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THE VASORELAXANT EFFECT OF *VISCUM ALBUM* LEAF EXTRACT IS MEDIATED BY CALCIUM-DEPENDENT MECHANISMS

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Summary: *Viscum album* leaf extract has a folk reputation as an antihypertensive agent in Nigeria. Evidence suggests that it has a relaxant effect on smooth muscle. The present study was designed to investigate the role of calcium in the vasorelaxant effect of this extract. Concentration response studies to noradrenaline, KCl and $CaCl_2$ were carried out in rat aortic rings with and without the extract in physiological salt solution (n=6 each). Also the role of intracellular calcium mobilisation was studied by measuring the phasic response to noradrenaline in Ca^{2+}-free EGTA physiological salt solution (n=6). The contractile responses to noradrenaline or KCl were attenuated ($P<0.05$) and shifted to the right in the presence of the extract. Also the contractile response to $CaCl_2$ in the presence of noradrenaline or KCl was attenuated ($P<0.05$) and shifted to the right, while the phasic response to noradrenaline was significantly ($P<0.05$) diminished These results suggest that the vasorelaxant effect of *Viscum album* extract may be mediated by a non-specific non-competitive inhibition of Ca^{2+} influx as well as inhibition of Ca^{2+} mobilization from intracellular stores. This implies that it may contain vasorelaxant agents that may have calcium antagonistic potential.

Key words: *Ca^{2+}; Vasorelaxant activity; Viscum album, .EGTA, KCl*

Introduction

The extract of the leaves of *Viscum album* (mistletoe; family: Loranthaceae) has been used for centuries in traditional medicine in many parts of the world. Studies suggest that it has a variety of biological effects. These include anticancer and immune stimulating properties (Grossarth-Maticek and Ziegler, 2006a and b; Zuzak et al., 2006; Lyu and Park, 2006; Ye et al., 2006; Maldacker, 2006; Harmsma et al., 2006), cardioactive (Tenorio-Lopez et al., 2006), vasorelaxant (Ekpenyong et al., 1999) and gastro-intestinal smooth muscle relaxant actions (Adeyemi , Okpo and Adepoju, 1996). In Europe a mistletoe preparation called Iscador has been reported to be effective in the treatment of cancer (Grossarth-Maticek and Ziegler, 2006a and b; Maldacker, 2006; Harmsma et al., 2006). In Nigeria, the extract of the leaves of *V. album* is used in traditional medicine for the treatment of several ailments including hypertension (Adeyemi , Okpo and Adepoju,1996). Indeed a preparation of *V. album* leaves packaged as "*nacu* tea" has been shown to exhibit a non-specific relaxant and a possible calcium antagonist activity in isolated gastrointestinal (GIT) smooth muscle preparations (Adeyemi , Okpo and Adepoju, 1996). Its vasorelaxant (Ekpenyong et al., 1999) effect and its reduction of coronary vascular resistance (Tenorio-Lopez et al., 2006) suggest that it may have vasodilator properties. It has been shown that the vasorelaxant effect is endothelium dependent involving the release of nitric oxide and not prostacyline (Ekpenyong et al., 1999). However, the extract showed relaxant effect in endothelium-denuded preparations (Ekpenyong et al., 1999) suggesting that it might also act directly on the vascular smooth muscle via calcium-dependent mechanisms.

The contractile mechanism in smooth muscle is activated by a rise in the concentration of free intracellular Ca^{2+} concentration ($[Ca^{2+}]i$; Bohr, 1963; Somlyo and Somlyo, 1968; Bohr, 1973). The source of $[Ca^{2+}]i$ is of dual origin: influx from the extracellular fluid or release from internal pools (Bohr, 1963; Bohr, 1973). Thus, vascular smooth muscle relaxant agents may produce their effects by inhibiting either or both sources of Ca^{2+} (Ebeigbe and Aloamaka, 1985). Consequently, this study was designed to test the hypothesis that *V. album* may exert its smooth muscle relaxant effect by inhibiting Ca^{2+}influx or its release from the cellular stores.

Materials and Methods

Plant collection and extract preparation

Fresh leaves of *V. album* were harvested as a parasitic plant on the oil palm tree (*Elaies guinensis*) at Ago-Iwoye, Ogun State, Nigeria and identified by Professor Olowokudejo of the Botany Department,

University of Lagos, Nigeria. The fresh leaves were dried at room temperature for two weeks and then ground into fine powder. 500g of the powder was extracted with 5 litres of distilled water using a soxhlet extractor for 48 hours. The aqueous extract obtained was concentrated to dryness using a freeze dryer to yield 98g of lyophilized extract. The extract constituted about 19.6% of the weight of the original material. It was stored at 4°C until it was used for the experiments. The samples used for each experiment were freshly prepared daily by dissolving appropriate weights of the extract in distilled water.

Preparation of aortic rings

Adult male Sprague-Dawley rats weighing 200-260g were killed by cervical dislocation and the thoracic aorta quickly exposed, carefully cleaned of fat and immediately placed in a Petri dish containing physiological salt solution (PSS) of the following composition ($mmol.l^{-1}$): NaCl, 119; KCl, 4.7; KH_2PO_4 , 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 14.9; $CaCl_2$, 1.6; glucose, 11.5. The aorta was cut into 2 mm ring segments. Each aortic ring was mounted between two hooks suspended in a 20ml jacketed tissue bath containing PSS. The hook was anchored onto the base of the organ bath while the rod was connected to a Grass FTO3 force transducer (FT.O3; Grass Instruments, Quincy, MA, USA) for the measurement of changes in isometric contraction (Obiefuna et al., 1991; Obiefuna, Sofola and Ebeigbe, 1991; Adegunloye and Sofola, 1997). The recording was displayed on a 4-channel Grass model 7D polygraph (model 7D; Grass Instruments, Quincy, Mass., USA). The PSS in the organ bath was continuously bubbled with a 95% O_2-5% CO_2 gas mixture and the temperature and pH were maintained at 37°C and 7.4 respectively (Obiefuna et al., 1991; Obiefuna, Sofola and Ebeigbe, 1991; Adegunloye and Sofola, 1997).

Each aortic ring was under a passive tension of 2g. They were equilibrated or stabilized for 90 minutes during which they were stimulated for 3-5 minutes with 10^{-7} $mol.l^{-1}$ noradrenaline at 30 minute intervals (Obiefuna et al., 1991; Obiefuna, Sofola and Ebeigbe, 1991; Adegunloye and Sofola, 1997). In experiments requiring high concentrations of KCl, high-K^+ PSS was prepared by equimolar substitution of NaCl with KCl.

Relaxation response studies to V. album

At the end of the 90 minute stabilization period the rings were pre-contracted with 10^{-7} $mol.l^{-1}$ noradrenaline or $60mmol.l^{-1}$ KCl. After the contraction had peaked *V. album* (2-16 mg/ml) was added cumulatively into the bath. The effect of each concentration was allowed to stabilize before the addition of the next concentration. The IC_{50} of *V. album* in noradrenaline pre-contracted rings was 5.2 ± 0.79 mg/ml while the IC_{25} in KCl pre-contracted rings was 7.1± 0.56mg/ml. These concentrations of *V. album* were used in subsequent experiments detailed below. The IC_{25} was used for the experiments with KCl because the calculated IC_{50} in KCl precontracted rings was very high and might not be achievable in the tissue bath.

Concentration response studies to noradrenaline and KCl.

The rings were exposed to cumulative concentrations of noradrenaline (10^{-9} to $10^{-5}mol.l^{-1}$) after incubating with the vehicle (0.2ml distilled water) for 15 minutes and the contractile responses recorded. The tissues were washed by flushing with PSS at 15 minute intervals several times until the pen returned to baseline. After adequate rest the tissues were incubated with the extract (5.2 mg/ml) for 15 minutes and the concentration response study to noradrenaline repeated. The same procedure was used for the concentration response study to KCl (10-$80mmol.l^{-1}$) with and without the extract (7.1 mg/ml) in another set of freshly stabilized aortic rings.

Concentration response to $CaCl_2$

Aortic rings were stimulated with 10^{-5} $mol.l^{-1}$ noradrenaline in normal PSS. After the contraction had peaked, the normal PSS was substituted with Ca-free PSS containing $1mmol.l^{-1}$ EGTA for 30 minutes. Midway into this period the tissue was stimulated with 10^{-5} $mol.l^{-1}$ noradrenaline to further deplete its calcium stores. At the end of 30 minutes the Ca-free EGTA PSS was replaced with Ca-free PSS without EGTA after which 10^{-5} $mol.l^{-1}$ noradrenaline was added, to open the ligand-gated Ca^{2+} channels (Obiefuna et al., 1991) followed almost immediately by the addition of 0.2 ml of the vehicle or extract (5.2mg/ml). The tissues were incubated with the vehicle or extract for 15 minutes and then $CaCl_2$ (2.5×10^{-4} to 1.6×10^{-1} $mol.l^{-1}$) was added cumulatively (Sofola, Obiefuna and Adegunloye, 1993; Adegunloye, Sofola and Coker, 1993).

Concentration response to $CaCl_2$ was carried out in a different set of freshly stabilized aortic rings with or without the extract (7.1 mg/ml) using the above protocol. However 80 $mmol.l^{-1}$ KCl was used to open the voltage-gated Ca^{2+} channels before the cumulative addition of $CaCl_2$.

Phasic contraction to Noradrenaline

Phasic contraction to 10^{-5} $mol.l^{-1}$ noradrenaline was carried out in Ca-free EGTA PSS (Ebeigbe and Aloamaka, 1985; Perry and Webb, 1991; Sofola, Obiefuna and Adegunloye, 1993; Adegunloye, Sofola and Coker, 1993). The tissues were stimulated with 10^{-5} $mol.l^{-1}$ noradrenaline in normal PSS. At the peak

of the contraction the normal PSS was replaced with Ca-free PSS containing 1mmol.l^{-1} EGTA for 10 minutes in order to deplete the cellular stores of calcium. This was followed by incubation of the rings in PSS containing 2mmol.l^{-1} $CaCl_2$ for another 10 minutes (calcium loading) after which this was substituted with Ca-free EGTA PSS and incubated with vehicle or *V. album* (5.2mg/ml) for 15 minutes before they were stimulated with 10^{-5} mol.l^{-1} noradrenaline and the contractile response recorded. Such phasic contraction is a useful index of Ca^{2+} mobilization from intracellular stores (Ebeigbe and Aloamaka, 1985; Perry and Webb, 1991; Sofola, Obiefuna and Adegunloye, 1993; Adegunloye, Sofola and Coker, 1993).

Drugs

Drugs used in this study were noradrenaline (arterenol) and N′N′-ethylene glycol tetraacetic acid (EGTA). Both were from Sigma. The drugs were diluted in distilled water. They were freshly prepared on the day of the experiment.

Statistical Analysis

The results are presented as mean ± SEM. The IC_{50} or IC_{25} was computed using a programme for logit transformation of concentration response curves (CRCs; Obiefuna et al., 1991; Obiefuna, Sofola and Ebeigbe, 1991; Adegunloye and Sofola, 1997). The student's t test for paired data was used for statistical analysis. P<0.05 was taken as statistically significant.

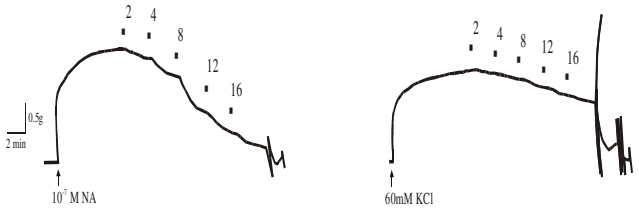

Fig.1. Typical tracings of relaxation responses to graded concentrations of *V. album* (2-16mg/ml) recorded in aortic rings pre-contracted with 10^{-7}M noradrenaline (NA) or 60mM KCl.

Results

Relaxation response to V. album

Fig 1 shows typical tracings of relaxation responses to *V. album* recorded in aortic rings pre-contracted with noradrenaline or KCl.

Concentration response to noradrenaline and KCl.

The concentration response of aortic rings to noradrenaline with and without *V. album* is shown in Figure 2. The curve obtained in the presence of *V. album* was significantly shifted to the right and attenuated compared to the control curve (P<0.05). The concentration response curve to KCl in the presence *V. album* was also significantly shifted to the right and attenuated compared to the control curve (P<0.05; Figure 3).

Concentration response to $CaCl_2$.

The concentration response curves to $CaCl_2$ in the presence and absence of *V. album* following stimulation by 10^{-5} mol.l^{-1} noradrenaline and 80 mmol.l^{-1} KCl respectively are shown in Figures 4 and 5. Again the curves were significantly shifted to the right and attenuated in the presence of *V. album* (P<0.05).

Phasic contraction to Noradrenaline

The phasic contraction to noradrenaline in the presence of *V. album* was significantly lower than that in its absence (P<0.05; Figure 6).

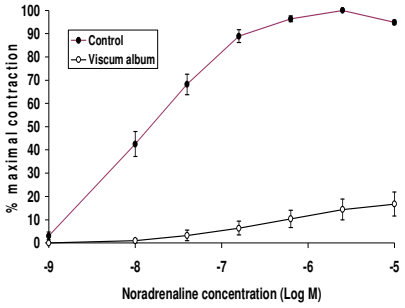

Fig.2. Concentration response curves for noradrenaline in aortic rings incubated with and without V. album (5.2mg/ml). Each point represents mean ± SEM of 6 experiments. P<0.05 at all concentrations.

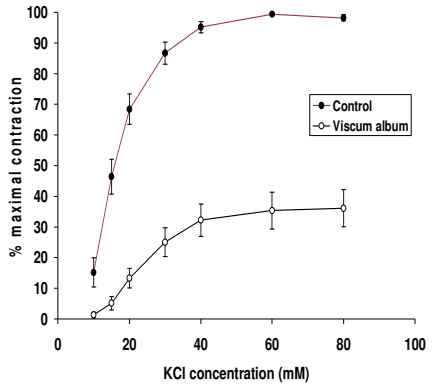

Fig. 3. Concentration response curves for KCl in aortic rings in the absence (control) and following exposure to V. album (7.1mg/ml). Each point represents mean ± SEM of 6 experiments. P<0.05 at all concentrations.

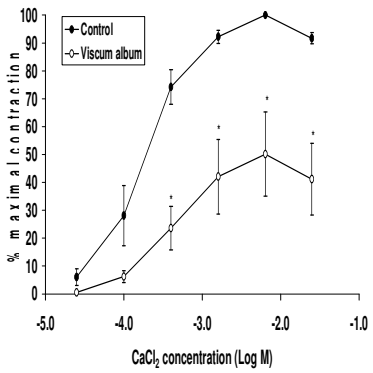

*Fig. 4. Concentration response curves for $CaCl_2$ in aortic rings incubated with and without V. album (5.2mg/ml) following opening of ligand-gated channels with 10^{-5} M noradrenaline. Each point represents mean ± SEM of 6 experiments. *P<0.05.*

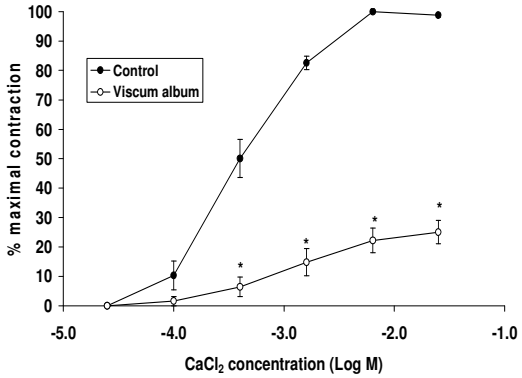

*Fig. 5. Concentration response curves for $CaCl_2$ in aortic rings incubated with and without V. album (7.1mg/ml) following opening of voltage-gated channels with 80mM KCl. Each point represents mean ± SEM of 6 experiments. *P<0.05.*

Discussion

The major finding of this study is that the aqueous leaf extract of *V. album* has a vasorelaxant effect that is mediated by Ca^{2+}-dependent mechanisms. Relaxation of noradrenaline and KCl pre-contracted aortic rings by the extract suggests that its relaxant effect is non-specific. However noradrenaline pre-contraction appeared to be more susceptible to *V. album* than KCl pre-contraction (Figure 1) as evidenced by the IC_{50} and IC_{25} value obtained respectively. Indeed IC_{25} was used for subsequent experiments with KCl because the calculated IC_{50} value obtained from the relaxation response experiments was very high. Such concentrations may not be suitable in small tissue baths used in this study.

The results show that the contractile responses to noradrenaline and KCl were attenuated by the extract as evidenced by the shifting of concentration response curve (CRC) of each contractile agent to the right and the depression of its maximal response in the presence of *V. album*. A similar pattern was observed in the CRCs to $CaCl_2$ in the presence of *V. album* following the opening of Ca^{2+}-channells by noradrenaline or KCl (Figures 4 and 5). Taken together, these observations are suggestive of non-competitive antagonism (Furchgott, 1966). The contractile mechanism in smooth muscle is activated by a rise in the concentration of free intracellular Ca^{2+} concentration ($[Ca^{2+}]i$; Bohr, 1963; Somlyo and Somlyo, 1968; Bohr, 1973). This may occur via Ca^{2+} influx through ligand-gated or voltage-gated Ca^{2+} channels (Bohr, 1963; Somlyo and Somlyo, 1968; Bohr, 1973) which were opened by the use of noradrenaline and KCl respectively in this study. Consequently, the present findings suggest that that the reduction in contractile response to these agonists by *V. album* may be through a non-specific non-competitive antagonistic action on Ca^{2+}influx.

However, since increase in $[Ca^{2+}]i$ may also occur through release from cellular stores the effect of *V. album* on this was studied by measuring the phasic response to noradrenaline. Such phasic contraction is a useful index of Ca^{2+} mobilization from intracellular stores (Ebeigbe and Aloamaka, 1985; Perry and Webb, 1991; Sofola, Obiefuna and Adegunloye, 1993; Adegunloye, Sofola and Coker, 1993). The results of the present study show that phasic response to noradrenaline in Ca^{2+}-free EGTA PSS was attenuated by the extract. This suggests that mobilization of intracellular Ca^{2+} for contraction by noradrenaline was inhibited.

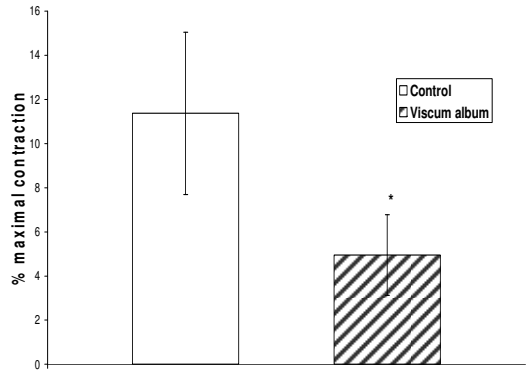

*Fig. 6. Bar graphs showing the effect of V. album (5.2mg/ml) on phasic contraction to noradrenaline in calcium-free PSS. n = 6 experiments. *P<0.05*

These results are consistent with an earlier study suggesting that a *V. album* preparation showed a non-specific relaxant and calcium antagonist activity in isolated gastrointestinal tract (GIT) smooth muscle preparations (Adeyemi , Okpo and Adepoju, 1996).

However, it is possible that *V. album* may effect its vasorelaxant action through other mechanisms as well. Earlier reports suggest that its vasorelaxant effect was endothelium dependent (Ekpenyong et al., 1999) occurring through the nitric oxide/soluble guanylate cyclase pathway (Ekpenyong et al., 1999; Tenorio-Lopez et al., 2006). Indeed since endothelium intact preparations were used in this study, some of the actions on Ca^{2+}influx or mobilization from cellular stores observed in this study for *V. album* may be partly mediated by nitric oxide (NO). This is because NO inhibits Ca^{2+} influx through ligand gated Ca^{2+}channels as well as release from cellular stores. Thus it is conceivable that *V. album* may achieve vasorelaxation through stimulation of the nitric oxide/soluble guanylate cyclase pathway as well as through Ca^{2+}-dependent mechanisms. These dual mechanisms suggest that *V. album* may be useful in the treatment of hypertension. Hypertension is characterized by an increase in peripheral resistance which may be due to elevated $[Ca^{2+}]i$ and/or endothelial dysfunction. *V. album* may be useful in its treatment conceivably by ameliorating these defects. However actual experiments will be needed to confirm this notion. In spite of this, the present findings provide some evidence suggesting that the use of this extract in Nigerian folk medicine for the treatment of hypertension may be justified.

In summary, this study was designed to investigate the role of calcium in the vasorelaxant effect of *V. album*. The contractile responses to noradrenaline and KCl were attenuated and shifted to the right in the presence of the extract. Also the contractile response to $CaCl_2$ in the presence of each agent (noradrenaline or KCl) was attenuated and shifted to the right, while the phasic response to noradrenaline was diminished. These results suggest that the vasorelaxant effect of *V. album* extract may be mediated by a non-specific non-competitive inhibition of Ca^{2+} influx as well as inhibition of Ca^{2+} mobilisation from intracellular stores. This study may provide a scientific basis for the use of this extract in Nigerian traditional medicine for the treatment of hypertension.

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