

Allelopathic effects on cowpea (*Vigna unguiculata* (L.) Walp) plant and cytotoxic activities of sterols and triterpene isolated from *Justicia anselliana* (NEES) T. Anders.

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Abstract

Purification of the allelopathic ethanol extract of *Justicia anselliana* aerial part led to the isolation of three known compounds: two sterols (stigmasterol and β -sitosterol) and one triterpene (lupeol). Their structures were determined by analysis of spectroscopic data and confirmed by GC/MS analysis. All isolated compounds were tested for their allelopathic effects on cowpea (*Vigna unguiculata*) growth and for their cytotoxic activity on human (HeLa, WI-38 and Mel-43) and mouse (J774) cells. All isolated compounds showed an inhibitory effect on the three parameters measured on cowpea (*Vigna unguiculata*) germination (rate of germination, shoot length and fresh weight). The highest rate of inhibition of cowpea's germination was observed with 200 ppm of lupeol ($-28.4\% \pm 0.7\%$) whereas all three parameters were inhibited very meaningfully by 200 ppm stigmasterol ($-26.6\% \pm 0.5\%$; $-24.2\% \pm 0.7\%$ and $-21.1\% \pm 0.7\%$ respectively for germination, shoot length and fresh weight of *Vigna unguiculata* (L.) Walp). None of the isolated compounds was toxic on J774 (murine macrophages), WI-38 (human lung fibroblasts), human HeLa (human cervix carcinoma cells) and melanoma Mel-43 cell. This is the first report of the allelopathic activities of lupeol, stigmasterol and β -sitosterol on cowpea (*Vigna unguiculata*).

Key Words: *Justicia anselliana*, purification, sterols, triterpene, allelopathy, *Vigna unguiculata* (cowpea), cytotoxicity

Introduction

Vigna unguiculata (L.) Walp (cowpea) is cultivated over more than nine million hectares in all the tropical area, in the mediterranean basin and in the United States (Guigaz, 2002). Worldwide, it is estimated that 37 million tones are annually produced (Langyintuo et al., 2003). Cowpea is a source of relatively low cost, high quality protein which contained adequate levels of most essential amino acids for pre-school children and all essential amino acids for adults. Their digestibility is higher than the one of other common legumes (Alessandra et al., 2004).

It is the most consumed leguminous: its seeds reduced into flour are used to make fritter, particularly in Benin and in some of the West African countries.

In south Benin, cowpea cultivation takes the lead on the economical level because of the

economical guarantee it offers to farmers. Its production occupies 90% of the working population and was done during the drop in water level, on the grounds set free by the water (Kossou et al., 2001).

In the Ouémé valley (south Benin) the presence of *Justicia anselliana* (Nees) T. Anders was very dangerous for cowpea. It belongs to the family of Acanthacea and helps in the enherbing of grounds for a percentage of 12 to 18% (Kossou et al., 2001). Nearly glabrous herb, upright or drooping, this plant is a 30 centimeters length species and can reach 60 centimeters (Hutchinson and Dalziel, 1963).

According to the empirical observations described by farmers, this grass forms a very intimate association with cowpea and at an advanced stage of its development; it leads to the discolouration of cowpea leaves. It thus puts an end to the cowpea

development. Allelopathy was defined as the effect of one plant (or microorganisms) on the growth of another plant through the release of chemical compounds into the environment (Rice, 1992; Rizvi et al., 1992). A contribution to the characterization of allelopathic potential of *Justicia anselliana* (Nees) T. Anders using some extracts from the dried materials (root and aerial part) of the weed proved that alcoholic extracts of its aerial part produced more significant effects on growth parameters such as seedlings elongation and fresh weight of the cowpea small plants than its root extracts (Ahanchede et al., 2003).

Material and methods

Vegetal Material

Aerial parts (dry season, in the beginning of flowering time) of *Justicia anselliana* were collected in Ouémé valley (south R. Bénin), in January 2005. Voucher specimen (AA6295/UNB) were deposited at the National Herbarium of the University of Abomey-Calavi (Republic of Benin). Seeds of cowpea (*Vigna unguiculata*) IT86D-719 variety were obtained from the International Institute of Tropical Agriculture (IITA) station of Benin.

Reagents and Standard Sterols and Triterpenes

Chloroform, toluene, hexane, methanol, ethanol and ethyl acetate of HPLC grade were purchased from Fisher Scientific. Anisaldehyde, reference sterols and triterpenes were obtained from Extrasynthèse (GENAY France).

Instrumentation

NMR spectra were recorded on a Bruker Advance DRX-400 spectrometer, in CDCl₃. MS analysis were achieved using an ion trap LCQ mass spectrometer (Finnigan MAT, San Jose, CA) equipped with an electrospray ionisation source. Vacuum liquid chromatography (VLC) was performed on silica gel 60 (0.063–0.200 mm; 70–230 mesh ASTM; MERCK). Medium performance liquid chromatography (MPLC) was performed with a glass column (omnifit : OM 6427 15 x 750 mm) on silica gel (15–25 µm, Merck LiChrorep® Si 60) as stationary phase; thin layer chromatography (TLC) with silica gel 60 F254 plates (Merck). Gas chromatography/mass spectrometry (GC/MS) was realised on a Trace GC 2000 series (ThermoQuest, Italy), equipped with an autosampler AS2000 (ThermoQuest). The GC system was interfaced to a Trace MS mass spectrometer (ThermoQuest) operating in the electron-impact mode. The capillary column (DB-XLB; column length 15 m × 0.25 mm with a 0.25 µm film thickness) was from J&W Scientific

To establish allelopathy according to the postulate of Putnam (Putnam, 1985), it is important to know the substances inducing damages and to establish their relationship with the inhibition or the stimulation of the cowpea growth. As sterols and triterpenes may have allelopathic effects (Macias et al., 1997, Fischer and Quijano, 1985) and in order to develop a natural herbicide of non toxic origin, we decided to isolate and purify the sterols and triterpenes from *Justicia anselliana* aerial part, study their cytotoxic activity on human and mouse cells and their allelopathic effects on the *Vigna unguiculata* (cowpea) growth

(Agilent Technologies, USA) and helium was used as carrier gas at a flow rate of 1.2 ml/min. For compounds identification, samples (1 ml, in dichloromethane) were injected in the split mode (split ratio: 1/9). The injector temperature was set at 250°C and the oven was programmed from 50°C to 320°C (10°C/min) and this last temperature was maintained for 3 min. The electron energy was 70eV and the ion source was at 250°C. Samples were analysed in a full-scan mode (250–650 amu).

Extraction and Isolation Procedure

Dried and powdered aerial part of *Justicia anselliana* (500 g) was mixed with 2.5 l of ethanol for 72 h at room temperature and percolated at 1 ml/min. After evaporation under reduced pressure, the ethanol extract (60.5 g) was successively extracted in a soxhlet apparatus with nHex (5.4 g; A), chloroform (18.9 g; B), ethyl acetate (8.4 g; C) and of methanol (26.2 g; D). Every extraction lasted 4 hours and the extracts were dried in vacuum under reduced pressure.

The ethyl acetate fraction (C, 6 g) was submitted to a silica gel VLC with a gradient of nHex–CHCl₃–EtOAc–MeOH to give 30 fractions (500 ml each). Thin-layer chromatography (TLC) analysis using Tol–EtOAc–MeOH (80:18:2) as mobile phase and revealed with sulphuric anisaldehyde showed that fraction n°8 (C₈:506.6 mg; eluted with CHCl₃–EtOAc 95:5) contained three major spots. Purification was achieved by medium performance liquid chromatography (MPLC) performed with Tol–EtOAc (92:2) as mobile phase at a flow rate of 1 mL/min (4 min/tube) to afford compounds **1**(104.4 mg), **2**(98.7 mg) and **3**(99.5 mg).

Bioassays with Cowpea Seeds

The allelopathic activities of different fractions (600 ppm concentration) (The bioassay with purified compounds was done at 200 ppm.) were tested on cowpea (*Vigna unguiculata* (L.) Walp) seeds. 60 mg of each fraction were dissolved

separately in 20 ml of the solvent in which they were extracted. 1 ml of this solution was poured on to sterilized paper filters (Watman n°1) carefully disposed in previously sterilized Petri dishes. In control Petri dishes, only 1 ml of the respective pure organic solvents was used. After organic solvent evaporation, 5 ml of double distilled water was added by Petri dish with 4 seeds of cowpea. Each extract and control treatments were replicated six times. The Petri dishes were then covered, conditioned in aluminium papers and introduced to the incubator to 25±1°C during 5 days. The rate of inhibition or stimulation in terms of germination, shoot length and fresh weight of the whole small plants of five days was calculated by considering the control as zero (Rawat et al., 1998) with the formula:

$I=100 \times (\text{Extract value} / \text{control value})$
(Parvez and al., 2004).

Cytotoxicity Assay

Cell Lines and Cultivation : J774 (murine macrophages) cells were cultured with Flow RPMI 1640 medium supplemented with 10% heat-inactivated foetal bovine serum, 0,33% L-glutamine, 1% non essential amino acids, 1% sodium pyruvate and penicillin-streptomycin (100 UI/ml-100 µg/ml). WI-38 cells (human lung fibroblasts), HeLa (human cervix carcinoma cells) and melanoma Mel-43 cell lines were grown in Gibco DMEM supplement with 10% heat-inactivated foetal bovine serum and penicillin (100 UI/ml). Cells were incubated in a humidified atmosphere with 5% CO₂, at 37 °C.

MTT Assay

Stock solution of extracts and pure compounds were prepared at 10 mg/ml in DMSO. The effects of extracts and isolated compounds, on all cell

lines were evaluated using MTT (Sigma) colorimetric assay based on cleavage of the reagent by mitochondrial deshydrogenases in viable cells (Mosmann, 1983). 5000 HeLa, Mel-43, WI38 and J774 cells in 200 µl medium were seeded in each well of 96-well plates. Cells were first incubated 24h, then, the medium was removed and replaced by 200 µl/well fresh medium containing various concentrations of extracts, compounds or DMSO at the same final concentration. Each concentration was tested in at least 6 wells. After 72 h treatment, the medium was removed and replaced by 100 µl of DMEM (without serum) containing 10 µl of MTT solution (3 mg/ml in PBS). After 45 min. in the incubator, the medium containing MTT was removed, and 100 µl of DMSO were added to each well. Plates were shaken and absorbance recorded at two wavelengths (570-620 nm) against a background control as blank. The relative absorbance was expressed as a percentage of the corresponding control considered as 100%. Camptothecin (Sigma) was used as positive cytotoxic reference compound (Stevigny et al., 2002). The results are expressed by IC₅₀ values (concentration of compounds causing 50% inhibition of cell growth) calculated from graphs using at least five different concentrations of each extract and isolated compound.

Statistical Analysis

The statistical analysis was realized with the software package (Statistical Analysis Systems) (SAS) (SAS / Stat User's Guide, 1990). The data were analysed by ANOVA (Analysis of variance). When a significant difference was observed at the level of 5%, the test of Newman Keuls was used to separate the averages (Steel and Torrie, 1980).

Results and discussion

Effects of Extracts on Seeds Germination;

Only the hexane fraction (A) of the aerial part of *Justicia ansellian* presented a stimulation effect on the shoot length (+ 10.2±0.2%) and the fresh weight (+ 45.2±1.2%) of *Vigna unguiculata* (L.) Walp at 600 ppm (**Fig. 1**). At the same dose the other fractions [chloroforme (B), ethylacetate(C),

methanol (D) and ethanol (E)] inhibited all three parameters in the order B>E>D>C, the ethyl acetate fraction (C) being the most active on all three parameters (-95.9±1.1%; -97.8±0.4% and -88.9±0.2% respectively for germination, shoot length and fresh weight of *Vigna unguiculata* (L.) Walp)

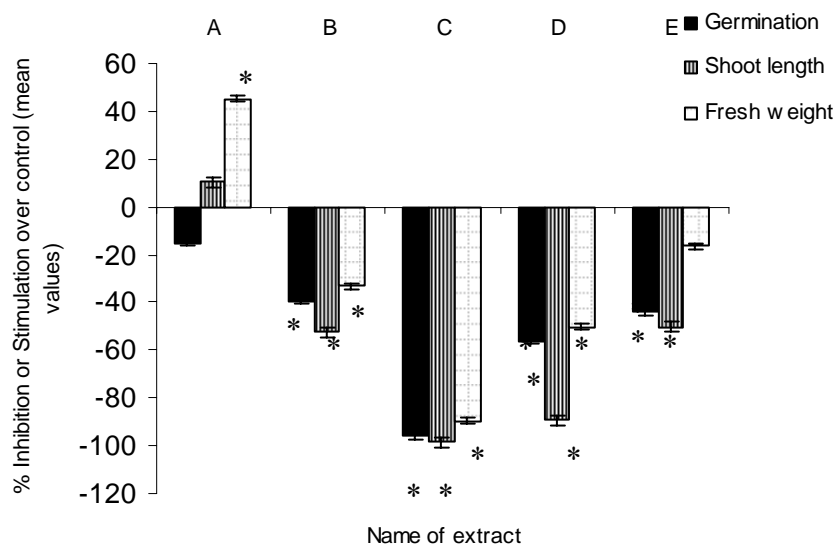


Figure 1: Effects at 600 ppm of *Justicia anselliana* soxhlet fractions on cowpea (*Vigna unguiculata*). $N = 6$, $P^* < 0.05$. A = hexane; B = $CHCl_3$; C = EtOAc; D = MeOH; E = EtOH. The upwards evolution corresponds to a stimulation of the parameters measured while the downwards evolution indicates an inhibition.

Isolation and Characterization of Sterols and Triterpene from *Justicia anselliana*;

The ethyl acetate fraction (C) was subject to vacuum liquid and medium performance liquid chromatography to afford one triterpene “lupeol (1)” and two sterols: stigmasterol (2) and β -sitosterol (3). Structural identification of 1-2-3 (Fig. 2) was carried out by analysis of their spectroscopic data (1H and ^{13}C -NMR and MS), comparison with

previously reported values (Table 1) (Yue et al., 2004; Mahato et al., 1994; Connolly and Hill, 1991; Saxena and Sosanna, 2005; Peter and Katalin, 2004) and GC-MS with standards (Table 2). 2D-NMR spectra allowed us to assign completely the 1H and ^{13}C -NMR of lupeol, stigmasterol and β -sitosterol. These compounds are reported for the first time in *Justicia ansellian*.

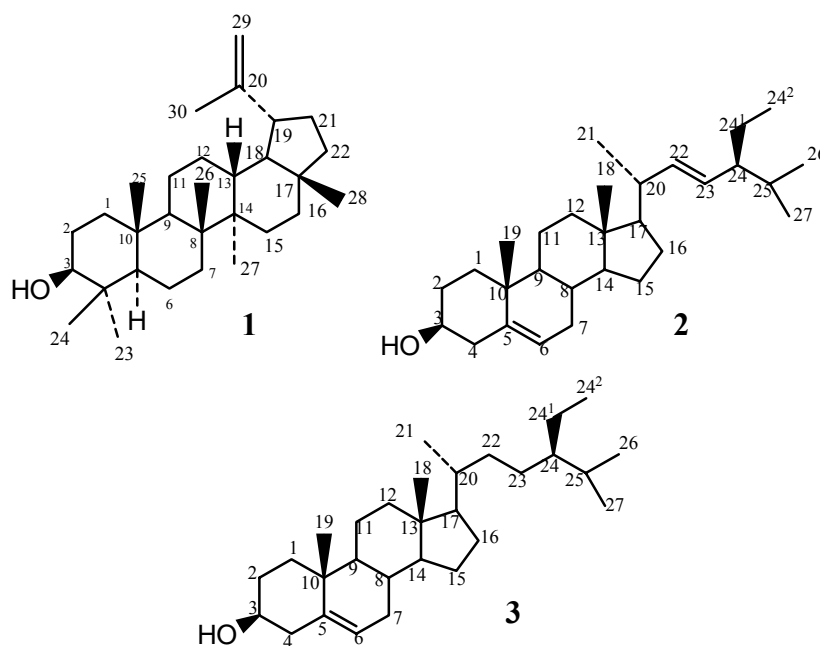


Figure 2: Structure of compounds 1 (lupeol), 2 (stigmasterol) and 3 (β -sitosterol).

Table 1: NMR data of **1**, **2** and **3** (400.13 MHz for ^1H and 100.62 MHz for ^{13}C ; in CDCl_3 and using tetramethylsilane (TMS) as an internal standard.)

Position	1		2		3	
	$^{13}\text{C}\delta(\text{ppm})$	$^1\text{H}\delta(\text{ppm})$	$^{13}\text{C}\delta(\text{ppm})$	$^1\text{H}\delta(\text{ppm})$	$^{13}\text{C}\delta(\text{ppm})$	$^1\text{H}\delta(\text{ppm})$
1	38.7	38.7m 0.90m	37.3	1.84 m 1.08 m	37.3	1.01 m
2	27.5	1.59m 1.67m	31.9	1.83 m 1.51 m	31.6	1.37 m
3	79.0	3.20dd	71.8	3.51 m	71.8	3.82 m
4	38.9	–	42.3	2.30 m 2.23 m	42.3	2.62 m
5	55.3	0.68m	140.8		140.8	
6	18.3	1.40m 1.50m	121.7	–5.33 m	121.7	–5.32 t
7	34.3	1.32m 1.42m	31.9	1.97 m 1.50 m	31.9	1.93 m
8	40.8	–	31.9	1.46 m	31.7	1.54 m
9	50.5	–1.29m	50.2	0.94 m	50.2	0.94 m
10	37.2	–	36.5		36.5	
11	20.9	–1.20m 1.40m	21.2	–1.50 m	21.1	–1.44 m
12	25.2	1.07m 1.68m	39.7	2.00 m 1.18 m	39.8	1.69 m
13	38.1	1.68m	42.2		42.3	
14	42.8	–	57.0	–1.01 m	56.7	–1.10 m
15	27.5	–1.00m 1.68m	24.4	1.58 m 1.06 m	24.3	1.51 m
16	35.6	1.37m 1.48m	28.9	1.72 m 1.28 m	28.3	4.61 m
17	43.0	–	56.8	1.75 q	56.1	1.74 m
18	48.3	–1.37m	12.3	0.70 s	11.9	0.67 s
19	48.0	2.38ddd	19.4	1.01 s	19.4	0.98 s
20	151.0	–	40.5	2.06 m	36.2	1.90 m
21	29.9	–1.37m 1.92m	21.2	1.03 d	18.8	0.92 d
22	40.0	1.37m 1.19m	138.3	5.17 dd	34.0	1.62 m
23	28.0	0.97s	129.3	5.04 dd	26.1	1.65 m
24	15.4	0.76s	51.3	1.54 m	45.9	1.58 m
25	16.1	0.83s	31.9	1.55 m	29.2	1.58 m
26	16.0	1.03s	21.1	0.85 dd	19.0	0.82 d
27	14.6	0.94s	19.0	0.80 dd	19.8	0.80 d
28	18.0	0.79s				
29	109.3	4.54d 4.67d				
30	19.3	1.68s				
24 ¹			25.4	1.43 m 1.18 m	23.1	1.52 m
24 ²			12.1	0.81 t	12.3	0.84 t

Table 2: Retention times and some characteristic fragment ions observed in the EI mass spectra after GC-MS of isolated or reference compounds.

compound	Molecular Masse	[M] ⁺	[M-CH ₃] ⁺	[M-H ₂ O] ⁺	[M-CH ₃ -H ₂ O] ⁺	^a TR (min)
1	426	426	411	408	393	27.7
lupeol	426	426	411	408	393	27.7
2	412	412	399	393	379	26.8
stigmasterol	412	412	397	394	379	29.8
3	414	414	399	396	381	27.2
β-sitosterol	414	414	399	396	381	27.2

^a retention time; **1** (lupeol), **2** (stigmasterol) and **3** (β-sitosterol).

Allelopathic Effect of Isolated Compounds; All the isolated compounds show an inhibitory activity on all three parameters measured on the cowpea (*Vigna unguiculata*) germination at 200 ppm (**Fig. 3**).

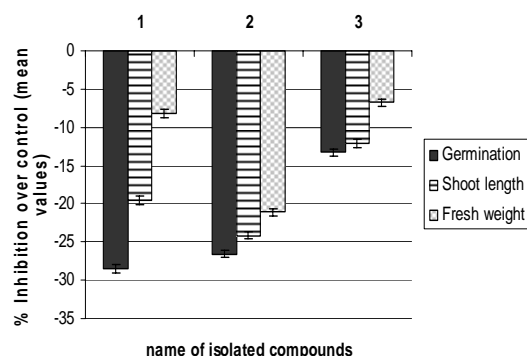


Figure 3: Effects at 200 ppm of sterols and triterpene isolated from *Justicia anselliana* on the cowpea (*Vigna unguiculata*). N=6, P* < 0.05. **1** (lupeol), **2** (stigmasterol) and **3** (β-sitosterol). The upwards evolution corresponds to a stimulation of the parameters measured while downwards evolution indicates an inhibition.

Lupeol inhibited mainly germination (-28.45 % ± 0.75%) while β-sitosterol greatly inhibited the shoot length and stigmasterol was very effective on all the three parameters.

Cytotoxic Effects of Fractions; The IC₅₀ (50% inhibitory concentration) values of MTT test (**Table 3**) show that hexane fraction (A) has an inhibitory effect only on J774 (IC₅₀=2.2±1.1 μg/mL) and HeLa (IC₅₀=37.6±1.2 μg/ml) cells, while the ethanol extract (E) inhibited J774 (IC₅₀=3.9±1.0 μg/ml) cells only. The other fractions were not toxic to the tested cell lines (IC₅₀ > 100). These results indicate that the allelopathic effect of C extract does not seem to be due to a cytotoxicity.

Table 3: Cytotoxic activities of fractions and isolated compounds.

Name of fractions and compounds	IC ₅₀ (μg/mL) ^a			
	J774 ^b	HeLa ^c	WI-38 ^d	Mel-43 ^e
Hexane (A)	2.2± 1.1	37.6±1.2	> 100	> 100
CHCl ₃ (B)	> 100	> 100	> 100	> 100
EtOAc (C)	> 100	> 100	> 100	> 100
MeOH (D)	> 100	> 100	> 100	> 100
EtOH (E)	3.9±1.0	> 100	> 100	> 100
Compound				
Lupeol (1)	> 100	> 100	> 100	> 100
Stigmasterol (2)	> 100	> 100	> 100	> 100
β-Sitosterol (3)	> 100	> 100	> 100	> 100

^aIC₅₀ (Concentration of drug causing 50% inhibition of mitochondrial activity); ^bmurine macrophages; ^chuman cervix carcinoma cells;

^dhuman lung fibroblasts; ^emelanoma.

We can conclude that three molecules (lupéol, stigmasterol and β -sitosterol) were isolated and identified for the first time from *Justicia ansellina*. The cytotoxicity tests indicate that these compounds do not possess cytotoxic activities on mammalian cells. Tested separately, these molecules showed weak effects of inhibition on cowpea germination. As they are less active than the C extract, other compounds may also be active or they act in synergy.

To our knowledge, this is the first report of the allelopathic activities of lupeol, stigmasterol and β -sitosterol on the cowpea but the allelopathic effects with relative lower concentrations of sterols and triterpenes on other plants are already reported in the literature (Macias et al., 1997; Fischer and

Quijano, 1985). This preliminary work can constitute a report accelerated in view of a survey more deepened of the allelopathic effects of *Justicia ansellina* on cowpea.

Abbreviations Used

CG, gas chromatography; MS, masse spectrometry; SAS, statistical analysis systems; ANOVA, analysis of variance; HPLC, high-performance chromatography; TLC, thin layer chromatography; EtOAc, ethyl acetate, EtOH, ethanol; MeOH, methanol; CHCl₃, chloroform; nHex, n-hexane; Tol, toluene; EI, electron impact; MPLC, medium performance liquid chromatography; VLC, vacuum liquid chromatography.

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