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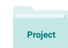

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Full length research paper

Evaluation of the biochemical and anti-snake venom effects of *Calliandra Portoricensis* extract fractions in wistar rat models challenged with venom of carpet viper (*Echis Ocellatus*)

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To evaluate the anti-snake venom effects of the flavonoid, polyphenolic and whole ethanolic fraction of the leaves of *Calliandra portoricensis* on some biochemical indices in Albino wistar rat challenged with carpet viper snake venom. Phytochemical screening was carried out using standard methodologies. Thirty (30) albino wistar rats of both sexes weighing between 100-150g were divided into five groups of six rats each. Groups 1 and 2 served as normal control and viperian venom control respectively. Groups 3, 4 and 5 were each injected intramuscularly with 0.2 ml of 1mg/ml, equivalent of 0.2mg, of viperian venom and subsequently injected with 0.5 ml of 100mg/100g body weight (b.w.) anti-dote fractions of flavonoid, polyphenolic and whole ethanolic extracts of *Calliandra portoricensis* respectively. The animals were anaesthetized with chloroform and then sacrificed. Blood was collected by cardiac puncture into pre-labelled sterile bottles for haematological analyses. Phytochemical screening revealed the presence of flavonoids and polyphenols and more specifically 2- hydroxyl, 4-methoxy benzoic acid. AST and ALT activity showed a significant increase ($p<0.05$) in all treated groups when compared to normal control. A significant increase ($p<0.05$) in creatine kinase (CK) activity was observed in venom control compared to normal control. These values decreased significantly ($p< 0.05$) in the fractions and extract treated groups, more so in the polyphenol and whole extract groups. The HGB level showed a significant decrease ($p<0.05$) while RBC and WBC counts showed a significant increase ($p<0.05$) in the venom control groups compared to normal control group. The HGB and RBC levels were reversed towards normal by treatment with the fractions and whole extract whereas only the flavonoid fraction reversed the WBC levels towards normal. The LDL:HDL ratio was found to be 1.44, 9.47, 0.44, 0.11 and 0.37 for groups 1, 2, 3, 4 and 5 respectively. A marked increase ($p<0.05$) in SOD and GPx activity was observed in groups 3, 4 and 5 when compared to the normal and viperian control groups. The phytochemical constituents in the plant extract may significantly lower the high lipid peroxidation and ameliorate hematotoxic effects induced by viperian venom. This may account for the protection against cardio-toxicity and the shock that normally accompanies carpet viper envenomation.

Key words: Snake venom, Envenomation, *Calliandra portoricensis*, Phytochemicals, Biochemical indices, Wistar Rats.

INTRODUCTION:

Deaths due to bites by venomous snakes are common features in the North eastern and central parts of Nigeria. Snake bites in these areas are beginning to constitute a major socio-economic problem, with the ever increasing number of victims of snakebite, due to the favourable breeding and multiplication environment for the snake species (Swaroop and Grab, 1954). Poisonous snakes are a particular problem in Africa and Southeast Asia (Wagstaff et al., 2006) and because not all victims of snakebite get to hospital, only estimates of illness and death are available. One estimate quoted by the World Health Organisation (WHO) is that 2.5 million snake bites

occur worldwide each year and 125,000 are fatal (Wagstaff et al., 2006). In West Africa, carpet viper (Echis ocellatus) is the most medically important viperian species (Wagstaff et al., 2006).

Following a viperian envenomation various local tissue alterations occur such as haemorrhage, edema and myonecrosis leading to tissue loss or organ dysfunction (Gutierrez, 1995) and usually these effects develop very rapidly after the snake envenomation, making neutralization by anti-venoms very difficult, especially if serotherapy is delayed due to either late access to medical care or scarcity of anti-venoms. A known conventional medical procedure involving the use of polyvalent anti-venom (PVA) to neutralize the venom has been in use. However, the use of PVA has many limitations. They are difficult to preserve in rural settings given the erratic nature of power supply; where they are available the PVA is very expensive and beyond the reach of rural people who make up the greater percentage of snakebite victims. Furthermore, life saving anti-venom contains an immunoglobulin pool of unknown antigen specificity and known redundancy which necessitates the delivery of large volumes of heterologous immunoglobulin to the envenomed victim, thus, increasing the risk of anaphylactic reactions, serum sickness and other adverse effects (Wagstaff et al., 2006).Thus the preferred therapy, accessible and affordable, in rural settings is phytotherapy.

Traditional herbalists in south eastern Nigeria have found the leaves and roots of *Calliandra portoricensis* (Jacq.) Benth., of the family Leguminosae, very useful and effective in neutralizing viperian venom. The extracts of *Calliandra portoricensis* appear to be specific on the haemotoxic venom of vipers like the rattlesnake, puff adder, and the carpet viper. In distribution, the plant is native to Central America but is abundant in parts of closed forest in Ghana and South eastern part of Nigeria (Irvene, 1961). In traditional preparations used for treating snakebites, the roots are ground to powder or the leaves squeezed in alcohol like wine or alcoholic beverage. It is believed that extracting the secondary metabolites in alcohol enhances the fast absorption of active principles into the blood stream.

Within 15 minutes of entry into the blood stream goose pimples appear on the skin of the snakebite victim, signifying the onset of action of the secondary metabolite and vanish within seconds (Alam *et al*., 1994; Alam and Gomes, 1998). It is thought that the ‘drug’ increases the permeability of the capillary membranes as well as that of the gastrointestinal tract (GIT) resulting in the flooding of the GIT with the complex formed by the secondary metabolite and the viperian venom. This results in concomitant stimulation of the emetic centre of the brain. Vomition and diarrhoea ensue with the resultant elimination of the haemotoxic venom from the body. Although, the active ingredient responsible for the anti-venom activity of *Calliandra portoricensis* is not known yet, a phytochemical screening of the plant leaf extract revealed the presence of flavonoids and polyphenols, and more specifically 2-Hydroxy-4-methoxy benzoic acid (Alam *et al*., 1994; Alam and Gomes, 1998), a secondary metabolite which probably carries out nucleophilic attack on some of the functional groups of the numerous hydrolytic enzymes and non-enzymatic polypeptides involved in carpet viper venom toxicity, thereby neutralizing the venom (Onyeama, 2011). It is also suggested that due to high content of polyphenols in *Calliandra portoricensis* the neutralization of viperian venom may be due to the complexation between the polyphenols and venom peptides (Houghton,1993).This study therefore is designed to ascertain (i) the phytochemistry and quantify the phytoactive ingredients of *Calliandra portoricensis* (ii) obtain polyphenol and flavonoids-rich solvent fractions and whole ethanolic extract to determine the effects of these extracts on biochemical indices of toxicity and (iii) evaluate the effects of the extracts on phospholipase A_2 enzyme activity in rats challenged with viperian venom.

MATERIALS AND METHODS

Source of Venom

Lyophilized snake venom of carpet viper was purchased from South African venom suppliers with email address: bewild@worldonline.co.za. The venom sample was preserved as a yellowish crystal inside a tube surrounded by a light-blue layer of silica gel to maintain a dry environment. This was then kept in a desicator at $8^{o}C$ till it was used.

Source of Plant

Calliandra portoricensis (Jacq.) Benth. 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 *Calliandra 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, Ebonyi State, Nigeria.

Phytochemical Screening (Analysis)

Harborne’s phytochemcial methods (Harborne, 1973) were used to identify alkaloids, flavonoids, saponin, tannins, and polyphenols (Sofowora, 1982). Sulphuric acid spray indicated the presence of glycosides while phlobatanins, anthraquines and hydroxyathraquines were identified by the method of Trease and Evans (1983).

Fractionation by Selective Solvent Extraction

The crude methanolic extract of the plant was fractionated by column chromatography using a Glass

Column packed with silica gel adsorbent material. A Flavonoid-rich extract fraction was selectively isolated with ethyl acetate and confirmed using the method described by Harborne (1973). Polyphenol fraction was isolated using 30% methanol and confirmed by the Folin-ciocatean spectrophotometric AOAC technique (AOCS, 1990).The methanol strength was increased to 70% to elute the column to afford a fraction which crystallised to give white needle-like crystalline solids. This was identified as 2-hydroxy, 4-methoxy benzoic acid according to the method described by Alam *et al*. (1994) and Alam and Gomes (1998).

Determination of Vitamins and Minerals

Determination of vitamins C and E was carried out using the method of the Association of Vitamin Chemists as described by Kirk and Sawyer (1989). The versanate EDTA titrametric method (Udoh and Ogunwale,1986) was employed to determine the calcium and magnesium content of the plant. The heavy metals in the plant were determined using atomic absorption spectrometric technique (AAS) (James,1995).

Animal treatment

30 albino wistar rats were assigned randomly into five treatment groups of 6 rats each. Group 1 (normal control) received water, the medium for reconstituting the fractions and extracts, intramuscularly. Group 2 (venom control) received 0.2ml of 1mg/ml of viperian venom. Groups 3, 4 and 5 were each injected intramuscularly with 0.2 ml of 1mg/ml (equivalent of 0.2mg) of viperian venom and subsequently injected with 0.5 ml of 100mg/100g body weight (b.w.) anti-dote fractions of flavonoid, polyphenolic and whole ethanolic extracts of *Calliandra portoricensis* respectively. This “antidote” was given 4 hours after the administration of the venom. The venom and the extracts were administered intramuscularly. Two hours after the “medication” with plant extracts, the rats were sacrificed after anaesthesia using chloroform vapour. Blood samples were collected from each group via cardiac puncture into sample tubes. One group of sample tubes contained anticoagulant, Ethylene diamine tetra acetate (EDTA) to prevent blood clotting and allow accurate measurement of haematological parameters. The other group of sample tubes contained whole blood that was allowed to clot and serum separated from them by centrifugation. The sera were then used to determine the lipid oxidation indices, aminotransferases, creatine kinase, phospholipase A_2 enzyme and oxidative stress enzymes activities.

LD_{50} of Plant Extract and Venom

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

Statistical Analysis

Analysis of variance (ANOVA) was used in analyzing the data generated by this study. Results were expressed as means ± standard deviation. Values of $P<0.05$, were regarded as being significant.

RESULTS

Table 1 shows the phytochemical compounds in *Calliandra portoricensis,* with flavonoids, polyphenols and reducing compounds being the dominant compounds. The mineral elements in the plant are given in table 2 with Ca and Mg being dominant. The result presented in table 3 revealed significant increases ($P<0.05$) in AST and ALT activities in the venom-treated rats and further significant increases ($P<0.05$) in the flavonoid-rich, polyphenol-rich and whole CP-extract-treated rats when compared to the control. There was a significant decrease ($P<0.05$) in CK activity in the flavonoid-rich and polyphenol-rich fractions and a further highly significant decrease ($P<0.05$) in the whole ethanolic extract when compared to normal control and the venom control groups. The computed AST:ALT ratio (Table 4) for the venom control group showed an increased value compared with the normal control. On the other hand the flavonoid-rich fraction group showed a significant decrease ($P>0.05$) in AST:ALT ratio, while the polyphenol-rich fraction and whole ethanolic extract groups showed a further statistically significant decrease ($P<0.05$) in AST:ALT ratio when compared to venom control.

Table 1 - 4

Table 5 represents venom PLA_2 activity in the serum of experimental rats. The data showed the PLA_2 enzyme activity in the venom control group (0.2104 µmol/min/ml) to be significantly higher ($P<0.05$) than the PLA_2 activity in the flavonoid-rich, polyphenol-rich and whole ethanolic extract group.

Table 5

The anti-oxidant enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), showed marked decreases ($P<0.05$) in the venom-treated rats (Figure 1-3)). These oxidative stress (antioxidant) enzymes activities increased significantly ($P<0.05$) in the extract-treated groups of rats. The administration of the plant extracts on the various groups of rats had significant positive effects on the anti-oxidant enzymes activities.

Fig 1-3

Assessment of the haematological indices indicated a significant decrease ($P<0.05$) in the HGB level of venom-treated rats, but a significant increase ($P<0.05$) in the Flavonoid-rich, polyphenol-rich and whole extract treated

rats compared to the venom-treated group (Table 6). The red blood cell counts increased in the venom-treated rats but the CP-extract treated groups maintained red blood cell counts close to the control group suggesting a positive effect on the neutralization of the viperian venom. The administration of venom and the various CP-extracts made no impact on the MCV and MCH. The flavonoid-rich and whole extract treated rats showed a significant decrease ($P<0.05$) on the PCV compared to the venom control and close to normal values.

White Blood Cells (WBC) showed a significant increase ($P<0.05$) in the venom control (Table 7). This was brought back to near normal value by treatment of the venom-challenged rats with the flavonoid-rich fraction. The granulocytes, especially the neutrophils, showed a preponderant increase in all the treatment groups when compared to the control. This increase was significant ($P<0.05$) compared to lymphocyte value which was non-significant ($P>0.05$) (Table 7).

The values of platelet count (Table 8) showed a significant decrease ($P<0.05$) probably due to the overwhelming haemotoxic action of the venom on platelets which the various CP-extracts failed to reverse. Although non-significant changes ($P>0.05$) were observed in the mean platelet volume (MPV) and platelet distribution width (PDW), the plateletcrit (PCT%) showed significant decrease ($P<0.05$).

Tables 6-8

The effect of treatment on the lipid profile of rats is shown in Figure 4. The venom-treated group showed a significant increase ($P<0.05$) in total cholesterol level. There was a steady increase in serum triacylglycerols (TG) that was significant ($P<0.05$) in all groups when compared to the control group. The high density lipoprotein (HDL) content of the venom-treated rats decreased significantly ($P<0.05$), but increased significantly ($P<0.05$) in the whole extract treated group The LDL:HDL ratio increased significantly ($P<0.05$) in the venom-treated rats and decreased significantly ($P<0.05$) in the whole extract treated group (Table 9**).** It is believed that the increase in the LDL: HDL ratio which predisposes coronary artery disease may be contributory to the cardiovascular disturbances involved in viperian envenomation.

Fig 4

Table 9

DISCUSSION

The outcome of the evaluation of the biochemical and anti-snake venom effects of *Calliandra portoricensis* extracts revealed increases in AST and ALT in the various treatment groups (Table 3). The computed AST:ALT ratio (Table 4) gave a significant value ($P<0.05$) in the serum of rats treated with the venom. The serum of rats treated with the various plant extract fractions showed a non-significant value ($P>0.05$) of AST:ALT ratio (Table 4). Although results on the effect of viperian venom administration on aminotranferase activities on tissues have not been reported in literature, the use of AST:ALT ratio may generally be used as a biomarker to monitor pathologies implicating the heart or liver as has been reported elsewhere (Howcroft,1987). According to these reports, an increase in AST:ALT ratio points to a pathology involving the heart while the reverse implicates the liver (Howcroft,1987).The increase in this ratio observed in the venom-treated animals therefore provided experimentally determined evidence, at molecular level, that viperian venom toxicity was indeed cardiovascular (Table 4).

Thus the increase in AST and ALT activities and the decrease in AST:ALT ratio pointed to increase in the detoxification ability and capacity of the liver to handle the venom-extracts complex which brought about increase in the ALT activity and the consequent decease in the AST:ALT ratio in rats treated with the various CP-extract fractions (Table 4).

Phospholipase A_2 enzyme (PLA_2) appears to be the lethal component of viperian venom (Onyeama *et al*., 2012). There were significant decreases in PLA_2 activity ($p<0.05$) in the CP-extract groups (Table 5). This decrease in PLA_2 activity may be attributed to the complexation of the venom PLA_2 with the polyphenolics and the reduction in the free radicals as a result of the flavonoid component of the plant extract (Onyeama *et al.* 2012). As a result of the complexation of the PLA_2 with the polyphenolics (Houghton,1993), there was a diminished PLA_2 activity and the subsequent inactivation of the venom by the CP-extract fractions. It can thus be inferred that these extracts neutralized PLA_2 enzyme activity which elicits all the damages and changes at molecular level, once there is viperian venom in the blood stream.

The anti-oxidant activities in the sera of CP-extracts treated groups showed statistically significant increases ($P<0.05$). In this study, viperian venom appeared to have obliterated or overwhelmed the naturally-existing anti-oxidant enzymes activities in rats. This appeared much more marked on SOD (Figure 1) activity and consequently on CAT (Figure 2) and GPx (Figure 3) indirectly. The increases in the CP-extract groups could be attributed to the heavy quantitative content of flavonoid-rich extract, the complexation of polyphenolics with PLA_2 (Houghton,1993) which is responsible for the generation of massive amounts of free radicals in viperian envenomation. The complexation of the polyphenols with Ca^{2+}-dependent PLA_2 and the marked anti-oxidant activity of the flavonoid content of *Calliandra portoricensis* seemed to have worked synergistically and in tandem with one another to enhance overall, the anti-oxidant potentials of the plant extract.

Haematological indices suggested a normalization of the blood parameters after treatment with CP whole extracts and fractions (Table 6 and 7). The spectrum of changes in lipid parameters especially the elevation in HDL (Figure 4) on the one hand and the rapid return of RBCs and WBCs to near normal values on the other have shown that *Calliandra portoricensis* extracts had a neutralizing effect on the venom. The decrease in

LDL:HDL ratio ((Table 9) proves that *Calliandra portoricensis* extracts removed the possibility of artherogenesis which the venom can predispose a victim to after recovery. In fact epidemiological studies indicate that high plasma HDL levels are strongly correlated with a low incidence of cardiovascular complication (Voet and Voet,1990).

CONCLUSION

Exposure of albino wistar rats to viperian venom alters negatively, their enzyme, lipid and haematological parameters. The venom is both cardiotoxic and haemotoxic and may bring life to an acute fatality if not treated immediately. The alteration of these biochemical parameters accounts for the viperian venom toxicity. Phytochemical fractions of flavonoids, polyphenolics and whole extract of *Calliandra portoricensis* have been able to reverse these altered biochemical changes to a very significant ($P<0.05$) extent. The effect of CP-extract fractions especially in lowering serum lipid level in envenomed rats with elevated plasma lipid levels, the marked decrease in PLA_2 enzyme activity and the reduction in free radicals as a result of the flavonoid component of the plant extract may be attributed to the complexation of the venom PLA_2 by the anti-oxidative secondary compounds of *Calliandra portoricensis*. Thus, we can conclude that this anti-venom property of *Calliandra portoricensis* can be exploited for therapeutic benefits.

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