAT activity of the extracts showed a consistent dose-dependent increase in activities. However, among the test samples it was observed that CAT activity increased with increased temperature of drying. Hence, extracts of *H. sabdariffa* calyx dried at 40 °C had significantly higher ($p < 0.05$) activity of catalase enzyme at a range of 0.73 ± 0.00unit/mg Protein–0.91 ± 0.04unit/mgProtein. The SOD activity increased in a dose-dependent manner for all the extracts dried at the different temperatures. Among the test samples, the extract dried at 30 °C had the highest effect on SOD at a range of 73.62 ± 0.26 unit/mg – 90.08 ± 0.13 unit/mg. *The* in vitro determination of the effects of the extracts of the differentially dried calyx of *H. sabdariffa* on lipid peroxidation showed that the lipid peroxidation inhibition activity of *H. sabdariffa* calyx dried at 40 °C increased in a dose-dependent manner (14.19 ± 0.26% - 30.69 ± 0.66%). It was significantly higher ($p < 0.05$) when compared to that of the other test samples, but significantly lower ($p < 0.05$) than that of the control. The pattern is the same for the IC_{50} value (Fig. 4b), such that among the test samples, the calyx dried at 40 °C had the lowest value (209 ± 11.86 μg/mL).

3.4. The extracts of the differentially dried Hibiscus sabdariffa calyx exhibited ameliorative effects against aluminium chloride-induced neurodegeneration in the brain of the experimental Wistar rats

To assess the protective effects of the extracts of the differentially dried calyx of *Hibiscus sabdariffa* against neurodegeneration, we induced neurodegeneration in the experimental Wistar rats with aluminium chloride and treated with different doses of the extracts. Our results, as indicated in Fig. 5a, show that lipid peroxidation (MDA) significantly ($p < 0.05$) increased in the brain of the experimental rats induced with $AlCl_3$ (7 mg/kg/day) when compared to the controls rats. However, treatment with quercetin alone, and the extracts of the differentially dried calyx of *H. sabdariffa* at low and high doses were able to restore the elevated lipid peroxidation to relatively low level. Interestingly, the extract dried at 30 °C proved to be the most potent in the restoration of

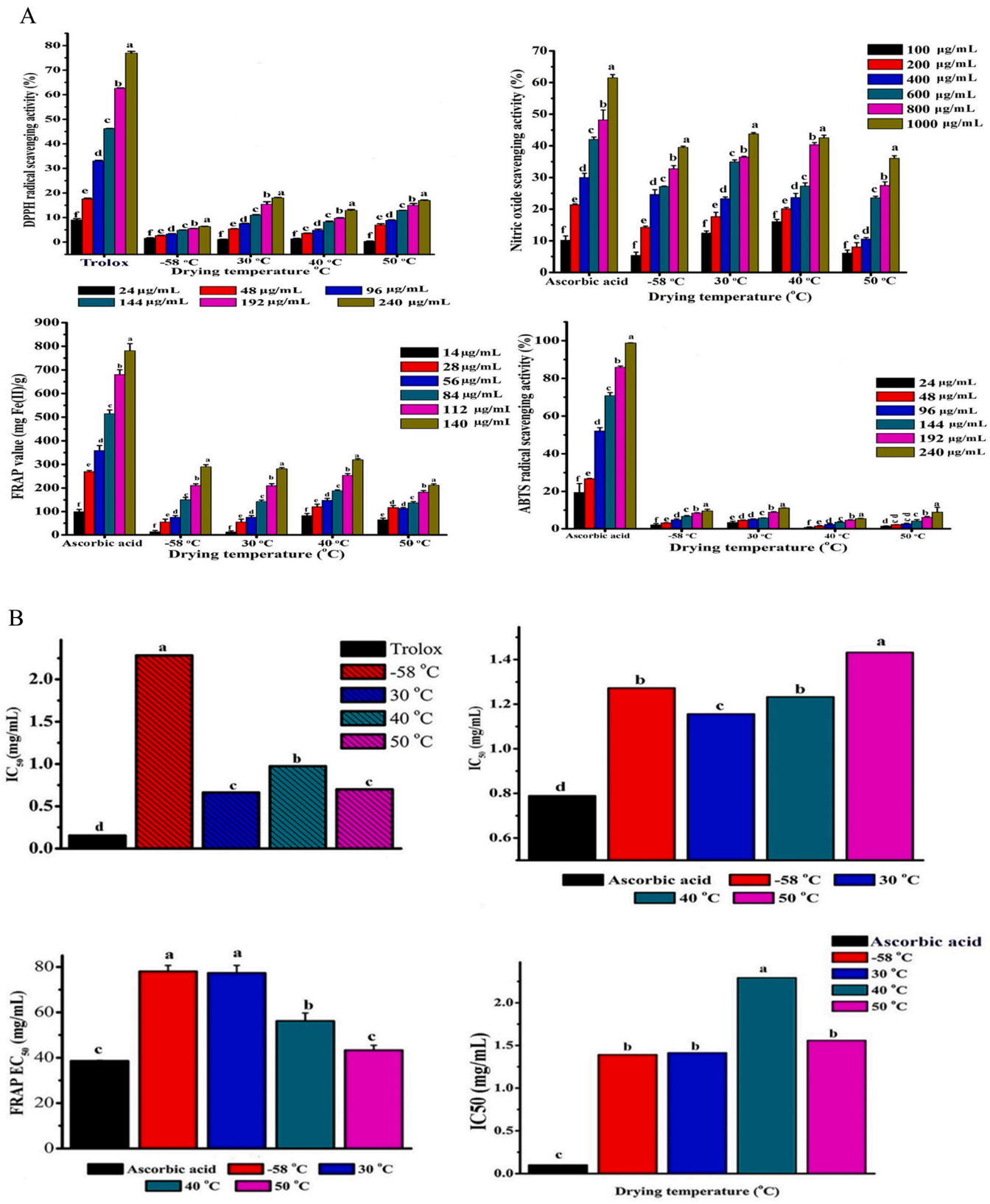

Fig. 3. **A**: DPPH, Nitric oxide, FRAP, and ABTS radical Scavenging activities of extract of *H. sabdariffa*. The bars represent Mean ± SD of triplicate determinations (n = 3). Data were analysed using one-way ANOVA, followed by Turkey's multiple comparisons post hoc test. Bars with different alphabets **(a, b, c, d, e, f)** are significantly different at $p < 0.05$. **B**: IC_{50} of DPPH, Nitric oxide, FRAP, and ABTS radical Scavenging activity of extract of *Hibiscus sabdariffa*. The bars represent Mean ± SD of triplicate determinations (n = 3). Data were analysed using one-way ANOVA, followed by Turkey's multiple comparisons post hoc test. Bars with different alphabets **(a, b, c, d)** are significantly different at $p < 0.05$.

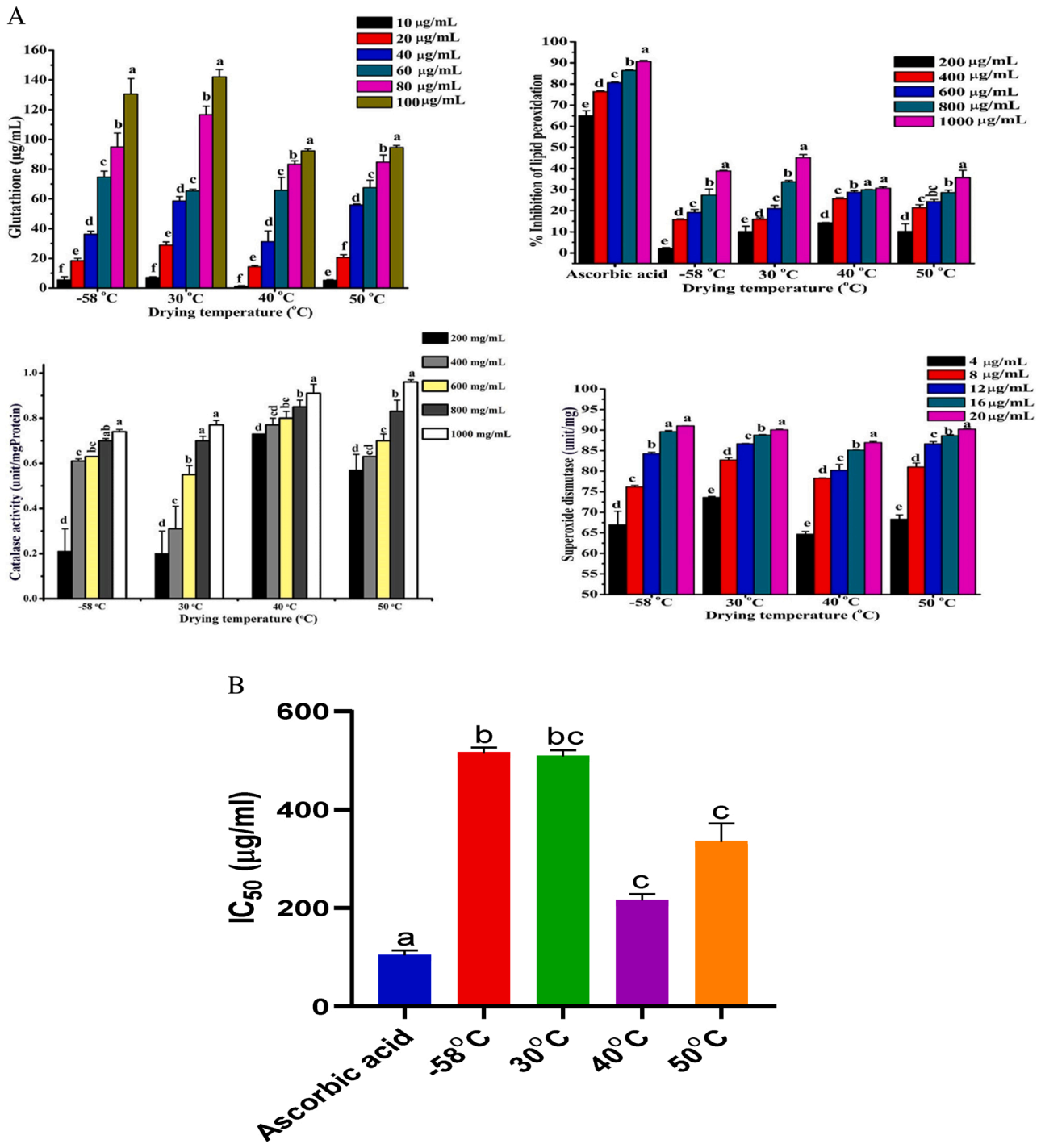

Fig. 4. **A**: Effect of *Hibiscus sabdariffa* extract on glutathione reductase, Lipid peroxidation inhibition, catalase, and superoxide dismutase activity. The bars represent Mean ± SD of triplicate determinations (n = 3). Data were analysed using one-way ANOVA, followed by Turkey's multiple comparisons post hoc test. Bars with different alphabets **(a, b, c, d, e, f)** are significantly different at $p < 0.05$. **B**: IC_{50} of Lipid peroxidation inhibition of extract of *Hibiscus sabdariffa* calyx. The bars represent Mean ± SD of triplicate determinations (n = 3). Data were analysed using one-way ANOVA, followed by Turkey's multiple comparisons post hoc test. Bars with different alphabets **(a, b, c)** are significantly different at $p < 0.05$.

lipid peroxidation in the brains of the experimental rats. Also, compared to the control group, the induction with $AlCl_3$ resulted in decreased levels of GSH and GPX in the rat brains. Among all treatment groups, we observed that at doses of 500 and 1000 mg/kg body weight, the extracts of the calyx of *H. sabdariffa* dried at 30 °C exhibited the highest capacity to increase the activities of GSH and GPx, and was comparable to that of the positive control (Quer) and baseline groups. The SOD and CAT activities of the test rats also decreased significantly ($p < 0.05$) after induction when compared with the negative control. Treatment of the test rats with only quercetin, and the extracts of the differential dried *H. sabdariffa,* at low and high doses, were able to increase the SOD and CAT activities of the brain of the rats to a relatively normal level.

In Fig. 5b, our results show that the test rats induced with $AlCl_3$ had significant increases ($p < 0.05$) in the percentage inhibition of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) activities when compared with the negative control group. Treatment with the

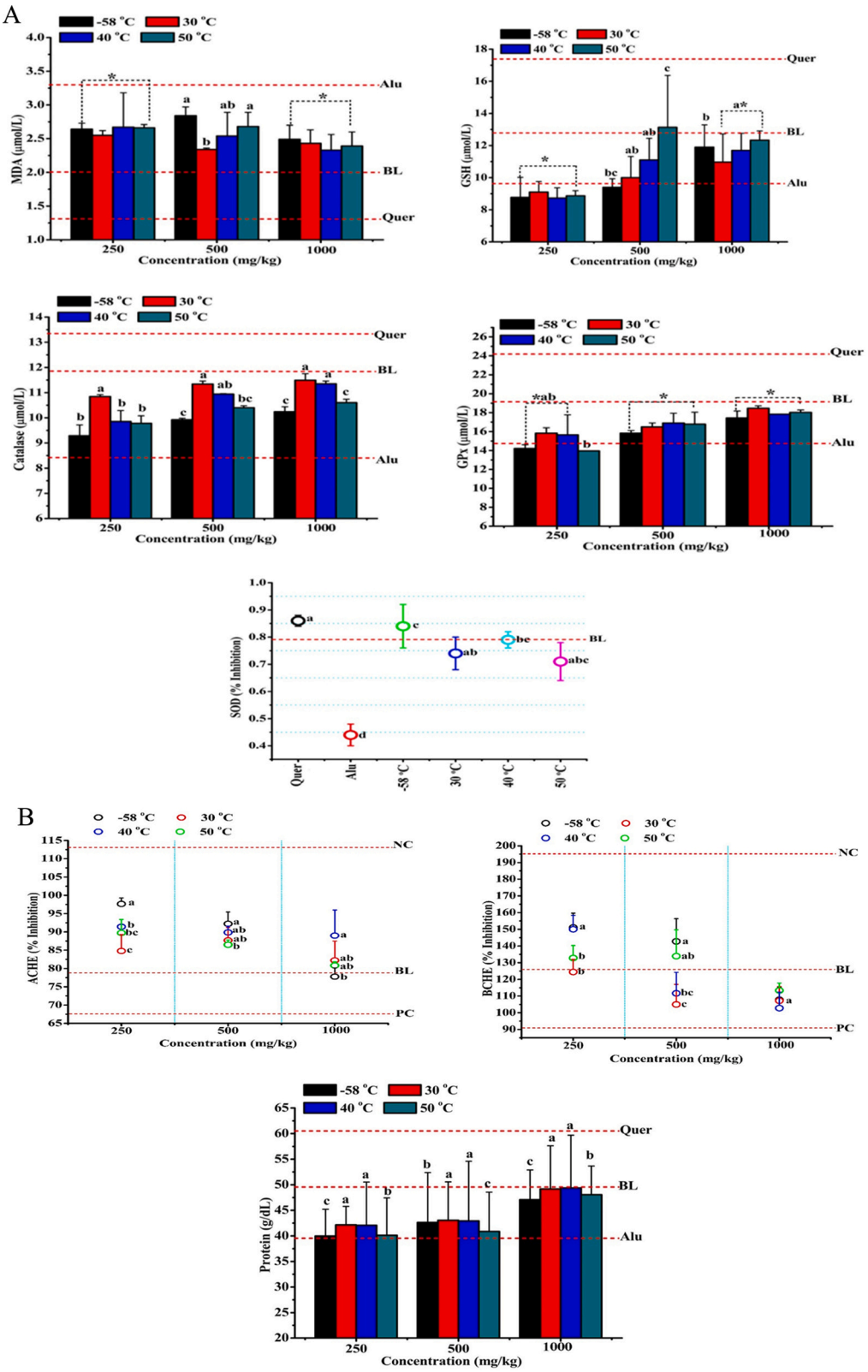

Fig. 5. A: Malonaldehyde (MDA), Catalase (CAT), Glutathione (GSH), Glutathione peroxidase (GPX), and Superoxide dismutase (SOD) activity after oral administration of extracts of *H. sabdariffa* calyx dried at different temperatures. Key: BL- Baseline; Alu - Aluminium; and Quer - Quercetin. The dotted lines represent the levels of the antioxidant activities for the indicated group. Values are presented as mean ± SEM (n = 8). Compared with control, * indicates no significance difference at $p > 0.05$, and bars with different alphabets **(a, b, c)** are significantly different ($p < 0.05$). **B:** Acetylcholinesterase (ACHE) activity, butyrylcholinesterase (BchE) activity, and protein content in rat brain after oral administration of extracts of differentially dried *H. sabdariffa* calyx. Key: BL- Baseline; Alu - Aluminium; and Quer – Quercetin; PC – Positive control (Quercetin); and NC – Negative control ($AlCl_3$). Values are presented as mean ± SEM (n = 8). Bars with different alphabets **(a, b, c)** are significantly different ($p < 0.05$).

antioxidant quercetin alone, and the extracts of the differentially dried calyx of *H. sabdariffa,* at low and high doses, were able to significantly ($p < 0.05$) decrease the percentage inhibition of AChE and BChE of the rat brain to near normal. It is worthy of note that extract dried at 30 °C proved to be most potent in the reduction of the percentage inhibition of AChE and BChE activity of the brain of the test rats. Also, we found that induction with $AlCl_3$ resulted in a significantly ($p < 0.05$) lower protein levels in the brain of the test rats, while treatment with the extracts caused a dose-dependent increase in the protein levels of the test rats, as compared with those of the control rats.

4. Discussion

The phytochemical profiling of medicinal plants and herbs is an important step in the evaluation of their bioactivities. However, variations in the drying temperatures play a key role in determining the retention and amounts of phytochemicals present in the medicinal plants. Our preliminary qualitative phytochemical screening of the aqueous extracts of the calyx (dried at different temperatures) of *H. sabdariffa* revealed the presence of some secondary metabolites such as cardiac glycoside, coumarin glycoside, phenols, tannins, terpenoids, alkaloids, flavonoids, steroids, anthraquinones, and saponins. We observed that the phytochemicals were mostly retained at the different drying temperatures employed in this study. However, coumarin glycoside was completely absent when the calyx of *H. sabdariffa* were dried at − 58°C, 30 °C, 40 °C and 50 °C. An increase in temperature resulted in the loss of steroid, as observed in the extract of *H. sabdariffa* calyx dried at 50 °C. The phytochemical constituents in the aqueous extract of *H. sabdariffa* calyx, as we observed, was similar to that earlier reported in the literature [37]. However, there are slight variations between the data from this study and that reported by Jamini et al., [37]. This may be attributed to variations in genetic composition, type of soil, and extractive solvent. Importantly, our results indicated that temperature variations significantly influenced the availability of phytochemicals in *H. sabdariffa* calyx, and thus its bioactivities. Alkaloids, saponins, tannins, flavonoids and phenols have been generally implicated in various therapeutic activities [13]. Specifically, flavonoids are well-known for their antiviral, anti-inflammatory, antioxidant and cytotoxic activities [38]. It has been demonstrated in several studies that dietary sources of saponins provide a preventive strategy in the lowering of the risk of several metabolic disorders [39]. Overall, the phytochemical screening clearly indicated that the extracts of *H. sabdariffa* calyx possess good amounts of phytochemical components that could pass as potent nutraceuticals.

In order to assess the variation in the concentration of these phytochemicals after drying the calyx at different temperatures, a quantitative screening was performed. The result of the total phenolic content showed significant differences among the extract of *H. sabdariffa* calyx (dried at different temperatures). The highest polyphenol, saponin, tannin, flavonoid, phenolic and alkaloid contents were recorded in the extract from the calyx of *H. sabdariffa*, dried at 30 °C. The observed increase could be attributed to the loss of water activity and removal of destructive enzymes in the calyxes; hence, the retention of high levels of phytochemicals in the extracts. Previous studies have shown that the application of temperature higher than 50 °C in the drying of *H. sabdariffa* calyx could result in the loss of essential oil yields and important phytochemical constituents such as total phenol [40]. According to Hatami et al., [41], *H. sabdariffa* contains a high level of total phenolic content (TPC) which is responsible for some of its biological activities. However, the amount of TPC is also very susceptible to changes in processing conditions such as high temperatures and availability of oxygen. The application of heat has been reported to cause significant degradation of phenolic compounds in medicinal plants and herbal tea, ginseng, and wax apple [42]. The result of the total phenolic content (TPC) showed a remarkable increase in the extract of the calyx that were dried at 30 °C, when compared with that of the calyx dried at other temperatures. This finding is similar to that of the study carried out by Chua et al. [43], where it was reported that the drying of *Strobilanthes crispus* at 60 °C, resulted in the retention of more of the phenol content than in those dried at 80 °C, 100 °C and 120 °C. Hence, they observed that drying at a longer time is as good as exposing them to a longer period of heat, light, and oxygen; hence, a higher chance of the degradation of the phytochemicals. Also, we observed that the total flavonoid content was mostly retained in the extract of the calyx dried at 30 °C. This finding correlates with those reported by Riaz and Chopra [44] which demonstrated that the flavonoid content of the calyx of *H. sabdariffa,* dried in a microwave, was higher than that dried in a hot-air oven at a temperature of 100 °C. A previous report also indicated that a temperature higher than 50 °C leads to a decrease in the yield of essential oil and total phenolic content of different plant species [45].

The retention of alkaloid was more evident in the extracts of *H. sabdariffa* calyx dried at 30 °C. Among phytochemicals in *H. sabdariffa* with biological and nutraceutical properties, tannin is reportedly lower in content [46]. Compared with the control, the tannin content decreased in all the extract samples. Nevertheless, our results show that tannin was mostly retained in the extract of the calyx dried at 30 °C. This is in agreement with the report from an earlier study which stated that different food processing methods often reduce the tannin content in plants [47]. High oxalate content in food possibly indicates toxicity, while lower oxalate content could be of an advantage. Our results show a significantly higher total oxalate content for the sample dried at − 58°C when compared to that for others at different drying temperatures. In this study, an increased temperature was observed to cause a reduction in the oxalate content. Previously, Bakr and Gawish [48], as well as Adeboye and Babajide [49], reported that the application of high temperatures in the processing of plant food products caused a reduction in the amount of oxalate. Similarly, Ojiako and Igwe [47] showed that proper and effective cooking of vegetables, before eating, significantly reduced the oxalate content. Our result also show that the extract of the calyx dried at 30 °C had the highest saponin content in comparison with the other samples dried at other temperatures. In a similar study by Bakr and Gawish [48], it was reported that different food processing methods often cause a reduction in tannin and saponin content in plants. Apart from the common phytochemical constituents, the calyx of *H. sabdariffa* are well known as an important source of dietary polysaccharides, vitamin B complex, and vitamin C. The application of temperature variations in this study revealed that the total vitamin C content in the extracts of *H. sabdariffa* calyx was mostly retained in the freeze-dried samples (calyxes dried at −58°C). Hence, a significant decrease in total vitamin C was observed as temperature increased. Ascorbic acid (vitamin C), which is considered as an important nutrient constituent in fruits and vegetables, is usually selected as a parameter for determination of nutritional value because of its labile nature compared to other nutrients in foods. The degree of loss of vitamin C during the process of drying of vegetables depends on their physical properties and the processing method employed [50]. Our findings corroborate with those of Sahar et al., [51] which stated that during the process of drying, a significant decrease in the ascorbic acid content was observed. Further, they reported that the highest vitamin C in dried *H. sabdariffa* leaves were obtained during freeze drying. Reports have shown that high temperatures and long drying time are the two main factors that influences vitamin C content in the final products [52]. A few studies have also reported that short drying time and lowered temperature maintains ascorbic acid [53]. Findings from this study suggest that the retention of vitamin C could be harnessed when *H. sabdariffa* calyx are dried at − 58°C.

In this study, we also evaluated the ability of the extracts from the dried calyx of *H. sabdariffa* to fight against free radicals. Free radicals could potentially react with biological macromolecules, with consequent oxidative damages to lipids, proteins and DNA [54]. The free radical scavenging constituents of medicinal plants have been regarded as potentially useful as therapies in the practise of ethnomedicine, and

the prevention and treatment of oxidative stress-induced disorders and diseases [9,55,56]. Here, the free radical scavenging potentials of the extracts of *H. sabdariffa* calyx, dried at different temperatures, was evaluated by determining their effects on the DPPH, ABTS, Nitric oxide (NO), and FRAP scavenging activities. A concentration dependent DPPH radical scavenging activity was observed among all the test samples. Although, significantly lower than the control, the extract of *H. sabdariffa* calyx dried at 30 °C recorded the highest DPPH radical scavenging activity. Our report correlates with those of Rabeta and Lai [57], and Rodrigues et al., [58] who reported that at temperatures higher than 50 °C there was a decrease in the yield of essential oil and total phenol from different plant species. DPPH assay is mostly attributed to phenolic compounds that participate in the scavenging of free radicals [59]. Oxidative stress, which results from the accumulation of ROS, has been linked with several diseases. Thus, the direct scavenging of DPPH could be partially related to the suppression of oxidative stress [60]. As shown in our results, all the extracts of *H. sabdariffa* calyx significantly decreased the release of nitric oxide in vitro. In addition, it was observed that the scavenging activity of nitric oxide by all the extracts of *H. sabdariffa* calyx increased in a dose-dependent manner. Chronic exposure to nitric oxide radical has been linked with several inflammatory conditions, and multiple sclerosis [61]. Thus, the scavenging of nitric oxide by *H. sabdariffa* may protect against some of these disease conditions. The FRAP analysis showed that all extracts exhibited ferric reducing power, which increases with increasing concentration. The reducing power capacity of the extracts may provide a significant indication of the potential antioxidant capacity of *H. sabdariffa*. Similar to the DPPH assay, the reducing power capacity of the extracts provides a significant indication of the potential antioxidant capacity of the calyx of *H. sabdariffa*. However, while DPPH assay is mostly attributed to phenolic compounds which participate in the scavenging free radicals, the reducing power involves the inactivation of metal ions such as ferric ions, which is also evaluated by phenolic acid [62]. The ABTS radical scavenging capacity of the extracts also increased in a concentration-dependent manner. However, among the differentially dried *H. sabdariffa* calyx, the ABTS scavenging activity of the extracts dried at − 58°C, 30 °C and 50 °C were not significantly different. Although, significantly lower than the control, the ABTS scavenging activity of the extracts of *H. sabdariffa* is still appreciable, and could contribute to the fight against oxidative stress.

The release of GSH could initiate the prevention of oxidative stress, since it is important in preventing and delaying damage to cells. Our results show that GSH concentration increased with an increased concentration of the extracts of the calyx of *H. sabdariffa*, which indicates a high level of antioxidant activity. The inhibitory effects of the extracts on lipid peroxidation were found to also increase with increasing concentrations, and most effective when the calyx was dried at 30 °C. This is also true for SOD activity which is highest in the sample dried at 30 °C, and this indicate the ability of the phytocomponents of the extracts to serve as good donors of hydrogen ions. SOD is an endogenous antioxidant which is capable of detoxifying super anion radicals into hydrogen peroxide which is further converted, by catalase, into water and molecular oxygen [63]. The increase observed in SOD activity in this study, is indicative of the response to oxidative damage by the extracts. The highest protective effect against oxidative damage via increase in endogenous catalase enzyme (known to play a role in the oxidation of H_2O_2) activity was observed in the extract of the calyx (dried at 50 °C) of *H. sabdariffa*.

Our findings also show that induction of oxidative stress and neurodegeneration in the experimental rats, with aluminium chloride, resulted in a significant increase in the level of TBARS (LPO) and significant decreases in the levels of protein, GPX, GSH and SOD. Aluminium can effectively aggravate iron-initiated lipid peroxidation (LPO). Transferrin receptors are usually found in the brain where they bind to aluminium and provide access point for the movement of iron from the circulation into the brain. The binding of aluminium to transferrin receptors often competes with and reduce the binding of iron to protein, consequently, resulting in an increased concentration of free iron in intracellular spaces. Thus, transferrin receptor agonists could possibly prevent the binding of aluminium to transferrin receptors, and prevent the release of free radical formation; hence brain oxidative stress [63]. The extracts from the calyx of *H. sabdariffa* were found to be very potent in ameliorating and protecting against the effects of the induced disease conditions. This is because treatment with the extracts resulted in a significant decrease in the level of TBARS (LPO) and significant increases in the levels of protein, GPX, GSH and SOD. These could be attributed to the antiradical and chelating effects of the phytocomponents of the extracts such as flavonoids, glycosides, and tannins. It is interesting to know that the extract of the calyx dried at 30 °C proved to be the most potent extract in the restoration of lipid peroxidation in the brain of the experimental rats. This, possibly, points at the prophylactic effect of *H. sabdariffa* in the management of neurodegeneration.

SOD activity is a sensitive index in oxidative damage because it scavenges superoxide anion and in turn forms hydrogen peroxide from it, which eventually leads to a diminishing effect of toxicity [9,56,64]. Catalase, an enzymatic antioxidant, is widely distributed in all animal tissues where it contributes to the decomposition of hydrogen peroxide and protect the tissue from highly reactive hydroxyl radicals [56,65]. As expected, post-treatment of rats with only quercetin and extracts of *H. sabdariffa* calyx were able to restore the decreased SOD and CAT activities in the brain of the rats, and was comparable to the control. GSH and GPX play an important role in the detoxification of potentially toxic compounds [66,67]. The levels of GSH and GPX in the rat brains were effectively restored in both experimental and positive control groups. Reduced glutathione, and glutathione reductase are essential antioxidant enzymes because they have been implicated in the direct destruction of reactive oxygen species. Consequently, the reduction in their activities may bring about a number of deleterious effects, like the accumulation of superoxide radicals and hydrogen peroxide, which is associated with neurodegenerative diseases [68]. This study inferred that administration of *H. sabdariffa* in healthy rat could prevent brain oxidative damages through the stimulation of an increase in the activities of GSH and GPX, and a concomitant decrease in TBARS level. Therefore, our findings suggest that *H. sabdariffa* could, possibly, initiate the prevention of cognitive decline during aging and neurodegenerative disease.

In line with the above findings, our results also show that induction with $AlCl_3$ caused significant increases in the percentage inhibition of AChE and BChE activities while treatment with the antioxidant quercetin alone, and the extracts of the differentially dried calyxes of *H. sabdariffa* caused significant decreases in the percentage inhibition of AChE and BChE of the rat brain to near normal. In the brain, acetylcholine and butylcholine are the major neurotransmitters that are responsible for cognitive function, wearing, and memory loss, while the enzymes responsible for their hydrolysis are acetylcholinesterase (Ache) and butyrylcholinesterase (BChe) [69]. Inhibition of AChE and BChE can induce cholinergic crisis which could affect the nicotinic synapses of the central nervous system in the brain, leading to neurodegeneration. Thus, increases in the percentage inhibition of these enzymes due to induction with aluminium chloride could possibly trigger cholinergic crises in the brains of the rats. AChE and BChE primarily helps in terminating neuronal transmission and signalling between synapses. This is often necessary to prevent the dispersal of ACh and BCh, overstimulation of cholinergic receptors, as well as the activation of nearby receptors. This is very vital for the proper functioning of the neurons in the brain. The reduction of the percentage inhibition of these enzymes (AChE and BChE), as occasioned by the treatment with the extracts of the differentially dried calyx of *H. sabdariffa* portends a protective or ameliorative effect of the extracts against the neurotoxic effects of $AlCl_3$. Among the different extracts, it is interesting to know that extract dried at 30 °C was more potent in the reduction of the percentage inhibition of AChE and

BChE activity of the brain of the test rats. This is possibly connected to our findings so far which show that the extract of the calyx of *H. sabdariffa* dried at 30 °C has the highest amount of phytocomponents, with better antioxidant potential. The in vitro administration of quercetin has been shown to exhibit AChE and BChE activities along with Fe^{2+}chelating activity [70]; hence, its activity in the rat brain was expected. The increased vulnerability of the $AlCl_3$ treated rat brain to oxidative stress was evident in the increased oxidative stress and decreased antioxidant enzymes observed in the negative control group. Our results also revealed that the experimental rats exposed to $AlCl_3$ had a significant decrease in the levels of protein in their brains, when compared to those of the control group. However, treatment with the extracts of *H. sabdariffa* calyx resulted in increases in the protein levels. The effects of the extracts were also shown to increase with increasing doses.

5. Conclusion

In this study, we report that the extracts of the differentially dried calyx of *H. sabdariffa,* possess good amounts of useful phytochemicals, and exhibited a good potential to fight against oxidative stress and protect against aluminium chloride-induced neurotoxicity in the brains of the experimental rats. It is also worthy of note that the most effective or potent extract of *H. sabdariffa* calyx, among the ones studied, is the extract from the calyx that were dried at 30 °C.

Institutional review board statement

The experimental procedure of this study was approved and carried out at the laboratory of the Department of Biochemistry, Faculty of Life Sciences, Ambrose Alli University, Ekpoma, Edo State, Nigeria.

Consent to participate

Not Applicable.

Consent for publication

Not Applicable.

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CRediT authorship contribution statement

JOE and MAA conceived, designed and performed the experiments; JOE, KO and MAA performed the analysis and interpretation of the data, while KO prepared the draft of the manuscript. All authors have reviewed and approved the final draft of the manuscript. All authors agree to be accountable for all aspects of work ensuring integrity and accuracy.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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