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Original Research Article

Isolation and identification of bioactives organic compounds from the bark and seeds of *Santaloïdes afzelii* (Connaraceae)

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ABSTRACT

Santaloïdes afzelii (R.Br. ex Planch.) Schellenb (Connaraceae) is used in Africa as an analgesic, aphrodisiac and in the treatment of stomachaches, muscular pains and in medico magic rites. The present study aims to identify the bioactive organic compounds from the bark and seeds of *Santaloïdes afzelii* collected in Korhogo in the north of Côte d'Ivoire. Microbiological testing againts *Staphylococcus aureus* of the hydro ethanolic crude extracts of the bark and seeds showed low activities compared to the activities of oxacillin and cefoxitin. The chemical investigations of these extracts led to the identification of gallic acid, *epi*-catechin and dimers of procyanidin.

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1. Introduction

The rich and diverse tropical flora contains a multitude of plants that contribute to meeting the nutritional, medicinal and economic needs of the populations (Baumer, 1995). Thus, research on medicinal plants is of great interest. The biological activities of plants are due to the essential oils in their various organs (Mohammadhosseini et al., 2017; Nunes and Miguel, 2017; Wansi et al., 2019) or their secondary metabolites (Venditti and Bianco, 2018; Mohammadhosseini et al., 2019). According to World Health Organization (WHO), more than 21,000 plants are being used for medicinal purpose by more than 80% of the world population (OMS, 2000). Thus, this organization promotes and develops traditional medicine. However, a lack of scientific knowledge on certain plants limits their wide use.
Santaloïdes afzelii (R.Br. ex Planch.) Schellenb (Christophe, 2006) which belongs to the plant family Connaraceae is widely dispersed in the North of Côte d'Ivoire (Guy, 1954). It is used in traditional medicine for the treatment of various diseases (Ambé, 2001) such as stomachaches and muscular pains (Bouquet and Debray, 1974) and is said to have magic medicinal properties (Arbonnier, 2009), aphrodisiacs and analgesic activities (Bouquet and Debray, 1974). Preliminary phytochemical studies carried out on plants of the family of Connaraceae showed the presence of triterpenes (Oliveira et al., 2012) and phenolic compounds including flavonoids (Kalegari et al., 2011). Phenolic compounds represent a very important category of secondary metabolites with multiple biological properties.
Our previous study on the leaves of *Santaloïdes afzelii* showed the presence of flavonoids and gallic acid (Soro et al., 2012). This study aims to evaluate the antibacterial activity of the bark and seeds extracts and to identify organics compounds.

2. Experimental

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2.1. Plant material

Bark and seeds of *Santaloides afzelii* were collected at the beginning of the dry season in Korhogo in the north of Côte d'Ivoire. A voucher sample was identified by Prof. L. Aké-Assi, National Floristic Center (CNF) of Abidjan, Félix HOUPHOUËT-BOIGNY University (UFHB) where the sample was deposited with the reference (Occurrence ID : UCJ004099 ; Recorded by : CNF-UFHB). The collected plant materials were washed under running water and shed dried. The dry samples were crushed and stored at 10 °C until use.

2.2. Phytochemical analysis

2.2.1. Extraction method and separation process

The samples from bark and seeds of *Santaloïdes afzelii* were extracted separately. The dried and pulverized samples of bark (400 g) and seeds (300 g) were exhaustively extracted with hexane at room temperature by constant stirring. Each residue was extracted with 70% EtOH (3 x 500 mL) at room temperature by constant stirring for 24 hours. After filtration using cotton followed by watmann paper, the extracts were concentrated under reduced pressure at 40 °C to afford a brown residue. The residue of each plant was suspended in water and partitioned successively with CH_2Cl_2 (3 x 200 mL) and AcOEt (3 x 200 mL). The obtained fractions were separately dehydrated with anhydrous sodium sulfate and evaporated under vacuum after filtration to give dichloromethane fraction and ethyl acetate fraction.

2.2.2. Isolation procedure

The ethyl acetate fraction (3 g) of the bark of *Santaloïdes afzelii* was subjected to column chromatography on silica gel 60 with solvents gradients CH_2Cl_2-AcOEt and AcOEt-MeOH to give 7 fractions (E_1-E_7). Fractions E_2 (340 mg) and E_6 (343 mg) were purified by flash column chromatography on silica gel 60, eluting with CH_2Cl_2-MeOH to afford compound **2** and compound **3,** respectively.
The ethyl acetate fraction (0.8 g) of the seeds of *Santaloïdes afzelii* was subjected to column chromatography on silica gel 60 with solvents gradients CH_2Cl_2-AcOEt and AcOEt-MeOH to give 8 fractions (G_1-G_8). Fraction G_3 was purified by flash column chromatography on silica gel 60, eluting with CH_2Cl_2-MeOH to afford compound **2**.
Another portion (0.52 g) of the AcOEt extract of the seeds of *Santaloïdes afzelii* was fractionated by Sephadex gel exclusion chromatography with MeOH (100 %) as eluent to give 5 fractions (G'_1 to G'_5). Compound **1** was obtained by purification on silica gel column of the fraction G'_1 eluting with CH_2Cl_2-MeOH.

2.2.3. Spectroscopy and chromatographic analysis

The structures of the isolated compounds were established by NMR spectral experiments, ultraviolet (UV) spectrometry, liquid chromatography with UV photodiodearray detection (LC-UV) and combination of HPLC with mass spectrometry ((LC-MS).

A Brucker Avance 400 spectrometer was used for ^1H and ^{13}C-NMR spectra recorded at 400 and 100 MHz, respectively. The spectra were recorded at 23 °C. The NMR data were measured in CD_3OD or DMSO-d_6. The chemical shifts were expressed from TMS signal at 0 ppm. The chemical shifts are expressed in ppm and the multiplicities are given as singlet (s), doublet (d), triplet (t) and multiplet (m). The coupling constants are reported as *J* value in Hz. The reproducibility of ^{13}C NMR shift was about ±0.05 ppm, depending on cell and concentration. Chemical assignments were made using DEPT 135, HMBC, HSQC techniques or common chemical shift assignments rules.
ESI-MS was recorded on a Shimadzu GC MS-QP 2010 with electron-impact ionization (70 eV). HRMS in the positive ion mode was performed using a QTOF Ultima Global hybrid quadrupole time-of-flight instrument (Waters-Micromass).
Flash column chromatography was performed on Macherey-Nagel Silica gel 60 (1540 µm). Experiment TLC plates (Macherey-Nagel, ALUGRAM® SILG/UV254, 0,2 mm silica gel 60Å) were visualized under UV light at 254 nm and/or by dipping the TLC plates in a solution of phosphomolibdic acid (3 g) in EtOH (100 mL) followed by heating with a heat gum.
Analytic high-performance liquid chromatography (HPLC) was performed using a RP-18 (5 µm) Lichro CART® 150-4,6 mm at 25 °C. The binary elution system was composed with acetonitrile (solvent A) and 0.2 % TFA/water (solvent B). Separations were performed at room temperature by solvent gradient elution: 10-20 % B for 40 min, 20-30% B for 5 min, 30-40% B for 5 min, 40-45% B for 5 min and then returned to the initial conditions (10 % B) in 5 min to re-equilibrate the column. The flow rate for both analyses and washing cycles was 0.8 mL/min. The concentration of each sample was 0.1 mg/mL in methanol and detection wavelengths were 254, 280, 325 and 530 nm.

2.2.4. Bacterial strains

The bacterial strains used for biological tests were provided by the antibiotic unit of natural substances and survey of resistance of micro-organisms for anti-infective (ASSURMI), Department of Bacteriology at Pasteur Institute of Côte d'Ivoire (IPCI). The strains used were *Staphylococcus aureus* sensitive to methicillin (*S. aureus Meti S*), *Staphylococcus aureus* resistant to methicillin (*S. aureus Meti R*), *Pseudomonas aeruginosa* sensitive to ceftazidime and imipenem (*P. aeruginosa* Cefta S & Imp S), *Pseudomonas aeruginosa* resistant to ceftazidime and imipenem (*P. aeruginosa* Cefta R & Imp R). Referenced strains of *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 were also tested.
For getting young colonies for the tests, the different bacterial strains were subcultured by streaking method and incubated in an oven at 37 °C for 18 to 24 hours.

2.2.5. Antimicrobial efficiency test of extracts

The efficiency test was used to detect biological activity of extracts was developed by Golly et al. (2012). For this test, the agar and Mueller Hinton broth were the main culture media. The mixture of DMSO/distilled water in proportion 1:1 (v/v) was used as solvent to prepare the solution of leaves extracts. Non-impregnated discs of

6 mm of diameter, purchased from Biorad® were also used. The tests were performed on bacterial inoculums of 5.10^6 CFU/mL.
Each disc was impregnated with 40 µL of extract or fractions solutions at 200 mg/mL concentration. After drying, the discs were placed on the agar previously seeded with micro bacterial strains and incubated at 37 °C for 18 to 24 hours (Zakaria et al, 2006). The observation of an inhibition zone reflected the existence of antimicrobial activity. Observation of an inhibition zone can be used to judge the efficiency of substances in extract or fractions. Control tests were carried out using discs impregnated with 40 µL of appropriate solvent used to prepare the extract or fractions.
Tests on young colonies using oxacillin (OX-5 µg) and cefoxitin (FOX-30 µg) for *S. aureus* and the ceftazidime (CAZ-30 µg) and imipenem (IMP 10 mg) for *P. aeruginosa* were made under the same conditions.

3. Results and Discussion

3.1. Extraction and separation result

Table 1 presents the yields from extraction and separation of bark and seeds samples of *Santaloïdes afzelii*. The results show that the yield of hydroalcoholic extracts and ethyl acetate from the barks is higher than those of seeds. The yields of the hexane extract from the seeds is higher than that of the bark. The yields of the dichloromethane fractions from bark and seeds are similar. The hydro ethanolic extract and ethyl acetate fraction give the best yields.

Table 1
Extraction and separation results.

Samples	Bark		Seeds	
Crushed plant	400,00 g		300,00 g	
Hexane extract	2,31 g	0.57%	6,45 g	2.15%
EtOH/H_2O extract	21,00 g	5.25%	10,00 g	3.33%
CH_2Cl_2 fraction	0,64 g	0.16%	0,45 g	0.15%
AcOEt fraction	3,28 g	0.82%	1,40 g	0.46%

3.2. Antimicrobial efficiency test

Table 2 presents the results of the antimicrobial efficiency tests. Hydro ethanolic extracts from barks and seeds of *Santaloïdes afzelii* have significantly sensitive against *Staphylococcus aureus* with inhibition diameters varying from 11 mm to 12 mm. These extracts are inactive against *Pseudomonas aeruginosa*. The activities of these extracts are low compared to those of oxacillin and cefoxitin.

3.3. HPLC analysis

The HPLC analysis at 280 nm (Fig. 1 and Fig. 2) of the ethyl acetate fractions from bark and seeds of *Santaloïdes afzelii* indicate 4 major compounds **1**, **2**, $\mathbf{3_{B1}}$ and $\mathbf{3_{B2}}$. Accordingly, Fig. 3, Fig. 4 and Fig. 5 showed the HPLC analysis of compounds **1**, **2** and **3**, respectively.

3.4. UV and mass analysis

The UV/Visible and mass analysis of compounds **1**, **2**, $\mathbf{3_{B1}}$ and $\mathbf{3_{B2}}$ are summarized in Table 3. The UV spectra (Table 3) of compounds **1**, **2**, $\mathbf{3_{B1}}$ and $\mathbf{3_{B2}}$ showed absorbance bands at 271, 278, 280 and 208 nm, respectively. Compounds $\mathbf{3_{B1}}$ and $\mathbf{3_{B2}}$ have the same UV spectrum and mass value. The obtained absorbance bands are characteristics of phenolic compounds (Pretsch et al., 2009; Matti, 2014).

3.5. Identification of isolated compounds

3.5.1. Compound **1**

Compound **1** is a yellow-white powder soluble in methanol. The UV absorption spectrum (Table 3) shows a maximum at 270 nm characteristic of an aromatic carbonyl (Pretsch et al., 2009).
The high-resolution mass spectrum shows a molecular ion at *m/z* 193.0142 $[M+Na]^+$ suggesting a molecular mass of 170.0142 corresponding to the empirical formula $C_7H_6O_5$.
The ^{13}C, ^1H, and DEPT 135 NMR characteristics of compound **1** in CD_3OD are reported in Table 4. The ^{13}C-NMR spectrum of compound **1** showed six carbon signals: two equivalent aromatic carbons attached to hydrogen at δc 110.33 ppm (C-2/C-6), one carbonyl group at δc 170.41 ppm (C=O) and one aromatic quaternary carbons at δc 121.98 ppm (C-1). The same spectrum showed three quaternary aromatic carbons attached to oxygen at 139.60 ppm (C-4) and 146.41 ppm (C-3/C-5). The ^1H-NMR spectrum of compound **1** showed one signal at δ_H 6.96 ppm (H-2/H-6) as singlet. The spectral data of compound **1** correspond to those of gallic acid (Fig. 6) (Korul'kina et al., 2004; Soong and Barlow, 2006).

3.5.2. Compound **2**

Compound **2** is a white powder soluble in methanol. The UV spectrum (Table 3) has a maximum at 278 nm. The high-resolution mass spectrum shows a molecular ion at *m/z* 313.0803 $[M+Na]^+$, suggesting a molecular mass of 290.0803 corresponding to the empirical formula $C_{15}H_{14}O_6$. The ^{13}C, ^1H, and DEPT 135 NMR characteristics of compound **2** in DMSO-d_6 are reported in Table 5. The ^{13}C and DEPT 135 NMR spectral data show fifteen (15) signals including seven (7) quaternary aromatic carbons, five (5) CH aromatic carbons, two (2) CH and one (1) CH_2. The ^{13}C-NMR spectrum of compound **2** shows two aromatic carbons at δc 95.04 ppm (C-6) and δc 93.78 ppm (C-8). The quaternary aromatics carbons attached to oxygen in the ring A are at δc 155.31 ppm (C-5), δc 156.12 ppm (C-7) and δc 156.40 ppm (C-9). The quaternary aromatic carbons in ring B is at δc 130.53 ppm (C-1'). The quaternary aromatic carbons attached to oxygen in ring B are at δc 144.79 ppm (C-3'/C-4'). These carbons are characteristic of flavanols. The spectral data of compound **2** correspond to those of catechin (Shen et al., 1993; Lin, et al., 2009). The coupling constant between H-2 and H-3 (J = 7.68 Hz) shows a 2,3-cis configuration (Lokvan et al., 2004) and confirms that compound **2** is the *epi*-catechin (Fig. 6).

3.5.3. Compound **3**

Table 2
Antimicrobial tests of hydro ethanol extracts of bark and seeds of *Santaloïdes afzelii*.

Bacterial strains	Tested substances					
	Observed inhibiton diameter (mm)					
	EtOH/H_2O extract from bark	EtOH/H_2O extract from seeds	Oxacillin	Cefoxitin	Celfazidime	Imipenem
Sa S	12	12	43	32	-	-
Sa R	11	12	0	0	-	-
Sa AT	12	12	28	30	-	-
Pa S	0	0	-	-	30	30
Pa R	0	0	-	-	0	0

Not tested (-); Ethanol (EtOH); Eau (H_2O); *Staphylococcus aureus* sensitive to methicillin (Sa S); *Staphylococcus aureus* resistant to methicillin (Sa R); referenced strains of *Staphylococcus aureus* ATCC 25923 (Sa AT); *Pseudomonas aeruginosa* sensitive to ceftazidime and imipenem (Pa S), *Pseudomonas aeruginosa* resistant to ceftazidime and imipenem (Pa R); referenced strains of *Pseudomonas aeruginosa* ATCC 27853 (Pa AT).

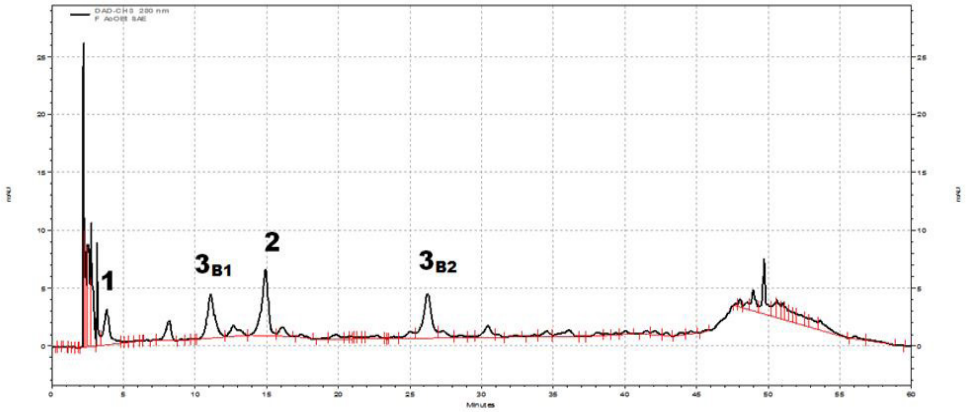


Fig. 1. HPLC chromatogram for AcOEt fraction of the bark.

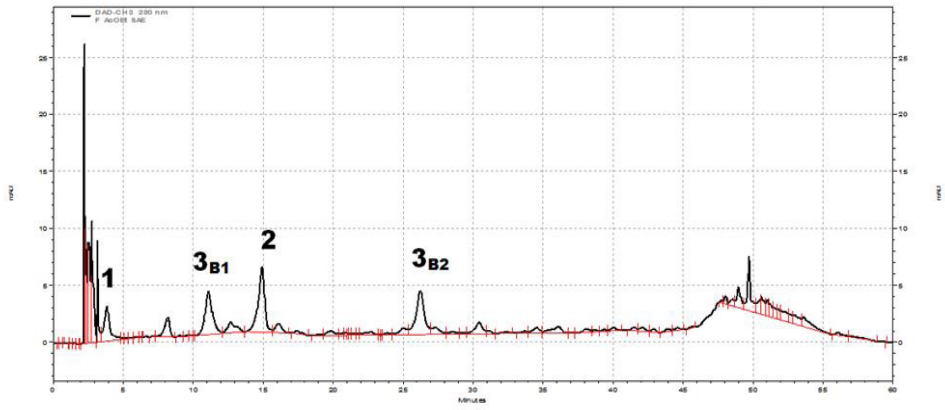


Fig. 2. HPLC chromatogram for AcOEt fraction of the seeds.

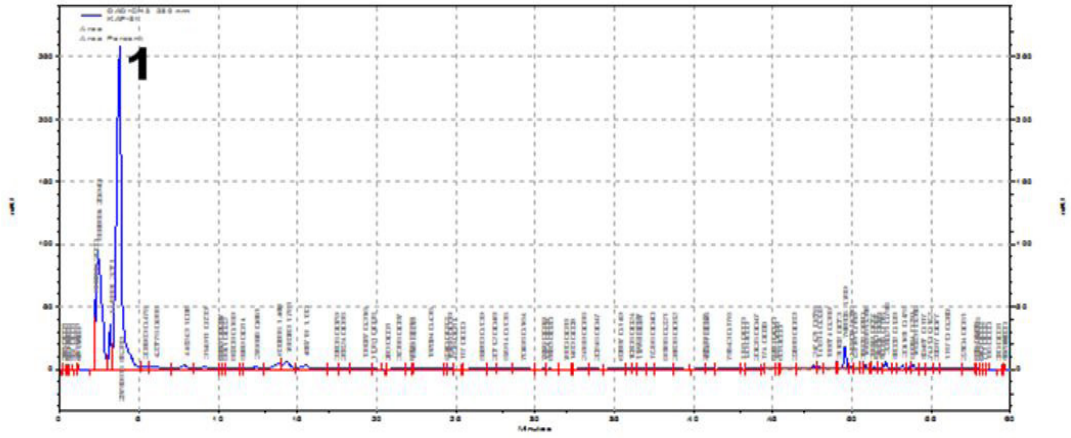


Fig. 3. HPLC chromatogram for isolated compound **1**.

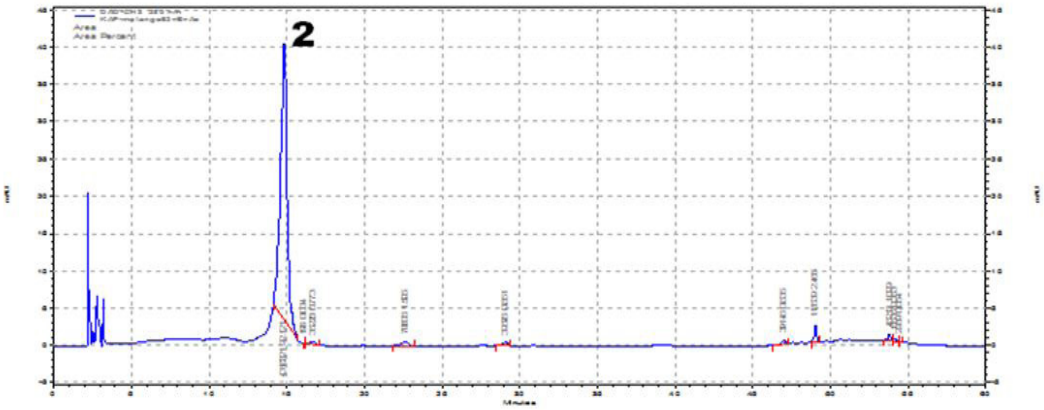


Fig. 4. HPLC chromatogram for isolated compound **2**.

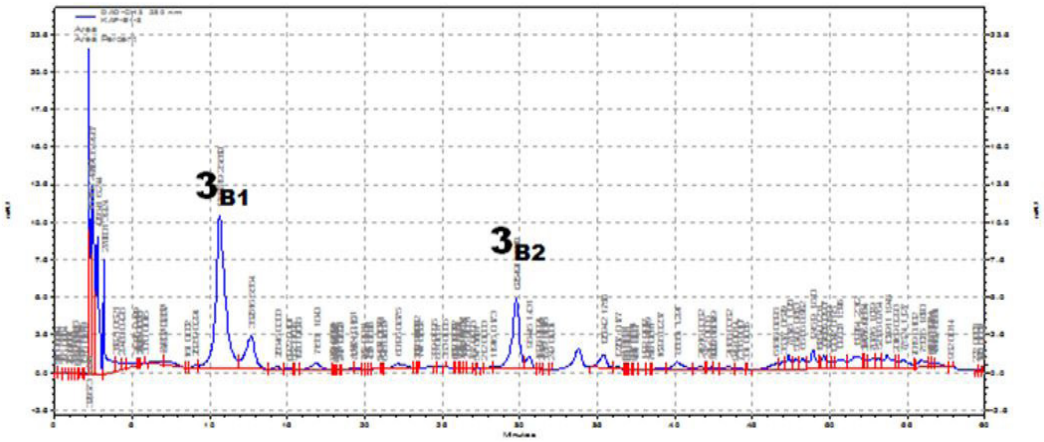
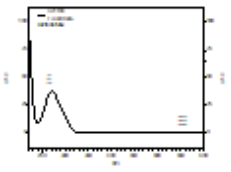
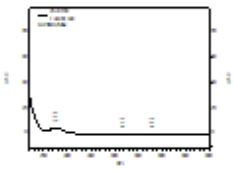
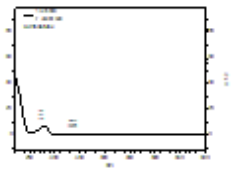
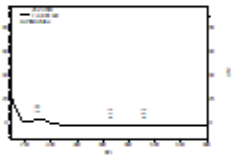
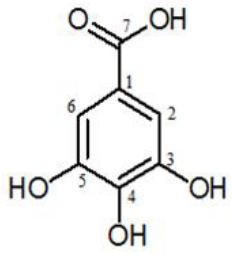


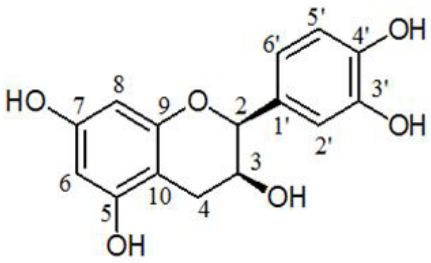
Fig. 5. HPLC chromatogram for isolated compounds 3B1 and 3B2.

Table 3
UV/Visible and mass analysis of compounds 1, 2, 3B1 and 3B2.

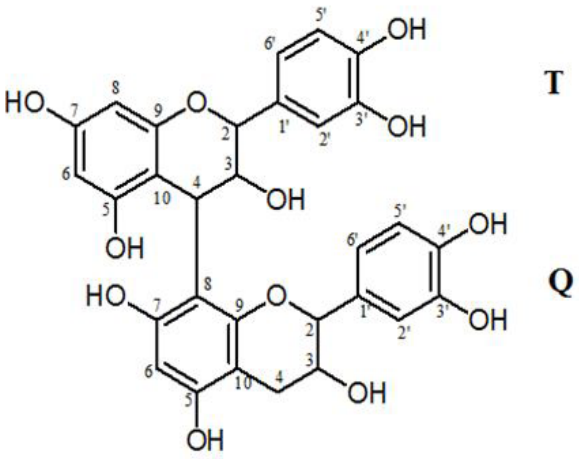
	Compound **1**	Compound $\mathbf{3_{B1}}$	Compound **2**	Compound $\mathbf{3_{B2}}$
UV analysis spectrum				
Rt (min)	3,85	11,05	14,95	26,80
λ_{max}	271 nm	280 nm	278 nm	280 nm
HREI-MS m/z $[M+Na]^+$	193,0142	601,1323	313,0803	601,1323
Mass	170.0142	578,1323	290,0803	578,1323
Formula	$\mathbf{C_7H_6O_5}$	$\mathbf{C_{30}H_{26}O_{12}}$	$\mathbf{C_{15}H_{14}O_6}$	$\mathbf{C_{30}H_{26}O_{12}}$



1 : gallic acid



2 : epi-catechin



T

Q

3 : dimer procyanidin

Fig. 6. Molecular structures of isolated compounds.

Table 4
^{1}H and ^{13}C NMR data of compound **1** in the CD_3OD.

Carbon position	^{1}H NMR (δH ppm)	^{13}C NMR (δC ppm)	DEPT 135
1	-	121.98	C
2	6,96 (s)	110.33	CH
3	-	146.41	C
4	-	139.6	C
5	-	146.41	C
6	6,96 (s)	110.33	CH
7	-	170.41	C=O

Table 5
^{1}H and ^{13}C NMR data of compound **2** in the DMSO-d6.

Carbon position	^{1}H NMR (δH ppm)	^{13}C NMR (δC ppm)	DEPT 135
2	4,48 (d) J=7,68 Hz	80.95	CH
3	3,82 (m)	66.25	CH
4	2,35 (dd) J=16,04 Hz / 8,4 Hz	27.82	CH2
	2,65 (dd) J=16,04 Hz / 5,4 Hz		
5	-	155.31	C
6	5,89 (d) J=2,20 Hz	95.04	CH
7	-	156.12	C
8	5,69 (d) J=2,30 Hz	93.78	CH
9	-	156.4	C
10	-	99	C
1′	-	130.53	C
2′	6,60 (d) J=2,00 Hz	118.36	CH
3′	-	144.79	C
4′	-	144.79	C
5′	6,72 (dd) J=8,16 Hz	114.46	CH
6′	6,69 (d) J=8,16 Hz / 2,00 Hz	115.01	CH

Compound **3** is a brown powder soluble in methanol. The UV spectrum (Table 3) shows a maximum at 280 nm. The high-resolution mass spectrum of compound **3** indicates a molecular ion at *m/z* = 601.1323 $[M+Na]^+$ suggesting a molecular mass of 578.1323 corresponding to the empirical formula $C_{30}H_{26}O_{12}$. This mass indicates exactly that compound **3** is a dimer of procyanidin (Svedström et al., 2002). The UV absorption spectra at 280 nm of compound **3** are similar to that of flavan-3-ols (compound **2**). Indeed, when the hydroxyl group at the position 5 is substituted or when the hydroxyl group at the position 3 of the flavan-3-ol backbone is esterified by the gallate, the absorption limit shifts to a shorter wavelength (≈ 6 nm). Polymerization between flavan-3-ols does not change the UV absorption spectra (Sun et al., 2007). Compound **3** could therefore be a dimer of flavan-3-ol. The HPLC spectrum (Fig. 5) of compound **3** indicates two (2) peaks, which could indicate the presence of dimers B2 and B1.
Condensed tannins are naturally present in the bark of plants (Zhang et al., 2010; Wei et al., 2010). So, the presence of catechin in the seeds and bark of *Santaloïdes afzelii* can suggest the presence of catechin tannins or condensed tannins in barks. The ^{13}C, ^{1}H, and DEPT 135 NMR characteristics of compound **3** in CD_3OD are reported in Table 6. To facilitate a better understanding of future NMR assignments of chemical shifts, one monomer was designated as T and the other as Q. The ^{1}H NMR spectra (Fig. 7) of compounds **3** and compound **2** (catechin) showed similar signals. These signals, in the form of multiplets, indicate the possible presence of dimer of procyanidin (Fig. 6). The signals of the aromatic CH carbons correspond to those of procyanidin (Tarascou, 2005). The signals at 28.53, 37.20, 68.84, 73.32, 77.12 and 82.86 ppm are characteristic of the six carbons (C4Q, C4T, C2Q, C2T, C3Q and C3T) of the two heterocycles (ring C) of the procyanidin dimers of type B. The spectral data of compound **3** correspond to those of dimer procyanidin type B (Foo and Lu, 1999; Stark et al., 2005).

Table 6
^{1}H and ^{13}C NMR data of compound **3** in the CD_3OD.

Carbon position	^{1}H NMR (δH ppm)	^{13}C NMR (δC ppm)	DEPT
C2T/C2Q	4,80 (d) / 4,60 (d)	77,12/82,86	CH/CH
C3T/C3Q	4,00 - 4,10 (m)	73,32/68,84	CH/CH
C4T/C4Q	4,46 (d) / 2,6 / 2,8 (m)	37,20/28,53	CH/CH2
C5T/C5Q	-	157,80/156,99	C/C
C6T/C6Q	5,85 (s) / 5,78 (s)	95,87/96,32	CH/CH
C7T/C7Q	-	157,80/156,99	C/C
C8T/C8Q	5,70 (s) /-	95,54/106,61	CH/C
C9T/C9Q	-	157,8/155,68	C/C
C10T/C10Q	-	100,85/101,22	C/C
C1′T/C1′Q	-	132,24/132,24	C/C
C2′T/C2′Q	6,52-6,82 (m)	119,90/119,42	CH/CH
C3′T/C3′Q	-	145,55/145,86	C/C
C4′T/C4′Q	-	145,92/145,25	C/C
C5′T/C5′Q	6,52-6,82 (m)	116,13/115,75	CH/CH
C6′T/C6′Q	6,52-6,82 (m)	116,13/117,76	CH/CH

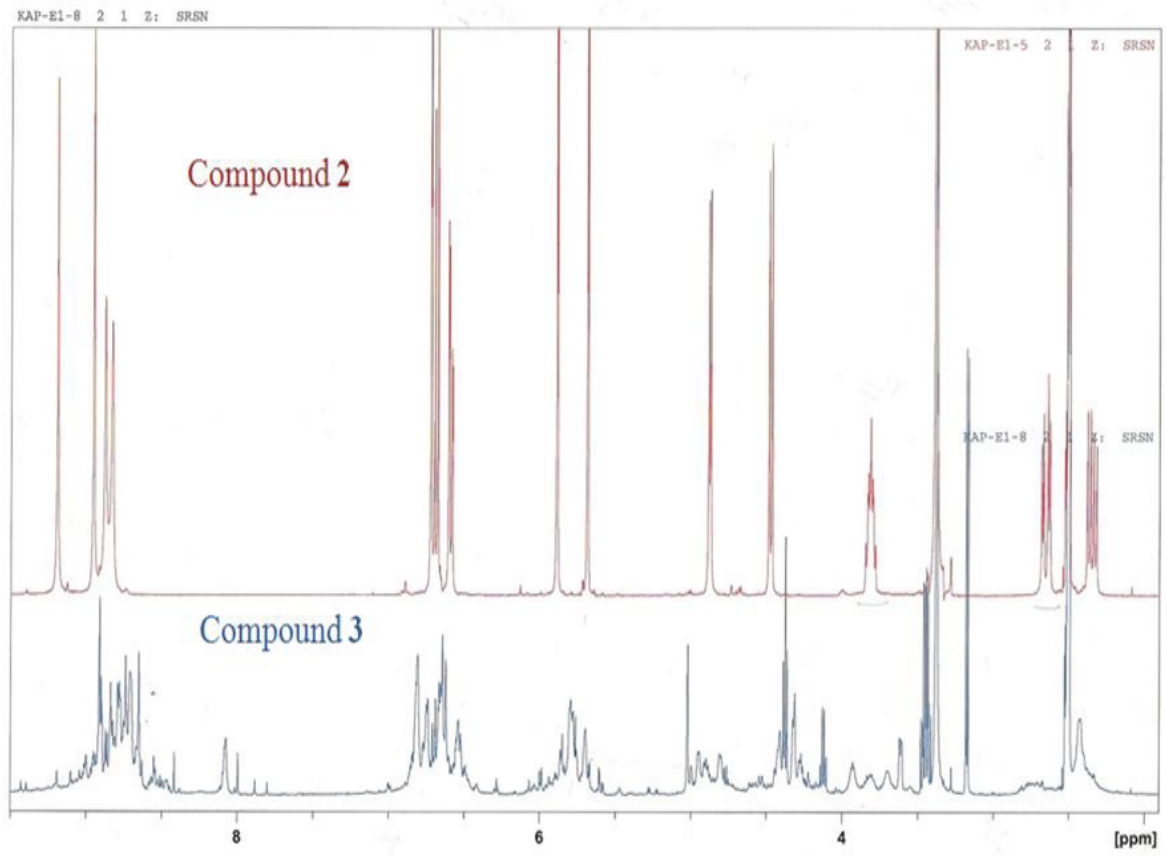


Fig. 7. ^{1}H NMR spectrum of compounds **2** and **3**.

4. Concluding remarks

The hydro ethanolic extracts of seeds and bark of *Santaloïdes afzelii* showed low activities against three strains of *Staphylococcus aureus.* Chromatographic and spectroscopic investigation led to the isolation and identification of gallic acid (**1**), *epi*-catechin (**2**) and dimers procyanidines (**3**). These compounds are known in the literature for their antioxydant, anticancer, antiinflammatory and antibactrial properties. These results justificated the antibacterial activity of the hydro ethanolic extracts of this plant.

Conflict of interest

The authors declare that there is no conflict of interest.

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