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## Antibacterial and phytochemical evaluations of *Garcinia orthoclada* Baker

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#### Abstract

*Garcinia orthoclada* Baker is a medicinal plant endemic to Madagascar. Antibacterial study of this species revealed that the ethyl acetate-soluble and butanol-soluble parts of the crude ethanolic extract from small branches of *G. orthoclada* were active against the five Gram-positive and Gram-negative bacteria *Bacillus cereus*, *Proteus mirabilis*, *Salmonella enterica* subsp., *Shigella flexneri* and *Pseudomonas aeruginosa*, using disc diffusion method at the concentration of 1 mg/disc. The bioactive ethyl acetate-soluble extract was phytochemically investigated by a combination of open column chromatography and gas chromatography–mass spectroscopy (GC-MS) technique, which led to the identification of seventeen known compounds, consisted of nine triterpenoids, five monoalkenes, one cyclane, one diterpene and one phenol. Some of the compounds identified have been reported to exhibit antibacterial activity. The present study demonstrates the potentials of *G. orthoclada* as a source of antibacterial agents.

**Keywords:** *Garcinia orthoclada*, Clusiaceae, antibacterial activity, GC-MS

#### 1. Introduction

The increasing emergence of bacteria and fungi resistant to commercially available drugs represents a major problem to public health worldwide since it is the main culprit of the unsuccessful treatment of microbial infections. Therefore, there is an urgent need for the development of new and potent antimicrobial agents. Plants are considered by researchers as an undeniable source of substances with antimicrobial activity as inferred from their widely use in traditional medicine in different countries to treat infectious diseases. In this regard, many plants have been selected for *in vitro* antimicrobial screening study through ethnopharmacological approach and random collection<sup>[1, 2]</sup>.

Madagascar is known as a country of biodiversity hotspot. The flora of Madagascar comprises 11 220 vascular plants, of which 10 650 are Angiosperms and 84% are endemic<sup>[3]</sup>. This biological richness constitutes a promising reservoir of natural products of great significance as drugs and lead structures. However, a large majority of these plants remains unexplored. In the present study, *Garcinia orthoclada* Baker is the subject of biological and phytochemical evaluations.

The pantropical genus *Garcinia* (Clusiaceae) comprises more than 250 species of dioecious trees and shrubs that are especially encountered in lowland tropical forests<sup>[4]</sup>. This genus is represented in Madagascar and in the Comoros islands by 32 recognized species, 31 of which are endemic<sup>[5]</sup>. *G. orthoclada* is endemic to Madagascar and traditionally used for the treatment of syphilis, back pain, haemoptysis and uro-genital infections<sup>[6-8]</sup>. To our knowledge, no previous biological or phytochemical works have been carried out on this species. Important chemical constituents described from the genus *Garcinia* include a variety of phenolic compounds such as xanthenes and benzophenones, and a series of lanostane triterpenoids<sup>[9-11]</sup>. The aim of this research is to investigate the antibacterial activity and the chemical constituents of *G. orthoclada* small branches.

#### 2. Materials and methods

##### 2.1 General

Silica gel 60 (EMD Chemicals, 0.04-0.063 mm) was used for column chromatography. All solvents were distilled. GC-MS analyses were conducted using an Agilent 7890A gas chromatograph equipped with an Optima 5 fused silica gel capillary column (60 m x 0.25 mm internal diameter, 0.25 µm film thickness) and directly interfaced to a Hewlett-Packard 5975C

mass spectrometer. The carrier gas was helium. The GC was operated in split mode at a split of 20 ml/min. Mass spectra were recorded in EI mode at 70 eV. Identification of compounds was achieved by comparison of the EI-MS by computer matching against commercial (Wiley 275 and NIST 98) MS libraries and a home-made library.

## 2.2 Collection of plant material

The small branches of *G. orthoclada* were collected in October 2014 in the rainforests of Ambatovy in the Alaotra Mangoro region of Madagascar. The species was identified by one of us (S.R.) at the Botany and Ethnobotany Department of the National Center of Applied Pharmaceutical Research (CNARP), Antananarivo, Madagascar, where a voucher specimen (ROL735) is also deposited.

## 2.3 Preparation of extracts

The dried small branches of *G. orthoclada* were ground into powder and extracted with EtOH by maceration for 48 hours at room temperature, resulting in the crude ethanolic extract after evaporation under reduced pressure of the solvent. The crude ethanolic extract was subjected to successive liquid-liquid partitioning between hexane and water, ethyl acetate and water, and *n*-butanol and water to furnish hexane-soluble, ethyl acetate-soluble, butanol-soluble and water-soluble extracts.

## 2.4 Phytochemical screening

The detection of main classes of phyto-constituents such as alkaloids, flavonoids, cardenolides, iridoids, saponins, anthraquinones, triterpenoids, steroids, leucoanthocyanins, tannins and coumarins were conducted on the crude ethanolic extract according to methods previously published [12, 13]. Appearance of specific colors or precipitates indicates the presence of the targeted metabolites.

## 2.5 Antibacterial assay

One Gram-positive, *Bacillus cereus* (ATCC 14579) and four Gram-negative, *Proteus mirabilis* (ATCC35659), *Salmonella enterica* subsp. (ATCC13076), *Shigella flexneri* (ATCC 12022) and *Pseudomonas aeruginosa* (ATCC10145) bacterial strains were used for susceptibility assays using the disc diffusion method [14]. Sterilized filter paper discs of 6 mm (Biomérieux, Marcy l'Étoile, France) were saturated with 10 µl of the samples dissolved in appropriate solvents (1

mg/disc). The impregnated disks were placed on to agar plates seeded with respective test organisms. The plates were then incubated at 37°C for 24 hours. The antibacterial activity corresponds to the diameter of inhibition zones surrounding paper disk. Neomycin 30 µg (Bio-Rad, Marnes-la-Coquette, France) was used as positive control. Tests were performed in triplicate. A test sample is considered as active if the diameter of inhibition zone is greater than 6 mm.

## 2.6 Fractionation and characterization of compounds

The ethyl acetate fraction was first separated by column chromatography over silica gel eluted with dichloromethane/methanol (19:1 to 9:1) to give 10 fractions (I-X). Compounds 5 and 6 were characterized by GC-MS analysis of fraction I. Repeated column chromatography over silica gel of fraction V and GC-MS analyses of selected resulting fractions allowed the characterization of compounds 1, 3 – 16. Compounds 2 and 17 were eluted from fraction VI which was separated by the same manner as the fraction V.

## 3. Results

### 3.1 Preparation of extracts

Maceration in EtOH of 1 kg of the plant material yielded 110 g (11%) of crude ethanolic extract. Liquid-liquid partitioning of the crude extract furnished 42.5 g (38.6%) of hexane-soluble, 23.2 g (21.2%) of ethyl acetate-soluble, 4.7 g (4.2%) of butanol-soluble and 6.8 g (6.2%) of water-soluble extracts.

### 3.2 Phytochemical screening

The phytochemical screening was performed on the crude ethanolic extract by means of different chemical assays. It revealed the presence of triterpenoids, steroids, cardenolides, flavonoids, coumarins, tannins and leucoanthocyanins.

### 3.3 Antibacterial assay

The hexane-soluble, ethyl acetate-soluble, butanol-soluble and aqueous-soluble extracts were evaluated for their antibacterial activity (Table 1). The ethyl acetate-soluble and butanol-soluble extracts showed antibacterial activity against the five bacterial strains with diameters of inhibition zones ranging between  $7.5 \pm 0.7$  and  $14 \pm 1.4$  mm at a concentration of 1 mg/disc. The hexane-soluble and water-soluble extracts inhibited the growth of *S. flexneri* ( $9.5 \pm 0.7$  mm) and *P. mirabilis* ( $7 \pm 0$  mm) at the same concentration, respectively.

**Table 1:** Antibacterial activity of extracts from *G. orthoclada*

Test organisms	Diameter of inhibitory zone (mm)				
	HE	EE	BE	WE	C
<i>Bacillus cereus</i>	–	$8.5 \pm 0.7$	$9 \pm 0$	–	$18 \pm 0$
<i>Proteus mirabilis</i>	–	$10 \pm 0.7$	$11.5 \pm 0.5$	$7 \pm 0$	$24 \pm 0$
<i>Salmonella enterica</i> subsp.	–	$7.5 \pm 0.7$	$8 \pm 0$	–	$17 \pm 0$
<i>Shigella flexneri</i>	$9.5 \pm 0.7$	$14 \pm 1.4$	$14 \pm 0$	–	$25 \pm 0$
<i>Pseudomonas aeruginosa</i>	–	$9 \pm 0$	$10 \pm 0$	–	$22 \pm 0$

Values are given in Mean  $\pm$  SD (n=3); -: inactive; HE: hexane-soluble extract; EE: ethyl acetate-soluble extract; BE: butanol-soluble extract; WE: water-soluble extract; C: Control (Neomycin)

This is the first report on the antibacterial activity of this species.

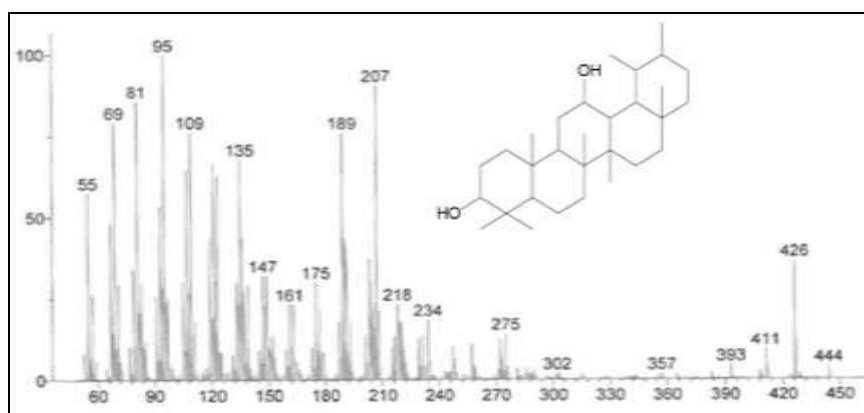
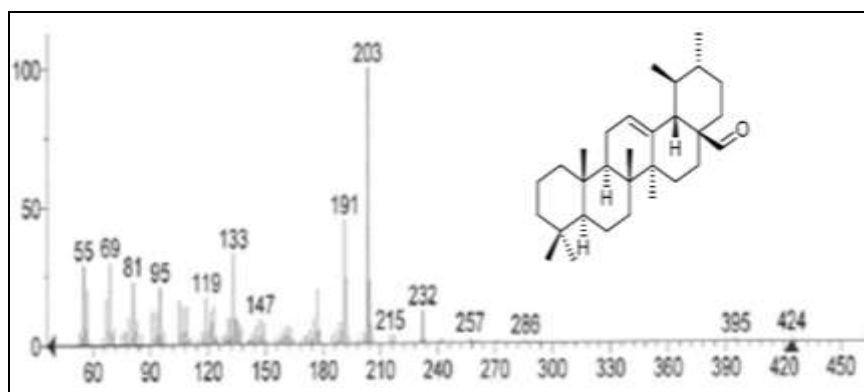
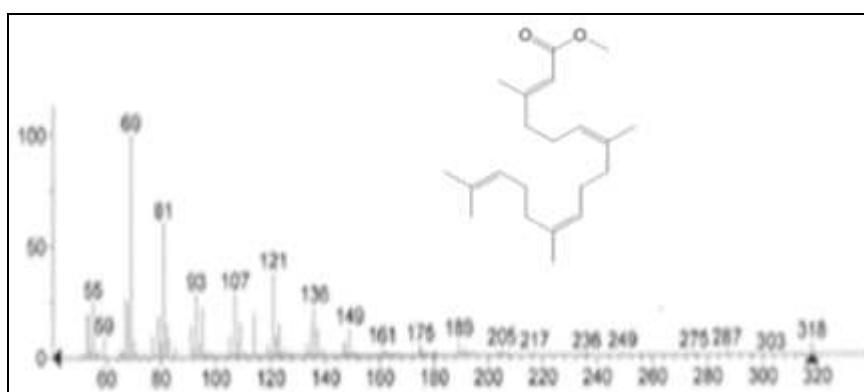
## 3.4 Fractionation and characterization of compounds

The bioactive ethyl acetate-soluble extract was subjected to open column chromatography over silica gel. GC-MS analyses of selected resulting fractions led to the

characterization of seventeen known compounds consisted of nine triterpenoids 1-9, five monoalkenes 10, 13 - 16, one cyclane 12, one diterpene 11 and one phenol 17 (Table 2). The hydrocarbons and diterpene derivatives 10 – 16 had 16 to 26 carbon atoms in their structures. This is the first report of the isolation compounds 1 – 17 in *G. orthoclada*, and to our knowledge, the first report of long-chain hydrocarbons isolation from a plant of the genus *Garcinia*. The mass spectra of the four unusual compounds 1, 4, 11 and 17 identified from *G. orthoclada* are shown in figures 1-4.

**Table 2:** Compounds identified in the ethyl acetate-soluble extract by CC fractionation followed by GC-MS analyses of selected fractions

No.	RT on GC-MS (minute)	Compound name	Molecular formula	Molecular weight
1	38.97	Ursane-3,12-diol	C <sub>30</sub> H <sub>52</sub> O <sub>2</sub>	444
2	37.94	Betulin	C <sub>30</sub> H <sub>50</sub> O <sub>2</sub>	442
3	36.90	Oleanolic acid	C <sub>30</sub> H <sub>48</sub> O <sub>3</sub>	456
4	36.79	Urs-12-en-28-al	C <sub>30</sub> H <sub>48</sub> O	424
5	33.96	Friedelin	C <sub>30</sub> H <sub>50</sub> O	426
6	33.37	Friedelinol	C <sub>30</sub> H <sub>52</sub> O	428
7	31.14	$\alpha$ -Amyrin	C <sub>30</sub> H <sub>50</sub> O	426
8	30.32	$\beta$ -Amyrin	C <sub>30</sub> H <sub>50</sub> O	426
9	30.06	Lupeol	C <sub>30</sub> H <sub>50</sub> O	426
10	22.46	( <i>E</i> )-Hexacos-9-ene	C <sub>26</sub> H <sub>52</sub>	362
11	21.4	(2 <i>E</i> ,6 <i>Z</i> ,10 <i>Z</i> )-3,7,11,15-Tetramethyl-hexadeca-2,6,10,14-tetraenoic acid methyl ester	C <sub>21</sub> H <sub>34</sub> O <sub>2</sub>	318
12	20.91	Cyclotetracosane	C <sub>24</sub> H <sub>48</sub>	336
13	19.22	1-Docosene	C <sub>22</sub> H <sub>44</sub>	304
14	17.39	( <i>E</i> )-Eicos-3-ene	C <sub>20</sub> H <sub>40</sub>	280
15	15.38	1-Octadecene	C <sub>18</sub> H <sub>36</sub>	252
16	13.17	1-Hexadecene	C <sub>16</sub> H <sub>32</sub>	224
17	12.27	2,4-Di- <i>tert</i> -butylphenol	C <sub>14</sub> H <sub>22</sub> O	206

**Fig 1:** Mass spectrum of Ursane-3,12-diol 1**Fig 2:** Mass spectrum of Urs-12-en-28-al 4**Fig 3:** Mass spectrum of (*2E*,6*Z*,10*Z*)-3,7,11,15-tetramethyl-hexadeca-2,6,10,14-tetraenoic acid methyl ester 11

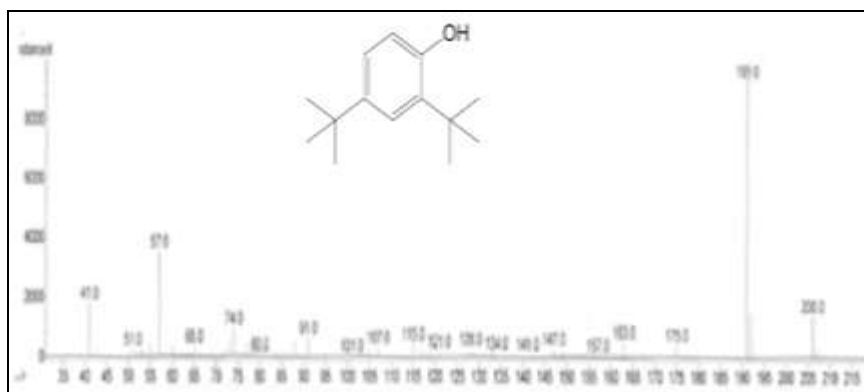


Fig 4: Mass spectrum of 2,4-di-tert-butylphenol 17

#### 4. Discussion

The use of plants is an attractive alternative to combat infectious diseases in connection with the various types of bioactive secondary metabolites they produce. In the present research, *G. orthoclada*, an endemic plant to Madagascar was evaluated for antibacterial activity and phytochemical composition.

The hexane-soluble and water-soluble extracts of *G. orthoclada* small branches weakly inhibited the growth of *S. flexneri* and *P. mirabilis*, respectively. They had no effect on the other bacteria. In contrast, the ethyl acetate-soluble and butanol-soluble extracts showed moderate to weak antibacterial activity against the five pathogenic microorganisms tested. The differences in the antibacterial activities of these extracts could be ascribed to discrepancies in their chemical compositions. Previous studies on related species have yielded similar results, showing low antibacterial activity for hexane-soluble and water-soluble extracts [15, 16]. The ability of ethyl acetate and *n*-butanol to extract phytoconstituents of various classes might be given as a fitting explanation to these observations. As indicated by the results of the phytochemical screening, the crude ethanolic extract of *G. orthoclada* was composed of different classes of secondary metabolites, some of which are known as antibacterial principles and are likely to be soluble in ethyl acetate and *n*-butanol.

The antibacterial profiles of the ethyl acetate-soluble and butanol-soluble extracts were identical for *S. flexneri* the most sensitive tested organism, followed by *P. mirabilis*, *P. aeruginosa* and *B. cereus*. The less sensitive strain was *S. enterica* subsp..

*S. flexneri*, the most sensitive bacteria in this work, is the most frequently isolated *Shigella* species from human infections along with *S. sonnei*. Shigellosis or bacillary dysentery still remains a major health problem in many parts of the world by causing 160 million cases and hundreds of thousands of deaths a year [17]. Therefore, elucidation of the active principles from *G. orthoclada* is worth a lot of attention to overcome shigellosis. The activity against *P. mirabilis* which is a main cause of uro-genital infections, lends support to one of the ethnopharmacological uses of this plant.

Furthermore, the effectiveness of the ethyl acetate-soluble and butanol-soluble extracts from *G. orthoclada* against the four Gram-negative bacteria is of great interest in the search for new antibacterial agents against these pathogenic organisms. Gram-negative bacteria are generally more resistant to antibiotics than Gram-positive bacteria due to differences in their cell-wall structure. In Gram-negative bacteria, the presence of a lipopolysaccharide covering makes difficult for hydrophobic molecules to go through the outer membrane [18].

Although the antibacterial activity of extracts against the tested Gram-negative bacteria was weaker than the standard neomycin, *G. orthoclada* represents a natural resource which can be exploited in order to combat various human ailments. Since the extracts subjected to antibacterial assays in this study are still unpurified, it is expected that the isolation work carried out on them will afford substances with improved antibiotic property.

From the above results, it was evidenced that *G. orthoclada* displays antibacterial activity due to the presence of bioactive phytochemicals. A total of seventeen compounds were identified in the bioactive ethyl acetate-soluble extract using GC-MS technique. Antibacterial assays on these compounds could not be performed, but it is anticipated that some of them are partly responsible for the overall antibacterial activity of the ethyl acetate-soluble extract on the basis of literature data. For example,  $\beta$ -amyrin has been reported to have antibacterial activity [19]. Friedelin has been previously isolated from a related species *G. smeathmannii* and has displayed antibacterial activity against several Gram-positive and Gram-negative bacteria [20]. In addition, the antibacterial activity of the ethyl acetate-soluble and butanol-soluble extracts of *G. orthoclada* against *P. mirabilis* and *S. flexneri* may be due to the presence of lupeol which has been shown to inhibit the growth of these two bacteria [21]. The allelochemical 2,4-di-tert-butylphenol is a natural compound occurring in plants and microorganisms. It has demonstrated an anti-methicillin resistant *Staphylococcus aureus* activity [22].

#### 5. Conclusion

The present study demonstrates the antibacterial activity of *G. orthoclada* against the Gram-positive *Bacillus cereus* and the Gram-negative *Proteus mirabilis*, *Salmonella enterica* subsp., *Shigella flexneri* and *Pseudomonas aeruginosa* bacterial strains which are responsible for several pathogenesis. Seventeen chemical constituents were characterized in the bioactive ethyl acetate-soluble extract from *G. orthoclada* small branches using GC-MS technique. They are reported for the first time from this species and some of them have been indicated to display antibacterial activity. These findings provide an important basis for further investigations on this species which will focus on bio-guided isolation of antibacterial agents.

#### 6. Conflict of interests

Declared none

#### 7. Acknowledgement

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