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Allanblackia floribunda Oliver: an aphrodisiac plant with
vasorelaxant properties

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Abstract

Ethnopharmacological relevance: *Allanblackia floribunda* Oliver is one of the most commonly used medicinal plant in Cameroon. The stem bark of the plant is traditionally used for its aphrodisiac and antihypertensive properties.

Aim of the study: To validate the traditional uses of *Allanblackia floribunda* stem bark ethanol extract through the evaluation of their aphrodisiac and vasorelaxant properties.

Materials and methods: The extract abilities to increase sexual desire and the frequencies of erection (mount), intromission and prolonged latency of ejaculation were studied on adult male rats. The vasodilator effect was investigated using isolated rat aorta rings. Tests were conducted using fractions obtained by reverse phase column-chromatography (CC), after the acquisition of the HPLC fingerprint of the ethanol extract, resulted the most active in previous studies.

Results: The CC allowed the isolation of five fractions whose aphrodisiac and vasodilator activities were tested and compared with those of the whole extract. Four compounds were identified and characterized, three of them, Fukugiside, Morelloflavone and Volkensiflavone, are secondary metabolites known to be in *Allanblackia floribunda*; the forth, Spicataside, is a biflavonoid glycoside known to be present in the genus *Garcinia* but never found neither in *Allanblackia floribunda* nor in *Allanblackia* genus.

The crude ethanolic extract (CEE) induced a relaxation on aorta rings with $EC_{50} = 11 \pm 2$ $\mu\text{g}/\text{mL}$ and Morelloflavone displayed a similar activity with $EC_{50} = 42 \pm 6$ $\mu\text{g}/\text{mL}$; for all the other compounds only the vasodilation % at the maximum concentration testable (90 $\mu\text{g}/\text{mL}$) was determined: 30 ± 8 (Fukugiside), 24 ± 6 (Spicataside), 33 ± 4 (Morelloflavone+Volkensiflavone), 47 ± 1 (Volkensiflavone). Regarding the activity on

male sexual behaviour, only CEE and Fukugiside showed activity in the 9 parameters evaluated.

Conclusions: These results may support the traditional uses of *Allanblackia floribunda* as aphrodisiac plant with antihypertensive properties suggesting the phytocomplex as responsible for the claimed activity.

Keywords: *Allanblackia floribunda* bark extract, aphrodisiac, vasodilation, aorta.

1. Introduction

Allanblackia, a medium-sized fruit tree native to sub-Saharan Africa, belongs to the *Clusiaceae* family. It is an evergreen plant growing well in the rainforest, typical of some regions of west, central and east Africa, mostly from Sierra Leone to Cameroon and Gabon, Congo and Uganda. In Ghana, it grows in the Western, Central, Ashanti and Eastern Regions in forest stands as well as on cocoa farms (Irvine, 1961). Nine species in the genus have been recorded (Bamps, 1969, Denys, 1980), namely *Allanblackia floribunda* Oliver or tallow-tree, *Allanblackia gabonensis* (Pellegr.) Bamps, *Allanblackia kimbiliensis* Spirlet, *Allanblackia kisonghi* Vermeesen, *Allanblackia marienii* Staner, *Allanblackia parviflora* A. Chev., *Allanblackia stanerana* Exell & Mendonça, *Allanblackia stuhlmanii* Engl. and *Allanblackia ulugurensis* Engl.

Allanblackia trees are a fundamental source of traditional medicine for many populations in Africa, especially in Cameroon, Nigeria, Gabon and Congo. Particularly, seeds and fruit's pulp have been used as support in times of food scarcity, wood for the construction of local houses, twigs as candlesticks and the smallest as chew-sticks or toothpicks. However, between all of these, the most relevant application consists of producing edible oil from *Allanblackia* seeds. (Crockett, 2015). The decoction of stem bark and leaves of *Allanblackia floribunda* are used in the treatment of dysentery, toothache, asthma, bronchitis, urethral discharge and cough in Gabon and Congo. In Gabon, the bark is also pounded and rubbed on the body to relieve painful conditions (Ayoola et al., 2008). In Ghana the decoction of the stem bark relieves body pains, hypertension and toothache, the bark is used also to prepare a paste which could be used to treat boils, dysentery and respiratory tract infections as cough, asthma and bronchitis (Abbiw, 1990, Nuga et al., 2015). The nuts of the plant produce fine oil assumed to relieve rheumatism in Tanzania.

Furthermore, a decoction is taken for dysentery and as a mouthwash for toothache and, in Ivory Coast, for stomach pains (Steentoft, 1988). The bark decoction of stem and root is also used in Central African Republic and West Africa to treat toothache, dysentery and as analgesic (Lewis et al., 1977). In Akwa Ibom state of Nigeria, the local communities use the leaves as well as bark and root of the plant to treat dysentery, diarrhoea, skin diseases and some other microbial infections (Ajibesin et al., 2008). In Congo, a decoction of the bark or leaves is used for stomach-pain, cough, asthma, bronchitis and other bronchial affections while the lees from this preparation are rubbed on painful areas after scarification (Bouquet, 1969). Actually, *Allanblackia floribunda* is one of the most commonly used medicinal plant in Cameroon (Balick et al., 1996). In addition, in Cameroon, the stem bark of the plant mixed with *Capsicum frutescens* or *Solanum anguivi* is used for the treatment of cough (Betti, 2004). Moreover, information provided by practitioners of traditional medicine suggests that the plant possesses useful aphrodisiac and antihypertensive properties (Bilanda et al., 2010). The efficacy of this plant in the improvement of male sexual behaviour has been described in rats (Kada et al., 2012) to confirm folkloric claims by local people in Cameroun.

Multiple mediators contribute to all the biological effects described. Among these, nitric oxide (NO) has been suggested to be the primary physiological mediator of penile erection (Azadzo et al., 1992); also, NO synthesized by the endothelium of small vessels is involved in the control of vascular tone and plays an important role in the regulation of blood pressure (Moncada et al., 1993, Zhao et al., 1996).

Nevertheless, since vascular activities claimed for *Allanblackia floribunda* have never been proved, the aim of this work is the validation of the traditional use of this plant as an antihypertensive and aphrodisiac remedy. Thus, based on previous results obtained for

the aqueous extract (Bilanda et al., 2010, Kada et al.,2012) the *in vivo* aphrodisiac effects of the ethanol extract and fractions of *Allanblackia floribunda* and *in vitro* effects on the contractile responses of isolated aorta, will be investigated.

2. Experimental

2.1. Plant Material

Stem bark of *Allanblackia floribunda* was collected at Okola, Central Region of Cameroon in February 2007 and identified by Professor Pom Henry of the Cameroon National Herbarium (CNH), Yaoundé, where the voucher specimen (1380/HNC) was deposited by R. Letouzey. The stem bark was washed with tap water, cut into small pieces and oven dried for 7 days at room temperature.

2.2 Extraction procedure

Dried powder (1 Kg) of *A. floribunda* was exhaustively extracted with 2 L of 95% aqueous ethanol at room temperature for 3 days. The mixture obtained was filtered and then concentrated under reduced pressure to give 94 g of crude extract (yield 9.40 % on a dry mass basis). The extract obtained was reconstituted in distilled water to give the doses required for each experiment.

2.3. Chromatographic analyses

2.3.1 . Apparatus and chromatographic conditions

The chromatographic analyses were performed with an Agilent technologies HP-1100 (Palo Alto, CA-USA) equipped with quaternary pump model, rheodyne injector valve with a 20 µl loop, degasser for the mobile phase, the temperature control apparatus model

and UV-detector with variable wavelength model. Data management has been carried out by HPLC ChemStation software version A.04.01. An Agilent Technologies LiChrospher 100 RP-18 column (5 µm) 4 x 250 mm was used to analyse crude ethanol extract (CEE) of *A. floribunda* and the fractions obtained by column chromatography (CC). The analyses were carried out using a step gradient system of 0.02% trifluoroacetic acid (TFA) in H₂O (solvent A) and CH₃CN (solvent B), as follow: 10% to 55% of solvent B in 25 min, 55% to 100% of solvent B in 2 min; flow rate:1 ml/min; the UV absorbance was measured at 225 nm.

CEE and fractions obtained by CC were weighed and solubilized to reach a concentration of 10 mg/ml (1/10 MeOH/H₂O), vortexed for 2 min, sonicated for 5 min and finally centrifuged for 5 min (4000 rpm).

2.3.2 Preparative Column chromatography

150 g of LiChroprep RP-18 25-40 µm (Merk) were packed in a glass column (300 mm x 50 mm) using a dry packing method; 500 mg of *A. floribunda* ethanol extract were dissolved in n-hexane (45 ml), vortexed for 5 min, sonicated for 5 min and centrifuged for 10 min (4000 rpm); this operation was repeated for 4 times. Dry defatted extract (0.502 g) was re-solubilized in methanol (45 ml) and adsorbed on celite: after solvent removal the dry powder was directed charged into the glass column. A stepwise gradient H₂O-MeOH with 0.02% of TFA was used. Gradient elution: 70/30→60/40 in 1 CV (column volume= 150 ml, each achieved in 3,75 min), 60/40→50/50 in 3CV, 50/50→40/60 in 3 CV, 40/60→100 in 4 CV, at a flow rate of 40 ml/min. The elution was monitored at 225 and 366 nm; fractions with same chromatographic profile were collected affording 5 final fractions namely F1 (0.218 g), F2 (0.071 g), F3(0.066 g), F4(0.093 g), F5(0.029 g).

2.4 Characterization of isolated compounds

2.4.1 HPLC-ESI-MS analysis

A Thermo Scientific LCQ FLEET system equipped with LCQ FLEET ion trap mass spectrometer, Surveyor MS Pump/Autosampler/PDA Detector (Thermo Fisher Scientific Inc., Waltham, MA) was used for mass spectrometric measurements. The chromatographic separation of CEE of *A. floribunda* was performed on an Atlantis C18 2.1 x 150mm column (Waters Corporation, Milford, MA). The analyses were carried out using a step gradient system of 0.1% of formic acid in H₂O (solvent A) and CH₃CN (solvent B), as follow: 10% to 55% of solvent B in 25 min, 55% to 100% of solvent B in 2 min; flow rate: 0.3 ml/min. The injection volume was 5 µl. The ESI parameters were set as follows: spray voltage 3.5 kV, capillary temperature 260 °C, sheath gas flow rate 50 arbitrary units, and auxiliary gas flow rate 20 arbitrary units. The Thermo Fisher Scientific Xcalibur 2.1 software was used for data acquisition and processing. The ESI-MS and ESI-MS/MS were acquired in positive mode.

2.4.2 NMR analysis

Elucidation of the isolated compounds was carried out using Nuclear Magnetic Spectroscopic analyses.

NMR spectra were acquired at 400 MHz for ¹H using a Bruker Avance 400 MHz spectrometer equipped with Bruker's TopSpin 1.3 software package. The abbreviations s, d, t, q, br s, and m stand for the resonance multiplicities singlet, doublet, triplet, quartet, broad singlet, and multiplet, respectively. Sample temperatures were controlled with the variable-temperature unit of the instrument.

*2.5 Evaluation of the aphrodisiac and vasodilator activities of *Allanblackia floribunda**

2.5.1 Animals

Healthy, 3-months-old male Wistar rats weighing 240–250 g and 2.5-months-old females weighing 200-210 g were obtained from the Animal House of the Laboratory of Animal Physiology of University of Yaoundé 1. The animals were housed in clean cages placed in well-ventilated housed conditions (temperature 25°C; photoperiod: 12 h natural light and 12 h dark). They were allowed free access to food and tap water. All males were trained for sexual experience and only those exhibiting good copulatory behaviour were selected for the study. The studies were conducted according to the guidelines of the Cameroon National Ethical Committee on the use of laboratory animals for scientific research (Ref No. FW-IRB00001954).

For vasodilation activity study male Wistar rats weighing 180-200 g were purchased from Envigo (S. Pietro al Natisone, Italy). As few animals as possible were used. The purposes and the protocols of our studies have been approved by Ministero della Salute, Rome, Italy.

2.5.2 Drugs

The drugs used for the study include sildenafil citrate (Viagra) (Micron Pharmaceuticals India), estradiol benzoate (Sigma Chemicals, USA), progesterone (Sigma chemicals, USA).

2.5.3 Animal grouping and drugs

Forty male sexually experienced rats were randomized into eight groups of 5 animals and orally treated as follows: group I (control) received 10 mL/kg body weight of distilled water; group II received 5 mg/kg body weight of Viagra; group III received 150 mg/kg body weight of the ethanol extract of *Allanblackia floribunda* and group IV to VIII received 150 mg/kg body weight of fractions 1 to 5 (F1 to F5) of the plant extract respectively. All animals from each group were monitored for sexual behaviour 30 minutes after the administration of the treatment. Sexual experience was induced by exposing each male rat to an oestrous female three times a week for 3 weeks. Only males showing mounting behaviour were selected for the study.

2.5.4 Copulatory behaviour test procedure

This experiment began at 05:30 PM local time in a quiet room under dim light. An ovariectomized receptive female rat brought to oestrus by sequential subcutaneous injection of oestradiol benzoate (10 µg/kg body weight) and progesterone (0.5 µg/kg body weight) respectively 48 h and 4 h prior to the test (Amin *et al.*, 1996) was introduced to the male 10 min (adaptation period) after administration in a cage of dimensions (48.5 cm × 33.5 cm × 22.5 cm). After monitoring for 30 minutes,, the following male sexual behaviour parameters were recorded or calculated: penile licking (the number of time animals licked their penile), mount frequency (MF) and intromission frequency (IF), (the number of mounts and intromissions from the time of introduction of the female until ejaculation), mount latency (ML) and intromission latency (IL), (the time interval between the introduction of the female and the first mount or intromission by the male), ejaculation latency (EL), (the time interval between the first intromission and ejaculation), post-ejaculatory interval (PEI), (the time interval between ejaculation and

the first intromission of the following series). Sexual behaviour parameter computed is copulatory efficiency = Number of intromissions/ number of mounts. The test was terminated when the male failed to evince sexual interest or when the female did not show receptivity.

2.5.5 Statistical analysis

Data are expressed as mean \pm SEM (Standard Error of Mean). Statistical analyses used repeated measures analysis of variance (ANOVA) to account for the different treatments and were complemented with the Student-Newman-Keuls Test. Differences were considered statistically significant at $P < 0.05$. All analyses were performed using the Graphpad Instat software Version 3.10.

2.5.6 Vasodilator activity

Male Wistar rats were anaesthetized with isoflurane, decapitated and exsanguinated. The aorta was removed immediately, dissected free of fat and connective tissue and cut into rings of about 3–4 mm in width. Endothelium-intact rings were mounted under 1.0 g tension in organ baths containing 30 ml of Krebs-bicarbonate buffer with the following composition (mM): NaCl 111.2, KCl 5.0, CaCl₂ 2.5, MgSO₄ 1.2, KH₂PO₄ 1.0, NaHCO₃ 12.0, glucose 11.1, maintained at 37 °C and gassed with 95% O₂-5% CO₂ (pH 7.4). The rings were allowed to equilibrate for 60 min and endothelium integrity was assessed with 10 μ M acetylcholine (ACh) in rings precontracted by 1 μ M phenylephrine. A relaxation $\geq 75\%$ of phenylephrine-induced tone was considered sign of functional endothelium. Aortic rings were then washed and equilibrated for another 60 min period and then contracted with 1 μ M L-phenylephrine. When the response to the agonist reached a

plateau, cumulative concentrations of the extract were added. Results are expressed as EC_{50} (\pm SEM) $\mu\text{g/mL}$. Responses were recorded by an isometric transducer connected to the MacLab System PowerLab. Addition of the vehicle (DMSO) had no appreciable effect on contraction level

3. Results and discussion

A. floribunda is used in center Cameroon for its aphrodisiac and antihypertensive properties (Bella, 2008; Bilanda et al., 2010; Kada et al., 2012), as suggested from practitioners of traditional medicine.

In order to validate the traditional uses, the stem bark extract and the fractions obtained after CC, were investigated for:

- (i) their ability to increase sexual desire, frequencies of erection (mount), intromission and prolonged latency of ejaculation;
- (ii) their antihypertensive activity through the vascular relaxation of endothelium-intact rat aorta rings.

Based on previous paper of Kada et al. (Kada et al., 2013), the most potent crude ethanol extract (CEE) was selected for further bioguided fractionation with the final aim to obtain pure compounds and evaluate which may be responsible for the aphrodisiac and antihypertensive activities. The RP-HPLC analysis of CEE allowed the acquisition of the fingerprint (Figure 1) which showed four main peaks (A, B, C and D) with a quite good resolution and apparently easy to be fractionated and separately collected. The analytical method was transferred to a preparative flash column chromatography (CC) which allowed the separation of five fractions, as reported in Figure 2.

The CEE was analysed also in LC-MS, as described in the materials and methods section. The molecular weight of the four peaks, reported in order of their retention time, were found to be: 718, 702, 556 and 540, respectively. Following their fragmentation patterns recorded in positive ionization mode, compounds with MW of 718, 556 and 540 correspond to Fukugiside, Morelloflavone and Volkensiflavone respectively, three biflavonoids already identified in the stem bark of *A. floribunda* in previous works (Kuete et al., 2011, Locksley and Murray, 1971), while compound with MW of 702 was identified as Spicataside, a glycosylated flavonoid isolated for the first time from the bark of *Garcinia spicata* (Konoshima et al., 1970) and, so far as we know, never found in *A. floribunda* nor in the genus *Allanblackia* (Table 1; Figure 1S-4S Supplementary Material).

Molecular formulas are reported in Figure 3. The final structure elucidation was performed by NMR analysis. Variable temperature NMR experiments confirmed that at room temperature isolated biflavonoids and their glycosylated derivatives exist as a mixture of conformers.

¹H-NMR spectra of peak C, recorded in dms_o-d₆ at 25°C and 80°C, were in agreement with those reported in literature for Morelloflavone (Li, et al., 2002). The measured specific optical rotation ($[\alpha]_D^{25} = 2.1^\circ$, *c* 0.1, MeOH) showed that our sample was an almost racemic mixture of 2*R*,3*S*-2*R*,3*S*-morelloflavone ($[\alpha]_D^{25} = +188^\circ$, *c* 0.1, MeOH), and 2*S*,3*R*-morelloflavone. Peak A revealed to be morelloflavone-7''-O-β-D-glucoside, which is known as Fukugiside (Elfita et al., 2009). Its protonic spectrum resembled that of Morelloflavone, but typical signals for a glucosyl moiety were present. ¹H-NMR analysis of Peak D and B furnished spectra which were in agreement with those reported for Volkensiflavone (Chen et al., 1975) and volkensiflavone-7''-O-β-D-glucoside (Jamila

et al., 2014), respectively. The measured optical activities indicated that all biflavonoid derivatives were isolated as racemic mixture.

The 5 fractions were analysed, as described in the experimental section, in order to evaluate their sexual behaviour and their ability to promote vascular relaxation of endothelium-intact rat aorta rings. Results, in comparison with those of CEE, are reported in Table 2 and Table 3, respectively. Due to the poor amount of fraction 5 (Volkensiflavone), results were considered not significant and thus were not reported in Table 2.

A. floribunda crude ethanol extract (150 mg/kg body weight, CEE) significantly increases ejaculatory latency, mount frequency, ejaculatory frequency and copulatory efficiency, when compared with animals treated with distilled water, while a significant decreasing ($p < 0.001$) of post ejaculatory interval is observed at the same dose. Fraction 1, containing pure compound identified as Fukugiside, decreases significantly ($p < 0.001$) mount latency and intromission latency compared to animals treated with distilled water. The same fraction increases significantly mount and intromission frequency, penile licking, ejaculatory frequency and copulatory efficiency compared to control group and decreases significantly ($p < 0.001$) post ejaculatory interval. Fraction 3, which is a mixture of Spicataside (major compound) and Morelloflavone, decreases significantly ($p < 0.01$) mount and intromission latency and increases significantly ejaculatory frequency when compared to control group. Fraction 2, which contains only Spicataside, and fraction 4, which contains a mixture of Morelloflavone (major compound) and Volkensiflavone, did not show any significant statistical variation with all the parameters evaluated compared to animal who received distilled water. Viagra (5 mg/kg) increases significantly all parameters investigated when compared to distilled water.

F1 and F3 produce significant reductions in mount latency (ML) and intromission latency (IL), compared with controls. These parameters are considered to be inversely proportional to sexual motivation or desire (Yakubu et al., 2005). Since sexual desire depends on testosterone (McGinnis et al., 1989), our observations suggest that F1 and F3 are able to increase testosterone levels.

Moreover, F1 increases significantly ejaculation latency and copulatory efficiency, while F3 only increases significantly the ejaculatory latency. These parameters are useful indices of sexual vigour and potency (Tajuddin et al., 2004; Mbongue et al., 2005). The significant increases of these parameters in rats receiving *A. floribunda* extract and both fractions F1 and F3 suggest that the plant exerts a positive effect on sexual performance (Ratnasooriya and Dharmasiri, 2000); Fukugiside and the mixture Spiacataside + Morelloflavone, the bioactives compounds present in F1 and F3, may be responsible for the increasing of sexual performances (Ratnasooriya and Dharmasiri, 2000).

Also, premature ejaculation is one of the important causes of sexual dysfunction. The prolonged ejaculatory latencies associated with the extract and fractions are indications of improved sexual function in rats (Yakubu and Afolayan, 2008). The post- ejaculatory interval (PEI) is an index of potency, libido and rate of recovery from exhaustion after a first series of mating (Tajuddin et al., 2004). The decreased PEI observed with CEE and F1 may be due to the enhanced potency and libido or to the decreased exhaustion in the first series of mating or both. Frequencies of mount (MF) and intromission frequencies (IF) indicate libido and sexual potency. The significant increase in MF and IF in animals treated with CEE and F1 suggests that *A. floribunda*, (Yakubu and Afolayan, 2008) contains bioactive compound/s able to increase erection process, particularly the biflavonoid Fukugiside responsible for the activity of F1. The absence of activity

observed with other compounds isolated from the plant suggests a synergism of action. Also, when a single compound is tested at the same dose than a whole crude extract, it is likely that the compound is rather toxic than active.

The evaluation of the compounds in smaller doses (and in a dose-response manner) in Wistar rats will be evaluated in future studies.

The crude ethanolic extract (CEE) and F3 (Spicataside + Morelloflavone) induced a relaxation on aorta rings with $EC_{50} = 11 \pm 2 \mu\text{g/mL}$ and $EC_{50} = 42 \pm 6 \mu\text{g/mL}$ respectively; for all the other fractions only the % of vasodilation at the maximum concentration testable was determined (Table 3). As reported in literature for all flavonoids (Singh et al., 2014) vascular relaxation induced by tested extract and fractions involves endothelium dependent NO-pathway: indeed CEE tested on endothelium-denuded rat aorta strips induced no relaxation (data not shown). The EC_{50} values of the two active samples are significantly different (**, P value = 0.0016, t-test); the greater CEE activity may be due to the presence in the phytocomplex of synergies that are lacking in the isolated fraction.

Conclusion

The significant increases in sexual behaviour parameters observed for ethanol extract and F1 and F3, are convincing evidence that the plant has potent aphrodisiac properties and produces significant and sustained increases in sexual activity. The compounds presents in the extracts and fractions may act like Viagra who was used here as a reference drug, by increasing the available NO in the corpus cavernosum and spongiosum and therefore promoting a prolong erection. This hypothesis is confirmed by the results obtained on

vascular relaxation of endothelium-intact rat aorta rings via endothelium-dependent NO pathway.

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