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The Chief Editor,

American Journal of Tropical Medicine and Public Health.

Dear Colleague,

We the authors of this work titled," Anti – diabetic activity of aqueous extracts of *Cinchona calisaya* bark and *Vitex doniana* leaves in Alloxan – Induced diabetic rats," are by this letter requesting that this manuscript be reviewed for publication in your widely read American Journal of Tropical Medicine and Public Health. We confidently declare that there's no competing interest.

It will be immensely appreciated if this article is favourably considered.

Thank you.

Dr. C.N.Ezekwesili (Corresponding Author.)

Anti-diabetic activity of aqueous extracts of *Vitex doniana* leaves and *Cinchona calisaya* bark in alloxan – induced diabetic rats.

By

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ABSTRACT

Vitex doniana Sweetand *Cinchona calisaya* WEEDare tropical medicinal plants endued with important pharmacological properties. The effects of aqueous extracts of *V.itex doniana* leaves and *C.inchona calisaya* bark on alloxan-induced diabetes mellitus in Wistar albino rats were evaluated. Diabetes mellitus was induced by a single intraperitoneal(i.p) injection of 150.0.0 mg/kg body wt of alloxan monohydrate. The aqueous extracts of *V.doniana* leaves and *C. calisaya* bark were administered intraperitoneally to four diabetic groups at same doses of 50.0 and 100.0 mg/kg body wt. The actions of the extracts were compared with that of the standard oral hypoglycaemic agent,glibenclamide.Both extracts caused significant ($p < 0.001$) decreases in blood sugar levels of the rats at both doses testedall the tested doses. At 50.0 mg/kg body wt. *V. doniana* leaf extract produced 82.9% reduction in blood sugar level (i.e from 492.8 to 84.5 mg/dl) after four days of daily injection whereas, *C.calisaya* caused 64.4% decrease. Unlike *C. calisaya* bark, *V.doniana* at both doses tested, was more potent than the reference drug, glibenclamide (0.3 mg/ kg body. wt.). The anti-diabetic activity of *V. doniana* did not vary with the dose, whereas the observed effect of *C.calisaya* decreased with increase in dosewas dose – dependent. *C. calisaya* exhibited higher anti-diabetic activity at a lower dose of 50.0 mg/kg body wt. Both medicinal plants therefore possess valuable anti-diabetic property. Their effects on the anti-oxidant status were also investigated. *V. doniana* and *C. calisaya* extracts caused increases in the activity of SOD and lipid peroxidation when compared with control,but the increases were lower than that produced by alloxan, indicating attenuation of free radical generation. Quantitative pPhytochemical analysesscreening of both extracts showed the presence of saponins(0.92%), flavonoids(7.05%), alkaloids(1.8%), and cardiac glycosides(2.8%) in *V. doniana*, whereas saponins(2.0%), flavonoids(5.0%), alkaloids(6.0%), and cardiac glycosides(3.54%) were detected in *C. calisaya*., tannins, and carbohydrates.

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Keywords: Anti-diabetic activitys, *Vitex doniana*, *Cinchona calisaya*, Hypoglycaemic, Anti-oxidant.

INTRODUCTION

Diabetes mellitus is a metabolic disorder characterized by hyperglycaemia together with biochemical alterations of glucose and lipid metabolism (Pari *et al*., 2002). This chronic disorder arises as a result of insufficient production of the pancreatic β-cells hormone insulin or inadequate utilization of insulin. Two major types of diabetes mellitus reported to increase mortality and morbidity are the type I commonly known as insulin – dependent diabetes mellitus (IDDM) and type 2, also called non-insulin – dependent – diabetes mellitus (NIDDM).

At least 80% of Africans depend on herbal medicine for their health care(Iwu *et al*., 1999; Kilani, 2006; Ajose, 2007; Okwu and Uchegbu, 2009). The high cost, low availability, uncertainty of use during pregnancy and undesirable side effects of synthetic drugs have been some of the factors leading to a preference for hypoglycaemic drugs of plant origin, which are believed to be suitable for chronic treatments (Berger, 1985; Okigbo and Mmeka, 2006). Herbal preparations with anti-diabetic potentials are growing in popularity.

Vitex doniana Sweet (Verbeanaceae) is a perennial forest plant widely distributed in tropical West Africa. In ethnomedicine, *V. doniana* leaf is employed in the treatment of stomach and rheumatic pains, inflammatory disorders, diarrhoea and dysentry (Agunnu *et al*., 2005; Iwueke *et al*., 2006).Likewise *Cinchona calisaya WEED* (Rubiaceae) commonly known as quinine bark, is one of the famous rain forest plants which are now widely cultivated in many tropical countries for their medicinal and commercial values. Extracts of *C. Calisaya* bark have been reported to possess anti-malarial (Bareness *et al*., 2006) and anti-parasitic activities (Rojas, 2006)

In Nigeria, ~~from information available from the indigenous traditional healers,~~ *V.doniana* leaves and *C. Calisaya* bark are useful in the management of diabetesis~~ics~~ (personal communication), although these medicinal properties have not been established scientifically.

The aim of this study ~~is~~, therefore,is to investigate antidiabetic potential~~these acclaimed values~~ of these forest plants.

Oxidative stress, caused by imbalance between the production of reactive oxygen species (ROS) and a biological system's ability to readily detoxify or scavenge released reactive intermediates, has been

implicated by many researchers in the pathogenesis of some chronic conditions such as Alzheimer's disease, Parkinson's disease, diabetes mellitus (David *et al*., 2005), rheumatoid arthritis and neurodegeneration (Valko, *et al*., 2007). In view of this, the effects of the extracts of the two plants on some anti-oxidant mechanisms of the body were also studied. Phytochemical analyses of the extracts were also carried out.

2.0 Material and Methods

2.1 Plant material.

Fresh leaves of *V.doniana* and *C. calisaya* bark were collected from Abagana, Anambra State, Nigeria, in January,2011. The plants were identified and authenticated at the International Centre for Ethnomedicine and Drug Development (INTERCEDD) where voucher specimens, InterCEDD 207 and 304 respectively were deposited.

2.2 Experimental animals

Forty adult male albino rats (Wistar) weighing between 100.0 – 175. 0 g were purchased from the Department of Veterinary Medicine, University of Nigeria, Nsukka. The animals were housed in standard animal cages, fed with commercial feed and water, *ad libitum,* and acclimatized for 7 days.

2.3 Chemicals.

Alloxan monohydrate, adrenaline and thiobarbituric acid were manufactured by Sigma, Germany. All other chemicals used in our study were of analytical grade.

2.4 Preparation of extracts.

Five hundred grammes(500 g) each of dried and pulverized *V. doniana* leaves and *C. calisaya* bark were macerated twice in 2.0 L of cold distilled water for 18 ± 1 h. Filtrations were done using cheese cloth and then Whatman No. 1 filter paper. Combined filtrates were evaporated in a water bath at 80^{o}C. The crude extracts obtained (36.4% and 21.7% yields for *V. doniana* and *C. calisaya* respectively) were stored in the refrigerator and used for the experiments.

2.5 Determination of extracts' effects on alloxan – induced diabetic rats.es mellitus.

Forty adult male albino rats (Wistar) were weighed and their base line blood sugar levels recorded

using a glucometer (Accucheck Active). A single dose intraperitoneal injection of alloxan monohydrate (50.0 mg/kg body wt.) was given to thirty-five rats, whereas five rats served as the negative control group. Glucose solution (75%) was used to prevent the initial hypoglycaemia usually caused by alloxan monohydrate. Two days after alloxan monohydrate administration, the blood sugar levels of the rats were recorded and the diabetic rats (with blood sugar levels ≥ 250.0 mg/dl) were divided into six groups of five animals each according to their body weights. Four test groups received daily treatment with the aqueous extracts of *V. doniana* leaves and *C. calisaya* bark at the same doses of 50.0 and 100.0 mg/kg body wt each whereas, the remaining animals served as the untreated diabetic (positive control) group. The reference group (five rats) were treated with glibenclamide (0.3 mg / kg body wt), whereas the negative control group received distilled water(1 ml/kg. body wt).. Extracts and drugs were solubilized in distilled water and tween -80(1%) respectively and the duration of treatment was four days. All administrations were done intraperitoneally. The fasting blood sugar levels of all the rats were measured daily before any treatment. The animals were sacrificed on the last day and blood samples were collected for anti-oxidant enzymes assays.

2.6 Superoxide dismutase (SOD) activity assay.

Whole blood SOD activity was assayed utilizing the method of Okpuzor *et al*.,(2009). Whole blood (1.0 ml) was diluted within 9.0 ml of distilled water to make a one in ten dilution of the blood. An aliquot of 0.2 ml of diluted blood was added to 2.5 ml of 0.05 M sodium carbonate buffer (pH 10.2) and left to equilibrate in the spectrophotometer(Ultrospec 3100 UV/Visible Spectrophotometer, Amershan Biosciences). and The reaction was started by addition of 0.3 ml of freshly prepared 0.3 mM adrenaline to the mixture which was quickly mixed by inversion.

The reference cuvette contained 2.5ml buffer, 0.2 ml of distilled water and 0.3 ml of substrate (adrenaline). The increase in absorbance at 480mm was monitored at 30 seconds interval for 150 seconds.

2.7 Determination of catalase activity.

Serum catalase activity was determined according to Beer and Sizer (1952) as reported by Usoh *et al* .,2005, by measuring the decrease in absorbance at 240nm in a UVuv recording spectrophotometer(Ultrospec 3100 UV/Visible Spectrophotometer, Amershan Biosciences) by monitoring the decomposition of H_2O_2 as described by Aebi (1984). The reaction mixture (3.0 ml) contained 0.1 ml of suitably diluted serum in phosphate buffer (50.0 mM, pHPH 7.0) and 2.9 ml of 30.0 mM H_2O_2 in phosphate buffer.The reference reagent contained 0.1 ml of buffer and 2.9 ml of 30.0 mM H_2O_2 in buffer. An extinction coefficient for H_2O_2 at 240 nm of 40M M-I cm-I was used for the calculation. The specific activity of catalase was expressed as moles of H_2O_2 reduced / min / mg. protein

An extinction coefficient for H_2O_2 at 240 nm of 40M M-I cm-I was used for the calculation. The specific activity of catalase was expressed as moles of H_2O_2 reduced / min / mg. protein .

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2.8 Determination of lipid peroxidation.

The method of Biye and Aust (1978) as described by Usoh *et al*.,2005 was employed. Serum (0.4 ml) aliquots were collected into the test-tubes and were mixed with 1.6 ml of 0.25N HCL, 0.5 ml of 15.0% TCA (trichloroacetic acid(TCA)) and 0.5 ml of 0.375% of TBA (thiobarbituric acid(TBA)). The reaction mixtures were then placed in 100°C boiling water for 15 minutes, cooled and centrifuged at 3000 rpm for 10minutes. The optical densities of the supernatants were recorded at 532nm with Ultrospec 3100 UV/Visible spectrophotometer, Amershan Biosciences, against a reagent blank which contained only distilled water.

2.9 Phytochemical analyses.

Quantitative pPhytochemical anallysesscreening wereas carried out according to established procedures. The amounts of alkaloids present were determined using the method of Harbone,1973, whereas saponins were estimated according to AOAC method,1984. The method of Bohanam and Kocepal- Abyazam,1994 was employed for the estimation of the quantities of flavonoids present in the extracts, whereas glycosides were measured using the method of AOAC, 1984.by Sofowora (1980) and Cuiled (1982) for the presence of .alkaloids, flavonoids, saponins tannins, cardiac glycosides and carbohydrates.

3.0 RESULTS

3.1 **Anti-diabetic activity of the extracts.**

The average blood sugar level for the non-diabetic group was 85.05 ± 6.67 mg/dl.

Intraperitoneal administration of alloxan monohydrate (150.0 mg/kg body wt.) increased the average blood sugar levels of the animals (by up to 396.0%). Daily administration of 50.0mg/kg. body wt. of aqueous extract of *V. doniana* caused a significant(p< 0.01) decrease of 82.9% (from 492.8 ± 38.60 to 84.5 ± 6.8mg/dl) in the average blood sugar levels. Increasing the dose of the extract to 100.0 mg/kg. body wt. did not increase the efficacy of the extract. Aqueous *C. calisaya* bark extract at the tested doses, 50.0 and 100.0 mg/kg. body wt. reduced the blood glucose levels by 64.4 and 36.9% respectively(see Fig.1) It's effect at 50 mg/kg.body wt.was statistically significant(p< 0.05) when compared with the untreated group. Unlike *C. calisaya*, the extract of *V. doniana* was more potent than the reference drug, glibenclamide .(0.3 mg/ kg. body wt.,(79.91%, i.e from 408.8±38.3 to 98.5±8.5)) at both doses tested.

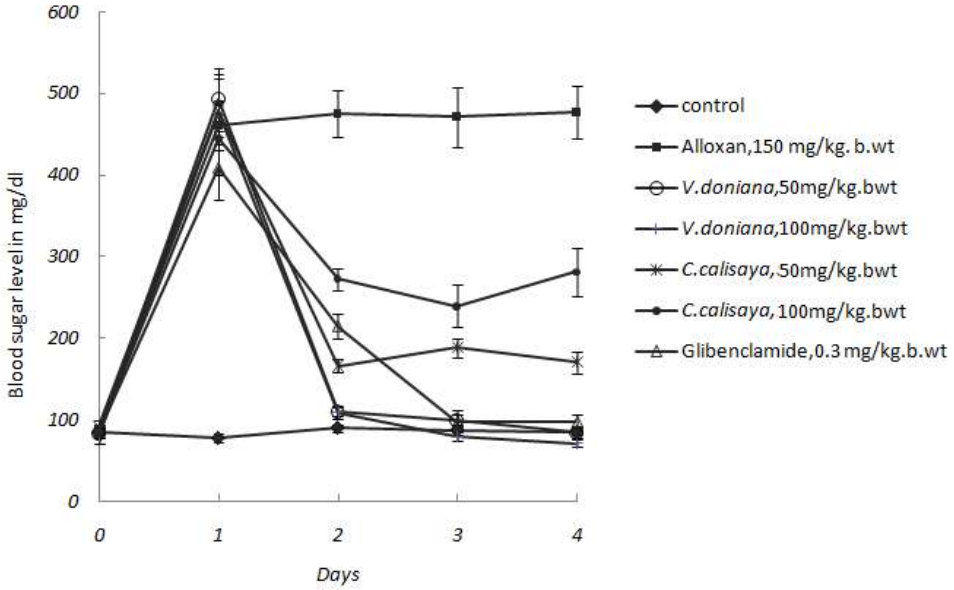
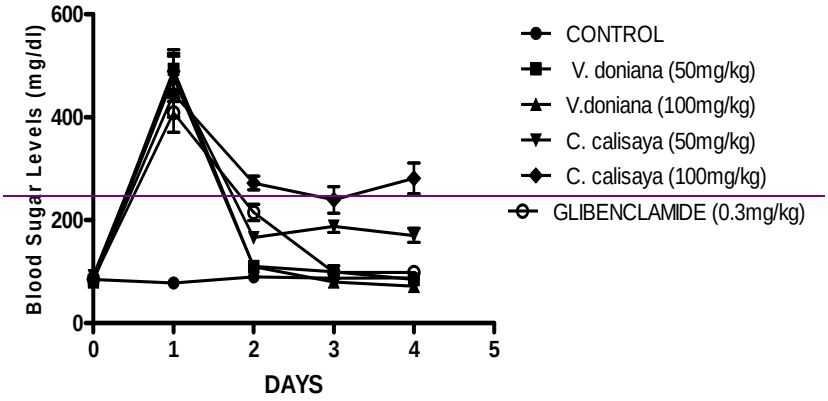


Fig.1



~~Fig. 1~~

3.2 Superoxide dismutase activity

There were significant ($p < 0.01$) elevations (up to 94.0%) in the activity of superoxide dismutase in all the extract- treated diabetic rats compared to the control animals (Fig.2). Highest increase (130.0%) was recorded in the rats treated with the standard oral hypogly-caemic agent~~s~~, glibenclamide. The

extracts of *V.doniana* and *C.calisaya* caused decreases in SOD activity when compared with the untreated diabetic rats. The effects of both *C. calisaya* and *V. doniana* extracts on SOD activity was dose – dependent.~~were dose –dependent.~~

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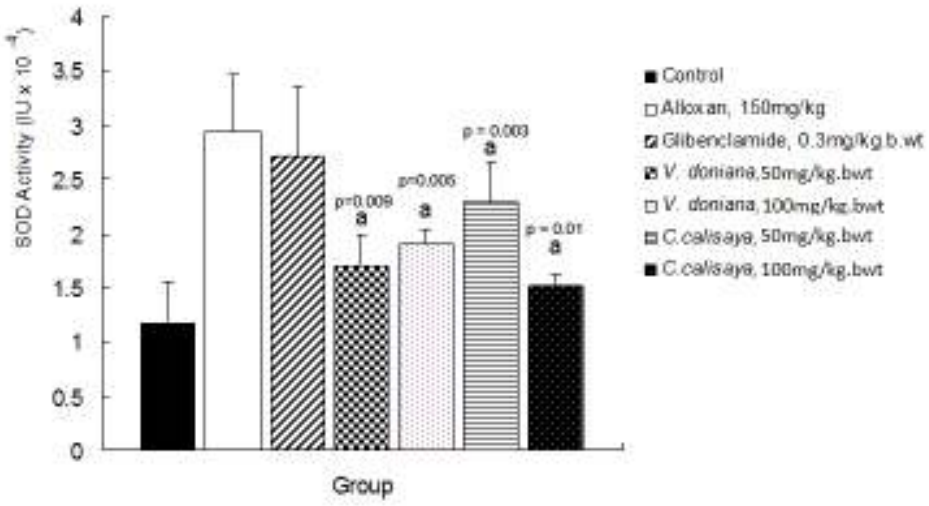
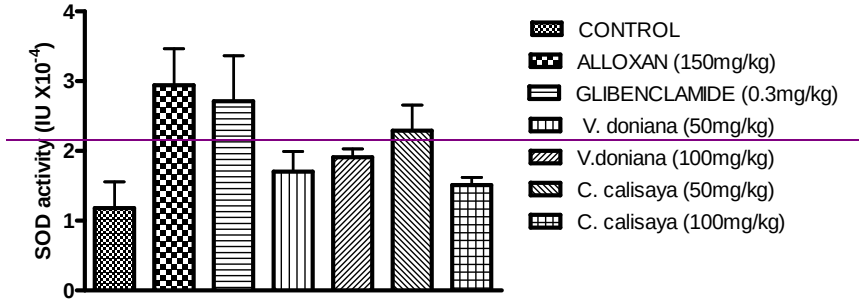


Fig.2

Effect of extracts on the SOD activities of diabetic rats. Data are expressed as mean ± SEM, n=5.

a indicates statistically significant difference from the control by student's T test, P < 0.01



~~Fig. 2~~

3.3 Catalase activity~~.~~

When compared with the control, slight and insignificant (p > 0,05) decreases in serum catalase activity were observed on ~~caused by the~~ treatment of the diabetic rats with *C. calisaya* extract (Fig.3),but untreated diabetic group gave significant(p<0.05) decrease in catalase activity.~~.~~Comparison with the untreated diabetic group showed that *C. calisaya* caused significant(p < 0.05) increase in catalase activity Glibenclamide and *V. doniana* extract (100~~.0~~ mg/kg. body wt. dose) significantly (p < 0.01) inhibited the action of this enzyme by 30.4% when compared with the control but insignificantly(p > 0.05) increasd the activity when compared with untreated diabetic rats. The effect of *V. doniana* extract insignificantly(p > 0.05) increased with dose.

~~The effect of *V. doniana* extract was dose – dependent.~~

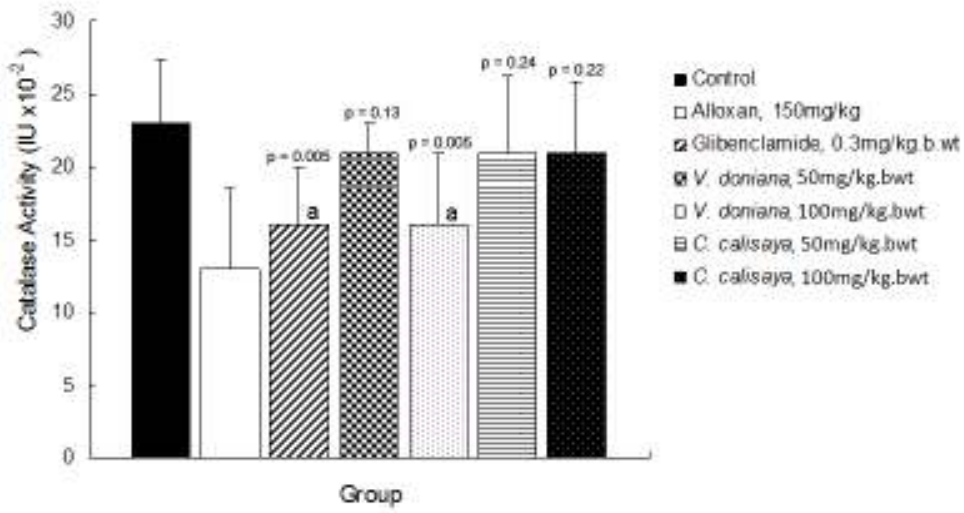


Fig.3

Effect of extracts on the Catalase activities of diabetic rats. Data are expressed as mean ± SEM, n=5.

a indicates statistically significant difference from the control by student's T test, P < 0.01

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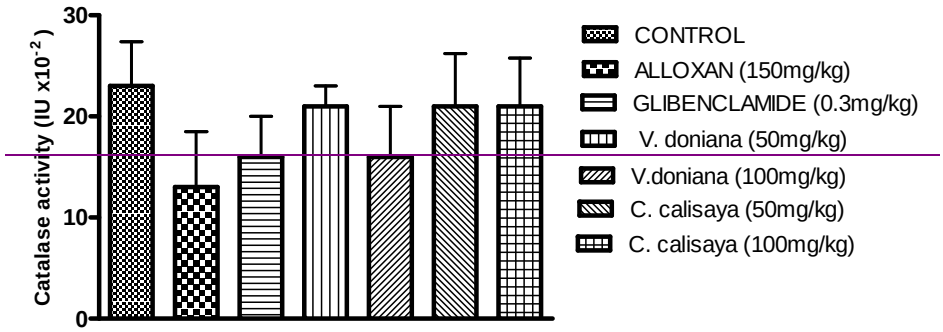

~~Fig. 3~~

3.4 Lipid – Peroxidation~~.~~

Malonyl-aldehyde (MDA) levels were significantly higher ($p < 0.001$) in the serum of all the diabetic rats treated with ~~the extracts of~~ *C. calisaya* and *V. doniana* extracts when compared with the control. The reference drug, glibenclamide 0.3 mg/ kg. body wt., caused the highest (up to 700%) increase in serum MDA levels of the rats. ~~The effect of~~ *C. calisaya* extract was more effective at a lower dose than at a higher dose, whereas varying the dose did not have any effect on the action of *V. doniana* on lipid – peroxidation (see Fig 3). C. calisaya and *V. doniana* at both doses tested produced significant($p < 0.001$) decreases in malonylaldehyde concentration when compared with the untreated diabetic group.

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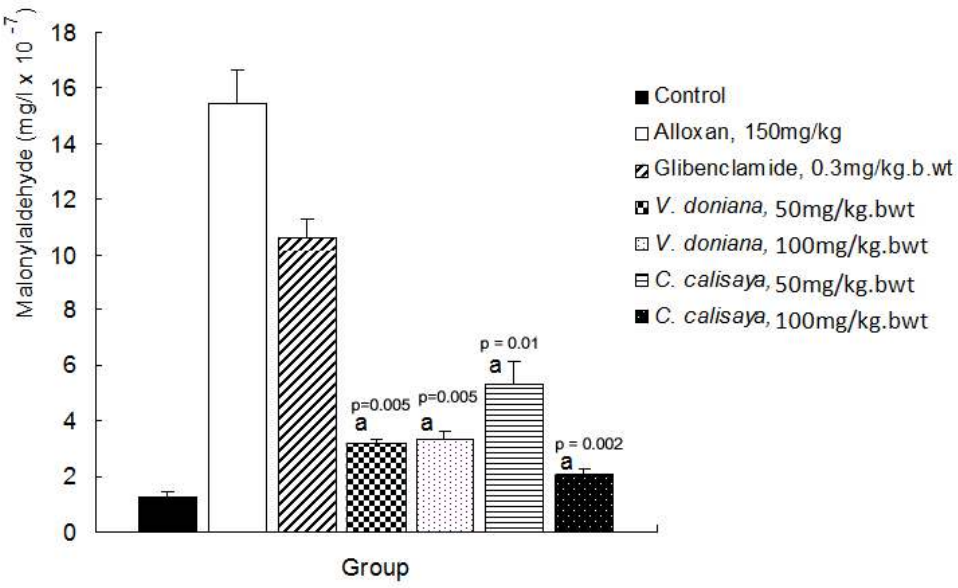

Fig.4

Effect of extracts on the MDA Levels of diabetic rats. Data are expressed as mean ± SEM, n=5. a indicates statistically significant difference from the control by student's T test, P < 0.01

~~Fig. 4~~

3.5 Phytochemical analysis

Results of the phytochemical analyses revealed the presence ~~of appreciable quantities~~ of alkaloids(1.8%), flavonoids(7.05%), saponins(0.92%), and cardiac glycosides(2.8%) ~~and carbohydrates~~ in *V. doniana* ~~both~~ leaf extract, ~~whereass of~~ *C. calisaya* bark contained alkaloids(6.0%), flavonoids(5.0%), saponins(2.0%), and cardiac glycosides(3.54%). ~~*V. doniana* leaves. Traces of tannins were observed.~~

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~~**Table1: Result of Phytochemical Screening**~~

~~**Phytochemical**~~	~~**Observation**~~	
	~~***V. doniana***~~	~~***C. calisaya***~~

Alkaloids	++	++
Flavonoids	++	++
Saponins	++	++
Tannins	+	+

Where + represents **trace amount**, and ++ represents **intensely present**.

4.0 DISCUSSION.

Findings from Oour investigation revealed that there were significant ($p < 0.001$) decreases in the blood sugar levels of the diabetic rats treated with 50.0 and 100.0 mg/kg body wt. doses of the aqueous extracts of both plants. *V. doniana* leaves and *C. calisaya* bark. Their potencyaction of *V.doniana* extracts was greater thanere comparable to that of the standard anti-diabetic drug, glibencldamide, at the dose of 0.3 mg/kg. body wt. These observations suggest that both valuable forest plants possess anti-diabetic activity and therefore justifiesy their traditional use of these plants in the management of the chronic disease diabetes mellitus. *C. calisaya* extract was more potent at a lower dose (50.0 mg/kg. body wt.) which produced 64.4% decrease in blood sugar level than at a higher dose (100.0 mg/kg. body wt.) that gave 36.9% reduction. On the other hand, the anti-diabetic effect of *V. doniana* was not in any way influenced by the increase in dose (Fig. 1). This implies that both extracts do not exhibit cumulative effects on the sugar levels of the rats (Salahdeen *et al*., 2004) probably because none of the processes involved in their absorption, distribution, metabolism, or elimination exhibit saturability.

Increased generation of free radicals together with reduced levels of body's endogenous antioxidant vitamins and enzymes are considered to be the main contributor to oxidative stress (Fakai *et al*., 2011Ellnan Wojitaszek et al., 2003). Free radicals' attack on membrane lipids, proteins and DNA is

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believed to be involved in many health disorders such as diabetes mellitus, cancer, neurodegenerative and inflammatory diseases (Charles and Brian,2010; David *et al*., 2005). The anti-oxidant enzymes include superoxide dismutase (SOD) and catalase. Superoxide dismutase generates H_20_2, while catalase consumes it. Precisely, catalase is the main regulator of H_20_2 metabolism (Lazlo *et al*., 2005).

Increased plasma lipid peroxidation and superoxide dismutase activity in type 2 diabetes mellitus have been well documented (Marjani, 2005; Akalin *et al*., 2008). Alloxan induces experimental diabetes mellitus in animals by enhancing the generation of H_20_2 ~~through the reaction of alloxan and reduced glutathione *in vitro*,~~ as well as decreasing the catalase enzyme activity (Kazunori *et al*., 2009). The higher SOD activity ~~seen~~ in diabetes mellitus in response to the increased generation of H_20_2 may be attributed to an adaptive mechanism in the tissues (Akalin *et al*., 2008). Results of our study showed that superoxide dismutase activity and plasma lipid peroxidation were significantly higher ($p < 0.01$ and $p < 0.001$ respectively) in all the diabetic rats compared to the control (Figs: 2 and 4). Lower catalase activity was observed in all the groups treated with the extract of *C. calisaya* and *V. doniana*(Fig.3).

~~Results of our studies showed that superoxide dismutase activity and plasma lipid peroxidation were significantly higher (p < 0.01 and p < 0.001 respectively) in all the diabetic rats compared to the control (Figs: 2 and 4). Lower catalase activity was observed (Fig. 3).~~

These observations corroborate previous reports by other researchers (Marjani, 2005; Akalin *et al*., 2008; Ebuehi *et al*., 2009) that many ~~species of the herbal~~ plants produce their pharmacological actions by restoring the oxidant – antioxidant balance of the body.

Treatment of the diabetic rats with either the standard oral hypoglycaemic drug, glibenclamide or the hypoglycaemic *V. doniana* and *C. calisaya* extracts caused increases in the activity of SOD and lipid peroxidation when compared with control,but the increases were lower than that produced by alloxan. Significant ($p<0.01$) inhibition of catalase enzyme activity was elicited by glibenclamide.

Our observations have, therefore, clearly indicated that alteration of the oxidants – antioxidants imbalance may be part of the mode of action of ~~these anti-diabetic extracts of~~ *V. doniana* and *C. calisaya*. It has been reported that many plants with hypoglycaemic action may act on blood glucose through inhibition of endogenous glucose production (Eddouks~~,~~ *et al*., 2003), or interference with gastrointestinal glucose absorption (Musabayane *et al*., 2006).~~;~~ The~~some~~ active ingredients present in some extracts may exhibit ~~may have~~ insulin – like activity~~substances~~ (Gray and Flatt., 1999)~~,; some may~~ inhibit insulinase activity, or ~~and some may~~ increase secretion of insulin from the β- cells of the pancreas (Trivedi *et al*., 2004; Yadai *et al*., 2008)~~, while others may~~ increased active β- cells' population in the pancreas as a result of~~by activating~~ regeneration of these cells by plant extracts has also been documented (Shannugasundaram *et al*., 1999; Jelodar *et al.*, 2007). All these mechanisms

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are yet to be investigated in order to elucidate the exact mechanism(s) of action of *V. doniana* and *C. calisaya* extracts.

Results of the qualitative phytochemical analyses showed that both extracts contained appreciable quantities of alkaloids, flavonoids, saponins, and cardiac glycosides. ~~and carbohydrates whereas trace amounts of tannins were observed (Table. 1).~~ One or more of these phytochemicals could be contributing to the observed activities of the extracts (Edeoga *et al*., 2005).

In conclusion, it is evident from preliminary~~our~~ findings that the aqueous ~~leaf~~ extract of *V. doniana* leaf and *C. calisaya* bark possess ~~strong~~ anti-diabetic activity against alloxan-induced diabetes mellitus. ~~,~~ Aa~~l~~teration of the oxidants – antioxidants imbalance created by alloxan may be contributing to their anti-diabetic activity. Subsequent work should focus more on the elucidation of their mechanisms of action, as well as isolation and characterization of the active ingredients.

5.0 COMPETING INTERESTS

Authors have declared that no competing interests exist.

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FIGURE CAPTIONS

Figure 1: Effects of aqueous extracts of *C. calisaya* bark and *V. doniana* leaves on blood glucose levels in alloxan – induced diabetic rats. Both extracts caused significant reductions in blood glucose levels at all the tested doses. Data are expressed as mean±SEM, n=5

Figure 2: Superoxide dismutase activity in extracts' treated – diabetic rats. Both extracts reduced alloxan – induced increases in superoxide dismutase activity. Data are expressed as mean±SEM, n=5. a indicates statistically significant difference from the control by ANOVA, $p<0.01$

Figure 3: Catalase enzyme activity in extracts' treated – diabetic rats. The extracts decreased the activity of this serum enzyme. Data are expressed as mean±SEM, n=5. a indicates statistically significant difference from the control by ANOVA, $p<0.01$

Figure 4: Malonylaldehyde concentrations in extracts ' treated – diabetic rats. Increased concentrations of malonylaldehyde were observed when compared with the control group. But extracts produced lesser increases relative to alloxan. Data are expressed as mean±SEM, n=5. a indicates statistically significant difference from the control by ANOVA, $p=0.001$. b indicates statistically significant difference from untreated diabetic rats (alloxan), $p<0.001$