Phytochemistry 70 (2009) 621–627

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Antiparasitic activity of prenylated benzoic acid derivatives from *Piper* species

Ninoska Flores [a,b], Ignacio A. Jiménez [a], Alberto Giménez [b], Grace Ruiz [b], David Gutiérrez [b], Genevieve Bourdy [b], Isabel L. Bazzocchi [a,*]

[a] *Instituto Universitario de Bio-Orgánica "Antonio González", Universidad de La Laguna, Avda. Astrofísico, Francisco Sánchez 2, La Laguna, 38206 Tenerife, Canary Islands, Spain*

[b] *Instituto de Investigaciones Fármaco Bioquímicas, Facultad de Ciencias Farmacéuticas y Bioquímicas, Universidad Mayor de San Andrés, Avda. Saavedra 2224, Miraflores, La Paz, Bolivia*

ARTICLE INFO

Article history:
Received 2 July 2008
Received in revised form 4 March 2009
Available online 8 April 2009

Keywords:
Piper heterophyllum
P. aduncum
Prenylated benzoic acids
Leishmanicidal
Trypanocidal
Antiplasmodial

ABSTRACT

Fractionation of dichloromethane extracts from the leaves of *Piper heterophyllum* and *P. aduncum* afforded three prenylated hydroxybenzoic acids, 3-[(2*E*,6*E*,10*E*)-11-carboxy-3,7,15-trimethyl-2,6,10,14-hexadecatetraenyl)-4,5-dihydroxybenzoic acid, 3-[(2*E*,6*E*,10*E*)-11-carboxy-13-hydroxy-3,7,15-trimethyl-2,6,10,14-hexadecatetraenyl]-4,5-dihydroxybenzoic acid and 3-[(2*E*,6*E*,10*E*)-11-carboxy-14-hydroxy-3,7,15-trimethyl-2,6,10,15-hexadecatetraenyl]-4,5-dihydroxybenzoic acid, along with the known compounds, 4,5-dihydroxy-3-(*E*,*E*,*E*-11-formyl-3,7,15-trimethyl-hexadeca-2,6,10,14-tetraenyl)benzoic acid (arieianal), 3,4-dihydroxy-5-(*E*,*E*,*E*-3,7,11,15-tetramethyl-hexadeca-2,6,10,14-tetraenyl)benzoic acid, 4-hydroxy-3-(*E*,*E*,*E*-3,7,11,15-tetramethyl-hexadeca-2,6,10,14-tetraenyl)benzoic acid, 3-(3,7-dimethyl-2,6-octadienyl)-4-methoxy-benzoic acid, 4-hydroxy-3-(3,7-dimethyl-2,6-octadienyl)benzoic acid and 4-hydroxy-3-(3-methyl-1-oxo-2-butenyl)-5-(3-methyl-2-butenyl)benzoic acid. Their structures were elucidated on the basis of spectroscopic data, including homo- and heteronuclear correlation NMR experiments (COSY, HSQC and HMBC) and comparison with data reported in the literature. Riguera ester reactions and optical rotation measurements established the compounds as racemates. The antiparasitic activity of the compounds were tested against three strains of *Leishmania* spp., *Trypanosoma cruzi* and *Plasmodium falciparum*. The results showed that 3-(3,7-dimethyl-2,6-octadienyl)-4-methoxy-benzoic acid exhibited potent and selective activity against *L. braziliensis* (IC_{50} 6.5 μg/ml), higher that pentamidine used as control. Moreover, 3-[(2*E*,6*E*,10*E*)-11-carboxy-3,7,15-trimethyl-2,6,10,14-hexadecatetraenyl)-4,5-dihydroxybenzoic acid and 4-hydroxy-3-(3-methyl-1-oxo-2-butenyl)-5-(3-methyl-2-butenyl)benzoic acid showed moderate antiplasmodial (IC_{50} 3.2 μg/ml) and trypanocidal (16.5 μg/ml) activities, respectively.

© 2009 Elsevier Ltd. All rights reserved.

1. Introduction

Parasitic diseases, including leishmaniasis, malaria and chagas, are serious problems for public health in the world, especially in tropical and subtropical regions. In the absence of effective vaccines, chemotherapy still plays a critical role in treating these diseases, but in many cases current therapies are inadequate and in some the situation is deteriorating because of drug resistance. Therefore, there is an urgent need to search for novel, effective and safe drugs for the treatment of these diseases. One of the main opportunities is through the discovery of new antiparasitic agents from natural origins (Ioset, 2008).

The genus *Piper* (Piperaceae) is a pantropical group with nearly 2000 species, constituting an important element of montane and lowland forests (Quijano-Abril et al., 2006). A recent phylogenetic analysis for the genus suggests three major clades in *Piper*, representing three large geographical regions: America (1300 sp.), Asia (600 sp.) and the South Pacific (100 sp.) (Jaramillo and Manos, 2001). Several *Piper* species have been included in the system of traditional medicine in Latin America (Gupta, 1995). Therefore, the leaves of *Piper hispidum* and *P. elongatum* are used as poultices for healing wounds and to treat the symptoms of cutaneous leishmaniasis "Uta" (Estevez et al., 2007), and the leaves of *P. aduncum* are used for inflammation, and as antiseptic (Orjala et al., 1994). Phytochemical studies of *Piper* species (Parmar et al., 1997) describe the isolation of metabolites with antifungal (Terreaux et al., 1998; Lago et al., 2004), antibacterial (Ramji et al., 2002), insecticidal (Siddiqui et al., 2004), cytotoxic (Chen et al., 2003) and antioxidant (Yamaguchi et al., 2006) properties. Regarding antiprotozoal metabolites from *Piper* species, flavanones (Portet et al., 2007), chalcones and dihydrochalcones (Torres-Santos et al., 1999; Hermoso et al., 2003; Flores et al., 2007), neolignans (Luize et al., 2006) and alkaloids (Rukachaisirikul et al., 2004) have been described.

* Corresponding author. Tel.: +34 922 318594; fax: +34 922 318571.
E-mail address: ilopez@ull.es (I.L. Bazzocchi).

0031-9422/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phytochem.2009.03.010

Previous investigations into *P. aduncum* report essential oils (Rali et al., 2007; Vila et al., 2005), chromenes (Moreira et al., 1998), prenylated benzoic acids (Orjala et al., 1993; Baldoqui et al., 1999; Lago et al., 2004) and dihydrochalcones (Orjala et al., 1994). However, the chemical constituents of *P. heterophyllum* have not been described.

Several *Piper* species are claimed to be used in traditional medicine for the treatment of parasitic diseases, and benzoic acid derivatives are frequent metabolites in this genus. However, a few reports on the antiparasitic activity of this class of compounds have been published (Lopes et al., 2008; Flores et al., 2008). As part of our research into the Bolivian *Piper* species, we have carried out a phytochemical analysis of the leaves of *Piper heterophyllum* and *P. aduncum*. We report herein the isolation and structural elucidation of three new prenylated benzoic acid derivatives (**1**–**3**), along with six known compounds (**4**–**9**). Their structures were elucidated by extensive spectroscopic analysis, and comparison with data reported in the literature. Concerning the absolute configuration of the stereogenic center in compounds **2** and **3**, Riguera's method (Seco et al., 2004), a modified Mosher ester procedure, together with optical rotation measurements established the new compounds as racemates. In the search for new antiparasitic agents, the compounds have been tested for their leishmanicidal, trypanocidal and antiplasmodial activities.

2. Results and discussion

Repeated chromatography of the dichloromethane extracts of the leaves of *P. heterophyllum* and *P. aduncum* on silica gel and Sephadex-LH-20, afforded three new prenylated hydroxybenzoic acid derivatives (**1–3**). In addition, six known compounds were isolated and identified by comparison of their spectral data with those reported in the literature as 4,5-dihydroxy-3-(*E*,*E*,*E*-11-formyl-3,7,15-trimethyl-hexadeca-2,6,10,14-tetraenyl)benzoic acid (arieianal) (**4**) (Green et al., 1999), 3,4-dihydroxy-5-(*E*,*E*,*E*-3,7,11,15-tetramethyl-hexadeca-2,6,10,14-tetraenyl)benzoic acid (**5**) (Seeram et al., 1996), 4-hydroxy-3-(*E*,*E*,*E*-3,7,11,15-tetramethyl-hexadeca-2,6,10,14-tetraenyl)benzoic acid (**6**) (Maxwell and Rampersad, 1989), 3-(3,7-dimethyl-2,6-octadienyl)-4-methoxy-benzoic acid (**7**) (Baldoqui et al., 1999), 4-hydroxy-3-(3,7-dimethyl-2,6-octadienyl)benzoic acid (**8**) (Seeram et al., 1996) and 4-hydroxy-3-(3-methyl-1-oxo-2-butenyl)-5-(3-methyl-2-butenyl)benzoic acid (**9**) (Orjala et al., 1993).

The molecular formula $C_{27}H_{36}O_6$ assigned to compound **1** was determined by analysis of the ^{13}C NMR data associated with HR ESI(+)MS. The IR spectrum indicated the presence of hydroxyl group (3407 cm^{-1}), carboxyl acid (3520–2560 and 1681 cm^{-1}) and aromatic ring (1612 and 775 cm^{-1}). The ^1H NMR (Table 1) spectrum exhibited signals for two aromatic protons at δ 7.44 (*d*, *J* = 2.8 Hz) and δ 7.47 (*d*, *J* = 2.8 Hz), suggesting a 1,3,4,5-tetrasubstituted aromatic ring in the molecule. The spectrum also displayed signals for four vinyl methyl groups at δ 1.56, 1.58, 1.65 and 1.72; seven allylic methylene groups, six of them as multiplets at δ 2.05–2.13 (8H) and 2.19–2.28 (4H) and one occurred as a doublet at δ 3.36 (2H, *J* = 6.7 Hz). Additionally, signals for four olefinic protons at δ 5.11 (2H, *m*), 5.31 (1H, *t*, *J* = 6.7 Hz) and 6.84 (1H, *t*, *J* = 7.6 Hz), were observed. The ^{13}C NMR (Table 1) spectrum exhibited signals for 27 carbon atoms, showing signals in the low-field region corresponding to two carboxyl acids (δ 171.3 and 172.6) and six aromatic carbons (δ 114.4, 120.2, 124.2, 127.5, 142.8 and 147.7), along with signals for eight olefinic and eleven sp^3-hybridized carbons. All these data suggested that **1** is a benzoic acid derivative with a geranylgeranyl chain. The assignment of the ^1H and ^{13}C NMR data were based on 2D experiments, and comparison with data reported for benzoic acid analogues (Green et al., 1999). The connectivity of aromatic and aliphatic moieties was revealed

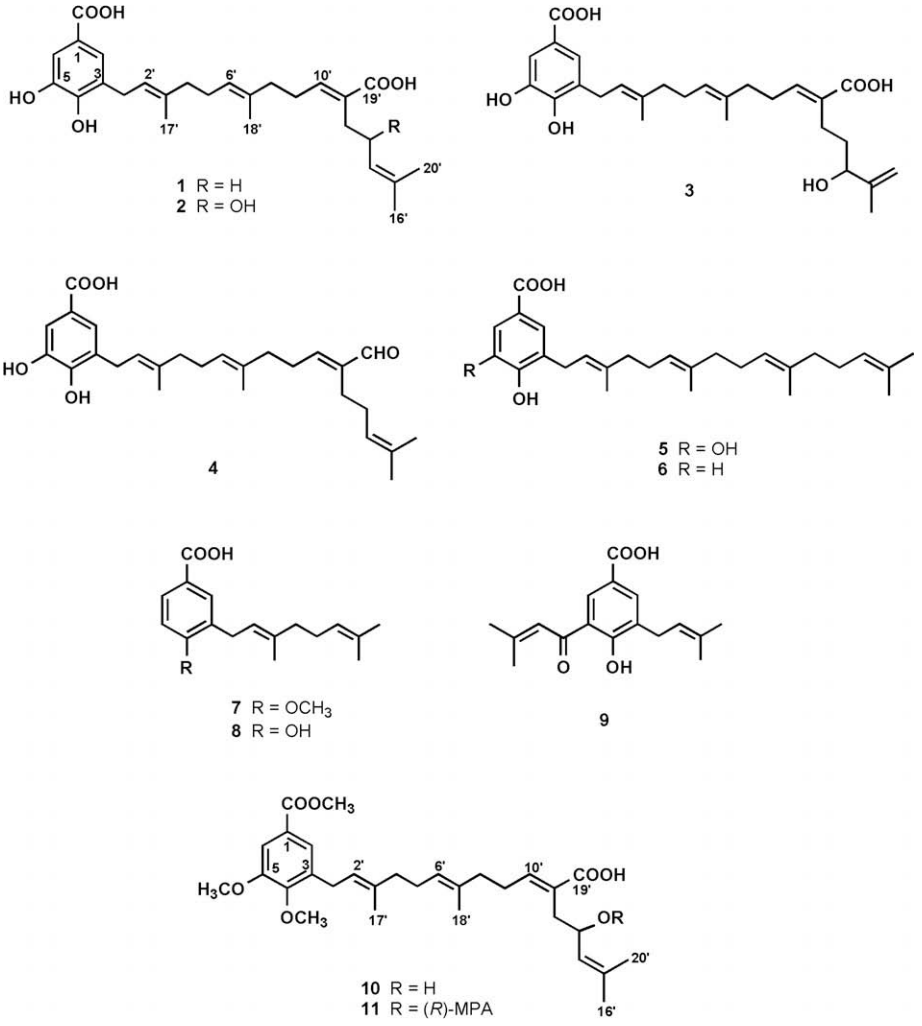
Table 1
^1H and ^{13}C NMR (400 MHz) data for compounds **1–3** in $CDCl_3$.

Position	**1**		**2**		**3**	
	δ_H[a]	δ_C[b]	δ_H[a]	δ_C[b]	δ_H[a]	δ_C[b]
1		120.2 *s*		120.4 *s*		123.1 *s*
2	7.47 *d* (2.8)	124.2 *d*	7.48 *s*	123.7 *d*	7.47 *d* (2.8)	123.8 *d*
3		127.5 *s*		127.2 *s*		127.3 *s*
4		147.7 *s*		147.4 *s*		147.7 *s*
5		142.8 *s*		143.0 *s*		143.1 *s*
6	7.44 *d* (2.8)	114.4 *d*	7.54 *s*	114.4 *d*	7.52 *d* (2.8)	114.3 *d*
COOH		171.3 *s*		171.3 *s*		171.3 *s*
1′	3.36 *d* (6.7)	28.0 *t*	3.42 *d* (5.2)	27.7 *t*	3.38 *d* (6.3)	27.8 *t*
2′	5.31 *t* (6.7)	121.7 *d*	5.34 *t* (5.2)	121.2 *d*	5.33 *t* (6.3)	121.4 *d*
3′		136.5 *s*		136.9 *s*		136.7 *s*
4′	2.05–2.13 *m*[*]	39.2 *t*	2.16–2.28 *m*[*]	39.0 *t*	2.05–2.16 *m*[*]	39.0 *t*
5′	2.05–2.13 *m*[*]	25.6 *t*	2.16–2.28 *m*[*]	25.6 *t*	2.05–2.16 *m*[*]	26.1 *t*
6′	5.11 *m*[*]	124.8 *d*	5.16 *t* (5.8)	125.0 *d*	5.15 *t* (6.9)	124.9 *d*
7′		133.5 *s*		133.5 *s*		133.6 *s*
8′	2.05–2.13 *m*[*]	37.9 *t*	2.16–2.28 *m*[*]	37.4 *t*	2.19–2.40 *m*	37.3 *t*
9′	2.19–2.28 *m*[*]	26.9 *t*	2.16–2.28 *m*[*]	28.9 *t*	2.05–2.16 *m*[*]	25.5 *t*
10′	6.84 *t* (7.6)	145.2 *d*	6.79 *t* (7.2)	141.4 *d*	7.13 *t* (6.4)	147.5 *d*
11′		130.8 *s*		126.1 *s*		124.3 *s*
12′	2.19–2.28 *m*[*]	26.5 *t*	2.53 *dd* (4.9, 13.1) 3.06 *dd* (3.8, 13.1)	32.2 *t*	2.05–2.16 *m*[*]	22.1 *t*
13′	2.05–2.13 *m*[*]	27.3 *t*	5.20–5.27 *m*[*]	75.0 *d*	1.70–1.78 *m*	27.2 *t*
14′	5.11 *m*[*]	123.4 *d*	5.20–5.27 *m*[*]	123.1 *d*	4.68 *dd* (2.2, 7.8)	81.9 *d*
15′		132.0 *s*		139.8 *s*		141.8 *s*
16′	1.65 *s*	25.4 *q*	1.76 *s*	25.5 *q*	4.96 *s*, 5.01 *s*	113.1 *t*
17′	1.72 *s*	15.7 *q*	1.74 *s*	15.9 *q*	1.73 *s*	15.8 *q*
18′	1.58 *s*	15.6 *q*	1.63 *s*	15.8 *q*	1.67 *s*	15.9 *q*
19′		172.6 *s*		172.0 *s*		167.8 *s*
20′	1.56 *s*	17.3 *q*	1.78 *s*	18.2 *q*	1.80 *s*	17.9 *q*

[a] Chemical shifts δ, in ppm relative to TMS, *J* in Hz in parentheses.
[b] Data are based on Dept, HSQC, and HMBC experiments.
[*] Overlapping signals.

by analysis of the HMBC spectrum (Fig. 1). Thus, correlation between signals at δ 7.47 (H-2)/7.44 (H-6) and δ 171.3 (COOH) indicated one of the carboxyl group was attached to C-1, and the position of the terpene side chain to the aromatic ring was determined by correlation of the signals at δ_H 7.47 (H-2) and δ_C 28.0 (C-1′). Moreover, the second carboxyl group was placed at C-11′ of the side chain, in agreement with the correlation observed between the signal at δ_H 6.84 (H-10′) and δ_C 172.6 (C-19′). In addition, a broad multiplete methylene at δ 2.19–2.28 correlated to both, the vinylic carbon of the conjugated olefin (C-10′) and the vinylic methine of the terminal isoprene unit (C-14′), establishing the C-10′, C-11′ and C-19′ as a conjugated system. The stereochemistry at C-2′ and C-6′ within the geranylgeranyl residue was determined as (*E*) on the basis of the ^{13}C NMR chemical shifts of the vinyl methyl groups C-17′ (δ_C 15.7) and C-18′ (δ_C 15.6), which both lie between 15 ppm as a result of the shielding γ-*cis* interactions. However, no such interaction exists for the C-16′, as it resonates at a lower field (δ 25.4) (Maxwell and Rampersad, 1989). The geometry of the C-10′, C-11′-double bond was established as *E* on the basis of the ^{1}H NMR chemical shift of the vinyl proton H-10′ (δ_H 6.84), which was further supported by comparison with the shifts reported for *Z* and *E*-2-methyl-2-butenoic acid and arieianal (Green et al., 1999). All these data allowed full assignment for all hydrogen and carbon atoms, and established the structure of compound **1** as 3-[(2*E*,6*E*,10*E*)-11-carboxy-3,7,15-trimethyl- 2,6,10,14-hexadecatetraenyl)-4,5-dihydroxybenzoic acid.

Compound **2** was obtained as a colorless oil, and its molecular formula $C_{27}H_{36}O_7$ was determined by analysis of ^{13}C NMR and HR EIMS spectra. Comparison of its ^{1}H and ^{13}C NMR data (Table 1) with those of compound **1** indicated the presence of an additional secondary hydroxyl group with signals at δ_H 5.20–5.27 and δ_C 75.0 and the loss of a methylene group (δ_H 2.05–2.13 and δ_C 27.3) for compound **2**, as the most notable differences. These data suggested a prenylated 3,4,5-trisubstituted benzoic acid with a hydroxylated geranylgeranyl chain for compound **2**, which was further confirmed by COSY, HSQC and HMBC experiments. The cross-peak observed in the HMBC (Fig. 1) between the signals at δ_H 5.20–5.27 (H-13′) and δ_C 126.1 (C-11′)/139.8 (C-15′) located the hydroxyl group at C-13′ on the geranylgeranyl chain. In order to determine the absolute configuration of the stereogenic center at C-13′, and prior to preparing the Riguera ester derivative, the phenolic hydroxyls and carboxyl acids of compound **2** were protected with a methyl moiety, yielding derivative **10**. Treatment of **10** with (*R*)-(-)-α-methoxyphenylacetic acid (MPA) afforded the corresponding diastereomeric esters (**11**). The magnetic nonequivalence of the signals assigned to the oxymethine and methoxyl groups belonging to the chiral auxiliary established the very low diastereomeric excess (3%). Therefore, this suggests compound **2** is almost a racemic mixture. Accordingly, the structure of **2** was assigned as 3-[(2*E*,6*E*,10*E*)-11-carboxy-13-hydroxy-3,7,15-trimethyl- 2,6,10,14-hexadecatetraenyl]-4,5-dihydroxybenzoic acid.



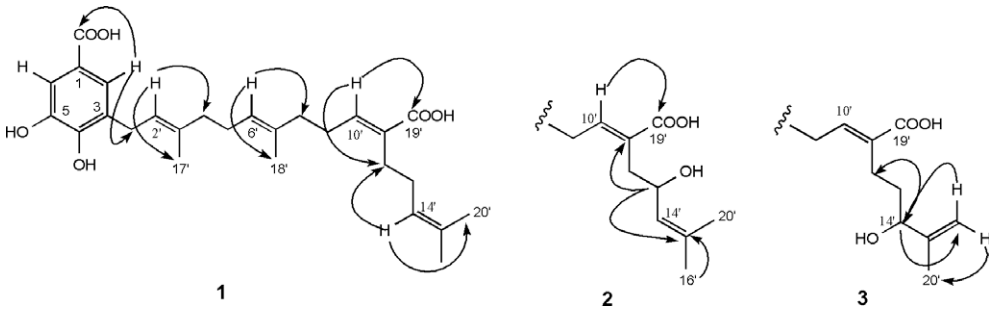


Fig. 1. Selected ^{1}H–^{13}C (HMBC) correlations for compounds **1**–**3**.

Compound **3** resulted to be an isomer of compound **2**, as indicated by the molecular formula $C_{27}H_{36}O_7$ determined by ^{13}C NMR and HR ESI(+)MS data. The ^{1}H NMR spectrum (Table 1) displayed signals for three vinyl methyl groups (δ_H 1.67, 1.73 and 1.80); eight methylene groups, six of them as multipletes at δ_H 1.70–1.78 (2H), 2.05–2.16 (8H), 2.19–2.40 (2H), one as a doublet at δ_H 3.38 (2H), and the other as two singlets (δ_H 4.96 and 5.01). Additionally, signals for three olefinic protons at δ_H 5.15 (1H), 5.33 (1H), 7.13 (1H) as triplets and one methine proton characteristic to a secondary hydroxyl group at δ_H 4.68, were observed. Comparison of its ^{1}H and ^{13}CNMR data (Table 1) with those of compound **2** suggested that one isoprene unit in the geranylgeranyl chain is the most notable difference between both compounds. The connectivity observed in the HMBC (Fig. 1) experiment of the vinylic protons at δ_H 4.96, 5.01 (H-16′) with δ_C 17.9 (C-20′)/81.9 (C-14′), and the cross-peak between the signals at δ_H 4.68 (H-14′) and δ_C 113.1 (C-16′)/22.1 (C-12′) located the hydroxyl group on C-14′, allylic to a terminal double bound. Analysis of the COSY, HSQC and HMBC experiments permitted the complete assignment of all the protons and carbons as shown in Table 1. Riguera's method have not been performed for **3** due to paucity of material. However, since its structure is closely related to that of **2**, and its optical rotation was close to 0°, this compound must also be almost racemic. Therefore, the structure of **3** was determined as 3-[(2*E*,6*E*,10*E*)-11-carboxy-14-hydroxy-3,7,15-trimethyl-2,6,10,15-hexadecatetraenyl]-4,5-dihydroxybenzoic acid.

In the search for potential antiparasitic agents, all compounds were assessed *in vitro* for their leishmanicidal (*Leishmania* spp.), trypanocidal (*Trypanosoma cruzi)* and antiplasmodial (*Plasmodium falciparum*) activities. The results obtained in the evaluation of the prenylated benzoic acids against promastigote forms of three strains of *Leishmania*, *L. amazonensis*, *L. braziliensis* and *L. donovani* are presented in Table 2. Compound **7** showed a high selectivity against *L. braziliensis* (IC_{50} 6.5 μg/ml), even being more active than pentamidine, used as positive control (IC_{50} 10.0 μg/ml). In addition, compound **9** exhibited significant activity with an IC_{50} of 17.8 μg/ml against the three strains of *Leishmania* used. The other assayed compounds showed slight or no activity with IC_{50} values ranging from 27.0 to 100 μg/ml. Regarding the trypanocidal activity, compound **9** proved to be active against epimastigote forms of *T. cruzi*, with an IC_{50} of 16.5 μg/ml, while the other compounds showed IC_{50} > 20 μg/ml. Benznidazole was used as positive control (IC_{50} 7.4 μg/ml). The compounds were also evaluated for their antiplasmodial activity. All the compounds were inactive (IC_{50} > 10 μg/ml), except for compound **1**, which exhibited a moderate activity against *P. falciparum* with an IC_{50} of 3.2 μg/ml, in comparison with chloroquine, used as a positive control (IC_{50} 0.1 μg/ml).

Table 2
Leishmanicidal activity of compounds **2**–**9** *in vitro* on promastigote forms of *Leishmania* spp.

Compounds	IC_{50} ± SD (μg/ml)[a]		
	L. amazonensis	*L. braziliensis*	*L. donovani*
1	> 100	> 100	> 100
2	63.1 ± 1.0	40.7 ± 0.8	46.8 ± 1.1
3	64.2 ± 0.5	39.0 ± 1.5	43.9 ± 1.6
4	77.0 ± 0.5	68.5 ± 1.5	53.9 ± 0.1
5	56.1 ± 3.6	33.7 ± 0.5	42.9 ± 2.5
6	56.8 ± 0.9	44.5 ± 0.9	41.8 ± 2.6
7	50.1 ± 0.9	6.5 ± 0.4	50.3 ± 1.1
8	27.0 ± 4.1	27.0 ± 1.9	27.0 ± 1.5
9	17.8 ± 1.1	17.8 ± 0.4	17.8 ± 0.7
Control[b]	10 ± 0.7	10 ± 0.7	10 ± 0.7

[a] Data are expressed as mean standard deviation of three determinations.
[b] Pentamidine was used as positive control.

The biological activity seems to be associated with shorter side chains in the molecule. It is also apparent that the presence of two isoprene moieties instead a single geranyl chain around the benzoic acid moiety improve the activity (**9** *versus* **8**). Moreover, concerning the regiosubstitution of the aromatic ring, the C-4 position plays an important role for the selectivity (**7** *versus* **8**). Among the longer chain compounds, an aldehyde group in position C-11′ makes the molecule more potent than the related carboxyl moiety (**4** *versus* **1**). Furthermore, it is important to point out that introduction of a hydroxyl group in positions C-13′ or C-14′ in the chain increase the activity (**2** *versus* **1** or **3** *versus* **4**). On the other hand, a geranylgeranyl side chain and a carboxyl acid as in **1** seems relevant for the antiplasmodial activity.

This is the second report of the antiparasitic properties of prenylated benzoic acid derivatives (Lopes et al., 2008). This study therefore points to the *in vitro* effectiveness of the antileishmanial benzoic acid derivative **7**, and the moderate activity of **9** and **1** as antiplasmodial and trypanocidal agents, respectively. These findings encourage further studies in order to evaluate a higher number of prenylated benzoic acid derivatives to perform SAR studies focusing on obtaining more potent and selective antiparasitic compounds.

3. Experimental

3.1. General

Optical rotations were measured on a Perkin Elmer 241 automatic polarimeter in $CHCl_3$ at 20 °C. UV spectra were obtained on a JASCO V-560 spectrophotometer in absolute EtOH. IR (film) spectra were measured on a Bruker IFS 55 spectrophotometer. NMR spectra were recorded in $CDCl_3$ on a Bruker Advance 400 spectrometer. The chemical shifts are given in δ (ppm) with residual $CDCl_3$

as internal reference and coupling constants in Hz. EIMS and HR EIMS were obtained on a Micromass Autospec Spectrometer, and HR ESIMS spectra were performed on LCT Premier XE Micromass Electrospray spectrometer. Silica gel 60 (particle size 15–40 and 63–200 μm, Machery–Nagel) and Sephadex LH-20 (Pharmacia Biotech) were used for column chromatography, while silica gel 60 F_{254} (Machery–Nagel) was used for analytical and preparative TLC. The spots were visualized by UV light and heating silica gel plates sprayed with H_2O–H_2SO_4–AcH (1:4:20). All solvents used were analytical grade (Panreac). The optically pure (*R*)-(-)-α-methoxyphenylacetic acid (MPA) was purchased from Sigma, whereas the other reagents were purchased from Aldrich and used without further purification.

3.2. Plant material

Leaves of *Piper heterophyllum* Ruiz & Pav. (voucher specimen GB-1922, March 1997) and *Piper aduncum* L. (voucher specimen GB-1892, January 1997), were collected in Ixiamas, Abel Iturralde Province, La Paz, Bolivia. Voucher specimens are deposited in the Herbario Nacional de La Paz, Universidad Mayor de San Andrés, La Paz, Bolivia, and were identified by Dr. R. Callejas, Herbarium of the Antioquia University, Colombia.

3.3. Extraction and isolation

The dry leaves (400.0 g) of *P. heterophyllum* were crushed and extracted with EtOH, using a Soxhlet apparatus for 48 h. After removing the solvent in vacuum, the extract was partitioned into a CH_2Cl_2–H_2O (1:1, v/v) solution, yielding the organic (50.0 g) and aqueous (1.9 g) extracts. The soluble fraction in CH_2Cl_2 was fractionated by vacuum–liquid chromatography (VLC) on silica gel and eluted with gradient systems of increasing polarity of *n*-hexane:EtOAc (0–100%), yielding five major fractions (A–E), combined according to TLC detection. Fraction C (2.0 g) was submitted to column chromatography (CC) on Sephadex LH-20 (1.30 × 4 cm), using hexane:$CHCl_3$:MeOH (2:1:1) as eluent affording five fractions, C1–C5. Fraction C4 (130 mg) was purified by prep. TLC (CH_2Cl_2:MeOH 5%) to give **6** (5 mg). Fraction D (4.5 g) was submitted to flash CC over silica gel eluted with a gradient of *n*-hexane:CH_2Cl_2 to give seven fractions D1–D7. Fraction D2 (547.0 mg) was chromatographed using flash CC over silica gel (CH_2Cl_2:*iso*-PrOH of increasing polarity 1–20%) to obtain six fractions, D2.1–D2.6. Fraction D2.4 (63 mg) was purified by prep. TLC (*n*-hexane:Et_2O, 3:7) to give **2** (9.9 mg) and **3** (4.0 mg). Fraction D4 (178 mg) was submitted to prep. TLC (CH_2Cl_2:Et_2O, 7:3) to afford **4** (8.4 mg) and **5** (12.0 mg). Fraction D5 (1.1 g) was subjected to flash CC over silica gel (CH_2Cl_2:*iso*-PrOH of increasing polarity 1–25%) to obtain six fractions D5.1 to D5.6. Compound **1** (6.6 mg) was isolated from fraction D5.5 (25 mg) after purification by prep. TLC (*n*-hexane:EtOAc, 1:1).

Leaves (154.0 g) of *P. aduncum* were crushed and extracted with EtOH–H_2O (70:30, v/v) at room temperature for 48 h. After removing the solvent in vacuum, the extract was partitioned in CH_2Cl_2-H_2O (1:1, v/v) solution, yielding the organic (7.6 g) and aqueous (0.5 g) extracts. The organic extract was fractionated by VLC on silica gel and with gradient systems of increasing polarity *n*-hexane:Et_2O (0–100%), yielding six fractions (A–E). Fraction B (2.0 g) was subjected to flash CC, which was eluted with Et_2O containing increasing amounts of EtOAc, to give three major fractions, B6 (156 mg), B8 (302 mg) and B9 (72 mg). B6 and B8 were submitted to CC on Sephadex LH-20, using hexane:$CHCl_3$:MeOH (2:1:1) as eluent, yielding compounds **7** (20 mg) and **9** (33 mg), respectively. Compound **8** (7 mg) was isolated from fraction B9 after purification by prep. TLC (hexane:Et_2O 1:1).

3.4. 3-[(2E,6E,10E)-11-carboxy-3,7,15-trimethyl-2,6,10,14-hexadecatetraenyl)-4,5-dihydroxybenzoic acid (*1*)

Colorless oil; UV λ_{max}^{EtOh} nm (log ε): 213 (6.3), 258 (6.4); IR $\nu_{max}^{film} cm^{-1}$: 3520–2560, 3407, 2925, 2854, 1681, 1612, 1442, 1380, 1300, 1216, 1196, 775; for ^1H (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data, see Table 1. Positive HR ESIMS *m/z* 479.2415 (calc. for $C_{27}H_{36}NaO_6$, 479.2410).

3.5. (±)-3-[(2E,6E,10E)-11-carboxy-13-hydroxy-3,7,15-trimethyl-2,6,10,14-hexadecatetraenyl]-4,5-dihydroxybenzoic acid (*2*)

Colorless oil; $[\alpha]_D^{20}$: −3.5 ($CHCl_3$, *c* 0.31); UV λ_{max}^{EtOh} nm (log ε): 213 (6.3), 262 (6.4); IR $\nu_{max}^{film} cm^{-1}$: 3513–2593, 3309, 2925, 2854, 1731, 1679, 1604, 1442, 1302, 1215, 1016, 756; for ^1H (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data, see Table 1. EIMS *m/z* (rel. int.): 454 [M^+−H_2O] (3), 436 (13), 410 (18), 392 (5), 377 (4), 287 (7), 271 (8), 232 (15), 205 (37), 161 (53), 123 (100), 93 (31), 81 (41). HR EIMS: [M^+−18] 454.2355 (calc. for $C_{27}H_{34}O_6$, 454.2350).

3.5.1. Preparation of derivative *10*

To a solution of compound **2** (5.6 mg, 0.012 mM) in acetone (1 ml), potassium carbonate (24 mg) and dimethyl sulfate (0.02 ml), were added and the reaction was stirred for 72 h at room temperature. The reaction mixture was concentrated to remove the organic solvent. Water (3 ml) was added and the product was extracted using dichloromethane (3 × 3 ml). The organic layer was dried over $MgSO_4$, filtered and concentrated to yield an oil, which was purified by preparative TLC (CH_2Cl_2/acetone, 9:1) to afford **10** (3.5 mg, 0.0068 mM).

3.5.2. (±)-(2E,6E,10E)-12-[2,3-Dimethoxy-5-(methoxycarbonyl) phenyl]-2-(2-hydroxy-4-methyl-3-pentenyl)-6,10-dimethyl-2,6,10-dodecatrienoic acid (*10*)

$[\alpha]_D^{20}$: −2.9 ($CHCl_3$, *c* 0.3); IR ν_{max}^{film} cm^{-1}: 3520–2567, 2926, 2854, 1732, 1680, 1612, 1442, 1216, 775; ^1H NMR spectral data (400 MHz, $CDCl_3$): δ 1.62 (3H, *s*, H-18′), 1.76 (3H, *s*, H-17′), 1.77 (3H, *s*, H-16′), 1.79 (3H, *s*, H-20′), 2.04–2.25 (8H, *m*, H-4′, H-5′, H-8′ and H-9′), 2.50 (1H, *dd*, *J* = 3.8, 12.6 Hz, H-12′), 3.03 (1H, *dd*, *J* = 6.1, 12.6 Hz, H-12′), 3.39 (2H, *d*, *J* = 5.7 Hz, H-1′), 3.88 (3H, *s*, OMe), 3.91 (3H, *s*, OMe), 3.92 (3H, *s*, OMe), 5.16 (1H, *t*, *J* = 5.2 Hz, H-6′), 5.29 (1H, *t*, *J* = 5.6 Hz, H-2′), 5.20–5.26 (2H, *m*, H-13′ and H-14′), 6.70 (1H, *t*, *J* = 7.3 Hz, H-10′), 7.47 (1H, *s*, H-2), 7.53 (1H, *s*, H-6). ^{13}C NMR spectral data (100 MHz, $CDCl_3$): δ 16.0 (*q*, C-18′), 16.3 (*q*, C-17′), 18.4 (*q*, C-20′), 25.7 (*q*, C-16′), 26.5 (*t*, C-5′), 28.5 (*t*, C-1′), 28.8 (*t*, C-9′), 32.4 (*t*, C-12′), 37.7 (*t*, C-8′), 39.6 (*t*, C-4′), 52.1 (*q*, COO*Me*), 55.9 (*q*, OMe-5), 60.6 (*q*, OMe-4), 74.3 (*d*, C-13′), 111.2 (*d*, C-6), 122.4 (*d*, C-2′), 123.8 (2 × *d*, C-14′, C-2), 125.3 (*d*, C-6′), 125.4 (*s*, C-1), 126.6 (*s*, C-11′), 131.1 (*s*, C-3), 133.5 (*s*, C-7′), 136.2 (*s*, C-3′), 139.5 (*s*, C-15′), 140.1 (2 × *s*, C-5, C-10′), 152.4 (*s*, C-4), 167.0 (*s*, *C*OOMe), 171.0 (*s*, C-19′). EIMS *m/z* (rel. int.): 496 [M^+−H_2O] (2), 468 (5), 465 (4), 394 (2), 350 (6), 287 (2), 205 (33), 123 (100), 93 (29), 81 (39). HR EIMS: [M^+−18] 496.2827 (calc. $C_{30}H_{40}O_6$, 496.2820).

3.5.3. Preparation of Riguera's esters

A solution of (*R*)-MPA (8 mg, 0.0482 mM), **10** (3 mg, 0.0058 mM), 1,3-dicyclohexylcarbodiimide (6 mg) and a catalytic amount of 4-dimethylaminopyridine in dry dichloromethane was stirred at room temperature for 24 h, and the solvent was removed to give a thick oil, which was purified by preparative TLC (*n*-hexane-EtOAc, 6:4) to give **11** (2.1 mg, 0.0032 mM).

3.5.4. (R)-α-Methoxy-α-phenylacetyl derivative of *10* (*11*)

$[\alpha]_D^{20}$: −14.9 ($CHCl_3$, *c* 0.1); ^1H NMR spectral data (400 MHz, $CDCl_3$): δ 1.63 (6H, *s*, H-18′), 1.76 (6H, *s*, H-17′), 1.78 (6H, *s*,

H-16′), 1.79 (6H, *s*, H-20′), 2.04–2.27 (16H, *m*, H-4′, H-5′, H-8′ and H-9′), 2.47 (1H, *dd*, *J* = 3.7, 12.5 Hz, H-12′), 2.52 (1H, *dd*, *J* = 3.8, 12.6 Hz, H-12′), 3.01 (1H, *dd*, *J* = 6.0, 12.6 Hz, H-12′), 3.04 (1H, *dd*, *J* = 6.1, 12.5 Hz, H-12′), 3.38 (4H, *d*, *J* = 5.7 Hz, H-1′), 3.29 (3H, *s*, OMe, MPA), 3.36 (3H, *s*, OMe, MPA), 3.86 (3H, *s*, OMe), 3.89 (3H, *s*, OMe), 3.91 (6H, *s*, OMe), 3.93 (6H, *s*, OMe), 4.70 (1H, *s*, MPA), 4.71 (1H, *s*, MPA), 5.16–5.31 (6H, *m*, H-2′, H-6′ and H-14′), 5.37 (2H, *m*, H-13′), 6.70 (2H, *m*, H-10′) 7.25–7.55 (14H, *m*, H-2 and H-6, MPA). EIMS *m/z* (rel. int.): 662 [M^+] (1), 616 (1), 496 (1), 410 (5), 287 (2), 205 (23), 161 (46), 123 (100. HR EIMS: [M^+] 662.3461 (calc. for $C_{39}H_{50}O_9$, 662.3455).

3.6. (±)-3-[(2E,6E,10E)-11-carboxy-14-hydroxy-3,7,15-trimethyl-2,6,10,15-hexadecatetraenyl]-4,5-dihydroxybenzoic acid (**3**)

Colorless oil; $[\alpha]_D^{20}$: + 5.2 ($CHCl_3$, *c* 0. 16); UV λ_{max}^{EtOh} nm (log ε): 212 (6.2), 260 (6.3); IR ν_{max}^{film} cm^{-1}: 3490–2592, 3360, 2925, 2854, 1714, 1679, 1630, 1444, 1379, 1194, 775 cm^{-1}; for ^1H (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data, see Table 1. Positive HR ESIMS *m/z* 495.2357 (calc. for $C_{27}H_{36}NaO_7$, 495.2359).

3.7. Leishmanicidal activity

The *in vitro* leishmanicidal activity was evaluated against promastigote forms of *Leishmania braziliensis* 2903, *L. amazonensis* PH8 and *L. donovani* PP75 (all from IBBA, Instituto Boliviano de Biología de Altura), which were grown at 28 °C in stationary culture and were seeded at 1×10^4/100 μl/well in 96-well flat bottom microtiter plates in *Leishmania* medium based on RPMI 1640. Test samples or standard drugs dissolved in DMSO were added at a further 100 μl/well to give a final concentration of 100 μg/ml and serial dilutions thereof. All assays were carried out in triplicate. Leishmanicidal activity was expressed as IC_{50} values (the concentration of a compound which caused a 50% reduction in parasite viability). Pentamidine (10 μg/ml) (Sigma–Aldrich) was used as a positive control (Deharo et al., 2004).

3.8. Trypanocidal activity

Epimastigote forms of *Trypanosoma cruzi*, Tulahuen strain, were cultivated at 26 °C in liver infusion tryptose medium (LIT), supplemented (5%) with heat inactivated (56 °C for 30 min) foetal calf serum (technically modified from Chataing et al., 1998). Parasites in logarithmic growth phase were distributed in 96-well flat bottom microtiter plates at a concentration of 5×10^4/ml. Each well was incubated for 72 h with increasing concentrations of the sample, ranging from 10 μg/ml up to 100 μg/ml. The activity was measured by optic counting with an inverted microscope and comparison with control wells. Benznidazole (7.4 μg/ml) was used as the reference drug for this assay. All assays were carried out in triplicate.

3.9. Antiplasmodial activity

F-32 Tanzania (chloroquine sensitive) strains of *Plasmodium falciparum* (Kindly provided by Dr. Fandeur, Pasteur Institute, Cayenne, France) were cultured (Trager and Jensen, 1976) on glucose-enriched RPMI 1640 medium, supplemented with 10% human serum at 37 °C. After 24 h of incubation at 37 °C, the medium was replaced by fresh medium together with the compound to be evaluated at three different concentrations (0.1, 1 and 10 μg/ml), and incubation was continued for a further 48 h. On the third day of the test, a blood smear was taken from each well and parasitemia was calculated for each concentration of sample compared to the control. IC_{50} values were determined graphically by plotting concentrations *vs.* percent inhibition. Chloroquine (0.1 μg/ml) was used as a positive control. All test were performed in triplicate.

Acknowledgment

This investigation was supported by International Foundation of Sciences (Sweden, Grant F/3408-1), DGCYT (CTQ2006-13376/BQU), Program of Swedish Cooperation (UMSA-SIDA/TB-BRC, ASD91) and PCI-Iberoamérica (A/010794/07, AECI) projects. International collaboration was supported by project X.5 "Búsqueda, Obtención y Evaluación de Nuevos Agentes Antiparasitarios" CYTED (Iberoamerican Program of Science and Technology for Development, Subprogram X).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2009.03.010.

References

Baldoqui, D.C., Kato, M.J., Cavalheiro, A.J., Da Silva, B.V., Young, M.C., Furlan, M., 1999. A chromene and prenylated benzoic acid from *Piper aduncum*. Phytochemistry 51, 899–902.
Chen, J.J., Duh, C.Y., Huang, H.Y., Chen, I.S., 2003. Cytotoxic constituents of *Piper sintenense*. Helv. Chim. Acta 86, 2058–2064.
Chataing, B., Concepción, J.L., Lobatón, R., Usubillaga, A., 1998. Inhibition of *Trypanosoma cruzi* growth *in vitro* by *Solanum* alkaloids: a comparison with Ketoconazole. Planta Med. 64, 31–36.
Deharo, E., Ruiz, G., Vargas, F., Sagua, H., Ortega, E., Rojas, A., Jiménez, A., 2004. Técnicas de Laboratorio Para la Selección de Sustancias Antichagas y Leishmanicidas. Prisa Ltda.-CYTED, La Paz, Bolivia.
Estevez, Y., Castillo, D., Pisango, T., Arevalo, J., Rojas, R., Alban, J., Deharo, E., Bourdy, G., Sauvain, M., 2007. Evaluation of the leishmanicidal activity of plants used by Peruvian Chayahuita ethnic group. J. Ethnopharmacol. 114, 254–259.
Flores, N., Cabrera, G., Jiménez, I.A., Piñero, J., Giménez, A., Bourdy, G., Cortés-Selva, F., Bazzocchi, I.L., 2007. Leishmanicidal constituents from the leaves of *Piper rusbyi*. Planta Med. 73, 206–211.
Flores, N., Jiménez, I.A., Giménez, A., Ruiz, G., Gutiérrez, D., Bourdy, G., Bazzocchi, I.L., 2008. Benzoic acid derivatives from *Piper* species and their antiparasitic activity. J. Nat. Prod. 71, 1538–1543.
Green, T.P., Treadwell, E.M., Wiemer, D.F., 1999. Arieianal, a prenylated benzoic acid from *Piper arieianum*. J. Nat. Prod. 62, 367–368.
Gupta, M.P., 1995. Plantas Medicinales Iberoamericanas. Presencia Santa Fé de Bogota, Colombia.
Hermoso, A., Jiménez, I.A., Mamani, Z.A., Bazzocchi, I.L., Piñero, J.E., Ravelo, A.G., Valladares, B., 2003. Antileishmanial activities of dihydrochalcones from *Piper elongatum* and synthetic related compounds. Structural requirements for activity. Bioorg. Med. Chem. 11, 3975–3980.
Ioset, J.R., 2008. Natural products for neglected diseases: a review. Curr. Org. Chem. 12, 643–666.
Jaramillo, M.A., Manos, P.S., 2001. Phylogeny and patterns of floral diversity in the genus *Piper* (Piperaceae). Am. J. Bot. 4, 706–716.
Lago, J.H.G., Ramos, C.S., Casanova, D.C.C., Moradin, A.A., Bergamo, D.C.B., Furlan, M., Cavahleiro, A.J., Bolzani, V.S., Young, M.C.M., Guimaraes, E.F., Kato, M.J., 2004. Benzoic acid derivatives from Piper species and their fungitoxic activity against *Cladosporium cladosporioides* and *C. Sphaerospermum*. J. Nat. Prod. 67, 1783–1788.
Lopes, A.A., Lopez, S.N., Regasini, L.O., Batista Jr., J.M., Ambrosio, D.L., Kato, M.J., Bolzani, V.S., Cicarelli, R.M.B., Furlan, M., 2008. In vitro activity of compounds isolated from *Piper crassinervium* against *Trypanosoma cruzi*. Nat. Prod. Res. 22, 1040–1046.
Luize, P.S., Ueda-Nakamura, T., Filho, B.P., Cortéz, G.D., Nakamura, C.V., 2006. Activity of neolignans isolated from *Piper regnellii* (MIQ.) C.DC. var. *pallescens* (C.DC.) YUNCK against *Trypanosoma cruzi*. Biol. Pharm. Bull. 10, 2126–2130.
Maxwell, A., Rampersad, D., 1989. Novel prenylated hydroxybenzoic acid derivatives from *Piper saltuum*. J. Nat. Prod. 52, 614–618.
Moreira, D.L., Guimaraes, E.F., Kaplan, M.A.C., 1998. A chromene from *Piper aduncum*. Phytochemistry 48, 1075–1077.
Orjala, J., Erdelmeier, C.A.J., Wright, A.D., Rali, T., Sticher, O., 1993. Five new prenylated *p*-hydroxybenzoic acid derivatives with antimicrobial and molluscicidal activity from *Piper aduncum* leaves. Planta Med. 59, 546–551.
Orjala, J., Wright, A.D., Behrends, H., Folkers, G., Sticher, O., 1994. Cytotoxic and antibacterial dihydrochalcones from *Piper aduncum*. J. Nat. Prod. 57, 18–26.
Parmar, V.S., Jain, S.C., Bisht, K.S., Jain, R., Taneja, P., Jha, A., Tyagi, O.D., Prasad, A.K., Wengel, J., Olsen, C.E., Boll, P.M., 1997. Phytochemistry of the genus *Piper*. Phytochemistry 46, 597–673.

Portet, B., Fabre, N., Roumy, V., Gornitzka, H., Bourdy, G., Chevalley, S., Sauvain, M., Valentin, A., Moulis, C., 2007. Activity-guided isolation of antiplasmodial dihydrochalcones and flavanones from *Piper hostmannianum* var. Berbicense. Phytochemistry 68, 1312–1320.

Quijano-Abril, M.A., Callejas-Posadas, R., Miranda-Esquivel, D.R., 2006. Areas of endemism and distribution patterns for neotropical *Piper* species (Piperaceae). J. Biogeog. 33, 1266–1278.

Rali, T., Wossa, S.W., Leach, D.N., Waterman, P.G., 2007. Volatile constituents of *Piper aduncum* and *Piper gibbilimbum* C.DC (Piperaceae) from Papua New guinea. Molecules 12, 389–394.

Ramji, N., Ramji, N., Iyer, R., Chandrasekaran, S., 2002. Phenolic antibacterials from *Piper betle* in the prevention of halitosis. J. Ethnopharmacol. 83, 149–152.

Rukachaisirikul, T., Siriwattanakit, P., Sukcharoenphol, K., Wongvein, C., Ruttanaweang, P., Wongwattnavuch, P., Suksamrarn, A., 2004. Chemical constituents and bioactivity of *Piper sarmentosum*. J. Ethnopharmacol. 93, 173–176.

Seco, J.M., Quinoa, E., Riguera, R., 2004. The assignment of absolute configuration by NMR. Chem. Rev. 104, 17–117.

Seeram, N.P., Jacobs, H., McLean, S., Reynolds, W.F., 1996. Prenylated hydroxybenzoic acid derivatives from *Piper murrayanum*. Phytochemistry 43, 863–865.

Siddiqui, B.S., Gulzar, T., Mahmood, A., Begun, S., Khan, B., Afshan, F., 2004. New insecticidal amides from petroleum ether extract of dried *Piper nigrum* L. Whole fruits. Chem. Pharm. Bull. 52, 1349–1352.

Terreaux, C., Gupta, M., Hostettmann, K., 1998. Antifungal benzoic acid derivatives from *Piper dilatatum*. Phytochemistry 49, 461–464.

Torres-Santos, E.C., Moreira, D.L., Kaplan, M.A.C., Meirelles, M.N., Rossi-Bergman, B., 1999. Selective effect of 2′, 6′-dihydroxy-4′-methoxychalcone isolated from *Piper aduncum* on *Leishmania amazonensis*. Antimicrob. Agents Chemother. 43, 1234–1241.

Trager, W., Jensen, J.B., 1976. Human malaria parasites in continuous culture. Science 193, 673–675.

Vila, R., Tomi, F., Mundina, F., Santana, A.I., Solis, P.N., Lopez Arce, J.B., Balderrama, S.L., Iglesias, J., Gupta, M., Casanova, J., Cañigueral, S., 2005. Unusual composition of the essential oils from the leaves of *Piper aduncum*. Flavour Frag. J. 20, 67–69.

Yamaguchi, L., Lago, J.H.G., Tanizaki, T.M., Mascio, P.D., Kato, M.J., 2006. Antioxidant activity of prenylated hydroquinone and benzoic acid derivatives from *Piper crassinervium* Kunth. Phytochemistry 67, 1838–1843.