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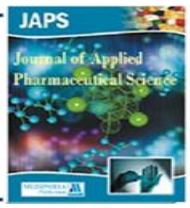

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Evaluation of the Effects of *Calliandra Portoricensis* Extracts on Oxidative Stress Enzymes in Wistar Rats Challenged with Venom of *Echis Ocellatus*

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ABSTRACT

Evaluation of the effects of *Calliandra portoricensis* extracts on oxidative stress (anti-oxidant) enzymes was carried out using wistar rat model. The anti-oxidant enzymes evaluated were superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx). These extracts were used to ascertain their potency in enhancing these enzymes activities in envenomed rats. A total of 30 rats were randomly assigned into 5 treatment groups of 6 rats each. Group 1 (control) received nothing. Group 2 received viperian venom only. Groups 3, 4 and 5 received same amount of venom in addition to calculated doses of flavonoid-rich, polyphenol-rich and whole ethanolic extracts respectively. The dosages of the venom and the *C. portoricensis* extracts were administered intramuscularly. After about 4 hours, the rats were sacrificed, serum collected and the oxidative stress enzymes assayed. Marked decrease of SOD, CAT and GPx enzymes activities occurred in the group 2 rats while increases ($P<0.05$) were observed in groups 3, 4 and 5 experimental rats. These findings suggest that *C. portoricensis* extracts actually attenuated the obliterating effects of the viperian venom on the naturally-occurring anti-oxidant enzymes in experimental wistar rats.

Keywords: *Calliandra portoricensis*, Venom, Oxidative stress enzymes.

INTRODUCTION

Herbal medicinal products are assuming greater roles in the lives of people across the world in the age of global upsurge of microbial drug resistance, toxicity, side effects and escalating cost of synthetic products. They constitute the first line of treatment for many of the world's population, being readily available, traditional and relatively inexpensive. In South eastern Nigeria, traditional herbalists have effectively used extracts of *Calliandra portoricensis* to treat the lethal envenomation of carpet viper (*Echis ocellatus*). The bite of this snake and other venomous snakes is a major socio-medical problem (Swaroop and Grab, 1954) and Nigeria appears to have the highest mortalities in Africa and Asia due to snakebites (Sawaii and Homma, 1975). In the North eastern and central areas of Nigeria, the environment seems favourable for the breeding of snakes because of its rocky and marshy ecosystem, hence the increasing number of snakebite cases in these areas. Whatever are the figures quoted for snakebite cases, these appear to be approximate because not all victims of such bites get to hospital.

However, one estimate quoted by World Health Organisation (WHO) is that 2.5million occur globally each year and 125,000 are fatal (Wagstaff *et al*, 2006).

Following a viper strike various tissue alterations like haemorrhage, edema and myonecrosis occur with resultant tissue loss or organ dysfunction (Ownby, 1982; Gutierrez, 1995). If serotherapy is not executed promptly due to late access to medical care or scarcity of anti-venoms (Gutierrez *et al*, 1998) neutralization cannot be achieved and death supervenes in few hours. In rural communities where most of the snakebite cases occur, treatment with the conventional polyvalent anti-venom (PVA) may not be feasible. This is due to difficulties in preserving the PVA as a result of erratic power supply; where available the PVA is very expensive and beyond the reach of rural victims. The preferred therapy, accessible and affordable in rural settings is phytotherapy.

Haemorrhage, edema, myonecrosis and other tissue alterations follow serious viperian strike which invariably gives rise to massive accumulation of free radicals which contributes to the venom toxicity. This study therefore is designed to ascertain the effects of selective solvent extracts of *C. portoricensis* on toxic free radicals which among other indices, are an indicator in carpet viper venom toxicity. This article investigates the effects of *C. portoricensis* extracts, which are used widely in traditional recipe to treat carpet viper envenomation in south eastern Nigeria, on oxidative stress enzymes.

MATERIALS AND METHODS

Snake venom of carpet viper was purchased from South African venom suppliers cc;bewild@worldonline.co.za. This was kept in a desiccator at 8^{o}C till it was used.

Source of Plant

Calliandra portoricensis was sourced from the extensive secondary forest of Oji-River in Enugu State where it is used traditionally in the treatment of snakebite of the viperian species. Taxonomically, the plant was identified and confirmed to be *C. portoricensis* by Professor Jonathan C.Okafor, Professor of Botanical Taxonomy, Ebonyi State University, Abakaliki, Nigeria. Voucher specimen "CP-O No.1" has been preserved for reference in the Botany Herbarium of Ebonyi State University, Abakaliki.

Plant Extract

Three hundred and fifty grammes (350g) each of fresh and dry leaves and roots were crushed separately and extracts prepared from them by refluxing them in 80% ethanol for 72 hours in a Soxhlet extractor. The extracts were then concentrated in rotary evaporator and dissolved in 0.9% saline for use.

Phytochemcial Screening

Qualitative and quantitative screening of the plant extracts was carried out using the methods of Harborne (1973) for alkaloids, flavonoids, saponins, tannins, polyphenols and reducing compounds; Sofowora (1982) for glucosides, Trease and Evans (1983) for phlobatinins, anthraquines and hydroxymethyl anthraquines. Two (2) g of the concentrated extracts were dissolved in 10ml of 0.9% saline and 2ml used for each component of the qualitative and quantitative analysis.

Selective Solvent Extraction

A measured weight of the processed sample was boiled in 100ml of 2M HCl solution under reflux for 40 minutes. After cooling and filtering, the filtrate was treated with equal volume of ethyl acetate. This technique has preferential selection for flavonoids in the ethyl acetate phase (Harborne, 1973). The total phenols (polyphenols) were extracted using the Folin-ciocatean spectrophotometer technique (AOAC, 1990) since methanol selectively extracts phenols.

Animal Treatment

A total of 30 albino wistar rats weighing between 90-120gms were used. The rats were assigned into five treatment groups with group one as control receiving no venom challenge and extract treatment. Treatment group 2 was given 0.2ml of 1mg/ml of viperian venom.

Groups 3, 4, and 5 received same dose of venom. Four hours after the venom challenge, calculated dose (0.5ml of 100mg/100g body weight) of flavonoid-rich, polyphenol-rich and whole ethanolic extracts were given to the rats in groups 3, 4 and 5 respectively. Both the venom and the extracts were given intramuscularly. The choice of the intramuscular route was informed by the need to follow or mimic the natural path of snake envenomation in man and animals.

Two hours after the "medication" with plant extracts, the rats were sacrificed by euthanasia and blood samples collected from the various groups via cardiac puncture into sample tubes. The samples were allowed to stand for one hour to clot. Serum was later separated from the clot by spinning at 5000 revolutions per minute (rpm). Serum was decanted and the quantity collected was used for oxidative stress (anti-oxidant) enzymes assays.

This group of three enzymes superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx)) forms the anti-oxidants through which free radicals formed in the body are moped up and removed or neutralized in the system. In the present study, Cayman's superoxide dismutase assay kit which utilizes a tetrazolium salt for detection of superoxide radicals generated by xanthine oxidase and hypoxanthine was used. One unit of SOD is the amount of enzyme needed to exhibit 50% dismutation of the superoxide radical.

Cayman's catalase assay kit which was used to ascertain the catalase enzyme activity utilizes the peroxidatic function of CAT. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced was measured colorimetrically with 4-amino-3-hydrazino-5-mercapto 1, 2, 4-triazole (purpaled) as the chromogen (Johnson and Borg, 1998; Wheeler *et al*, 1990). Purpaled specifically forms a bicylic heterocycle with aldehydes, which upon oxidation changes from colorless to a purple colour.

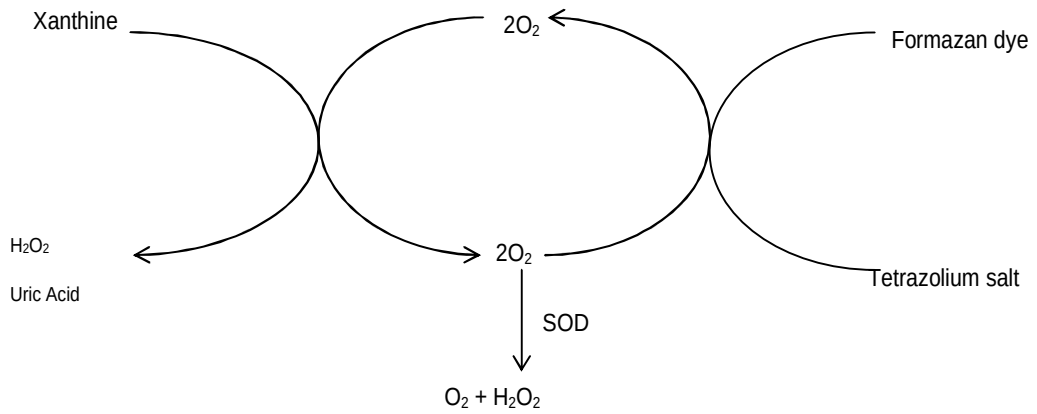

Fig. 1: Scheme of the Superoxide Dismutase Assay.

Cayman's GPx assay measures GPx activity indirectly by a coupled reaction with glutathione reductase (GR). Oxidized glutathione (GSSG) produced upon reduction of hydroperoxide by GPx was recycled to its reduced state by (GR) and NADPH.

$$R\text{-}O\text{-}O\text{-}H + 2GSH \xrightarrow{GPx} R\text{-}O\text{-}H + GSSG + H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

The oxidation of NADPH to NADP+ is accompanied by a decrease in absorbance at 340nm. Under conditions in which the GPx activity is rate limiting, the rate of decrease in A_{340} is directly proportional to the GPx activity in the sample (Paglia and Valentine, 1967).

Table. 1: Qualitative Constituents of Fresh *Calliandra portoricensis.*

S/No	Chemical Constituents	Fresh Root	Fresh Leaf	Fresh mixed sample
1	Alkaloids	+	+	++
2	Glycosides	+	+	+
3	Saponins	+	-	+
4	Tannins	-	+	+
5	Flavonoids	++	++	++
6	Reducing compounds	++	++	++
7	Polyphenols	++	+++	+++
8	Phlobatannins	-	-	-
9	Anthraquines	-	-	-
10	Hydoxymethyl anthraquine	+	+	-

Keys: + Present, ++ Present in moderately high level, +++ Present in high level, - Absent

Table. 2: Qualitative Constituents of Dry *Calliandra portoricensis.*

S/No	Chemical Constituents	Dry Root	Dry Leaf	Dry mixed sample
1	Alkaloids	+	+	+
2	Glycosides	+	+	++
3	Saponins	+	+	+
4	Tannins	-	-	-
5	Flavonoids	++	+	+
6	Reducing compounds	++	++	+
7	Polyphenols	++	++	+++
8	Phlobatannins	+	-	-
9	Anthraquines	-	+	+
10	Hydoxymethyl anthraquine	-	-	-

Keys: + Present, ++ Present in moderately high level, +++ Present in high level, - Absent

LD_{50} of Plant Extract and Viperian Venom

The determination of the LD_{50} of plant extract and the venom was done using the method of Lorke (1983). LD_{50} was determined to form a basis of dosage for subsequent assays employing sublethal doses of plant extract and venom.

The LD_{50} of *C. portoricensis* which was carried out in 2 stages (tables III and IV) clearly determined the dose of the extract at which 50% of the experimental animals died. The geometric mean of the non-lethal dose and the lowest lethal dose was calculated as the LD_{50} (Lorke, 1983).This geometric mean gave a value of 150mg/kg body weight (b.w).The LD_{50} of the venom was similarly determined and gave a value of 250µg/kg b.w.

Table. 3: Determination of LD_{50} of *C. portoricensis* (Stage 1)

Substance	Doses (mg/kg b.w)	Mortality
Ethanolic extract	10,000	3/3
Ethanolic extract	625	3/3
Ethanolic extract	39	1/3

Table. 4: Determination of LD_{50} of *C. portoricensis* (Stage 2)

Substance	Doses (mg/kg b.w)	Mortality
Ethanolic extract	5	0/2
Ethanolic extract	39	0/2
Ethanolic extract	150	1/2

Statistical Analysis

Analysis of variance (ANOVA) was used in analyzing the data generated from this study. Results of the study were expressed as mean of the values of each treatment group ± standard deviation. Data between treatment groups were analyzed using two-way analysis of variance. Values of $P<0.05$ were regarded as being significant.

RESULTS

The result presented in table V showed that there were marked decreases in SOD, CAT and GPx activities ($P<0.05$) in the venom-treated rats. The anti-oxidant enzymes activities in the treatment groups 3, 4 and 5 rats increased significantly ($P<0.05$). Therefore the administration of the flavonoid-rich, polyphenol-rich and whole ethanolic extracts on these groups of rats had positive significant effects on the anti-oxidant enzymes activities (table V).

Table. 5: Effect of Treatment on the Serum Anti-oxidant Enzymes Activities (SOD, CAT and GPx) of Experimental Rats (n=30).

Test /Tissue	Control	Venom-treated	Flavonoid-rich extract	Polyphenol-rich extract	Whole ethanolic extract
SOD	0.98±0.59	0.24±0.22	2.44±0.21*	1.73±0.30*	4.53±1.52*
CAT	87.39±0.15	85.64±0.80	88.73±0.98	87.50±0.36	90.83±0.60*
GPx	61.52±6.98	19.82±2.63	407.66±22.83*	209.04±12.76*	577.31±18.23*

Values are expressed as mean ± SD. Means on the same column with asterisk are significantly ($p<0.05$) different from values of control and venom-treated groups in the same row.
SOD = Superoxide dismutase (u/ml)
CAT = Catalase (nmol/min/ml)
GPx = Glutathione Peroxidase (nmol/min/ml)

DISCUSSION

The focus of this study was to assess whether intramuscular administration of the various extracts of *C. portoricensis* induced any positive changes in the serum, of oxidative stress enzymes, in envenomed rats. Viperian venom, from this study, appeared to have obliterated or overwhelmed the naturally-existing anti-oxidant enzymes activities in rats.

This appeared much more marked in SOD activity and consequently on CAT and GPx indirectly. The statistically significant increases ($P<0.05$) in the anti-oxidant enzymes activities in treatment groups 3, 4 and 5 rats could be attributed to the heavy quantitative content of flavonoid-rich extract and the complexation of polyphenolics with phospholipase A_2 (PLA_2) enzyme (Houghton, 1993) which is responsible for the generation of massive amounts of free radicals in viperian envenomation. The complexaton of the polyphenols with the Ca^{2+}-dependent PLA_2 enzyme and the marked anti-oxidant activity of the flavonoid content of the plant extract seemed to have worked synergistically and in tandem with one another to enhance overall, the anti-oxidant potentials of *C. portoricensis* extracts.

CONCLUSION

The assay of oxidative stress (anti-oxidant) enzymes indices revealed that *C. portoricensis* extracts increased the SOD, CAT and GPx activities in envenomed rats. Working in tandem with complexation of the polyphenolics and PLA_2 enzyme, theanti-oxidant enzymes were able to positively mop up and eliminate free radicals that are common features in carpet viper strike.

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