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International Journal of Pharmacognosy & Chinese Medicine
ISSN: 2576-4772

Antiplasmodial Activity of Griffipavixanthone and Morelloflavone the Main Compounds from *Garcinia Chromocarpa* Engl. (*Clusiaceae*)

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Research Article

Volume 6 Issue 1

Received Date: April 12, 2022

Published Date: May 02, 2022

DOI: 10.23880/ipcm-16000225

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Abstract

Ethnopharmacological relevance: The genus *Garcinia* is a rich source of species mostly used in traditional medicine to treat a many diseases. Bioactive constituents from this genus have been used as antimicrobial, anticancer, antimalarial, anti-inflammatory, hepatoprotective and anti-HIV. According to these properties, major compounds of this plant have been evaluated for their antiplasmodial activity.

Materials and methods: The methanol extract of the G. chromocarpa stem bark was used in this study. The chromatographic fractionation of this latest led to the isolation of two major compounds, identified by NMR and MS spectroscopic techniques. The crude extract and pure compounds were compared using the *in vitro* evaluation of the malaria parasite growth inhibition by colorimetric method (p-LDH assay). Furthermore ABTS•+ and DPPH• scavenging activities and cytotoxicity through WST1 assay were evaluated.

Results: Based on the chromatographic profile two major compounds were isolated. They were characterized by spectroscopic analysis interpretation and comparison with the data in the literature: one biflavonoid (1) and one xanthone (2). The compound 2 (Griffipavixanthone, IC50 ± S.D = 5.80±0.32 µg/mL) was found to be more active against the malaria parasite, chloroquine-sensitive (3D7) strain of P. falciparum, than the compound 1 (Morelloflavone, IC_{50} ± S.D = 9.20±0.96 µg/mL). The antiradical activities connected to the ability to scavenge free radicals were observed for the ethanol crude extract of the stem bark, and the leave of *G. chromocarpa*.

Conclusions: This work demonstrated for the first time the antiplasmodial activity of *G. chromocarpa* and its isolated major compounds. These latest are known in the genus *Garcinia* but, here, firstly described in *G. chromocarpa Engl*.

Keywords: Anthraquinones; Malaria; Antioxydant; Traditional Medicine; Phytochemistry

Introduction

Most of central Africa is endowed with tropical forests presenting a huge vegetal biodiversity. These plants constitute a rich, but largely untapped pool of natural products with potential socio-economic benefits.

Malaria remains one of the most serious world health problems and the major cause of mortality and morbidity in the endemic regions. In 2011, the United Nations Secretary-General declared a goal of reducing malaria deaths to zero by 2015 [1].

The large tropical genus Garcinia Linn is well known as a source of xanthones, biflavonoids and benzophenones [2]. Seeds and bark are the most commonly used, and *G. kola, G. lucida and G. mannii* are the most frequently and intensively exploited species.

In our previous studies, fingerprints and the LC-DAD method were developed and validated for the quality control of an antimalarial improved traditional medicine containing *G. kola* [3].

Research on new antimalarial substances must therefore be continued and several strategies should be explored.

Natural products isolated from plants used in traditional medicine, which have potent anti-plasmodial action *in vitro*, represent potential sources of new anti-malarial drugs [4]. Plants continue to serve as the basis for many pharmaceuticals used today [5].

Species of genus *Garcinia* are rich sources of bioactive constituents with antimicrobial, anticancer, anti-inflammatory, hepatoprotective and anti-HIV activities [6]. To our knowledge, only few investigations have been performed on the phytochemical composition and biological activities of *Garcinia chromocarpa.*

In continuing efforts to discover natural antimalarial agents, methanol and dichloromethane extracts of *G. chromocarpa* was studied for the first time against malaria parasite, and its phytochemical study allowed the isolation of their major compounds.

Materials and Methods

Plant Materials

Plant materials of *G. chromocarpa* were collected in Kinshasa-DRC in September 2015 and authenticated by Mr Nlandu at the Institut National d'Etudes et de Recherches en Agronomie (INERA) of the University of Kinshasa-DRC, where herbarium specimen is kept with the Voucher Number: PROCUV 2738. The leaves and stem-bark of *G. chromcarpa* were oven-dried below 50 °C and powdered for analysis.

Chemicals

All solvents used were of analytical and HPLC grade and purchased from Merck VWR (Leuven, Belgium). 2,2'-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), 2-Aminoethyldiphenylborat, Artemisinin (purity 98%), Folin-Ciocalteu's phenol reagent, and sodium persulfate were purchased from Sigma (Bornem, Belgium). 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2′,7′-dichlorofluorescein-diacetate (DCFH-DA) were purchased from Eastman Kodak (Rochester, NY, USA). Water was treated using a Milli-Q water ultra-purification system before use.

Equipment

For fractionation, both column chromatography using silica gel (0.040-0.063 mm, Merck-Darmstadt) and preparative HPLC system with a Diode-Array Detection using a Hypersil ODS® RP18 column (2.5x25 cm; 12-15 µ, Merck packing system) equipped with an Armen pump and a Büchi fraction collector were used.

Mass spectrometer Time of flight QToF 2 with the Lock Spray option was conducted using negative electrospray ionisation in ToF MS, with the mass range between 50-1200 Dalton, and the cone voltage of 25 V. The ionisation mode (ESI negative) with MS^E, MS/MS was applied with collision energy (V) between 20-35 ramp, at the scan time of 0.1 sec, and the leucine enkephalin as a lock mass.

NMR spectra were recorded using a Bruker Avance II spectrophotometer equipped with a 5 mm TCI cryoprobe operating at 500 MHz for ^1H, employing a standard Bruker pulse sequences (Wisembourg, France).

Preparation of Extracts

Crude methanol extracts were prepared for preliminary *in vitro* antimalarial assay. The dried and grinded stem bark (20 g) were extracted with methanol (2 ×500 mL) at room temperature. After filtration and concentration *in vacuo*, the crude extracts obtained were submitted for anti-malarial test *in vitro*. On the other hand the ethanol extract was prepared for the antioxidant assay: 10 g of the stem bark powder of *G. chromocarpa* were extracted three times with 100 mL of ethanol and concentrated.

Isolation of Compounds

For the isolation process, at the beginning, standard screening tests were used to detect phytochemical groups in the plant extracts [7]t. After the phytochemical screening, 20 g of *G. chromocarpa* stem bark powder were macerated during 8 hours with 200 mL of methanol and then percolated. The concentrated extract was fractionated two times using preparative HPLC analysis.

On the first fractionation by preparative HPLC, 500 mg of the extract were filtered (Acrodisc PSF GXF/GHP 0.45 nm filter) and injected into a HPLC Varian chain using a Hypersil ODS® RP18 column eluted with 0.05% TFA in water (mobile phase A) and MeCN (mobile phase B) under the following gradient elution mode and modification time (in min) A: B (v/v) 100:0 (0 min); 97:3 (1 min); 60: 40 (45 min); 60:40 (55 min), and 100:0 (56 min). The volume of injection was 6 mL, and the flow rate of 30.0 mL/min. 79 fractions (F1-F79) of 10 mL were collected and assembled after TLC analysis using ethyl acetate/methanol/formic acid/water (20:2.5:0.5:2) as mobile phase and 2-Aminoethyl diphenylborinate-Polyethylene Glycol (1:5, v/v) in 100 mL methanol as a developer.

On the other hand column chromatography was used for fractionation. 100 g of *G. chromocarpa* stem bark powder were first macerated with 500 mL of methanol for 48 h, and evaporated under reduced pressure to give 19.7 g crude extract (yield =9.7%, w/w). 5 g of this extract were eluted over silica gel using the mixture hexane-ethyl acetate (100:0 → 50:50). 157 fractions of 20 mL were collected (f1-f157) and analyzed by TLC using ethyl acetate/methanol/water (100:13.5:10) as a mobile phase and sulfuric vanillin as a spray reagent. Based on TLC analysis of fractions collected, 15 fractions (f43-57) were assembled and eluted over sephadex using methanol 100 %.

In Vitro Anti-Plasmodial Assays

Culture of the chloroquine-sensitive (3D7) strain of *P. falciparum* was maintained as described by Frédérich, et al. [8]. The *in vitro* antiplasmodial assay was conducted by measuring the pLDH activity as described by Jansen, et al. [9].

IC_{50} values were calculated by linear regression from a set of eight two-fold dilutions concentrations tested for each extract. Each analysis was performed in triplicate (n = 3). According to the WHO guidelines and the previous work of our laboratory (Jonville et al., 2008; Jansen et al., 2010), antiplasmodial activity was classified as following: $IC_{50} \leq 15$ µg/ml: promising activity ; IC_{50} = 15–50 µg/ml: moderate activity; $IC_{50} > 50$ µg/ml: weak activity; $IC_{50} > 100$ µg/ml: inactivity.

In Vitro Cytotoxicity Assay

The cytotoxicity of the plant extracts and pure compounds was assessed against WI-38 normal human fœtal lung fibroblasts as described by Stévigny, et al., [10] and as reported in our previous work [11].

In Vitro Antioxidant Activity

As reported in our previous work [11]; *G. chromocarpa* ethanol extracts and gallic acid were solubilized in DMSO. Gallic acid was used as positive control and ABTS•+ and DPPH• scavenging activities of extracts were expressed as IC_{50} values. Each sample was measured in triplicate.

ABTS Radical Scavenging Capacity: ABTS•+ radicals were generated by mixing potassium persulfate (2.45 mM) with ABTS (7 mM) and kept overnight in the dark. Working solution of ABTS•+ was obtained by adding 100% methanol to have an absorbance of 0.81 (±0.02) at 734 nm. Then 0.02 mL of standard or sample extracts were mixed with 1.98 mL ABTS•+ radical solution and incubated for 30 min in the dark. Absorbance was monitored at 734 nm with a Spectrophotometer Hewlett Packard 8453.

DPPH Radical Scavenging Capacity: Absorbance of the solution of 0.4 % of DPPH was adjusted to 0.75 ± 0.03 at 517 nm using fresh 80% (v/v) methanol. Then 0.02 mL of standard or sample were mixed with 1.98 mL of DPPH• solution and incubated for 30 min in the dark. Absorbance was monitored at 517 nm with a Spectrophotometer Hewlett Packard 8453.

Analysis of Total Phenolic Contents: The total phenolic contents (TPC) of ethanol crude extracts of the stem bark, the root bark and the leave of G. chromocarpa were determined using Folin-Ciocalteu method.

Results and Discussion

Phytochemicals

The TLC analysis of the two parts of the plant showed that the leave and the bark have the similar profile (Figure 1). We obtained good separation with the *G. chromocarpa* bark extract, so our work were mainly continued on the stem bark.

Phytochemical screening of the methanol crude extracts from the leaves and the stem bark revealed the presence of anthraquinones, flavonoids, saponins and terpenes. These compounds were already identified in the others species of *Garcinia*. From the Garcina plants growing in Africa, more than 130 secondary metabolites have been isolated. Benzophenones, flavonoids, triterpenoids and xanthones are

identified such as the major constituents.

There is no more phytochemical study on *G. chromocarpa* in the literature. Cyanidin was already identified in sterm bark and seeds of *G. chromocarpa* from Cameroon.

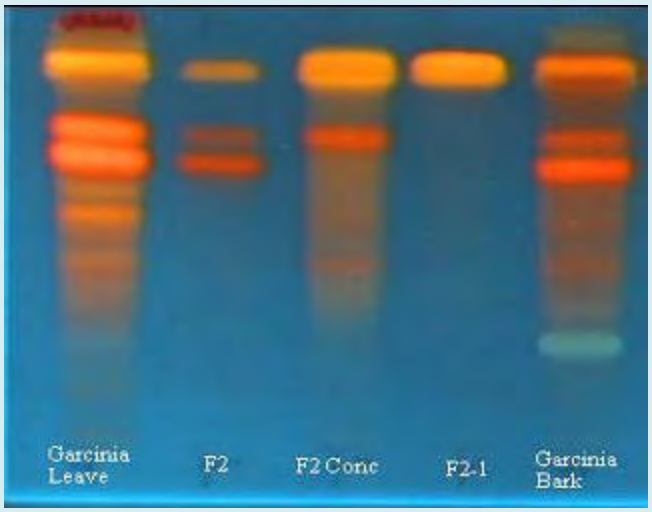

Figure 1*:* TLC of the Garcinia leaves and stem bark methanol extracts and fractions obtained by preparative HPLC (F2-1: Morelloflavone). Mobile phase: ethyl acetate /methanol/ formic acid/ water (20:5:1:4), spray reagent: 2-Aminoethyl diphenylborinate- Polyethylene Glycol (1:5, v/v) in 100 mL methanol.

Isolation and Structure Elucidation

As illustrated in figure 1, fraction (F2) from preparative HPLC fractionation, was concentrated to give F2 conc (9.38 mg). This latest was submitted to the second fractionation by preparative HPLC using the same system, to give a pure compound (F2-1= 3 mg). This pure compound has been identified by spectroscopic analysis. NMR and MS results were compared with the data in the literature. It was found to be a morelloflavone (compound 1).

Column chromatography fractionation with over Sephadex using methanol 100 % lead to the isolation of three compounds, but only one was pure (Figure 2). This compound has been identified as Griffipavixanthone (compound 2) by mean of NMR and MS, and compared with the data in the literature.

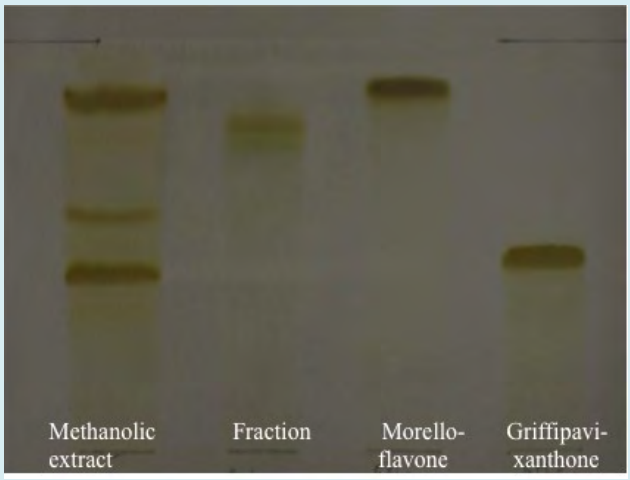

Figure 2: TLC of the G. chromocarpa stem bark methanol extract, fraction and isolated pure compounds. Mobile phase: ethyl acetate /methanol/ water (100:13, 5:10), spray reagent: ethanolic KOH 10 %.

Two major compounds were isolated from the methanolic crude extract of the *G. chromocarpa* stem bark (Figure 3). These compounds were (**1**) Morelloflavone a (3 → 8) biflavonoid and (**2**) Griffipavixanthone a bixanthone.

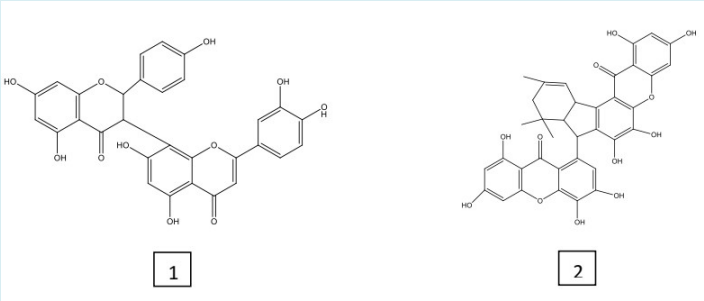

Figure 3: Chemical structures of two major compounds isolated from *G. chromocarpa.* (1): Morelloflavone and (2): Griffipavixanthone.

Griffipavixanthone is the bixanthone isolated from *Garcinia griffithii* [12]. Morelloflavone was already isolated from some species of the *Clusiacea* familly.

Antiplasmodial Activities of Crude Extracts and Pure Compounds

Crude extracts and isolated compounds were evaluated for their antiplasmodial activity *in vitro* against a chloroquine sensitive strain of *Plasmodium falciparum* (3D7) (Table 1). Artemisinin (98%, Sigma-Aldrich) was used as positive control.

Garcinia chromocarpa crude extracts	CH_2Cl_2 extracts IC50	MeOH extracts IC50	Yield CH_2Cl_2 extracts	Yield MeOH extracts
Stem bark	8.43±1.01	10.67±0.19	6.70%	15.40%
Leave	13.04±0.74	9.53±0.05	8.20%	26.20%
Garcinia chromocarpa Pure compounds	**IC50**			Yield (MeOH stem bark extract)
Compound 1 Morelloflavone (MM= 557)	9.2±0.96			3.70%
Compound 2 Griffipavixanthone (MM= 653)	5.80±0.323			1.90%

Table 1: In vitro antiplasmodial activity and yield (m/m) of crude extracts and pure compounds from G. chromocarpa (n = 3) against P. falciparum 3D7, IC_{50} ±S.D. (µg/mL).
Artemisinin: IC_{50} = 0.0072 ± 0.0018 µg/ml
Compound 2 was found to be more active than the compound 1.

Cytotoxicity Activity

The cytotoxicity test against the human non-cancer fibroblast cell line (WI38) through WST1 assay showed that the crude extracts and pure compounds had not any cytotoxicity, at the higher concentration tested of 100 µg/mL.

Antioxidant Activity

We evaluated the antioxidant potential of pure compounds and the ethanol crude extract of the stem bark, and the leave of *G. chromocarpa* using biochemical assays.

Scavenging of ABTS and DPPH radicals are the classic, simple and fast methods for evaluating radical- scavenging activity of pure compounds and complex samples [13].

The antioxidant activity of ethanolic extract and pure compounds was effective in the reduction of stable radicals $ABTS^{\bullet+}$ and $DPPH^{\bullet}$ (Table 2). These assays showed that pure compounds, the ethanol crude extract of the stem bark, and the leave of *G.* chromocarpa have the ability to scavenge free radicals connected with their IC_{50} values. When comparing crude extracts, the sterm bark was more active than leaves and for the pure compounds, griffipavixanthone was more active than morelloflavone.

Garcinia chromocarpa ethanol crude extracts and pure compounds.	TPC (GAE)	IC50 (µg/mL) ABTS	DPPH
Stem bark	157.73 ± 0.58	7.6 ± 0.9	15.89 ± 1.53
Leave	114.40 ± 0.46	11.12 ± 1.41	30.83 ± 4.8
Morelloflavone	-	50.85 ± 4.6	79.43 ± 18.97
Griffipavixanthone	-	2.05 ± 1.27	4.54 ± 1.06
Gallic acid (positive control)	-	0.71 ± 0.08	a. 0.10

Table 2: Total phenolic content (TPC) expressed in equivalent mg of gallic acid by g of crude material and IC_{50} ±S.D (n=6) expressed in µg/mL.

The high antioxidant activity of phenolic substances is often attributed to their -OH moieties, which are potent H donors because electron delocalisation across the molecule efficiently stabilises the resulting phenoxy radicals. For compounds such as xanthone, which is one of the cases in this work, another important feature is the planarity of the molecule, which permits conjugation and electron delocalisation. These factors are associated with an increase

in radical stability. Intramolecular H-bonds between neighbouring –OH and C=O groups increase aromatic-ring conjugation and raise the H- donation power of the molecule, because the resulting radicals are more delocalised. On the second case, the presence of a dihydroxy group ortho to the C=O moiety of the biflavonoids also increases antioxidant activity [14]. Griffipavixanthone and Morelloflavone compounds exhibit powerful antioxidant activities related to these structural features. Morelloflavone was found to be the most active radical scavenger of rotameric (3 → 8) biflavonoids from the bark of *Garcinia hombroniana* [6].

Results from the quantitative determination of total phenolic content are summarized in Table 2. Total polyphenol contents were calculated as gallic acid equivalents in grams per 100 grams of dry weight (g GAE/100g DW) and varied significantly ($p < 0.05$) between the leaves and sterm barks. The sterm barks showed the highest value of total phenol than the leaves. These results showed that there is a correlation between total phenols content and antioxidant capacities measured by ABTS and DPPH assays.

Conclusion

G. chromocarpa has been tested for the first time on *P. falciparum* and showed a significant activity. To the best of our knowledge any other phytochemical study on this plant could not be found in the literature.

As described in the table 1, the isolated compound (1) showed a good antiplasmodial activity, similar to the one of the initial methanol crude extract.

Compound 2 exhibited a higher antiplasmodial activity in vitro with IC_{50} ±S.D. of 5.80±0.32 µg/mL. This result is in accordance with data in the literature, in which we found that bixanthones exhibited potent bioactivities and promising pharmacological profiles such as cancer chemopreventive, anthelmintic, antimalarial, antitubercular and so on [15].

A high antioxidant activity of 2.05 ± 1.27 µg/mL (ABTS•+) and 4.54 ± 1.06 µg/mL (DPPH•) was observed for the compound (**2**), see Table 2. This compound has been described for the first time in the literature as a novel cytotoxic bixanthone isolated from Garcinia griffithii and Garcinia pavifolia, where the name griffipavixanthone has come from [16-18].

In this work, Griffipavixanthone and Morelloflavone were isolated for the first time from G. chromocarpa. Their antiplasmodial activity and the absence of cytotoxicity at the highest tested dose (100 µg/mL) have been demonstrated. This aspect could justify their use in traditional medicine but further studies are needed, especially *in vivo* studies to achieve improved traditional medicines.

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