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Flavonoid and triterpenes from the leaves of *Senecio gossypinus* Baker from Madagascar

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Abstract
Senecio gossypinus Bakeris an endemic species encountered in the northern, southern, western and central parts of Madagascar. Phytochemical screening of the EtOH extract from the leaves revealed the presence of flavonoids, sterols and polysaccharides. Fractionation of the hexane and EtOAc extracts led to the isolation of the mixture of α-amyrin (1) and β-amyrin (2), and kaempferol-3-*O*-α-L-arabinopyranoside (3), respectively. Compound 3 exhibited potent antimicrobial activity *in vitro* against *Pseudomonas aeruginosa, Proteus mirabilis, Streptococcus agalactiea, Staphylococcus aureus, Candida albicans* with inhibition zones of 13mm, 19mm, 15mm, 12mm and 9mm, respectively, at a test dose of 10 µg/disk using the disk diffusion method. Moreover, compound 3 showed moderate antioxidant activity against the free radical DDPH with an IC_{50} value of 1.90mg/mL. These results gave support to the traditional uses of the studied plant for the treatment of colic, bronchitis and wounds.

Keywords: *Senecio gossypinus*, flavonoid, triterpenes, antioxidant activity, antimicrobial activity

1. Introduction
*Senecio*s one of the most important genera in the Asteraceae family by encompassing 1468 species (Pelser *et al*., 2007) [1]. It is represented in Madagascar by 88 species, of which 78 are endemic. Plants of this genus are used in traditional medicine from different countries for the treatment of various human diseases including asthma, cough, bronchitis, eczema and wounds (Uzun *et al*., 2004; El-Shazly *et al*, 2002) [2, 3]. *Senecio* species have been reported to have antimicrobial activity against *Bacillus cereus, Staphylococcus aureus, Enterococcus faecalis* and *Candida tropicalis* (Uçuncu *et al*., 2010) [4]. Chemical works have resulted in the isolation of a variety of secondary metabolites such as pyrrolizidine alkaloids senecionine, seneciphylline and dodecanoic acid (Amira *et al*., 2005) [5].
S. gossypinus is a species endemic to Madagascar which grows in rocky ridges and ravines in the rainforests of the northern, southern, western and central regions of Madagascar at the altitude between 800 and 2000m. It is used in the indigenous system of medicine as a remedy for colic, bronchitis and wounds. To the best of our knowledge, there are no previous chemical and biological investigations on *S. gossypinus*. The present work aims at isolating chemical constituents from the leaves of this species and evaluating their antimicrobial and antioxidant activities.

2. Materials and Methods
2.1 Plant materials
The leaves of *S. gossypinus* were collected in July 02, 2014 at the beginning of the flowering period in Tolongoina, South-East part of Madagascar. The sample was identified at the Botanic and Zoological National Park of Tsimbazaza (Antananarivo), Madagascar and the National Museum of Natural History (Paris), France (7). A voucher specimen (No. 121A) has been deposited at the Plant-Health Laboratory (Fianarantsoa), Madagascar.

2.2 Phytochemical Screening
The presence of the main phytoconstituentclasses was sought using the chemical tests as described by Bassene (2012**)**: flavonoids (Wilstater test), tannins (Stiasny test followed by ferric chloride), alkaloids (Dragendorff's reagent), triterpenes (Liebermann - Buchard test), cardiotonic glycosides (Badjet and Kedde reactions), saponosides (foam index), cyanogenetic glycosides (Grignard test), and polysaccharides (Polysaccharide test).

2.3 Isolation
The dried and powdered leaves of *S. gossypinus* (300g) were extracted with EtOH (2L) at room temperature for 72 hours. After filtration, the EtOH solution was evaporated to dryness under reduced pressure to give 50.28g of crude extract. A part (20,6g) was submitted to successive liquid – liquid partition with hexane/water, DCM/water, EtOAc/water and n-BuOH/water to yield 2.20 g of hexane extract, 2.10 g of DCM extract, 7.08g of EtOAc extract, 7.10g of BuOH extract and 2.03g of water extract.

The hexane extract (2.0 g) was chromatographed over silica gel column eluted with gradient of different solvents: hexane/CH2Cl2 (100:0 to 0:100) followed by CH2Cl2/EtOAc (100:0 to 0:100) to yield 261 fractions. Fractions which showed similar TLC profile were combined to give 13 sub-fractions (I–XIII). A mixture of compounds 1 and 2 (26mg) was obtained from sub - fractions XII after washing it with hexane. TLC plates were developed using suitable eluents and visualized under UV light at 254nm and 365nm. Sulfuric vanillin was used as a spray reagent.

The EtOAc extract (1g) was separated over silica gel column chromatography eluted with solvents of increasing polarity. The elution with mixtures of DCM/EtOAc (100:0 to 0:100) allowed getting 201 fractions. The fractions n°181 to 190 were grouped and washed with acetone to leave 20mg of compound 3.

2.4 Structural Determination
The 1D NMR (1H NMR, 13C NMR), and 2D NMR (COSY, HSQC and HMBC) spectra
were recorded on a 600 MHz Bruker instrument using solutions of CD3OD with (TMS) as internal standard. Chemical shifts (δ) are reported in ppm and coupling constants in Hz.

Column chromatography was performed using silica gel 60 (Merck, 230 Mesh ASTM 0.063-0.200 mm). Thin layer chromatography (TLC) was conducted on silica gel 60 F254 (Merck).

2.5 Antibacterial Activity
The strains used to evaluate the antimicrobial and antifungal activities were obtained from the Biology Molecular Laboratory of CHU Fianarantsoa (Madagascar) and National Center for Environmental Research, Antananarivo (Madagascar). The Gram-positive bacteria were *Staphylococcus aureus* (ATCC9144), and *Streptococcus agalactiae* (ATCC 13813). The Gram- negative bacteria consisted of *Proteus mirabilis* (ATCC12453) and *Pseudomonas aeruginosa* (ATCC9027). The yeast was *Candida albicans* (ATCC10231).

The antibacterial and antifungal assays carried out on compound 3 were done in triplicate using the agar diffusion method. A solution of compound 3 at a concentration of 100mg/mL was prepared in DCM and 10 µL (10 µg/disk) was adsorbed onto cellulose disks of 6mm diameter. After evaporation of the solvent, the disks were placed in a Petri dish previously seeded with 5 mL of a suspension of the targeted microbial strain (0.2×104 bacteria/mL). The incubation was done at 37 °C for 24 h. Nalidixic acid, netilmicin and spectinomycin, were used as the positive control and assayed at the same dose. Methanol was used as negative control. The results were expressed in millimeter by measuring the diameter of inhibition zone for each disk: Ø < 7 mm: inactive, 7 mm ≤ Ø < 8 mm: slightly active, 8 mm ≤ Ø < 9 mm: significantly active, Ø ≥ 9 mm: very active.

2.6 Antioxidant Activity
2.6.1 Qualitative Analysis
Samples of extracts or pure substances were spotted on a silica gel plate which is then developed in appropriate eluent (DCM/MeOH, 80:20). The plate was sprayed with a 0.2% methanolic solution of the free radical 1,1-diphényl-2-picrylhydrazyle (DPPH). Active substance appears as yellow spot against violet background.

2.6.2 Quantitative Analysis
The determination of the IC_{50} value of the antioxydant activity against DPPH was conducted for compound **3** by using the method described by Molyneux (2004**)** [7] slightly modified. An ethanolic solution of DPPH was prepared by dissolving 4 mg of this product in 100 mL of ethanol. Fifty microliters of a sample solution at a given concentration are added to 950 µL of the DPPH solution. The sample and the standard (ascorbic acid) were tested in triplicate at different concentrations. The absorbance of each mixture was measured at 517 nm after 30 min incubation in the dark. The antioxidant activity against the free radical DPPH is expressed as the percentage inhibition (PI) using the following formula:

$$PI = 100 \frac{A_0 - A_1}{A_0}$$

A0: DPPH absorbance A1: sample absorbance

The curve representing the percent inhibition (PI) versus the concentration (C) was plotted with the Microsoft Excel 2016 software. The IC_{50} value was obtained by extrapolation of the 50% inhibition on the curve.

3. Results and Discussion
3.1 Phytochemical Screening
Phytochemical screening carried out on the ETOH extract revealed the presence of flavonoids, triterpenes and polysaccharides. Alkaloids, tannins, saponosides, coumarins, cardiotonic glycosides and cyanogenetic glycosides were not detected.

3.2 Isolation and Identification
Liquid-liquid partition followed by column chromatography over silica gel of the EtOH extract from leaves of *S. gossypinus* led to the isolation of a mixture of compounds 1 and 2, and compound 3 (Figure 1).

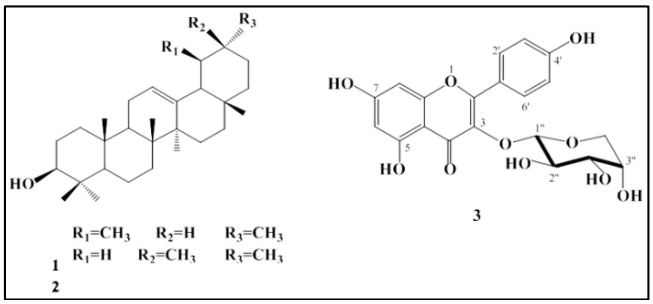

Fig 1: Structures of compounds 1-3

Compounds 1 and 2 were identified as α-amyrin and β-amyrin, respectively, by comparison of their NMR spectral data with those published in the literature (Liliana *et al*., 2012; Tian-Shung *et al.,* 1995) [8, 9]. α-amyrin and β-amyrin are two pentacyclic triterpenes widespread in plants. They have been shown to exhibit various pharmacological activities against a

variety of health-related conditions, such as microbial, fungal and viral infections, inflammation and cancer (Liliana *et al*., 2012) [8].

Compound 3 showed positive reaction for flavonoid in the Wilstater test by giving a red color. The molecule had a molecular formula C20H19O10 as determined by HRFABMS (m/z 419.0978 [M+H]+, calcd for C20H19O10 419.0978). The peak at m/z 287 [(M+H)-132]+ corresponded to the loss of one pentosyl moiety from the pseudo-molecular ion. Therefore, compound 3 was suggested to be a flavonoid glycoside.

The 1H NMR spectrum indicated the presence of a singlet at δ 12.62 which is attributable to a chelated proton of a hydroxyl group at the position C-5 of a flavonoid. Two other phenolic hydroxyl groups were present in the structure of compound 3, as expressed by the broad singlet of two protons at δ 10.20. Two pairs of doublet due to two *meta* coupled aromatic protons appeared at δ 6.42 (1H, d, *J*= 2.4 Hz) and δ 6.19 (1H, d, *J*= 2.4 Hz) and were assigned to H-8 and H-6 of the A-ring, respectively (Chainol K, 2007) [10].The A2B2 pattern at δ 8.07 (2H, m, *J*=8.9 Hz) and δ 6.87 (2H, m, *J*=8.9 Hz) is characteristic of a 1,4-disubstituted benzene ring and then corresponded to H-2',6' and H-3',5' of the B-ring, respectively. Analysis of the 13C NMR and HSQC spectra showed resonances for one ketone carbonyl carbon at δ 177.5, six sp2oxygenated carbons at δ 133.5, 156.2, 156.3, 160.0, 161.2 and 164.2, six sp2methine carbons at δ 93.6, 98.7, 115.2 (two carbons) and 131.0 (two carbons) and two aromatic quaternary carbons at δ 103.9 and 120.6 (Table 1). These spectral data were in favor of the kaempferol as the aglycon of the molecule.

The signals observed in the 1HNMR and 13CNMR spectra at δH 5.33 (1H, d, *J*=5.4 Hz) and δC

101.1 were due to the anomeric proton and carbon of a α-linked glycosyl moiety. The proton signals at δH 3.19 (1H, dd, *J*=11.4, 2.5 Hz, H-5"a), 3.51 (1H, brt, H-3"), 3.55 (1H, dd, *J*=11.4,

5.4 Hz, H-5"b), 3.64 (1H, m, H-4") and 3.73 (1H, m, H-2"), and sp3 oxygenated carbon resonances at δC 64.2 (C-5"), 66.0 (C-4"), 70.7 (C-2") and 71.5 (C-3") supported the pentosyl nature of the sugar which was established to be the α-arabinopyranose considering the chemical shift values, multiplicities and coupling constants, and by comparison with literature data (Kai- Jin Wang, 2007) [11].

The long-range correlation observed in the HMBC spectrum between C-3 (δ 133.5) and the anomeric proton (δ 5.33) indicated that the sugar moiety was attached to C-3 of the aglycon*via* a glycosidic linkage. Thus, the structure of compound 3 was determined to be kaempferol-3-O- α-L-arabinopyranoside. The spectral data of compound 3 were in good agreement with those published in the literature for kaempferol-3-*O*-α-L-arabinopyranoside (Begum *et al*., 2006; De Almeida *et al*., 1998) [13].

Table 1: 1H NMR (600 MHz) and 13C NMR (125 MHz) data for compound **3**

Position C/H	δC	δH (Multiplicity, *J* in Hz)
2		
3	156,2	
4	133,5	6,19 (d,J=2.4 Hz)
4a 5	177,5	6,42 (d, 2.4 Hz)
6	103,9	8,07 (m, J=8.9 Hz)
7	161,2	6,87 (m, *J*=8.9 Hz)
8	98,7	-
8a 1'	164,2	5,33 (d,J=5.4 Hz)
2',6'	93,6	3,73 (brt)
3',5'	156,3	3,51 (brt)
4'	120,6	3,64 (brm)
1"	131,0	3,55 (dd, J= 11.4, 5.4 Hz)
2"	115,2	3,19 (dd, J=11.4, 2.5 Hz)
3"	160,0	12,62(s)
4"	101,1	10,20(s)
5"	70,7	5,27 (brd)
OH-5 OH-7,4'	71,5	4,75 (brd)
OH-2"	66,0	4,58 (brd)
OH-3"	64,2	
OH-4"		

3.3 Antimicrobial Activity

Antimicrobial assay performed on compound **3** showed that it was very active against all the strains tested at a dose of 10µg/disk using the disk diffusion method, since the observed diameters of inhibition zones were greater than 9mm (Table 2). *P. mirabilis* (Gram negative) and *S. agalactiea* (Gram positive) were the most sensitive bacteria with inhibitory zones of 19 and 15 mm, respectively. *C. albicans* (yeast) was the less sensitive strain in this assay with inhibitory zone of 9 mm.

Table 2: Antimicrobial activity of compound 3

Microbial strains	Diameters of inhibition zone (mm) at a dose of 10 µg/disk
Pseudomonas aeruginisa	13
Proteus mirabilis	19
Streptococcus agalactiea	15
Staphylococcus aureus	12
Candida albicans	9

4. Antioxidant Activity

4.1 Qualitative Test

Compound 3 was active against the free radical DPPH by showing a yellow color against a violet background after spaying the TLC plate with a solution of the free radical DPPH.

4.2 Quantitative Test

The ability of compound 3 to scavenge the free radical DPPH was evaluated quantitatively by testing different concentrations ranging from 0.08 to 5mg/mL. The results are represented in figure 2 for compound 3 and the standard ascorbic acid. The IC_{50} values were determined to be 1.90mg/mL for the compound 3 and 0.108mg/mL for the ascorbic acid. Compound 3 showed a dose-response relationship, but its inhibition was less effective than that of the standard ascorbic acid.

Flavonoids are well-known to exert antioxidant activity against the free radical DPHH. The scavenging effect of flavonoids were attributable to the presence of the hydroxyl substituents in rings A, B and C, catechol group in ring B and C2-C3 double bond conjugated with a 4-oxo- functional group (Pietta, 2000) [14].

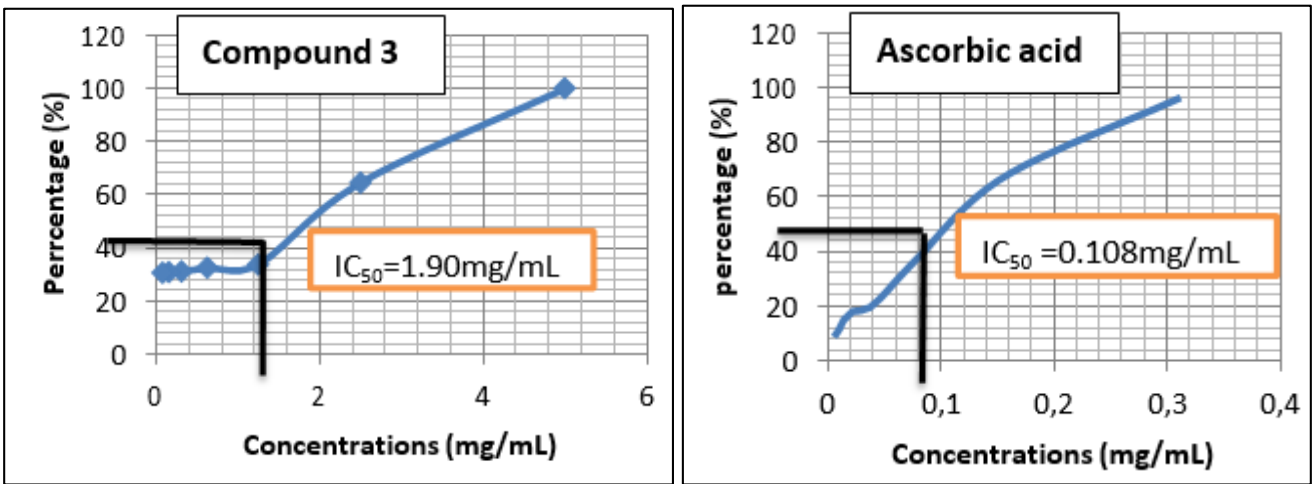

Fig 2: Antioxidant activity of compound **3** and ascorbic acid against DPPH

5. Conclusion

*S. gossynipus*is composed mainly of triterpenes, flavonoids and polysaccharides. The compounds identified in the present study were α-amyrin, β-amyrin and kaempferol-3-*O*-α-L-arabinopyranoside. They were isolated for the first time from this plant. Kaempferol-3-*O*-α-L- arabinopyranoside showed potent antimicrobial activity against *Pseudomonas aeruginis*, *Proteus mirabilis*, *Streptococcus agalactiea*, *Staphylococcus aureus* and *Candida albicans* and moderate antioxidant property against the free radical DPPH. These results give support to the traditional uses of this plant for the treatment of colic, bronchitis and wounds.

6. Acknowledgement

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