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A Rapid Determination and Quantification of Three Biologically Active Polyisoprenylated Benzophenones using Liquid Chromatography-Tandem Mass Spectrometry (MRM) Method in Five *Garcinia* species from Cameroon

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Following investigation of *Garcinia* genus, a sensitive, rapid and simple reversed-phase high performance liquid chromatography-electrospray ionization mass spectrometry method has been developed for the identification and quantification of three polyisoprenylated benzophenones, garcinol (1), isogarcinol (2) and 7-*epi*-clusianone (3), in the extracts of five *Garcinia* species from Cameroon. The separation of those compounds was achieved on a RP-18 column using a solvent system consisting of a mixture of acetonitrile-water-formic acid as a mobile phase in a gradient elution mode. The identification of the three compounds was determined on a triple quadrupole mass spectrometer with ESI interface operating in the negative mode. A multiple reaction monitoring (MRM) method was developed for the quantification of these polyisoprenylated benzophenones in the extracts of the *Garcinia* species. The method was validated through intra- and inter-day precision, with the relative standard deviation (RSD) less than 6%, limits of detection (LOD) and limits of quantification (LOQ) <1 ng. Overall recoveries ranged from 94% to 104%, with RSDs ranging from 0.8% to 4.5%. The results indicated that the fruits of *G. preussii* and the roots of *G. brevipedicellata* are good source of garcinol (1) and isogarcinol (2) respectively.

Keywords: *Garcinia* species, Garcinol, Isogarcinol, 7-*epi*-Clusianone, HPLC/ESI-MS/MS analysis, Multiple Reaction Monitoring.

Plants of the genus *Garcinia* (Clusiaceae) are used worldwide in traditional medicine for the treatment of diseases and are well known to be rich sources of secondary metabolites such as xanthenes, bioflavonoids, triterpenoids and benzophenones [1-6]. Phenolic constituents from *Garcinia* species have been reported to exhibit multiple pharmacological activities including anti-inflammatory, antimicrobial, anti-HIV, antibacterial, antioxidant and anticancer [7-13]. In Cameroon, the genus *Garcinia* has 21 species, the plant parts such as fruit, leaves, bark, root and stem are used in traditional medicine [14]. Phytochemical investigations revealed that *Garcinia* species from Cameroon are rich sources of polyisoprenylated benzophenones [10, 15-18].

As part of our continuing phytochemical investigation on *Garcinia* plants found in Cameroon [17, 19-20], we have developed and validated a highly sensitive and efficient method using HPLC/ESI-MS/MS in MRM mode for rapid determination of three benzophenones, garcinol (1), isogarcinol (2) and 7-*epi*-clusianone (3) (Figure 1) in various organs of five *Garcinia* species from Cameroon, *Garcinia lucida*, *Garcinia polyantha*, *Garcinia ovalifolia*, *Garcinia preussii* and *Garcinia brevipedicellata*.

Garcinol (1) exerts anti-proliferative, pro-apoptotic, cell-cycle regulatory and anti-angiogenic effects on oral cancer cells through inhibition of NF- κ B and COX-2 [21]. Isogarcinol (2), a natural compound is a new immunosuppressant and has anti-inflammatory effects [22]. 7-*epi*-clusianone (3) shows antiproliferative activity against cancer cell lines [23] and has influence on proliferation, clonogenic activity cell cycle progression and induction of apoptosis in two glioblastoma cell lines [24].

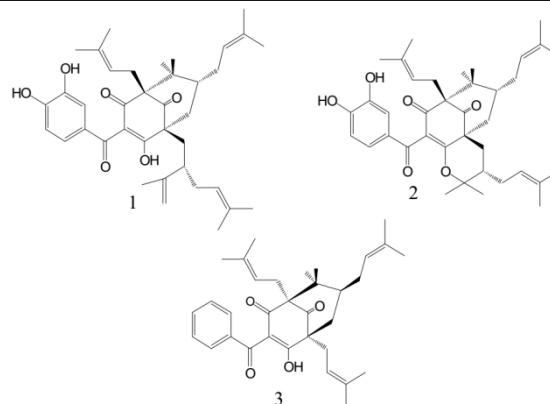


Figure 1: Structures of compounds 1-3 isolated from *Garcinia preussii*.

Clearly, these compounds have considerable clinical potential but they suffer from a lack of sensitive and reliable way to quantify these molecules in the extracts of the *Garcinia* species. To resolve these problems, a multiple reaction monitoring (MRM) method was developed for the quantification of these polyisoprenylated benzophenones.

Samples of different parts from the five species were extracted with methanol. HPLC/ESI-MS/MS analysis was performed in order to determine the quantification of the three polyisoprenylated benzophenones obtained from the fruits of *G. preussii*. The ESI mode was preferred over the APCI mode because it was found to be more sensitive during optimization of the HPLC-MS/MS method.

Table 1: Linearity of standard curves, LOD, LOQ and qualifier and quantifier ions for garcinol (1), isogarcinol (2) and 7-*epi*-clusianone (3).

Name of compounds and [M ⁺ H], m/z	Retention time (min)	Regression equation	R ²	Linear range ng/mL	LOQ ng	LOD ng	Quantifier	Qualifier
1: Garcinol, 601.5	6.62	y= 67.912x-135.18	0.9978	5.00-150.00	1	1	601.5→108.8	601.5→177.2
2: Isogarcinol, 601.5	4.89	y= 47.434x-19.949	0.9985	5.00-150.00	0.5	0.1	601.5→108.8	601.5→132.6
3: 7- <i>epi</i> -clusianone, 501.5	7.40	y= 108.09x-410.09	0.9991	5.00-150.00	1	0.5	501.5→145.02	501.5→417.30

Table 2: Intra- and inter-day precisions of the three benzophenone standards by HPLC/ESI-MS/MS.

Benzophenone	Spiked Conc. (ng/mL)	Intraday (n=6)			Interday (n=6)		
		Measured Conc. (ng/mL)	% Accuracy	% RSD	Measured Conc. (ng/mL)	% Accuracy	% RSD
1: Garcinol	5	5.1±0.24	101.1±4.04	4.0	5.2±0.14	103.3±2.26	2.2
	10	10.4±0.32	102.5±2.10	2.1	10.7±0.47	104.8±3.16	3.0
	50	50.9±0.42	103.6±1.54	1.5	51.0±0.37	104.2±1.84	1.8
2: Isogarcinol	5	5.2±0.16	104.3±2.80	2.8	5.3±0.31	102.6±5.61	5.5
	10	10.7±0.47	104.8±3.16	3.0	10.4±0.32	102.5±2.11	2.1
	50	50.6±0.41	102.9±3.45	3.3	51.0±0.29	103.9±3.15	3.1
3: 7- <i>epi</i> -clusianone	5	5.1±0.17	102.1±3.15	3.1	5.2±0.29	103.3±4.89	4.7
	10	10.4±0.34	103.7±3.45	3.3	10.6±0.72	104.2±4.81	4.6
	50	50.9±0.42	103.7±0.44	0.5	51.0±0.47	103.5±1.85	1.8

Table 3: The accuracy HPLC/ESI-MS/MS method for quantitative analysis of three benzophenones when three concentrations of standard benzophenones were spiked into *Garcinia prussii* bark.

Benzophenones	Measured concentrations of benzophenones (ng/mL)(n=4)		Recovery (%)	RSD (%)
	Spiked	Detected		
1: Garcinol	5	5.2±1.03	104±3.41	3.5
	10	10.1±0.21	101±1.42	1.4
	50	49.8±0.19	99.6±0.74	0.8
2: Isogarcinol	5	4.9±0.27	98±4.46	4.5
	10	10.4±0.21	104±1.43	1.4
	50	51.3±0.37	102.6±1.55	1.5
3: 7- <i>epi</i> -clusianone	5	5.0±0.13	100±2.09	2.0
	10	9.4±0.46	94±3.00	3.0
	50	49.1±0.19	98.2±0.77	0.8

In the present work, garcinol (1), isogarcinol (2) and 7-*epi*-clusianone (3) could be easily separated by the developed method. The presence of the selected compounds was detected in the MRM mode by mass fragmentography using two MRM transitions. Selection of transitions and their setting were determined using a 10 ng/mL solution. Standards were injected using HPLC without a column controlled by Agilent Technologies MassHunter workstation software for qualitative optimization. The method was validated for parameters such as linearity, precision and accuracy based on the International Conference on Harmonization (ICH) Guidelines [25]. Standard curves of the three benzophenones had acceptable linearity in the range of 5.00 – 150.00 ng/mL with correlation coefficients exceeding 0.9978. The minimum concentration levels at which analysts could be reliably detected (LOD) and quantified (LOQ) were 0.1 to 1 ng and 0.5 to 1 ng, respectively (Table 1). The %RSDs of intra- and inter-day precisions were less than 4% and 5% respectively (Table 2). Analytical recovery was performed using the blank matrix (bark of *G. prussii*) spiked with the three standards at three concentrations, 5, 10 and 50 ng/mL. The overall analytical recoveries of garcinol (1), isogarcinol (2) and 7-*epi*-clusianone (3) ranged from 94% to 104% with RSDs ranging from 0.8 – 4.5% (Table 3).

Thus, three polyisoprenylated benzophenones, garcinol (1), isogarcinol (2) and 7-*epi*-clusianone (3), could be separated, identified and quantified in five species of *Garcinia* using the developed method. The results were summarized in Table 4.

Garcinol (1) is detected in the different parts of each *Garcinia* species (Fruits, leaves, bark, roots) except in the bark of *G. prussii*. The higher concentration is found in the fruits and the leaves of *G. prussii* with 85.8 µg/mL and 13.1 µg/mL respectively, and also in

Table 4: Content (µg/mL) of garcinol (1), isogarcinol (2) and 7-*epi*-clusianone (3) in extracts of five *Garcinia* species (mean±SD, n=3).

Species	Parts	1: Garcinol ^a	2: Isogarcinol ^a	3: 7- <i>epi</i> -clusianone ^a
<i>G. prussii</i>	Fruits	85.8	0.3	0.7
	Leaves	13.1	0.2	0.3
	Bark	n.d.	n.d.	n.d.
<i>G. brevipedicellata</i>	Roots	8.3	4.6	0.04
	Bark	29.1	1.8	0.2
<i>G. lucida</i>	Leaves	9.6	0.3	n.d.
	Bark	19.4	0.4	0.04
<i>G. polyantha</i>	Fruits	3.5	0.1	n.d.
	Bark	2.7	0.2	n.d.
<i>G. ovalifolia</i>	Leaves	0.1	0.01	n.d.
	Bark	7.1	1.1	0.02

^aValues are mean ±CV% of four replicates in µg/mL, n.d.: not detected.

the bark of *G. brevipedicellata* (29.1 µg/mL) and *G. lucida*, (19.4 µg/mL) as compared to other plants organs (Table 4). This compound (1) is reported in very low amounts in the leaves of *G. ovalifolia* (0.1 µg/mL).

The concentration of isogarcinol (2) is the most important in *G. brevipedicellata* roots, *G. brevipedicellata* bark and *G. ovalifolia* bark with 4.6, 1.8 and 1.1 mg/mL respectively (Table 4). In comparison with the root extract, the contents of isogarcinol (2) in other plant parts (bark, leaves, roots, fruits) were very low (<1.9 µg/mL). The other organs plants contain low concentrations of the compound (2) whereas bark of *G. prussii* contains at best only traces.

The presence of 7-*epi*-clusianone (3) was only reported in low amounts between 0.7 and 0.02 µg/mL. The higher concentration is found in *G. prussii* fruits and leaves with 0.7 µg/mL and 0.3 µg/mL respectively. This compound (3) is absent or present at trace levels in *G. polyantha*, the leaves of *G. ovalifolia* and *G. lucida* and the bark of *G. prussii*.

Results indicated that the fruits of *G. prussii* could be a good source of garcinol (1) (85.8 µg/mL) and contain some 7-*epi*-clusianone (3) (0.7 µg/mL), whereas the roots of *Garcinia brevipedicellata* are a good source of isogarcinol (2) (4.6 µg/mL).

An efficient, sensitive and selective HPLC/ESI-MS/MS analytical method was developed for the identification and quantification of biologically active benzophenones in five *Garcinia* species from Cameroon. The developed method can be used to determine the concentration of polyisoprenylated benzophenones in different parts of many species of *Garcinia* for the selection of best plant organ to

be extracted for preparation of herbal formulations. This study describes the first report on comprehensive quantitative analysis of the garcinol (**1**), isogarcinol (**2**) and 7-*epi*-clusianone (**3**) in five *Garcinia* species of Cameroon, *Garcinia lucida*, *Garcinia polyantha*, *Garcinia ovalifolia*, *Garcinia preussii* and *Garcinia brevipedicellata*. This method can also be used for the screening and the quantification of plant extracts containing the above molecules.

Experimental

Plant material: *G. lucida*, *G. polyantha*, *G. ovalifolia*, *G. preussii* and *G. brevipedicellata* were collected in July 2008 at Ngoumé, located in the central part of Cameroon. The botanical identification was performed by Mr. Nana Victor, a botanist from the Cameroon National Herbarium (CNH), Yaoundé (Cameroon), where voucher specimens (55520/HNC, 50772/HNC, 30741/HNC, 30778/HNC and 45391/HNC for *G. lucida*, *G. polyantha*, *G. ovalifolia*, *G. preussii* and *G. brevipedicellata* respectively) were deposited.

Compound isolation from the fruits of *Garcinia preussii*: The air-dried powdered fruits (2 kg) were extracted by maceration with MeOH (3 x 3 L) for up to 48 h each at room temperature. After filtration, the MeOH extract was evaporated to dryness. The residue (310 g) was then dissolved in 800 mL of water and successively partitioned with hexane and EtOAc to give 90 g and 70 g, respectively. A portion of the hexane extract (50 g) was chromatographed over a silica gel column (20-63 μ m, 5 x 60 cm) using an EtOAc/Hexane step gradient (0:100-100:0 in 10% steps) to afford nine fractions (F1-F9). Fraction 2 (14 g) was separated by MPLC on LiChroprep RP-18 (40-63 μ m, 5 x 46 cm), with a MeOH/H₂O step gradient (60:40-100:0 in 10% steps, 3 mL/min) to afford five subfractions (F21-F25). This separation yielded garcinol (**1**, 7 g) and isogarcinol (**2**, 1.5 g) from F22 and F21 respectively. Further purification of F25 was carried out over a reverse phase C₁₈ column and separated with gradient mixtures of MeOH/H₂O (80:20 – 100:0 in 10% steps) to afford nine fractions (F251-F259). F258 provides 7-*epi*-clusianone (**3**, 250 mg). The structures and purity of isolated compounds were confirmed by NMR and MS as described previously [12].

Preparation of different extracts of *Garcinia* species for quantitative analysis: Samples of leaves, fruits, bark and roots from the five species were dried separately at 40°C for 3 days before milling into a fine powder. The dried powder was extracted with methanol during 6h. The methanol extract was filtered through filter paper and concentrated using rotary evaporator. Dried residues were weighed accurately, dissolved in 1 mL of methanol and sonicated for 15 min. The solutions were filtered through a 0.45 μ m syringe and then diluted with acetonitrile to the final working concentrations. All samples were prepared in triplicate.

HPLC/ESI-MS/MS Analysis: Instrument. LC analyses were carried out using a HP 1100 (Agilent technology) with a quattro micro mass spectrometer (Waters) as detector equipped with an electrospray ionization source and controlled by Masslynx software. The separation was performed on Kinetex 2.6 μ m C₁₈ 100 x 3.0 mm equipped with a guard column at 27°C.

Stock solutions of standards: Stock solutions (10.0 μ g/mL) garcinol (**1**), isogarcinol (**2**), 7-*epi*-clusianone (**3**) were prepared in volumetric flasks. Standard working solutions were then obtained by making appropriate dilutions of stock solutions using the mobile phase. The concentrations utilized for the preparation of a five point calibration curve ranged between 5.0 and 150.0 ng/mL for garcinol

(**1**), isogarcinol (**2**) and 7-*epi*-clusianone (**3**). The stock and working solutions were stored at -4°C.

HPLC/ESI-MS/MS conditions: Samples (10 μ L) were injected in triplicate. The elution was carried out with a binary mobile phase consisting of a gradient of MeCN + 0.1% formic acid (eluent B) and H₂O + 0.1% formic acid (eluent A) starting from 70% to 100% (eluent B) in 7 minutes at a flow rate of 0.5 mL/min at 27°C. The compounds were detected at the following transitions: (m/z) 601.5 \rightarrow 108.8 (quantifier) and 601.5 \rightarrow 177.2 (qualifier) for garcinol (**1**); 601.5 \rightarrow 108.8 (quantifier) and 601.5 \rightarrow 132.6 (qualifier) for isogarcinol (**2**); 501.5 \rightarrow 145.02 (quantifier) and 501.5 \rightarrow 417.3 (qualifier) for 7-*epi*-clusianone (**3**). Maximum resolution was obtained for all the molecules at fragmentor voltage of 150 V and collision energy of 17 eV. Identification and quantification of the three compounds were done on the basis of retention time and comparison of the presence of peaks in the MRM of sample and the standard spectra.

Optimization of MS conditions: The experiment was conducted in both polarities (positive and negative) of the atmospheric pressure chemical ionization (APCI) and electrospray ionization (ESI) modes. The response was found to be best in the negative ESI mode. For optimization of MS conditions, the fragmenting voltage, capillary voltage, collision energy and sensitivity for the fragmented ions of the three studied compounds were investigated. The optimal value of the fragmenting voltage was found to be 150 V under (-) ESI conditions, producing predominantly the analyte. The protonated species [M-H]⁺ of each compounds were selected as precursor ions. Quantification was performed in the MRM mode with the ion transitions m/z 601.5/108.8 as quantifier and m/z 601.5/177.2 as qualifier for garcinol (**1**) and isogarcinol (**2**) and with the ion transitions m/z 501.5/145.02 as quantifier and m/z 501.5/417.3 as qualifier for 7-*epi*-clusianone (**3**). (Table 1)

Preparation of calibration curves for standards: Before making a calibration curve, one blank injection was run to check the noise level of the system. Stock solutions were appropriately diluted for making a five point calibration curve for the three investigated molecules. The calibration equation of garcinol (**1**), isogarcinol (**2**) and 7-*epi*-clusianone (**3**) was obtained by plotting LC/MS peak area (y) versus the concentration (x, ng/mL) of calibrators as y = 67.912x-135.18 (R²= 0.9978), y= 47.434x-19.949 (R²= 0.9985) and y= 108.09x + 410.09 (R²= 0.9991), respectively. The equations showed very good linearity over the range used.

Method Validation: The established HPLC/ESI-MS/MS method is defined by repeatability, precision and reproducibility and was evaluated by linearity, LOD, LOQ, intraday and interday precision, and accuracy tests. The repeatability was evaluated by running the analyses in a single day at different times, involving the same instrument, laboratory and operator. The precision was estimated by evaluating intra-batch precision and inter-batch variability with respect to retention time and peak area of garcinol (**1**), isogarcinol (**2**) and 7-*epi*-clusianone (**3**). Intra-batch precision was determined after 8, 12 and 16 h from a set of six replicates in a single day carried out by the same operator. Inter-batch precision was evaluated from a set of six replicates analysed with the same mixture of standards on days 1, 3 and 6. The calibration curves were constructed with five concentrations of benzophenones standards in duplicate. Matrix effects and accuracy were determined using *G. preussii* bark by spiking with 1 mL of 70% MeOH containing appropriate concentrations of standards (Table 3), and repeated in four replicate.

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