Ameliorative Potential of Methanolic Extract of Clerodendrum splendens Leaf Against Stress Assault

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Abstract
Physiological stress leads to the compromising of the antioxidant status of the biosystem and progressing to the condition of oxidative stress. Thus, this study investigated the attenuating tendency of methanolic leaf extract of *Clerodendrum splendens* against physiological assault, by evaluating its effects on the oxidative stress markers and hepatopathic state. The assessment of the antioxidant potentials at 500mg/ml was typified using the total antioxidant capacity, and ABTS, DPPH and hydroxyl radicals scavenging activities. Others are reducing power, metal chelating and lipid peroxidation inhibition activities. Activities of *Clerodendrum splendens* leaf extract were evaluated on hepatophatic indices (ALT, ALP, AST and GGT, tatal bilirubin) as well as antioxidant status (glutathione transferase and superoxide dismutase, activities, and reduced glutathione). The total antioxidant activities of the extract was 149.97±1.233 mg/g AAE (Ascorbic Acid Equivalent). The extract exhibited significant, potent and appreciable antioxidant activities. The study confirmed that the extract contained highly antioxidative bioactive compounds contributing to its antioxidant and anti-inflammatory (anti-stress) potencies.
Keywords: Hepatopathy, Anti-stress, *Clerodendrum splendens* leaf extract, Antioxidant

INTRODUCTION

The medicinal efficacy of vegetable can be guaranteed as help in curing illness thereby reducing the cost on seeking orthodox medical therapy. *Clerodendrum splendens* is a popular vegetable for nutritional and pharmacological purposes. It is a flowering plant among the Yoruba populace (South-West, Nigeria) where it is used ground and used for soup delicacy. The species is a flowering plant of the family Lamiaceae. It is a twining climber.

A vast amount of research has been conducted to understand the intricate cascade of events that occur once the brain detects a disruption in homeostasis(a stressor) and the hormonal responses driven by these systems (Thomas and Lena, 2010; Kyrou and Tsigos, 2009; Charmandari and Tsigos, 2005). The key components of the "stress system" are the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (SNS). The hypothalamus is triggered to facilitate the secretion of corticotropin-releasing hormone (CRH or CRF, corticotropin-releasing factor) and arginine vasopressin (AVP), eliciting both the production of adrenocorticotropin hormone (ACTH) from the posterior pituitary and the activation of the noradrenergic neurons of the locus caeruleas/norepinepherine (LC/NE) system in the brain. The LC/NE system is primarily responsible for the immediate "fight or flight" response driven by epinephrine and norepinephrine, while ACTH drives the production of cortisol from the adrenal cortex. Under normal conditions, the production of CRH and ACTH fluctuate in a predictable circadian cycle and are inhibited by high levels of blood cortisol via a well-described negative feedback loop. Experimental and clinical evaluations are specific for a wide range of body changes also called adaptation syndrome, which are predictable rhythm and responses of the HPA axis (Thomas and Lena, 2010).

Cold and immobilization stressors can generate oxidative stress and skeletal muscle fatigue. Cold immobilization stress also called immobilization stress and cold restraint stress had been described by Popovic *et al*. (2009) as experimental induction of very extreme condition in rat model, but believed to have same effect on human as it revealed in animal models. Free radicals cause oxidative degradation of lipids, proteins, nucleic acids and carbohydrates molecules thereby compromising cell integrity and function. The large generation of free radicals, particularly reactive oxygen species and their high activity play important roles in the progression of a great number of pathological disorders like inflammation, atherosclerosis, stroke, heart disease, diabetes mellitus, multiple sclerosis, cancer, Parkinson's and Alzheimer's diseases (Aina and Oyedapo, 2013; Ozgen *et al.,* 2006 and Mensor *et al.*, 2001).

This study was aimed to evaluate the anti-stress properties of methanolic leaf extract of *Clerodendrum splendens* on hepatic function as well as modulation of the antioxidant status of the animals.

METHODOLOGY

Reagents and Chemicals used in this experiment were obtained from different sources such as British Drug House (BDH) and Sigma Aldrich limited; all were of good analytical grades. All the solutions, buffers and reagents were prepared using glass distilled water. Diazepam was used in this experiment as standard anti- stress drug (reference drug) that was purchased from Martadol Pharmaceutical Limited, Akure, Ondo State, Nigeria and was NAFDAC registered.

The *Clerodendrum splendens* leaf was collected in a location in Akure, Ondo State, Nigeria. Extraction of bioactive components involved (Jianping *et al.,* 2011); approximately 100g of oven-dried *Clerodendrum splendens*

leaf was soaked in 500 mL of 80% methanol (80:20, vol/vol) and adjusted to pH 1.5 with 1 M HCl. The mixture was then mixed thoroughly using a vortex mixer for 2 min and centrifuged at 6000g for 10 min at 4°C. The supernatant was collected, freeze-dried and reconstituted appropriately.

Phytochemical Screening
The phytochemical analysis of the extract was typified by qualitative evaluations for alkaloids, flavonoids, tannins and steroid.

***In Vitro* Antioxidant Assays**
Assay for Total Antioxidant Activity
To the reagent solution; sulphuric acid (0.6 M), sodium phosphate (28 mM) and ammonium molybdate (4 mM); 0.3 ml of sample was added and incubated at 95 ^{0}C in a water bath for 90 min. After cooling to room temperature absorbance was recorded at 765 nm against reagent blank. The absorbance of the sample was extrapolated on the ascorbic acid standard curve to obtain concentration of the sample in mg/ml then the total antioxidant activity (mg/g ascorbic acid equivalence) was calculated.

Assay of Reducing Power Activity
The reducing power of the extract was determined according to the method of Oyaizu (1986).

Assay of DPPH Radical Scavenging (1, 1-diphenyl-2-picrylhydrazyl) Activity
The DPPH radical scavenging activity of the extract was evaluated according to the method described by Leong and Shui (2002).

Assay of 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulphonic Acid) (ABTS) Radical Scavenging Activity
ABTS radical scavenging activity of the plant extract was determined according to the method of Re *et al*. (1999).

Assay of Metal Chelating Activity
The metal chelating activity was determined according to the method of Haro-Vicente *et al.* (2006).

Assay of Hydroxyl Radical Scavenging Activity
The hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and the extracts for hydroxyl radicals generated from the Fe^{2+} /ascorbate/EDTA/H_2O_2 system according to the method of Halliwell *et al*. (1987).

Assay of Lipid Peroxidation Inhibition Activity
The inhibition of lipid peroxidation was ascertained according to the method of Ohkawa *et al*. (1979).

Experimental Design
Immobilization stress (IS) combined with cold restraint stress (CRS)
Male albino rats were used according to the standard guidelines of the Care and Use of Experimental Animal Resources. 25 male albino rats weighing 200 ± 10 g were used to evaluate the ability of the *Clerodendrum splendens* leaf extract (reconstituted in water) to combat stress and were obtained from standard animal house. The rats were housed 5 per cage under constant environmental conditions (20–24 °C; 12 h light/dark cycle), and were given ad libitum access to standard pelleted food and water. After the administration of extract for 21 days, combined IS and CRS test was performed by immobilizing animals in the cold chamber at 4 ± 0.3 ºC; the plexiglass cage volume was adjusted to the size of the animal, to restrain completely their movements for 2 hours, except group 1. Group 2 animals were treated after stress with diazepam as standard anti-stress drug.
Group 1: Untreated, unstressed group-Negative control group (C-)
Group 2: Untreated, stressed group-Positive control group (C+)
Group 3: Diazepam treated (2.5 mg/ml/BW/0.2ml ip), stressed group (D2) (Ibukun and Oladipo, 2016)
Group 4: Treated (extract 5 mg/ml/BW/0.5ml orally), stressed group (C1)
Group 5: Treated (extract 10 mg/ml/BW/0.5ml orally), stressed group (C2)

Preparation of serum and tissue homogenates
Blood samples were collected by ocular punctures into plain bottles. Serum was prepared by aspiration of the clear liquid after clotting and centrifuged for 10 minutes at 3000g in a bench centrifuge.

In vivo Biochemical Estimation

Liver function Evaluations

Assay of Determination of Total Protein (TP) in liver tissues based on Weichselbaumin (1995). Aspartate Amino Transferase (AST) Activity in serum was evaluated based on the principle of Reitman and Frankel (1957). Alanine amino transferase (ALT) activity was carried out based on the principle described by Reitman and Frankel (1957). Alkaline phosphatase (ALP) activity was carried out based on the method of Englehardt *et al*, (1970). Assay of Determination of Bilirubin Concentration in serum and Gamma-Glutamyl Transferase (GGT) Activity in serum were conducted using a Randox Laboratory protocol.

Evaluation of Endogenous Antioxidant Enzyme in Serum

SOD activity was assayed by the method of Kakkar *et al,* (1984). Reduced glutathione (GSH) level in the serum was assayed following the method of Ellman (1959), modified by Hissin and Hilf (1973). GST catalyzes the conjugation reaction with glutathione in the first step of mercapturic acid synthesis. GST activity was measured by the method of Habig and Jakoby (1974).

All values are expressed as mean±standard deviation. Statistical evaluation was done using One Way Analysis of Variance (ANOVA) followed by Duncan's Multiple Range Test (DMRT). The significance level was set at $p<0.05$.

RESULT AND DISCUSSION

The total antioxidant capacity of *Clerodendrum splendens* leaf extract (Tab. 2) was 149.97±1.233 mg/g ascorbic acid equivalent (AAE), this result revealed that the extract had antioxidant activity and can potent scavenging free radicals. The iron reducing activities of extracts showed an ability to scavenge free radicals via reductive-oxidative process. DPPH radical scavenging activity showed the proton-donating ability of the extract. The extract was a fast and effective scavenger of the ABTS radical and this activity was comparable to that of trolox. Efficiency in quenching free radicals in the system is dependent on the overall antioxidant capacities. The metal (Fe^{2+}) ion chelating activities of the extract was typified by specific assay. The chelating ability of the leaf extract was found to be lower compared to the reference compound (EDTA) at the working concentrations. The hydroxyl ion radical scavenging activity at concentration (500 mg/ml) revealed competitive activity against the reference compound-mannitol. The lipid peroxidation inhibition activity exhibited by the extract was typified and revealed a lower activity against the reference-quercetin, at concentration of 500 mg/ml ($p<0.05$). The evaluations of the antioxidant activities of the extract were imperative to evaluating its possible in vivo modulation of the antioxidant status in bio-model. Table 2 revealed the activities of extract competitively with standard antioxidant compounds. It was obvious from the result that the extract had metal chelating activity as well as radicals scavenging abilities. The result also established the extract as a proton-donator, a reductive activity peculiar with good antioxidant compounds.

Table 1: Phytochemical constituents of the *Clerodendrum splendens* leaf extracts

Phytochemicals	
Flavonoids	-
Steroid	-
Terpenoids	++
Tannins	++
Saponin	++
Alkaloids	+

++ = Abundant, + = trace and - = absent.

Table 2: Antioxidant evaluations of *Clerodendrum splendens* leaf extract

500mg/ml	TOTAL ANTIOXIDANT(mg/g AAE)	ABTS RADICAL SCAVENGING (% activity)	DPPH RADICAL SCAVENGING (% activity)	METAL CHELATING (% activity)	REDUCING POWER (absorbance)	HYDROXYL RADICAL SCAVENGING (% activity)	LIPID PEROXIDATION INHIBITION
Clerodendrum splendens leaf	149.97±1.233	84.77±3.091	84.30±9.053	62.13±6.737	1.88±0.0012	89.22±4.663	79.16±1.923
ASCORBIC ACID	-	-	94.72±6.35	-	2.637±0.05	-	-
MANNITOL	-	-	-	-	-	96.34±9.16	-
EDTA	-	-	-	93.17±3.92	-	-	-
TROLOX	-	90.61±2.81	-	-	-	-	-
QUERCETIN	-	-	-	-	-	-	92.25±0.125

Values are expressed as mean ± standard deviation (n=3). Values with different superscript are significantly different (P<0.05).

Biochemical Estimations

These biochemical indices such as, alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyltransferase (GGT), glutathione transferase and superoxide dismutase (SOD), activities, and reduced glutathione (GSH) level total bilirubin and total protein concentration, were used in assessing the anti-stress property of the *Clerodendrum splendens* leaf extract.

Table 3: Biochemical evaluations of effects of *Clerodendrum splendens* leaf extract

GRPS	ALT (U/L)	TOTAL BILIRUBIN (mg/dl)	AST (U/L)	GGT (U/L)	ALP (U/L)	TOTAL PROTEIN (mg/dl)	SOD (µmol/min/ g tissue)	GSH (mg/g tissue)	GST (µmol/min /g tissue)
C-	18.26±4.55a	1.15±0.42ab	36.37±3.71a	12.61±0.42a	34.28±0.93a	17.05±0.81e	149.89±4.34d	51.19±4.01d	5.42±0.79e
C+	30.25±1.09d	3.38±0.12d	55.97±5.89d	44.83±0.02e	68.80±0.94d	3.31±0.89a	38.6±4.47a	12.846±0.65a	0.45±0.13a
D	24.22±4.26bc	1.36±0.15bc	44.28±1.74c	21.52±0.82b	36.02±2.14ab	12.83±0.30d	76.154±5.48b	20.182±1.20b	1.40±0.27b
C1	18.74±1.44a	1.12±0.12a	40.24±2.81b	34.29±2.11d	42.33±0.48c	10.38±0.32b	79.94±7.39b	25.92±3.31b	1.95±0.24c
C2	18.11±2.91a	1.09±0.11a	37.30±1.28a	22.39±0.42c	38.4±2.37b	14.92±1.28c	121.99±4.91c	29.44±2.18c	2.62±0.91d

Values are expressed as mean ± standard deviation (n=5). Values with different superscript are significantly different (P<0.05) for each assay.

Acute emotional stress (immobilization or restraint, alone or in combination with cold exposure) is one form of emotional stress, that has been widely used as a technique for and physiological changes coping ability of animals in response to stress (Pearl *et al.,* 1966). It had been reported that rats with restraint stress demonstrated elevation in the plasma levels of adrenocorticotropic hormone (ACTH) and corticosterone which occur during stress response via activation of the hypothalamic-pituitary-adrenal (HPA) axis (Lou *et al*., 2008). The advent of stress in the biosystem is envisaged to release corticosterone that is synthesized in response to adrenocorticotrophic hormone, stimulating the circulation of high-energy compounds such as glucose, free amino acids, and free fatty acids, initiating cellular proliferation (Ibukun and Oladipo, 2016).

Liver damage or hepatopathy could be confirmed by elevated activities of AST, ALP and ALT. The result of the present study is in agreement with some previous reports on the effect of stress on liver enzymes. Table 3 revealed the effect of *Clerodendrum splendens* leaf extract on physiological stress on liver enzyme markers; the alterations in the activities of GGT, ALT, AST and ALP. Pretreatment with doses of *Clerodendrum splendens* leaf extract and the standard drug (diazepam) (2.5 mg/ml/BW) in the stressed groups, significantly (p<0.05) reduced the elevated liver ALP, AST and ALT levels, which could be due to inhibition of stimulation of sympathetic nervous system by the polyphenolic and/or other antioxidants compounds. Previous research by Ibukun and Oladipo (2016) had demonstrated 2.5 mg/ml/BW of diazepam as better ameliorative dose in rat model compared to 5 mg/ml/BW. This study established the findings about the potency of 2.5 mg/ml/BW. The research showed significant amelioration of hepatopathy as a result of physiological stress by *Clerodendrum splendens* leaf extract typified by reduced activities of GGT, ALP, AST and ALT. The significant increase in the ability to reverse activities of stress indices of the liver by the leaf extract compared to diazepam could be as result of the phytoconstituents of the leaf extract which can be described as potent beyond stress amelioration but other pharmacological potencies.

Elevated levels of total bilirubin as a result of the unconjugated form indicate the severity of the acute hepatopathy resulting from physiological stress. The serum levels of the total bilirubin by anti-stress effect of the extracts were shown in Tab. 3. From the result, the activity of extracts is concentration dependent invivo. The advent of stress is characterized by increased unconjugated bilirubin which is a prominent element of the total bilirubin. The stressed untreated (C+) group had higher total bilirubin that was significantly different from the unstressed and untreated group (C-). The relationship between total bilirubin and stress could be traced to be oxidative proliferation of the erythrocyte membrane leading to denaturation of haemoglobin, this further led to cascade of reaction that generated total bilirubin. Moreover, hepatopathy would cause defect in the conjugation of bilirubin, leading to increased release of unconjugated bilirubin into the blood, justifying the hepatoprotective property of the *Clerodendrum splendens* leaf extract, which showed lower total bilirubin than the diazepam at both concentrations.

The modulation of oxidative stress markers in the blood revealed that the *Clerodendrum splendens* leaf extract facilitated the hepatic antioxidant system from the damage induced by the stress. Cells have cooperative defense systems for the reduction of oxidizing agents. The defense systems contain numerous enzymatic and non-enzymatic antioxidants, including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), reduced glutathione (GSH) and glutathione S-transferase (GST). However, when there is an over-flux of free radical, protective systems are overwhelmed. Catalase catalyzes the breakdown of hydrogen peroxide that is a reactive oxygen species to water and molecular oxygen, thus minimizing the proliferation of cells. Superoxide dismutase (SOD) is one important enzyme involved in the dismutation of superoxide radical. SOD protects against free radical injury by converting superoxide radical to hydrogen peroxide and prevents the formation of hydroxyl radical. In the stressed and untreated group (C+) there was significant decrease in the activities of SOD and catalase. During the advent of free radicals, the antioxidant system of the body is disrupted thus compromising the immune system. Initially, there will be increase in the activities of the enzymes. However, prolonged assault will result in proliferation of the hepatocytes as well as other sites of synthesis of these enzymes, thus leading to reduction in the concentrations as well as activities that are directly proportional. Excess free radicals have been shown to react with several amino acid residues *in vitro,* making active enzymes denatured (Stadtman and Berlett, 1998). The results revealed oxidative stress ameliorating activity of *Clerodendrum splendens* leaf which increase depended on dose. Thus, treatment with extract had a significant reduction in free radicals influence. The bioactive compounds of the extracts act as ligands that promote the genetic transcriptional and translational production of these endogenous antioxidant enzymes increasing the antioxidant status.

CONCLUSION

Clerodendrum splendens leaf extract revealed to have pharmacological secondary metabolites; saponins, terpenoids and tannins in abundant amount while alkaloids exist in trace amount. This indeed suggested *Clerodendrum splendens* leaf extract as relevant and potential antioxidant and anti-inflammatory agents. Thus, depicting *Clerodendrum splendens* leaf extract as possessing oxidative stress and hepatopathy attenuating activities. Immobilization stress combined with cold restraint stress had been confirmed as having deleterious effects on the physiological and immune systems against stressors and age-related macular degeneration. However, the activity of the concentration dependent *Clerodendrum splendens* leaf extract nullified the oxidative stress resulting from these assaults.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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