ORIGINAL ARTICLE

BIFLAVONOIDS ISOLATED FROM THE STEM BARK OF *Garcinia chapelieri H. Perr. Clusiaceae*

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ABSTRACT

Introduction: Madagascar has about 14,000 plant species, 80% of which are endemic and that it has an original flora by its biodiversity. The valorisation of these species through scientific research is necessary for its development. Indeed, in order to scientifically enhance the medicinal plants endemic to Madagascar, chemical and biological studies have been undertaken. **Objective:** The object of this study is to identify the active molecules of the acetate extract by NMR and to evaluate the antimicrobial and antioxidant potential of extracts of *Garcinia chapelieri* H. Perr. **Materials and methods:** The antioxidant and antimicrobial activities of the extracts (hexanic, ethyl acetate) and the chemical constituents of the most active extract of the bark were examined. Column chromatography and TLC methods allow the isolation of products. The structures of the isolated products were identified by concerted analysis of the 1D and 2D NMR spectra and by comparison with the literatures. **Results:** The results of antimicrobial activity showed the ineffectiveness of the extracts against most of the strains tested. In the quantitative test, the AcOEt extract is rich in antioxidant products than the hexanic extract. *Garcinia chapelieri* AcOEt extract ensure trapping of the DPPH 73.69 %.The value is around 1638.48 mM / mg / l of extract as equivalent to α-tocopherol with the concentration of 0.25 mg / ml. The fractionation of the ethyl acetate extract having a strong antioxidant activity led to the isolation of 4 known products ursolic acid (1) and the three flavonoids, including 5-hydroxy-4',7-dimethoxy-6,8-dimethylflavone (eucalyptin) (2) and the two biflavonoids morelloflavone (3) and 7 " - O-β-D-glycosylmorelloflavone (4).

Keywords: *Garcinia chapelieri H. Perr., triterpene, flavonoids, antifungal, antioxidant.*

1. INTRODUCTION

The genus *Garcinia* of the family Clusiaceae is represented by 250 species of which 31 are endemic to Madagascar [1]. Among these are *Garcinia chapelieri* which is a herb used in traditional medicine for the treatment of many human diseases such as yellow fever, stomach ache, toothache and skin irritation. Previous chemical study of this species has resulted in the isolation of two xanthones [2]. To our knowledge, it has not yet been the subject of biological work. The present study aims to evaluate the antioxidant activity by qualitative and quantitative tests with DPPH and the antimicrobial activity by the method of diffusion in agar medium of the extracts of the stem bark of *Garcinia chapelieri* in order to identify the constituents of the most active extract by concerted analysis of the NMR spectra and by comparison with the literatures.

2. MATERIALS AND METHODS

2.1 General procedures:

Silica gel 60 (Merck, 0.04-0.063 mm) was used for column chromatography. The thin layer chromatography was carried out on silica plates on a plastic or aluminum sheet (Macherey-Nagel, SIL G / UV254, 0.20 mm). All solvents are distilled before use. The 1D NMR spectra (^{1}H, ^{13}C, DEPT) and 2D (^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC, ^{1}H-^{13}C HMBC) are recorded on the Bruker 600 NMR apparatus operating at 600.19 MHz and 125.78 MHz using CD_3OD solvents, $CDCl_3$ and DMSO and TMS as internal reference.

2.2 Plant material:

The stem bark of *Garcinia chapelieri* was harvested in the Manombo-Farafangana region, 400 km from Fianarantsoa in the south-eastern part of Madagascar. The species was identified by botanists at the botanical and zoological park of Tsimbazaza Antananarivo and a voucher specimen has been deposited in the LCSN / COB laboratory.

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2.3 Extraction

The previously dried and ground bark (440 g) was extracted by maceration in solvents of increasing polarity hexane, ethyl acetate, methanol for 4 days. The solutions obtained are evaporated under reduced pressure to give hexane (1.31 g), ethyl acetate (7.76 g), methanolic (12.45 g) extracts.

2.4 Antimicrobial test

Microbial stem cells, two gram + (*Staphylococcus aureus*, *Shigella flexneri*) and two gram - (*Salmonella typhi*, *Escherichia coli*) were used to assess the antimicrobial activities of the extracts using the diffusion disc method [3]. Sterile discs of 6 mm in diameter (Biomérieux, Marcy l'Etoile, France) impregnated with 10 µl of extracts with a concentration equal to 100 mg / ml or 1 mg / disc are placed on the surface of the seeded agars. The petri dishes are then incubated at 37° C. The diameter of the inhibition zone (mm) around each disc is measured after 24 h. The reference antibiotics Neomycin (30 µg / disc), Gentamycin (30 µg / disc) and Tetracycline (100 µg / disc) are used as a positive control. The active extract has an inhibition zone greater than 7 mm.

2.5 Antioxidant test

The qualitative antioxidant test was carried out according to the bioautographic method [4]. Briefly, the extracts to be tested are deposited in solution on a silica plate. After development of the chromatoplate in an appropriate solvent, it is sprayed with a 0.2 % solution of 2,2-diphenyl-1-picrylhydrazyl (DPPH) in methanol. An active product has a yellow spot on a purple background.

The quantitative antioxidant activity was measured according to the method of Brand williams et al. (1995) [5,6]. A methanolic solution of DPPH was prepared by mixing 10 ml of a stock solution (24 mg DPPH / 100 ml MeOH) with 45 ml of methanol and kept at -20° C protected from light before use. In dry tubes, 200 µl of the test solution were introduced, then added 3800 µl of the DPPH solution. For each concentration, a control consisting of 3800 µl of DPPH, supplemented with 200 µl of methanol was prepared. After one hour of incubation in the dark at room temperature, a measurement of the absorbance at λ = 515 nm was carried out using a spectrophotometer. The antioxidant activity which expresses the capacity to trap the free radical is estimated by the percentage of discoloration of DPPH in solution in methanol. It is given by the following formula [7]:

$$\text{Inhibition (\%)} = (A_c - A_s / A_c) \times 100 \quad \textbf{(1)}$$

A_c = the absorbance of the control
A_s = the absorbance of the sample.

The results were expressed by the average of 3 measurements ± standard deviation.
The percentage of inhibition thus calculated was brought back to the standard calibration curve for α-tocopherol values between 100 mM to 600 mM to express the result in trolox equivalent mM / mg / l of extract.

2.6 Isolation

The ethyl acetate extract (4 g) was separated by chromatography on a silica column using the eluent hexane in gradient with ethyl acetate then methanol to give 377 fractions. Purification by crystallization of fractions having the same TLC appearance was carried out. Four products **1** (9 mg, white powder), **2** (17 mg, yellow powder), **3** (14 mg, yellow powder) and **4** (325 mg, yellow powder) respectively from fractions 77-79 eluted with hexane / AcOEt70 / 30, 93-102 eluted with hexane / AcOEt 70/30, 168-179 eluted with hexane / AcOEt 70/30 and 190-199 eluted with hexane / AcOEt 50/50 were obtained.

2.7 Spectral data

The structures of the isolated products were identified by concerted analysis of the ^{1}H, ^{13}C, DEPT, HMQC, HMBC NMR spectra and by comparison with data from the literature.

Product 1: White powder

δ (ppm) ^{1}H NMR (600.19 MHz, CD_3OD): 5.49 (1H, br.s, H-12), 3.45 (1H, dd, J = 5.7, 9.9 Hz, H-3), 2.63 (1H, d, J = 11.2 Hz, H-18), 1.24 (3H, s, H-23), 1.23 (3H, s, H-27), 1.05 (3H, s, H-25), 1.03 (3H, s, H -26), 1.00 (3H, br.s, H-29), 0.96 (3H, s, H-30), 0.90 (3H, s, H-24).
δ (ppm) DEPT Q (125.78 MHz, CD_3OD): 180.0 (C-28), 139.3 (C-13), 125.7 (C-12), 78.2 (C-3), 55.9 (C-5), 53.6 (C-18), 48.1 (C-9), 48.1 (C-17), 42.6 (C-14), 40.0 (C-19), 39.5 (C-20), 39.5 (C-8), 39.2 (C -1), 37.7 (C-4), 37.5 (C-22), 37.3 (C-10), 33.6 (C-7), 33.1 (C-21), 28.8 (C-23), 28.2 (C- 2), 25.0 (C-16), 24.0 (C-27), 23.7 (C-11), 21.4 (C-30), 18.8 (C-6), 17.5 (C-29), 17.5 (C-26), 16.6 (C-24), 15.7 (C-25).

Product 2: Yellow powder

δ (ppm) ^{1}H NMR (600.19 MHz, CD_3OD): 6.79 (s, H-1); 7.98 (d, H-2 ′); 7.00 (d, H-3 ′); 7.98 (d, H-6 ′); 7.00 (d, H-5′); 3.53 (7-OCH_3); 2.18 (8-CH_3); 3.71 (4′-OCH_3); 1.95 (6-CH_3).
δ (ppm) ^{13}C NMR (125.78 MHz, CD_3OD): 164.1 (C-2); 103.6 (C-3); 182.9 (C-4); 106.7 (C-4a); 156.1 (C-5); 113.4 (C-6); 162.3 (C-7); 109.4 (C-8); 151.8 (C-8a); 123.1 (C-1 ′); 128.4 (C-2 ′); 114.9 (C-3 ′); 162.3 (C-4 ′); 114.9 (C-5 ′); 128.4 (C-6 ′); 60.3 (7-OCH_3); 8.5 (6-CH_3); 55.7 (4'-OCH_3); 8.1 (8-CH_3).

Product 3: Appearance: Yellow powder

δ (ppm) ^{1}H-NMR (600.19 MHz, DMSO): Unit I: δ 5.72 (d, 1H, H-2); δ 4.89 (d, 1H, H-3); δ 5.93 (d, 1H, H-6); δ 5.98 (d, 1H, H-8); δ 7.15 (d, 2H, H-2 ′/ H-6′); δ 6.40 (d, 2H, H-3 ′/ H-5 ′); Unit II: δ 6.58 (s, 1H, H-3); δ 6.24 (s, 1H, H-6); δ 7.43 (s, 1H, H-2 ′); δ 6.91 (d, 1H, H-5 ′); δ 7.44 (d, 1H, H-6 ′).

δ (ppm) ^{13}C NMR (125.78 MHz, DMSO): Unit I: δ 81.4 (CH, C-2); δ 48.9 (CH, C-3); δ 197.0 (Cq, C-4); δ 164.2 (Cq, C-5); δ 97.6 (CH, C-6); δ 167.2 (Cq, C-7); δ 96.4 (CH, C-8); δ 163.4 (Cq, C-9); δ 103.7 (Cq, C-10); δ 128.3 (Cq, C-1 ′); δ 129.1 (2CH, C-2 ′/ C-6 ′); δ 115.2 (2CH, C-3 ′/ C-5 ′); δ 155.8 (Cq, C-4 ′); Unit II: δ 166.4 (Cq, C-2); δ 102.8 (CH, C-3); δ 182.1 (Cq, C-4); δ 162.3 (Cq, C-5); δ 99.2 (CH, C-6); δ 161.2 (Cq, C-7); δ 101.1 (Cq, C-8); δ 157.9 (Cq, C-9); δ 102.1 (Cq, C-10); δ 121.6 (Cq, C-1 ′); δ 113.9 (CH, C-2 ′); δ 146.3 (Cq, C-3 ′); δ 150.3 (Cq, C-4 ′); δ 116.7 (CH, C-5 ′); δ 119.7 (CH, C-6 ′).

Product 4: Appearance: Yellow powder

δ (ppm) ^{1}H NMR (600.19 MHz, DMSO): Unit I: δ 5.74 (d, 1H, H-2); δ 4.86 (d, 1H, H-3); δ 5.93 (d, 1H, H-6); δ 5.98 (d, 1H, H-8); δ 7.12 (d, 2H, H-2 ′/ H-6′); δ 6.38 (d, 2H, H-3 ′/ H-5′); Unit II: δ 6.42 (s, 1H, H-6); δ 6.64 (s, 1H, H-3); δ 7.32 (s, 1H, H-2 ′); δ 6.88 (d, 1H, H-5 ′); δ 7.26 (d, 1H, H-6 ′). Glucosyl 5.12 (H-1 ″); 3.28 (H-2 ″); 3.35 (H-3 ″); 3.46 (H-4 ″); 3.36 (H-5 ″); 3.64, 3.89 (H-6 ″)
δ (ppm) ^{13}C NMR (125.78 MHz, DMSO): δ 82.8 (CH, C-2); δ 51.0 (CH, C-3); δ 198.4 (Cq, C-4); δ 165.7 (Cq, C-5); δ 97.7 (CH, C-6); δ 168.3 (Cq, C-7); δ 96.5 (CH, C-8); δ 164.8 (Cq, C-9); δ 103.6 (Cq, C-10); δ 130.4 (Cq, C-1 ′); δ 129.4 (2CH, C-2 ′/ C-6 ′); δ 115.4 (2CH, C-3 ′/ C-5 ′); δ 158.5 (Cq, C-4 ′); Unit II: δ 166.1 (Cq, C-2); δ 103.3 (CH, C-3); δ 183.9 (Cq, C-4); δ 162.7 (Cq, C-5); δ 99.5 (CH, C-6); δ 161.6 (Cq, C-7); δ 104.1 (Cq, C-8); δ 156.6 (Cq, C-9); δ 106.4 (Cq, C-10); δ 121.5 (Cq, C-1 ′); δ 114.3 (CH, C-2 ′); δ 146.2 (Cq, C-3 ′); δ 151.1 (Cq, C-4 ′); δ 116.9 (CH, C-5 ′); δ 120.8 (CH, C-6 ′). Glucosyl 100.0 (C-1 ″); 73.3 (C-2 ″); 69.5 (C-3 ″); 76.8 (C-4 ″); 76.7 (C-5 ″); 61.1 (C-6 ″).

3. RESULTS

3.1 Antimicrobial test

For the evaluation of the antimicrobial potential of hexane and ethyl acetate extracts, four strains were tested, because each of them has specific cellular structures and metabolism. The results are shown in table 1.

Table 1: Diameter of the zone of inhibition of the hexane and AcOEt extracts of *Garcinia chapelieri.*

Products tested	**Diameter of inhibition (mm)**			
	Staphylococcus aureus	*Shigella flexneri*	*Salmonella typhi*	*Escherichia coli*
AcOEt extract	8 ± 0,66 (++)	0	0	0
Hexane extract	7 ± 0,33 (+)	12 ± 0,0 (+++)	0	0
Neomycin (30 µg/disc)	16 ± 4	NT	NT	NT
Tetracyclin (100 µg/disc)	NT	37 ± 1	34 ± 3	NT
Gentamycin (30 µg/disc)	NT	NT	NT	22 ± 1

• NT: not tested; ±: repeated tests 3X

The results showed the ineffectiveness of the extracts against most of the strains tested *Garcinia chapelieri* hexane extract is active on the germ of *Shigella flexneri* while ethyl acetate extract is active on *Staphylococcus aureus*.

3.2 Antioxidant test

The three extracts hexane, ethyl acetate and methanol contain antioxidant products (Figure 1). But the ethyl acetate extract is rich in antioxidant products from the stem bark of *Garcinia chapelieri*.

Eluent : Hexane/Acétone 2/8
Stationary phase : silica gel 60
Developer : DPPH/MeOH (2 mg/1 ml)
Ex_H : hexane extract
Ex_{Ace} : ethyl acetate extract
Ex_{Me} : methanol extract

Figure 1: Antioxidant activity of hexane, AcOEt, MeOH extracts from the stem bark of *Garcinia chapelieri.*

The quantification of the antioxidant activity of an extract or a product is measured by referring to the antioxidant activity of α-tocopherol by varying its concentration. This variation in α-tocopherol concentration as a function of absorbance is shown in Figure 2.

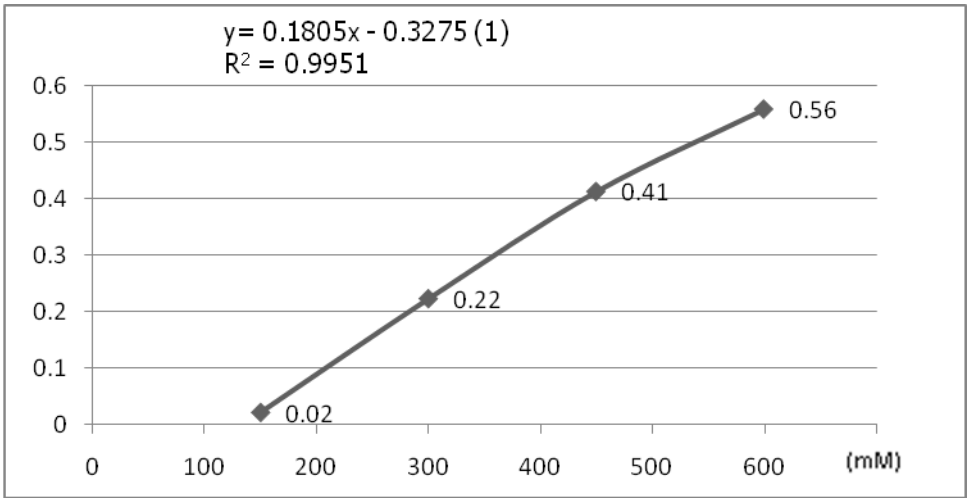

Figure 2: Standard curve of α-tocopherol concentration as a function of absorbance.

The results of the quantification of the hexane and ethyl acetate extracts are summarized in Table 2.

Table 2: Results of the quantitative test of the antioxidant activity of the hexane and ethyl acetate extracts.

Extracts	Concentration (mg/ml)	% inhibition	Vitamine Equivalent (mM/mg/l extract)
AcOEt	0.25	73.69	**1638.48**
Hexane	1	23.54	130.75
Control	méthanol+ DPPH	0	

According to this table, at a concentration of 0.25 mg / ml, the ethyl acetate extract inhibits the free radical of DPPH by 73.69 %. By bringing each DPPH absorbance value into the α-tocopherol trendline equation Y= 0.1805X – 0.3275 (R^2 = 0.9951), the ability to inhibit the free radical of DPPH for the AcOEt extract in α-tocopherol equivalent is equal to 1638.48 mM / mg / l of extract. The inhibitory activity of the AcOEt extract is very strong because almost all of the DPPH is trapped. On the other hand, the antioxidant activity of the hexane extract at 1 mg / ml equivalent to α-tocopherol equal to 130.75 mM / mg / l of extract is low because only 23.54 % of DPPH is trapped.

The strong antioxidant activity of AcOEt extract is due to the presence of polyphenols in the plant, thanks to its structure containing phenolic –OH.

3.3 Identification

The chemical isolation work was continued with the AcOEt extract which gave the best yield and is biologically active. Chromatography on a normal silica column of the AcOEt extract resulted in the isolation of four products **1**, **2**, **3** and **4**. They are identified by concerted analysis of the recorded NMR spectra and comparison with data in the literature. Product **1** is a triterpenic acid which is a ursolic acid [8, 9, 10]. Products **2**, **3** and **4** are known flavonoids which have been identified as 5-hydroxy-4',7-dimethoxy-6,8-dimethylflavone (eucalyptin) [11], morelloflavone, and 7-O-β-D-glycosylmorelloflavone [12, 13, 14], respectively. Their chemical structures are shown in figure 3.

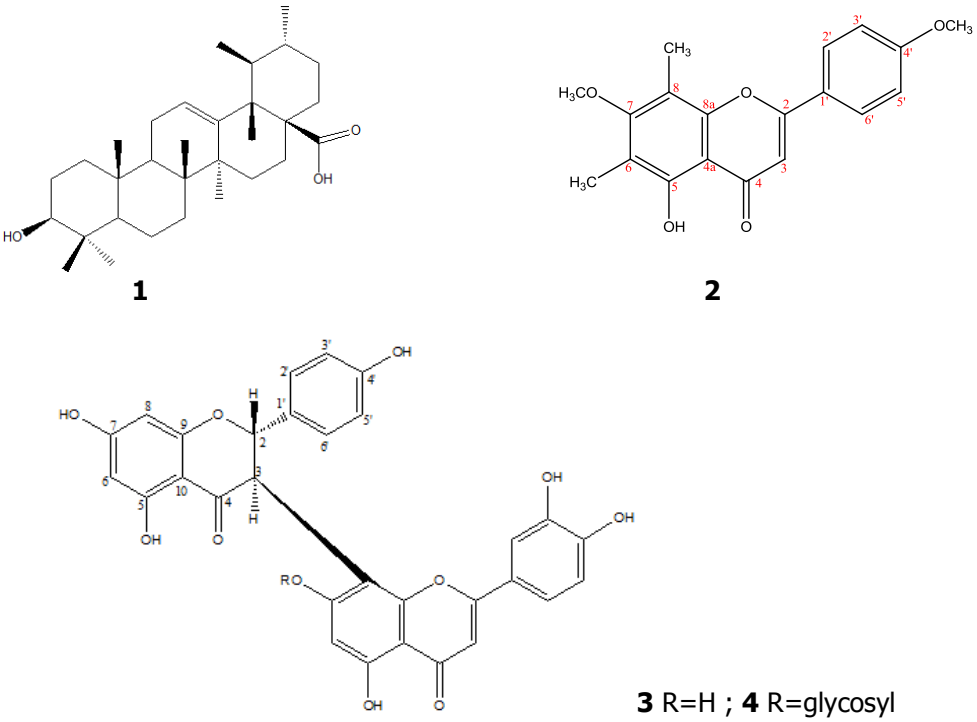

Figure 3: Chemical structures of products 1 to 4

4. DISCUSSION

One triterpene acid and three flavonoids have been isolated from the stem bark of *Garcinia chapelieri*. 5-Hydroxy-4',7-dimethoxy-6,8-dimethylflavone (eucalyptin) found from *Eucalyptus* species is isolated for the first time from the genus *Garcinia*. Species of the genus *Garcinia* are known for their richness in biflavonoids [15]. Morelloflavone (3) and 7'-glycosylmorelloflavone (4) have been reported in other species of the *Garcinia* genus such as *Garcinia brasiliensis*, *Garcinia griffithii*, *Garcinia buchananii* Baker [16] and *G. eugeniifolia* Wall. Biflavonoids can be chemotaxonomic markers of the genus *Garcinia*. Biflavonoids including morelloflavone (3) and 7'-O-β-D-glycosylmorelloflavone (4), are endowed with antioxidant activity [17]. Morelloflavone relieves atherosclerosis by inhibiting the oxidative modification of low density lipoproteins [18]. Ursolic acid has demonstrated multiple biological properties, such as anti-inflammatory activities due to its anti-cylooxygenase and anti-lipoxygenase, antiviral [19] and anti-tumor potential [20]; it also showed strong cytotoxic activity on colon cancer lines [21]. Ursolic acid also inhibits the activity of these enzymes in HL60 leukemia cells in vitro [22]. This triterpene is also used in cosmetology, because it has a potential to inhibit the induction of MMPs [23,24,25], which could also explain the beneficial role of this molecule in preventing skin aging. This work confirmed the traditional use of *Garcinia chapelieri* H. Perr. such as toothaches, stomach aches, irritations on the skin by anti-inflammatory and cosmetic drugs.

5. CONCLUSION

The data from this study shows that *Garcinia chapelieri* is a source of biologically active products with antibacterial and antioxidant properties. These results contribute to the scientific enhancement of this medicinal plant and endemic to Madagascar. In addition, it expands knowledge of the chemistry of the genus Garcinia in general, and *Garcinia chapelieri* in particular, by characterizing a triterpène (ursolic acid), a flavonoid and two biflavonoids.

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