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Research Article

Isolation of Diterpenoids from *Jatropha podagrica* against Hepatitis C virus

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ABSTRACT
One new lathyrane diterpenoid, Jatropodagrene (**1**) along with three known compounds have been isolated from the root bark of *Jatropha podagrica*. The structure of the new compound was established from its 1D and 2D NMR spectra and in comparison with data reported in previous literatures. Compound **1** was highly cytotoxic (98.86% inhibition) to the HCV virus, while compounds **2** (EC_{50}, EC_{90}, CC_{50} 5.8, 33.1, 22.6 µg/mL, respectively) and **3** displayed significant anti HCV activity. This is the first report of the anti hepatitis C virus activity of lathyrane skeleton and *J .podagrica*.

INTRODUCTION

Hepatitis C is attracting serious attention from scientists worldwide with a view to discovering new leads drugs for combating it. Hepatitis C virus (HCV) is a positive-stranded RNA virus, possessing a 9.6 kb genome encoding a single polyprotein that is subsequently cleaved by both host and virus protease into 10 sub units proteins, comprising six nonstructural and four structural proteins (Linderbach & Rice, 2005). It has been estimated that approximately 175 million people worldwide are chronically infected with HCV, and it is one of the leading causes of chronic hepatitis, cancer of the liver and cirrhosis (Lin *et al.*, 2006). Because the virus does not establish latency, it is possible to eradicate it (Schinazi *et al*, 2009; Shepard *et al*., 2005). To date, no vaccine against HCV has been developed owing to the presence of large numbers of HCV genotypes and quasi species, and lack of easily available animal models for vaccination tests (Bobeck *et al*., 2010). Therefore, it is obvious that new and better drugs obtained from natural sources are required in the battle against HCV. *Jatropha podagrica* Hook (JP) is used in parasitic skin infections, hepatitis, cancer related diseases (Aiyelaagbe *et al*., 2007). The paper reports the isolation of one new, two known diterpenoids and one flavonoid from JP and their HCV activity.

MATERIALS AND METHODS

General

The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$-d_6 at 400 MHz. The HRESIMS data were obtained using an Agilent MS TOF 1100 Series with electrospray ionization. The optical rotation was measured with a JASCO DIP 370 digital polarimeter. The ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$-d_6 (δH 7.25/δC 72.36) using a Brucker NMR spectrometer operating at 400 MHz for ^{1}H and ^{13}C nuclei. The NOESY spectrum was recorded at 25°C using a NOE pulse sequence with a mixing time of 500

ms, and the experiment was performed for 24 h with the concentration of the sample at 6 mg/mL. The HRESIMS data were obtained using an Agilent MS TOF 1100 Series with electrospray ionization. HPLC was carried out using a Waters 486 Series with a Phenomenex C18 column (10.0 × 250 mm) and an 1100 Series multiple wavelength detector.

Plant material and extraction

Plant material was collected from Edo State, Nigeria between January and June, 2013, identified and authenticated by Mr. Ugbogu O. A. and Shasanya O. S. of the Forest Research Institute of Nigeria (FRIN), where a voucher specimen (FHI 93265) was deposited in the herbarium. The shade dried plant material (400 g) was powdered and extracted with 1 liter methanol for 48 hr (3 X) by cold maceration. The MeOH extract (50g) was fractionated by VLC using a gradient system with hexane-EtOAc (100:0, 50:50, 0:100) and EtOAc-MeOH (50:50, 0:100). The hexane: ethylacetate (50%) fraction was further purified with LH-20 sephadex eluted with DCM:MeOH (1:1) to afford fractions I-VII. Two fractions that displayed anti HCV activity were purified by semi-preparative reversed-phase HPLC (diameter, 10 mm x length, 250 mm, 10 µm) eluted with CH_3CN/H_2O (70-100%) yielded **1** (t_R 13.8 min, 5 mg), **2** (t_R 6.0 min, 4.5 mg) and **3** (t_R 16.0 min, 6 mg). Compound **4** was obtained as colorless crystals from fraction III. Compound **1:** Light yellow solid: mp; 178-179ºC, $[\alpha]^{25}_D$-130º ($CHCl_3$; *c*0.001); UV (MeOH) λ_{max} nm (log e) 252; ^{1}H and ^{13}C-NMR data, see Table 1; HREIMS ($[M]^+$, *m/z* 330.1831, calcd.for $C_{20}H_{26}O_4$).
Compound (**2**): Yellow oil; $[\alpha]^{25}_D$-175º,: (c0:001, $CHCl_3$); UV (MeOH) λ_{max} nm (255; ^{1}H and ^{13}C-NMR data *m/z* (rel. int.): 332 $[M]^+$ (1), 314 $[M\ H_2O]^+$ (1), 299 (2), 271 (6), 161 (7), 95 (16), 81 (16) 55 (32) 43 (100) (Aiyelaagbe *et al*., 2007). Compound (**3**): White solid; mp 54-55ºC; $[\alpha]^{25}_D$-175º, (c0:0026, $CHCl_3$;); UV (MeOH) λ_{max} nm 265. ^{1}H and ^{13}C-NMR data (Aiyelaagbe *et al*., 2007). EIMS (probe) 70 eV *m/z* (rel. int.): 314 $[M\ H_2O]^+$ (2), 299 (2), 271 (11), 161 (34), 109 (26). Compound (**4**): Colorless crystals, mp 178-180ºC. UV (MeOH) λ_{max} nm: 215.0, 255.0, 308.5. EI-MS *m/z* (rel. int.): 222 [M^+, 100], 207 (39), 189 (5), 179 (12), 161 (14), 151 (14), 133 (11) (Ee *et al*., 2005).

Anti HCV assay

Antiviral and Cytotoxicity assays for HCV: Cells were maintained in Dulbecco's modified Eagles medium high glucose 4.5 g/L supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin and streptomycin, 1% L-pyruvate and 500 µg/mL of genticin (G418; Invitrogen). Genticin was used to select cells permitting the HCV RNA replication. The antiHCV activity was done using the method of Schinazi *et al*., (2010).

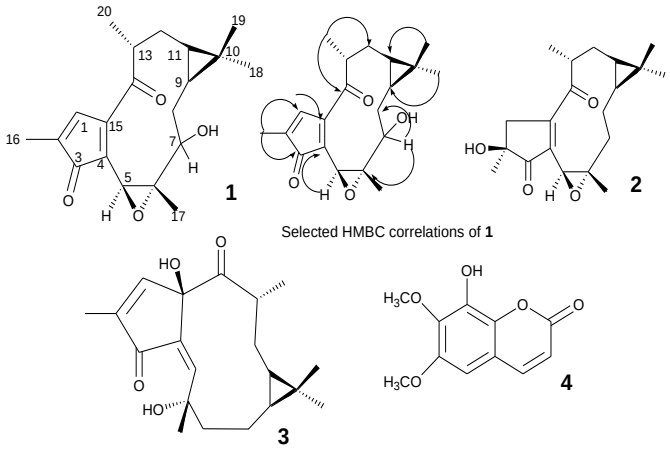
Cell Culture

Cells were seeded in 6-well plates at a density of 2.5 x 10^5 cells per well, 16 h before the beginning of treatment. Cells were treated with the molecules administered at different concentrations in complete medium that did not contain genticin. Administration of each compound was renewed every day for three consecutive days. Ribavirin (ICN Pharmaceuticals), mycophenolic acid (Sigma) and IFNa-2b (IntronA) were used in the same conditions as positive controls. Total RNA was extracted at the end of treatment (24 h after the last day of treatment) with the reagent (Eurobio), which is a mix of phenol and guanidinium thiocyanate. Northern blot analysis was then performed using 6022 V NorthernMaxTM-Gly kit (Ambion), following manufacturer's instructions. Five micrograms of total RNA were desaturate in global buffer at 5ºC for 30 min, separated by 1.1% agarose gel electrophoresis and then transferred for 12 hr onto a charged nylon membrane (Hybond$^+$, Marshal). Hybridization was carried out with three different [32P]CTP- labelled riboprobes obtained by *in vitro* transcription (Riboprobe *in vitro* transcription system; Promega). Two probes were complementary to the NS5A region of the HCV genome of negative polarity and positive polarity. A third probe was complementary to the beta-actin mRNA and obtained by *in vitro* transcription from a specific plasmid (pTRI beta actin human, reference 7424; Ambion). First, the blot was hybridized with the riboprobes directed against the negative strand of HCV RNA and beta-actin mRNA. After one night of hybridization at 68ºC, the membrane was washed, and then exposed to X-ray film and a phosphor screen (phosphorimager). This screen was then scanned and quantitative analysis was achieved using ImageQuant software. The amount of beta-actin mRNA was used as an internal loading control to standardize the amount of HCV RNA detected. The same membrane was subsequently hybridized with the negative sense riboprobe to determine the level of positive strand HCV RNA, using the same approach. For cell viability assays, cells were seeded in 96-well plates at a density of 12,500 cells per well. They were treated by the different extracts with the same concentration conditions as those used for the antiviral assays. Then, cell viability was measured by neutral red assay. Neutral red which specifically colours lysosomes and its accumulation depends on cellular membrane

integrity. The yield of neutral red incorporated in cells is proportional to the number of living cells. At the end of treatment, culture medium was removed; cells were washed by PBS and then coloured with neutral red at 0.005% for 3 hr at 37ºC. Cells were then fixed 1 min by formol calcium and lysed by a treatment with a v/v mixture of acetic acid and ethanol. After 15 min incubation, absorbance was read at 490 nm (Schinazi *et al*., 1992).

RESULTS

Compound **1** was obtained as a white solid; its molecular formula $C_{20}H_{26}O_4$ was established from its mass spectrum HREIMS ($[M]^+$, *m/z* 330.1830).The structure was unambiguously resolved from the analysis of its 1D and 2D NMR (HMQC) and (HMBC) spectral. Spectral obtained were compared with reported data in literature. The IR spectrum showed the presence of diagnostic carbonyl groups at 1730, 1710 cm^{-1} and unsaturation.



The ^{1}H-NMR spectral data (Table 1) revealed the presence of a cycloprapane moiety, one oleifinic proton (δ 6.90) and five methyl groups. On the other hand, the ^{13}C-NMR spectral data (Table 1) showed the presence of two double bonds in ring A, two carbonyl groups, five methyl, two methylene, five methines and five quaternary carbons.

Table 1: ^{1}H- and ^{13}C-NMR ($CDCI_3$) spectral data of compound **1**

No	δ ^{13}C	DEPT	δ ^{1}H (mult., J_{HH})	HMBC correlations (H→C)
1	135.6	CH	6.90 (1H, s)	C-1, 2, 3, 15
2	167.2	C	-	
3	207.5	C=O	-	
4	166.3	C	-	
5	60.3	CH	3.07 (1H, d, J =8.5Hz)	C-3, 4, 5
6	56.2	C		
7	36.3	CH	1.10 m	C-6, 7, 8, 9
8	40.5	CH_2	1.80 m	C-7, 8, 9
9	28.7	CH	0.19 m	C-8, 9, 10
10	17.7	C		
11	18.0	CH	0.57 m	C-10, 11, 12
12	40.3	CH_2	1.81 m	C-11, 12, 13
13	43.4	CH	3.24 m	C-12, 13, 14
14	208.2	C=O		
15	135.6	C		
16	18.5	CH_3	1.85 (3H, d, J= 5.6 Hz)	C-1, 2, 3
17	23.4	CH_3	1.41 s	C-6, 7
18	28.2	CH_3	0.99 s	C-9, 10, 19
19	15.5	CH_3	0.77 s	C-10, 11, 18
20	15.0	CH_3	1.16 (3H, d, J = 6.0Hz)	C-12, 13, 14
OH			3.88, s	

The ^{13}C-NMR showed the presence of two keto groups (δ 207.5 and δ 208.2) and epoxide group (δ 60.3.7 and δ 56.2). The downshift carbonyl group was consistent with the cyclopentanone chemical nucleus. The spectra data of Compounds **2-4** were compared with literature. As shown in Table 2, these compounds generally exhibited good inhibition of HCV of 98.86%, 89.71% and 82.61 % for **1**, **2** and **3** respectively, except compound **4** which had only low activity 17.02 and 7.49% for HCV and rRNA respectively). The most potent compound was **1** with pronounced cytotoxicity. It is noteworthy that compound **2** showed potent antiviral activity (EC_{50} , EC_{90}, CC_{50} of 5.8, 33.1 and 22.6 respectively (Table 3). Compound **4** did not display potent activity indicating that the lathyrane nucleus, epoxide and cyclopropane seem to be vital for antiHCV activity.

Table 2: Anti HCV activities of compounds (**1-4**)

Treatment	Concentration (µg/mL)	DCt HCV	DCt rRNA	% inhibition HCV	rRNA
1	10	6.49	UND	98.86	UND
2	10	3.29	1.34	89.71	60.29
3	10	2.53	1.14	82.61	54.47
4	10	0.25	0.11	17.02	7.49

UND; not determined

Table 3: EC_{50} and EC_{90} values of (**2-3**)

Treatment	Concentration (µg/mL)	DCt HCV	DCt rRNA	% inhibition HCV	% inhibition rRNA	EC values(µg/mL) $^aEC_{50}$	$^bEC_{90}$	$^cCC_{50}$
2	33	3.31	1.21	89.86	56.71	5.8	33.1	22.6
	10	1.37	0.79	61.20	41.93			
	3	0.80	0.33	42.33	20.21			
	1	0.38	0.02	22.92	1.37			
3	33	0.55	0.06	31.46	4.28	ND	ND	ND
	10	-0.73	-0.31	65.60	-24.17			
	3	-0.10	-0.45	-7.15	-36.79			
	1	0.09	-0.13	6.03	-9.65			

ND, not Determined.

$^aEC_{50}$: The concentration that affords 50% inhibition of viral growth.

$^bEC_{90}$: The concentration that affords 90% inhibition of viral growth

$^cCC_{50}$: concentration of the 50% cytotoxic effect

DISCUSSION

Compound **1** was isolated from *Jatropha podagrica* (400 g). A methanol extract of the root bark of *J. podagrica* was subjected to silica gel vacuum-liquid chromatography (VLC) followed by fractionation on an LH-20 Sephadex CC eluted with DCM:MeOH (1:1). Then purification steps used C18 column and semipreparative HPLC (Phenomenex C18 column 10.0 × 250 mm) at a flow rate of 3 mL/min to yield **1**. A comparison of these spectral data of **1** with those of the known constituents of *Jatropha* species suggested that the structure of **1** is closely related to that of **2** (Schmeda-Hirschmann *et al*., 1992). However, the double bond in ring A appeared in positions C-1, C-2 and C-4, C-15 in **1**. The presence of a 1,1-dimethylcyclopropane ring was evident from the corresponding ^1H and ^{13}C- NMR chemical shifts and from NMR comparisons with similar compounds. The chemical shift of the ^{1}HNMR in Table 1 shows the presence of one olefinic proton at the *β*-position of one of the keto groups. The 2D NMR indicated the presence of cyclopropane moiety (H-9 and H-11 with gem-dimethyl at C-10 position). The HMBC experiment (Table 1) showed the long range coupling between the protons and carbons. The C-4–C-15 double bond was assigned based on comparison of the NMR data with similar compounds in the literature (Schmeda-Hirschmann *et al*., 1992). Structure **1** was thus named japodagrene. The structure represents the lathyrane ring system which is common in the Euphorbiaceae family, but **1** is the first compound to have two double bonds on the cyclopentanone ring. Compounds **2-4** have previously been isolated (Aiyelaagbe *et al*., 2007; EEe *et al*., 2005). The potential anti-HCV activity of the isolated lathyrane diterpenoids, together with that of the fraxadin (**4**) were evaluated (Tables 2-3). The cytotoxicity of each compound was expressed as the concentration of

compound required to kill 50% (CC_{50}) of the DCt HCV cells.

In summary, Compounds **1-3** proved to be potential HCV inhibitors, with **1** as the most potent. Such activity profiles make them attractive candidate compounds for *in vivo* studies.

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