ANTIMALARIALS AND ANTIOXIDANTS COMPOUNDS FROM *PIPER TRICUSPE* (PIPERACEAE)

Alex Sáez Vega[a,b*], Benjamin Rojano[a,c], Silvia Blair[d] Cesar Segura[d], Bruno Figadere[e], Blandine Seon[e], Philippe Grellier[f] and Jairo Sáez[a],

[a]Instituto de Química, Química de Plantas Colombianas, Universidad de Antioquia, A.A: 1226, Medellín, Colombia.
[b] Grupo de procesos Ambientales y Biotecnológicos, Departamento de Ingeniería de Procesos, Universidad EAFIT, A.A: 3300, Medellín, Colombia. e-mail: asaez@eafit.edu.co
[c] Laboratorio Ciencia de los Alimentos, Escuela de Química, Universidad Nacional de Colombia, Sede Medellín, A.A: 3840, Medellín, Colombia
[d]Grupo Malaria, Sede de Investigación Universitaria, Universidad de Antioquia, Medellín Antioquia, Colombia.
[e]Laboratoire de Pharmacognosie, Associé au CNRS (BIOCIS), Université de Paris-Sud, F-92296, Chatenay-Malabry, France
[f]Laboratoire de Biologie fonctionnelle des protozoaires; Museúm National d´Histoire Naturelle 61 Rue Buffon 75005 Paris, France.

Summary

Dictyochromenol an unusual compound, rarely found in plants, and two known compounds obtained from petroleum ether extract of the whole plant *Piper tricuspe* showed antimalarial and antioxidant activity as well as cytotoxicity. The results show that the compounds are active against several *Plasmodium falciparum* strains with IC_{50}´s ranging from 1.37 to 29.78 μM and cytotoxic effects with IC_{50}´s ranging from 1.07 to 40.95 μM; the selectivity index based on IC_{50}´s suggests a high toxicity of the compounds. In addition, the compounds showed high antioxidant activity with IC_{50}´s ranging from 10.8 to 165.6 μM in the DPPH assay. Compounds were characterized by MS, ^{1}H and ^{13}C NMR and identified as Dictyochromenol, 3-Farnesyl-*p*-Hydroxy benzoic acid and 2´*E*,6´*E* 2-Farnesyl Hydroquinone. The ethnomedical claim of *Piper tricuspe* in the Colombian Pacific zone may be justified by the observed antiplasmodial activity of the extracts.

KEY WORDS: *Piper tricuspe*; Antioxidant; *plasmodium;* Dictyochromenol; Farnesyl derivatives

Malaria, a disease produced by the parasite *Plasmodium falciparum*, still has a great impact in human populations particularly in developing countries. Currently, the number of cases of malaria increases all over the world mainly because the spreading of *P. falciparum* drug resistant strains, caused in part by the failure of the current disease control programs, making the search of new alternatives urgent. Traditionally, plants have been source of antimicrobials and many reports have shown the biological activity of different plant-derived natural products. Natural products derived compounds have also been found as powerful antimalarials, such as for instance chloroquine a

synthetic derivative of Quinine which is obtained from the plant *Cinchona* and more recently artemisinin which is isolated from *Artemisia annua*, a traditional Chinese plant with demonstrated antimalarial activity (1).

Piperaceae family consists of many plants used in traditional medicine as treatment for several illness in native populations and several evidences have shown the biological activity of Piper`s family derived compounds. Several reports have demonstrated that *Piper* species produces compounds with antioxidant potential (2, 3) and specifically the polyprenylated hydroquinones (4), which have been recognized for their role as antioxidants in preventing formation of oxygen radical and hydrogen peroxide that induced cytotoxicity and tissue damage in various human diseases. The importance of the antioxidant constituents of plant materials in the maintenance of health and protection against heart diseases and cancer is also raising interest among scientists, food manufacturers and consumers as the trend for the future is toward functional food with specific health effects (5). Typical compounds that possess antioxidant activity including phenols, phenolic acids and their derivatives, flavonoids, tocols, phospholipids, amino acids and peptides, phytic acid, ascorbic acid, pigments, and sterols.

In this work, three pure compounds isolated from *Piper tricuspe* (*Piperaceae*) known as **"**Hojamano or costeño" plant traditionally used against snake bite and antimalarial in the Colombian Pacific zone (6) were tested *in vitro* in *P. falciparum* continuous culture and their cytotoxicity as well as their antioxidant potential determined. Two out of the three isolated compounds have been previously reported in *Piper* species (7, 8, 9). However, Dictyochromenol has only been identified in the brown alga *Dictyopteris undulata* as a piscicidal (10) and chemically synthesized (11, 12). Here, Dictyochromenol is reported for the first time in this plant family, despite of its presence that was suggested by Ampofo in *Piper auritum* (7). We report herein that the three compounds isolated from *P. tricuspe* possess an antioxidant activity comparable to other known antioxidant products like Tocopherol or Trolox in the DPPH assay. So far, there are no previous reports in the literature neither on the biological activity nor the chemical composition of *Piper tricuspe*.

Materials and methods

Plant Material. The botanical species *Piper tricuspe (Piperaceae)* was purchased from Tumaco (Nariño) Sud-western of Colombia. A voucher specimen was deposited in Herbarium of Antioquia University, Medellin, Colombia. (Voucher number: HUA: 80790).

General Experimental Procedures. Melting points were measured without correction on a Büchi apparatus. Optical rotations were obtained using a cell (1.5 mL) with 1 dm path length, on a Polartronic E (Schmidt-Haensch) polarimeter. UV spectra were obtained in MeOH, using a Jenway 6405 spectrophotometer. ^{1}H NMR (400 and 600 MHz) and ^{13}C NMR (100 MHz) spectra (all in $CDCl_3$) were recorded with Bruker AMX 400 and Bruker AM 600 NMR spectrometers, using TMS as internal standard. EIMS were obtained with a Nermag-Sidar R10-10C mass spectrometer. Silica gel 60 (Merck 0.063-0.200 mesh) was used for column chromatography, and precoated silica gel plates (Merck 60 F_{254} 0.2 mm) were used for TLC. TLC spots were sprayed with a mixture of sulphuric acid in acetic acid (1:9) and heating 100-105 °C.

Extraction and isolation. The whole plant of *Piper tricuspe* air-dried and powdered (1576g), was extracted with petroleum ether at room temperature, and concentrated in vacuo to give a crude extract (141.3g). The petroleum ether extract (20g) was subjected to passage over silica gel flash

column chromatography eluting with a step gradient of hexane-CH_2Cl_2, to obtain thirteen fractions as well fractionated means traditionally chromatographic column methods and preparative chromatography. Fractions were collected on the basis of their TLC profiles. Three compounds **1** (0.604g, 0.038%), **2** (1.17g, 0.074%), and **3** (1.94g, 0.123%) were eluted and identified as Dictyochromenol, 3-Farnesyl *p*-hydroxy benzoic acid and *2´E,6´E* 2-Farnesyl Hydroquinone respectively. The dichloromethane and methanolic extracts were tested by TLC and interesting compounds were not found.

Compound 1. Dycthiochromenol: A yellow oil, $[\alpha]_D^{29}$ = +11, ($CDCl_3$, *c* =0.182), U.V, (MeOH, c 0.0146), λmax : 235nm (3.34), 331nm (1.16) ^{1}HNMR ($CDCl_3$, 400 MHz), δ 5.58 (1H, d, J=9.8 Hz, H-3), 6.24 (1H, d, J=9.8 Hz, H-4), 6.6 (1H, d, J=2.0 Hz, H-5), 6.49 (1H, d, J=8.3 Hz, H-8), 6.6 (1H, dd, J=8.3 Hz, J=2.0 Hz, H-7), 1.69 (1H, m, H-1`), 2.1 (1H, m, H-2`), 5.1 (2H , m, H-3`, H-7`), 1.96 (1H, m, H-5`), 1.99 (1H, m, H-6`), 1.67 (3H, s, H-9`), 1.59 (3H, s, H-10`), 1.57 (3H, s, H-11`), 1.36 (3H, s, H-12`). ^{13}C NMR ($CDCl_3$, 100 MHz), δ 78.0 (s, C-2), 130.7 (d, C-3), 122.6 (d, C-4), 121.0 (s, C-4a), 115.0 (d, C-5), 149.6 (s, C-6), 116.0 (d, C-7), 112.0 (d, C-8), 146.1 (s, C-8a), 40.8 (t, C-1`), 22.5 (t, C-2`), 123.9 (d, C-3`), 135.1 (s, C-4`), 39.6 (t, C-5`), 26.6 (t, C-6`), 124.3 (d, C-7`), 131.0 (s, C-8`), 25.6 (t, C-9`), 17.6 (t, C-10`), 15.9 (t, C-11`), 25.9 (t, C-12`). HRTOF-MS: m/z 325.1 (M+H)+ (calcd. For $C_{21}H_{28}O_2$, 312.46).

Compound 2. 3-Farnesyl *p*-hydroxy benzoic acid: A pale brown oil; U.V, (MeOH, c 0.00067), λmax : 220nm (2.51), 259nm (1.87). ^{1}HNMR ($CDCl_3$, 400 MHz), δ 6.89 (1H, d, J=8.6Hz, H-5), 7.88 (1H, d, J=9.0Hz, H-2), 7.89 (1H, dd, J=8.0 Hz, J=2.0 Hz, H-6), 3.40 (2H, d, J= 7.0Hz, H-1`), 5.35 (1H, t, J= 6.2 Hz, H-2`), 2.05 (8H, m, H-4`, H-5`, H-8`, H-9`), 5.10 (2H, m, H-6`, H-10`), 1.6 (6H, s, H-12`, H-14`), 1.80 (3H, s, H-13`), 1.70 (3H, s, H-15`). ^{13}C NMR ($CDCl_3$, 100 MHz), δ 172.0 (-COOH), 127.0 (s, C-1), 130.4 (d, C-2), 115.7 (d, C-3), 159.5 (s, C-4), 121.6 (s, C-5), 132.5 (d, C-6), 29.5 (t, C-1`), 120.9 (d, C-2`), 139.2 (s, C-3`), 39.6 (t, C-4`, C-8`), 26.7 (t, C-5`), 26.4 (t, C-9`), 123.6 (d, C-6`), 135.6 (s, C-7`), 124.4 (d, C-10`), 131.2 (s, C-11`), 25.6 (q, C-12`), 17.6 (q, C-13`), 16.3 (q, C-14`), 16.2 (q, C-15`).

Compound 3. *2´E,6´E* 2-Farnesyl Hydroquinone: Amorphous pale yellow solid mp: 54-56 ºC; U.V, (MeOH, c 0.093), λmax : 235nm (3.09), 286nm (2.87), 292nm (2.87), ^{1}HNMR ($CDCl_3$, 400 MHz), δ 6.61 (1H, d, J=2.8 Hz, H-3), 6.57 (1H, dd, J=8.4 Hz, J=2.8 Hz H-5), 6.68 (1H, d, J= 8.4 Hz, H-6), 3.3 (2H, d, J= 7.1Hz, H-1`), 5.3 (1H, t, J= 6.8 Hz, H-2`), 2.05 (8H, m, H-4`, H-5`, H-8`, H-9`), 5.02 ((1H, m, H-6`), 5.1 (1H, t, J= 6.4 Hz, H-10`), 1.6 (3H, s, H-12`), 1.75 (3H, s, H-13`), 1.60 (3H, s, H-14`), 1.68 (3H, s, H-15`). ^{13}C NMR ($CDCl_3$, 100 MHz), δ 148.1 (s, C-1), 116.5 (d, C-3), 149.2 (s, C-4), 116.5 (d, C-5), 113.7 (d, C-6), 29.6 (t, C-1`), 121.2 (d, C-2`), 138.6 (s, C-3`), 39.6 (t, C-4`, C-8`), 26.6 (t, C-5,` C-9`), 123.6 (d, C-6`), 135.5 (s, C-7`), 124.3 (d, C-10`), 131.3 (s, C-11`), 25.6 (t, C-12`), 16.0 (t, C-13`), 16.0 (t, C-14`), 17.6 (t, C-15`). HRTOF-MS: m/z 337 (M+Na)+, 349(M+Cl)ˉ. (calcd. For $C_{21}H_{30}O_2$, 314.2246).

Cultivation of P. falciparum. Parasites were cultivated by the method of Trager and Gensen, (13) with minor modifications. Cultures were maintained in fresh group A-positive human erythrocytes suspended at 1% hematocrit in RPMI 1640 containing 10% human serum 3 g/L glucose, 45µg/L of hypoxanthine and 50 µg/L gentamicin. Flasks were incubated at 37ºC by the candle jar method. For testing CQ and compounds the stock culture was diluted in complete medium and normal human erythrocytes to a starting 1% hematocrit and 0.5 % parasitemia.

Parasites were noted to be late-ring and early throphozoites, with no evident schizonts. Stock solutions of the tested compounds and drugs were prepared at a concentration of 10 mg/mL (in DMSO), serially diluted in complete medium and dispensed into duplicate test wells to yield final concentrations ranging from 100 μg/mL to 0.005 μg/mL. Final volumes were 200 μL. Assays were read in beta counter and results statistically analyzed for significance.

Cytotoxicity test on mammalian cells. Cytotoxicity was determined using the colorimetric tetrazolium-dye (MTT) (14) briefly: Rat skeletal muscle myoblast L-6 cells were seeded into 96-well microplates at 5000 cells/well in 100 μL RPMI medium: After 24 h incubation the cells were washed and re-incubated with different concentrations of extracts for 5 days, at 37°C under a 5% CO_2 atmosphere. After incubation the medium was removed and 100 μL of new medium supplemented with 10 μL of MTT solution (5 mg/mL MTT dissolved in PBS and sterilised filter) was added to each well containing the cells for 3 h at 37ºC then 100 μL of SDS 10% was added to dissolve crystals and incubated overnight at 37ºC. Absorbance of each well was read at 580nm. The IC_{50} values were obtained from the drug concentration-response curve. The results were expressed as the mean ± the standard deviations determined from three independent experiments.

DPPH radical scavenging assay. Free stable DPPH (1,1-diphenyl–2-picrylhydrazyl) radical, was purchased from Aldrich Chem. Co (Millw WI), methanol from Merck (Darmstadt Germany), and 2,4,6-tri(2-pyridyl)-s-triazine (TPTZ) from Sigma-Aldrich Chem. Co. (USA). Radical scavenging activity against the stable radical DPPH was measured using the methods of Brand-Williams (15), with some modifications as described below. The antioxidant activities of compounds (dissolved in methanol) were assessed on the basis of the radical scavenging effect of the stable DPPH free radical. A volume of 990 μL from a 100 μM DPPH methanol solution was added to 10 μL of sample solutions of different concentrations and allowed to react at room temperature. After 30 min the absorbance values were measured at 517 nm and converted into the percentage antioxidant activity (AA). Trolox®, Tocopherol and Hydroquinone were used as reference compounds.

FRAP assay. The principle of this method is based on the increase in absorbance due to the formation of the tripyridiltriazine (TPTZ)-Fe (II) complex in the presence of reducing agents. The FRAP reagent contained 2.5 ml of a 10 μM TPTZ in 40 mM HCl plus 2.5 ml of 20 μM $FeCl_3$ and 25 mL of 0.3 μM acetate buffer, pH 3.6 and was prepared freshly and warmed at 37°C. Aliquots of 10 μL sample (samples dissolved in methanol) were mixed with 90 μL distilled water and 900 μL FRAP reagent. Temperature was maintained at 25°C. The readings at the absorption maximum (595nm) were taken every 30 min and were selected for calculation of FRAP values. Aqueous solutions of known ascorbic acid concentrations were used for calibration, and the results were expressed as FRAP values (g of ascorbic acid by 100 g of antioxidant). (16).

Results

Dictyochromenol (1), 3-Farnesyl *p*-hydroxy benzoic acid (2) and *2´E,6´E* 2-Farnesyl Hydroquinone(3), were obtained from petroleum ether extract of the whole plant *Piper tricuspe*

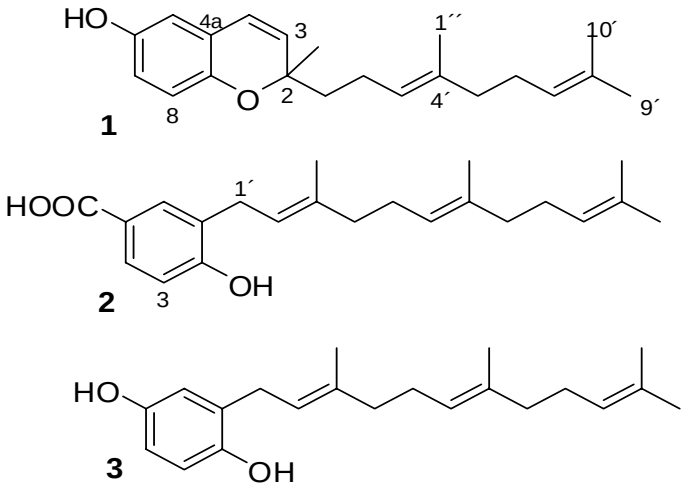

Figure 1. Molecular structure of *Piper tricuspe* compounds.

The in *vitro* antimalarial activity of the three *Piper tricuspe* compounds was quantified in *P. falciparum* continuous culture. One compound was found to have high antimalarial activity and cytotoxicity. Other two compounds displayed relatively low antiplasmodial activity and similar level of cytotoxicity. It is important to note that the level of antiplasmodial activity corresponded to the near same level of cytotoxicity as the selectivity index was close to unity.

Table 1. Summary of *Piper tricuspe* compounds biological activity in (μM)

Compound	Antiplasmodial activity (*P. falciparum* strain FCB-1)		Cytotoxicity to rat skeletal muscle myoblast L-6 cells		IIS
	IC_{50}	IC_{90}	IC_{50}	IC_{90}	
1	9.58	18.54	7.71	16.66	**0.8047**
2	29.78	62.88	40.95	68.95	**1.3749**
3	1.37	2.45	1.075	2.17	**0.7846**
Chloroquine	0.019	0.031	-	-	-

It was found that compound **3** has a relatively high activity with IC_{50} 1.37μM, other compounds ranged between 9.58 and 29.78 μM and Chloroquine had by far the lowest IC_{50} (0.019 μM). Remarkably the substitution in *m*-Hydroxy Farnesyl can be more important than the length of the chain itself, however the length of the chain is significant for the activity.

Compound **3** is conformed by two groups: Hydroquinone- and Farnesyl-group. Data not shown indicate that the hydroquinone cause hemolysis in the *P. falciparum* cultures suggesting that the citotoxic and antimalarial activity might be related to that nucleus, while the Farnesyl chain modulate the antimalarial activity of the molecule by suppressing its hemolytic activity.

Table 2 shows the antimalarial activity of the *Piper tricuspe* compounds to different *P. falciparum* strains; the results suggest that the different strains are differently inhibited by the evaluated compounds.

Table 2. *Piper tricuspe* Antimalarial activity to different *P. falciparum* strains in μM

	Thai	F32	PFB	K1
Compound	**IC_{50} (n=3)**	**IC_{50} (n=3)**	**IC_{50} (n=2)**	**IC_{50} (n=2)**
1	5.12±-0.5	10.56±1.2	3.52	8.96
2	73.14±0,9	41.97±3.1	85.86	89.67
3	0.93±0.03	1.14±0,1	0.55	0.58
Amodiaquine	0.010±0.0015	0.014±0.0023	0.015±0.0031	0.014±0.0028

The results in Table 3 show the antioxidant activity of the three compounds compared to common antioxidant standards by two different methods: on the one hand DPPH is a stable radical, of purple color in methanol, which is decolorized in presence of an antioxidant. This assay, is rigorously due to steric impediment in the DPPH molecule, where the most important part of the reaction occurs. The results in this assay showed that compound **3** (IC_{50} = 10.8 μM) had the highest hydrogen-donating capacity; compound **1** (46.9 μM) and **2** were weakly active (165 μM). On the other hand, the FRAP method is commonly used to measure the reduction potential in plasma and the reducing capacity of active compounds (16). It occurs by a single-electron transfer (SET) mechanism (17). The experimental results for all compounds are reported as a FRAP value. FRAP values are equivalent to g of ascorbic acid per 100 g of antioxidant compound. The FRAP values for compounds **1** and **3** were: 9.2 and 8.4 g/100g, respectively. Compound **2** showed very poor reducing capacity.

Table 3. Summary of *Piper tricuspe* antioxidant activity

	DPPH	FRAP (g./100g)
Compound	**IC_{50} (μM)**	**Ascorbic Ác. Equiv.**
1	46.9±0.35	9.2± 0.43
2	165.6±0.72	<1
3	10.8± 0.09	8.4± 0.11
Hydroquinone	5.9±0.01	18172.2±1.09
Trolox ®	9.2±0.01	7612.3±1.60
α-Tocopherol	10.2±0.01	3169.9±0.99

Discussion and conclusions

The obtained IS`s are lower than or near to 1 which suggests that the compounds are cytotoxic at least to this particular cell line. Further studies with other cell lines will help to define more precisely the cytotoxic potential of these compounds.

It is important to note that the level of antiplasmodial activity corresponded to the near same level of cytotoxicity as the selectivity index was close to unity. This suggests that these natural compounds cannot be suitable as antimalarial, however an analysis of the structure -activity relationship of the compounds can provide a key for increase their selectivity index.

In all the cases, compound **3** has the best activity against different strains of *P. falciparum* and other compounds showed variations in the activity against differents strains.

High antioxidant activity of the three compounds with IC_{50}`s ranging from 10.8 to 165.6 μM in DPPH assay indicated that the prenylated hydroquinone has the same level of activity than other known standard antioxidant products, such as Trolox®, Tocopherol and Hydroquinone. However in the FRAP assay, significant differences between the compounds and standard antioxidant products are found. Reducing capacity appears when hydroxyl group in *m*-Farnesyl is removed (e.g. compare Hydroquinone with its Farnesyl derivate).

These results suggest overall the farnesyl radical affect the antioxidant activity also in concordance with Yamaguchi (3) observations. This fact suggests that Farnesyl group affects SET reduction mechanism. Also, antioxidant activity decreases when dihydroxyl groups are replaced; apparently the hydroxyl group in *m*-Farnesyl in compound **3** seems to be essential for reducing capacity as suggested the antioxidant assays when this group is changed by acid group.

Due to high antioxidant activity of compound **3** in addition to its high antiplasmodial activity, this compound has probably a different mode of action than common oxidant drugs used against malaria (18). This suggests that Farnesyl Hydroquinone may have a mode of action like inhibitors of the mitochondrial NADH:ubiquinone oxidoreductase (complex I of the respiratory chain), due to its structural similarity to coenzyme Q such as homologs CoQo, CoQ_1 and CoQ_2, and the analogs duroquinone and specially decylubiquinone (19).

Acknowledgements

The authors are grateful to the Antioquia University, ECOS-NORD, ICFES, COLCIENCIAS, ICETEX for financial support, and to thank Drs. Isabelle Florant, and Sophie Susplugas of Laboratoire de Biologie fonctionnelle des protozoaires, Museúm National d´Histoire Naturelle for their help and assistance in radioactive experiments and for maintaining ***P. falciparum*** in continue culture.

References

1. Sharma P. and Sharma J.D. Plants showing antiplasmodial activity-From crude extracts to isolated compounds. Indian Journal of Malariology 1998; 35: 57-110.
2. Chanwitheesuk A, Teerawutgulrag A, Rakariyatham N. Screening of antioxidant activity and antioxidant compounds of some edible plants of Thailand. Food Chemistry 2005; 92: 491–497.
3. Yamaguchi L. F. Lago J. H., Tanizaki T. M., Di Mascio P., Kato M. J .. Antioxidant activity of prenylated hydroquinone and benzoic acid derivatives from *Piper crassinervium* Kunth. Phytochemistry 2006; 67: 1838–1843.
4. Tziveleka L-A., Kourounakis A. P., Kourounakis P. N., Roussis V. and Vagiasa C. Antioxidant Potential of Natural and Synthesised Polyprenylated Hydroquinones. Bioorganic & Medicinal Chemistry 2002; 10: 935–939.
5. Loliger J.. The use of antioxidants in food. In O. I. Aruoma, & B. Halliwell (Eds.), Free radicals and food additives. London: Taylor and Francis 1991; 129-150.
6. Otero, R. Mordeduras de serpientes y etnobotánica en la región noroccidental de Colombia. Toxinotas 2000; 04: 1-7.
7. Ampofo S. A. Roussis V. and Wiemer D. F. . New Prenylated Phenolics From *Piper Auritum*. Phytochemistry 1987; 26: 2367-2370.

8. Maxwell A. and Ampersad D. . Prenylated 4-hydroxybenzoic acid derivatives from *Piper marginatum*. Journal of Natural Products 1988; 51: 370-373.
9. Peña L. A. Avella E. Puentes de Díaz A. M.. Benzoquinona e hidroquinona Preniladas y otros constituyentes aislados de *Piper bogotense* C. DC. 2000; 29: 25-37.
10. Dave M. D., Kusumi T. Ishitsuka M., Iwashita T. Kakisawa H. A piscicidal Chromanol and chromenol from the brown alga *Dyctyopteris undulata*. Heterocycles 1984; 22: 2301-2307.
11. Goujon J. Y., Zammattio F. and Kirschleger B. Synthesis of various 2H-benzopyran compounds and their kinetic resolution by asymmetric hydrolysis of their racemic acetates mediated by lipases. Tetrahedron Asymmetry. 2000; 11: 2409-2420.
12. Aoki K. Takahasi M. Hashimoto M. Okuno T. Murata K. Suzuki M.. Total synthesis of both enantiomers of Dyctiochromenol and their (Z)-Isomers. Bioscience, Biotechnology and Biochemistry 2002; 66: 1915-1924.
13. Trager W and Gensen J. Human malaria parasites in continuous culture. Science 1976; 193: 673-675.
14. Mosmann T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 1983; 65: 55–63.
15. Brand-Williams, W., Cuvelier, M. E., Berset, C. Use of a free radical method to evaluate antioxidant activity. Lebensm.-Wiss. U. Technol. 1995; 28: 25-30.
16. Benzie, I.F.F.S., J. J. The Ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 1996; 239: 70-76.
17. Ronald L. Prior, X.W.y.K.S. Standardized Methods for the Determination of Antioxidant Capacity and Phenolics in Foods and Dietary Supplements. Journal of Agricultural and Food Chemistry, 2005; 53: 4290 - 4302.
18. Vennerstrom J. L., and Eaton J.W. Oxidants, Oxidant Drugs, and malaria. Journal of Medicinal Chemistry. 1988; 31: 1269-1277.
19. Estornell E., Fato R, Pallotti F, Lenaz G. Assay conditions for the mitochondrial NADH:coenzyme Q oxidoreductase. FEBS. 1993; 332: 127-131.