Cytotoxic Activities of Sterols and Triterpens Identified by GC-MS in *Justicia anselliana* (NEES) T. Anders Active Fractions and Allelopathic Effects on Cowpea (*Vigna unguiculata* (L.) Walp) plant.

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Summary

Purification of the allelopathic ethanol extract of *Justicia anselliana* aerial part led to actives purification fractions. GC-MS analysis of these fractions helped to identified ten known compounds: five sterols (campesterol; stigmasterol; β-sitosterol acetate; 9, 19-cyclolanostan-3-ol, 24-methylene; 9, 19-cyclolanost-24-en-3-ol), two triterpens (α – amyrin; lupeol), a diterpene (phytol) and two derivatives of alcane (heneicosane; ethyl palmitate). Eight of these compounds are reported for the first time on *Justicia anselliana*. Identification 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 tested compounds showed an inhibitory effect on the three parameters measured on cowpea (*Vigna unguiculata)* germination (rate of germination, shoot length and fresh weight), except the ethyl palmitate which stimuled the rate of germination and the shoot length of cowpea small plant. None of the identified compounds was toxic on J774 (murine macrophages), WI-38 (human lung fibroblasts), human HeLa (human cervix carcinoma cells) and melanoma Mel-43 cell.

Key Words: *Justicia anselliana;* identification; sterols; triterpens; allelopathy; *Vigna unguiculata* (cowpea); cytotoxicity

Résumé

La purification de l'extrait éthanolique de la partie aérienne de *Justicia anselliana* a permis d'obtenir des fractions actives dont l'analyse GC-MS conduit à l'identification de dix composés connus : cinq stérols (campestérol; stigmastérol; acétate de β-sitostérol; 9, 19-cyclolanostan-3-ol, 24-méthylène; 9, 19-cyclolanost-24-èn-3-ol), deux triterpènes (α – amyrine; lupéol), un diterpène (phytol) et deux dérivés d'alcane (hénéicosane; palmitate d'éthyle). Huit de ces composés sont pour la première fois identifiés dans *Justicia anselliana*. Des composés identifiés ont été testés pour leurs effets allélopathiques sur la germination du niébé (*Vigna unguiculata*) et pour leurs activités cytotoxiques sur des cellules humaines (HeLa, WI-38 et Mel-43) et de souris (J774). Tous les composés testés ont montré un effet d'inhibition sur les trois paramètres de germination du niébé mesurés (le taux de germination, l'élongation de la plantule et le poids frais de la plantule), à l'exception du palmitate d'éthyle qui stimule le taux de germination et l'élongation de la plantule du niébé. Les composés identifiés et testés ne sont pas toxiques sur les cellules J774 (murine macrophages de souris), WI-38 (fibroblastes embryonnaires humains de poumon), HeLa (cellules cancéreuses humaines du col de l'utérus) et melanoma Mel-43.

Mots Clefs : *Justicia anselliana;* identification; stérols; triterpènes; allélopathie; *Vigna unguiculata* (niébé); cytotoxicité

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1. Introduction

Vigna unguiculata (L) Walp (cowpea) is cultivated over more than nine million hectares in all the tropical area, in the mediteranean basin and in the United States [1]. Worldwide, it is estimated that 37 million tones are annually produced [2]. 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 that of other common legumes [3].

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 [4].

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% [4]. Nearly glabrous herb, upright or drooping, this plant is a 30 centimeters length species and can reach 60 centimeters [5].

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 and puts an end to the cowpea development. This can be the proof of allelopathic effects of *Justicia anselliana* (Nees) T. Anders on cowpea plant. 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 [6; 7].

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 [8]. To establish allelopathy according to the postulate of Putnam [9], it is important to know the substances inducing damages and to establish their relationship with the inhibition or the stimulation of the cowpea growth. Recently, we have isolated from the alcoholic extract of *Justicia anselliana*'s aerial parts, two sterols (stigmasterol and β-sitosterol) and one triterpen (lupeol) which separately, showed weak effects of inhibition on cowpea germination and did not have any toxic affect on mammalian cells tested [10]. These three molecules are less actives than the active fraction from which they were isolated. As other sterols and triterpens may also have allelopathic effects [11; 12] and in order to develop a natural herbicide of none toxic origin, we want in this work, to identify other sterols and triterpens from *Justicia anselliana* aerial part, to study their cytotoxic activity on human and mouse cells and their allelopathic effects on the *Vigna unguiculata* (cowpea) growth.

2. Material and methods

2.1. 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. Vouchers specimen (AA6295/UNB) was 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.

2.2. 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 triterpens were obtained from Extrasynthèse (GENAY France).

2.3. Instrumentation

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 (Agilent Technologies, USA) and helium was used as carrier gas at a flow rate of 1.2 ml/min. For compounds identification, samples (1 µ1, 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).

2.4. Extraction and identification 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 hexane (5.4 g; fraction A), chloroform (18.9 g; fraction B), ethyl acetate (8.4 g; fraction C) and of methanol (26.2 g; fraction D). Every extraction lasted 4 hours and the extracts were dried in vacuum under reduced pressure. Thin-layer chromatography (TLC) analysis showed that fraction C; eluted with toluene-ethyl acetate-methanol (80:18:2) as mobile phase and revealed with sulphuric anisaldehyde contained major spots regrouped in three zones (C_1, C_2, C_3). The ethyl acetate fraction (C, 6 g) was submitted to a preparative thin-layer chromatography (TLC) analysis using the same mobile phase and revealed with sulphuric anisaldehyde to give 3 fractions (C_1: 0.5 g at the Rf of squalene; C_2: 1.6 g at the Rf zone of α-amyrine, β-amyrine and / or lupéol and C_3: 2 g at the Rf zone of cholesterol, ergosterol, β-sitosterol and /or stigmasterol) which were recuperated and analysed by GC/MS.

2.5. Bioassays with cowpea seeds

The allelopathic activities of different fractions were evaluated at 600 ppm. The bioassay with purified compounds and components was done at 200 ppm. All fractions, purified compounds and components 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 (fraction A in hexane, fraction B in chloroform, fractions C in ethyl acetate, fraction D in methanol and fraction E in ethanol). 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 in each 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 [13] with the formula:

I-100 (with I= 100 x Extract value / control value)[14].

2.6. Cytotoxicity assay

2.6.1. 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% CO_2, at 37 °C.

2.6.2. MTT assay

Stock solution of extracts and pure compounds and component 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 [15]. 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 m1/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 m1 of DMEM (without serum) containing 10 m1 of MTT solution (3 mg/ml in PBS). After 45 min. in the incubator, the medium containing MTT was removed, and 100 m1 of DMSO were added to each well. Plates were shacked 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 [16]. The results are expressed by IC_{50} 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.

2.7. Statistical Analysis

The statistical analysis was realized with the software package (Statistical Analysis Systems) (SAS) [17]. 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 [18].

3. Results and discussion

3.1. 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).

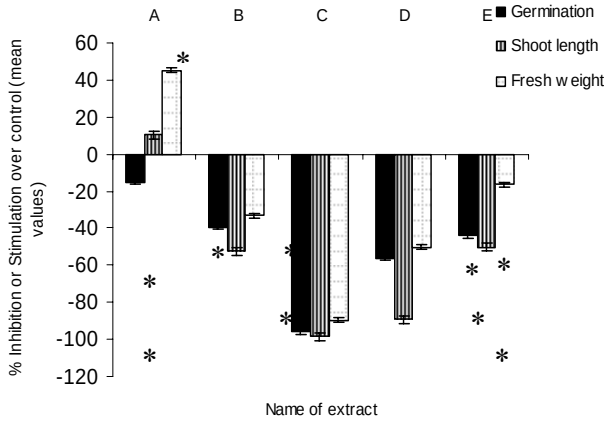


Figure 1: Effects at 600 ppm of *Justicia anselliana* soxhlet fractions on cowpea (*Vigna unguiculata*). *N =6, P* < 0.05. A = hexane; B = $CHCl_3$; C = EtOAc; D = MeOH; E = EtOH The upwards evolution corresponds to a stimulation of the parameters measured while the downwards evolution indicates an inhibition.*

3.2. Identification of sterols and triterpens in *Justicia anselliana;*

The ethyl acetate fraction (C) was subject to thin-layer chromatography (TLC) analysis. Elution with toluene-ethyl acetate-methanol (80:18:2) as mobile phase and revealation with sulphuric anisaldehyde revealed major spots collared in three zones (C_1, C_2, C_3). The ethyl acetate fraction (C, 6 g) was submitted to a preparative thin-layer chromatography (TLC) analysis using the same mobile phase and revealed with sulphuric anisaldehyde to give 3 fractions (C_1: 0.5 g at the Rf of squalene; C_2: 1.6 g at the Rf of and C_3: 2 g) which were analysed by GC/MS. The allelopathic tests of fractions C_1, C_2 and C_3 purified from fraction C (**Fig. 2**) showed that C is very rich in inhibitory allelochemicals but contained also stimulative allelochemicals of the germination parameters of cowpea.

Figure 2: Effects at 200 ppm of sterols and triterpen fractions (C_1, C_2 and C_3) purified from *Justicia anselliana* on the cowpea (*Vigna unguiculata*). N=*6, P*< 0.05. The parameters measured while downwards evolution indicates an inhibition.*

The GC/MS analysis of purification fractions led chromatograms C_1, C_2, C_3 (**fig. 3**). The analysis of these chromatograms helped to identify ten compounds: five sterols (campesterol; stigmasterol; β-sitosterol acetate; 9, 19-cyclolanostan-3-ol, 24-methylene; 9, 19-cyclolanost-24-en-3-ol), two triterpens (α – amyrin; lupeol), a diterpen (phytol), two derivatives of alcane (heneicosane; ethyl palmitate) (**table 1**). Only two of these compounds were previously isolated in the fraction C of ***Justicia ansellian*** plant [10].

3.3. Allelopathic effect of identified compounds

The identified compounds were tested for their allelopathic effects on cowpea. Nearly all tested compounds show an inhibitory activity on all three parameters measured on the cowpea (*Vigna unguiculata*) germination at 200 ppm (**Fig. 4**). Only ethyle palmitate stimulate the rate of germination (15.17 ± 1.20) and the shoot length (36.75 ± 0.17) of cowpea small plant of five days. This compound was only presented in the purified fraction C_1 (**Table 1**). Its presence can explained the stimulation effects of this fraction (**Fig. 2**).

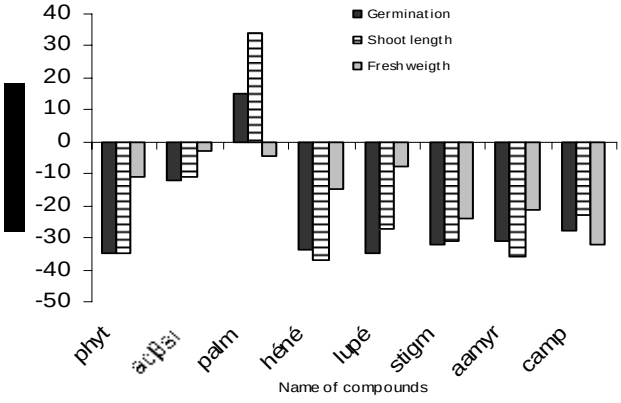


Fig. 4: Effects at 200 ppm of sterols and triterpene isolated from *Justicia anselliana* on the cowpea (*Vigna unguiculata*). N=*6, P*< 0.05.* phyt = phytol; acβsi = β-sitosterol acetate; palm = ethyle palmitate ; héné = heneicosane; lupé = lupeol; stigma = stigmasterol; aamyr = α – amyrin; camp = campesterol. *The upwards evolution corresponds to a stimulation of the parameters measured while downwards evolution indicates an inhibition.*

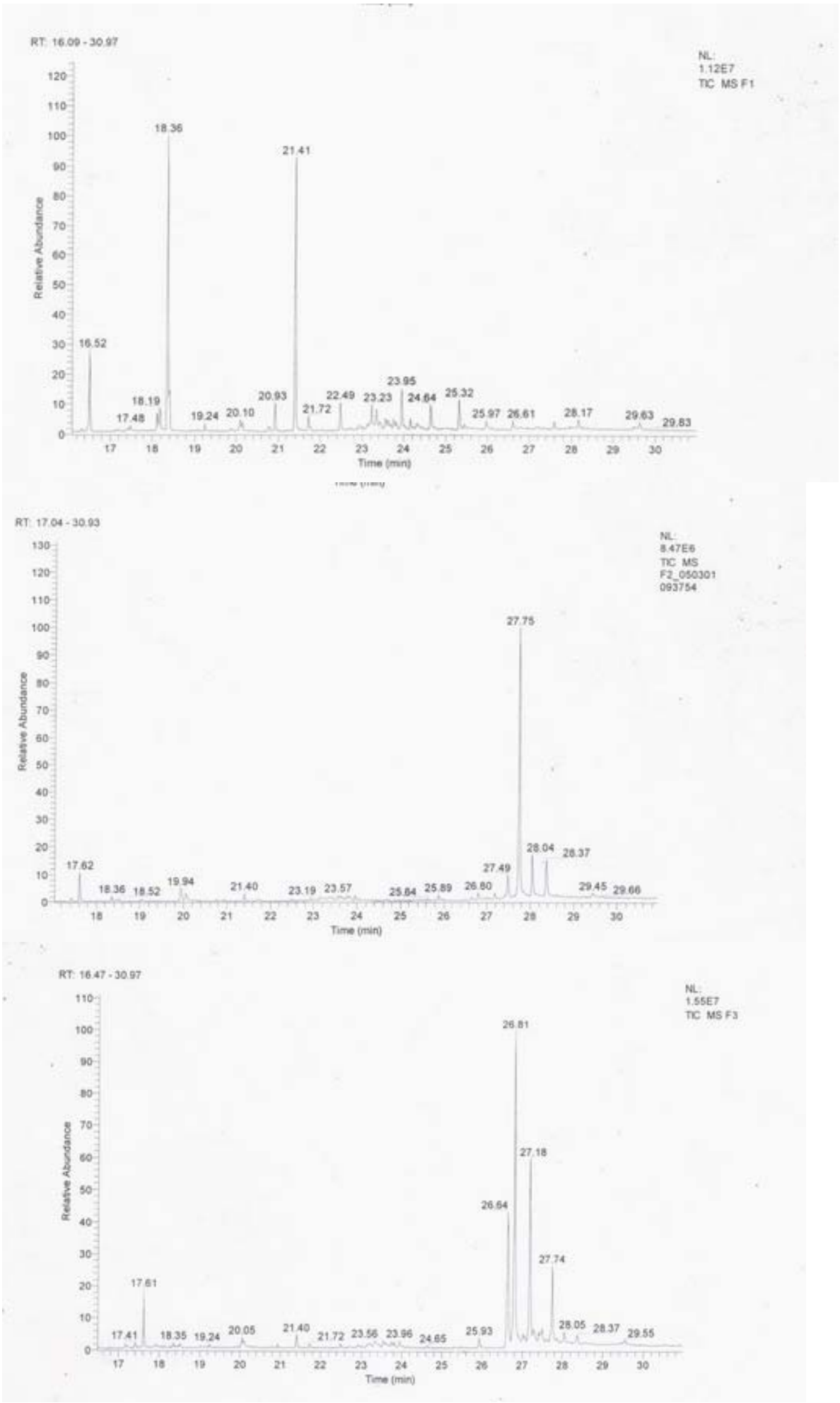
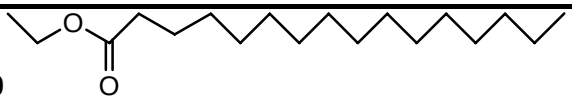
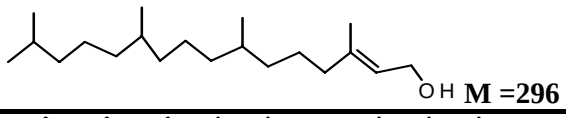
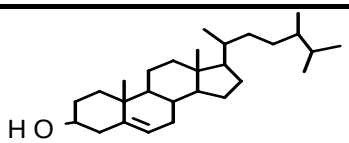
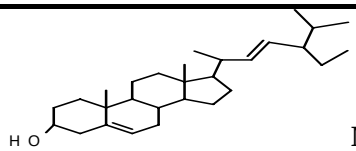
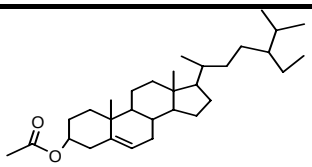
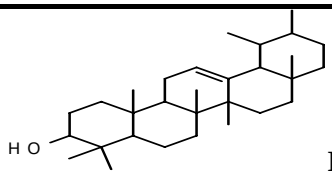
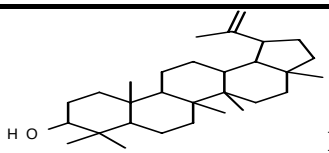
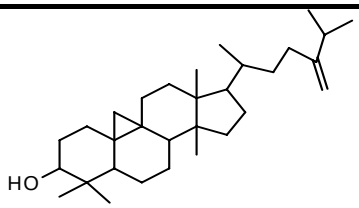
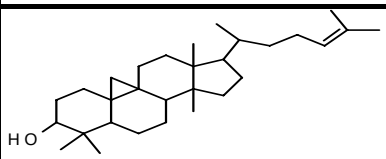


Fig. 3 : Chromatograms of C_1, C_2, C_3 fractions.

Table 1 : Compounds identified in fractions C_1, C_2 and C_3.

N°	Compounds	t_R^* (min)	Structures and Mass	Fractions		
				C_1	C_2	C_3
1	ethyl palmitate	16.52	 **M=260**	+	-	-
2	phytol	17.67	 **M =296**	-	+	+
3	heneicosane	18.36	 **M =296**	+	-	-
4	campesterol	26,60	 **M = 400**	-	-	+
5	stigmasterol	26.8	 **M = 412**	-	+	+
6	β-sitosterol acetate	27.18	 **M = 456**	-	-	+
7	α – amyrin	27.49	 **M = 426**	-	+	-
8	lupeol	27.74	 **M = 426**	-	+	+
9	9, 19-cyclolanostan-3-ol, 24-methylene	28.04	 **M =454**	-	+	-
10	9,19-cyclolanost-24-en-3-ol	28.37	 **M = 454**	-	+	-

t_R^* retention time

3.4. Cytotoxic effects of fractions and compounds

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

The cytotoxicity tests of identified compounds (**Table 2**) also indicates that these compounds do not possess cytotoxic activities on mammalian cells.

Table 2: Cytotoxic activities of factions and isolated compounds.

Name of fractions and compounds		IC_{50}(µg/mL)[a]			
		J774[b]	HeLa[c]	WI-38[d]	Mel-43[e]
Fractions	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
Compounds	lupeol	> 100	> 100	> 100	> 100
	stigmasterol	> 100	> 100	> 100	> 100
	β-sitosterol acetate	> 100	> 100	> 100	> 100
	α-amyrin	> 100	> 100	> 100	> 100
	campesterol	> 100	> 100	> 100	> 100
	hénéïcosane	> 100	> 100	> 100	> 100

4. Conclusion

We can conclude that seven molecules (β-sitosterol acetate; α-amyrin ; campesterol; heneïcosane ; 9, 19-cyclolanostan-3-ol, 24-methylene; 9,19-cyclolanost-24-en-3-ol) were identified for the first time from *Justicia ansellina*. Lupeol, stigmasterol and β-sitosterol were previously reported in this plant [10]. 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 [10].

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; $CHCl_3$: chloroform; Hex: hexane; Tol: toluene; EI: electron impact; MPLC: medium performance liquid chromatography; VLC: vacuum liquid chromatography.

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