**Journal of Medicinal Plants and By-products (2019) 2: 171-179**

**Original Article**

# Chemical Standardization of Extracts of *CalophyllumBrasiliense* with Antiplasmodial and Cytotoxic Activity

**Ana María Mesa Vanegas[1*], Silvia Blair Trujilo[2] and Carlos Peláez Jaramillo[3]**

[1]*Agrobiotechnology Research Group, Institute of Biology, Faculty of Exact and Natural Sciences, University of Antioquia, Medellín. Colombia*
[2]*Malaria Research Group. University Research Headquarters (SIU). Carrera 62 52-59. Tower 1.Lab. 610 SIU. Faculty of Medicine. University of Antioquia. A. A 1226.Medellín, Colombia*
[3]*Interdisciplinary group of molecular studies GIEM. Faculty of Exact and Natural Sciences, University of Antioquia. A. A 1226. Medellín, Colombia*

Article History: Received: 26 January 2019/Accepted in revised form: 14 April 2019


## Abstract

Malaria is considered one of the health problems in many parts of the world. The objective of this work is to implement standardized extraction strategies using analytical methodologies of the *Calophyllum brasiliense* Cambess. for the validation of antiplasmodial potential *in vitro*. Using HPLC chromatographic and chemical characterization techniques ($^{13}$C-NMR, $^{1}$H-NMR), the marker substance was identified and quantified on continuous cultures of *Plasmodium falciparum* chloroquine-sensitive strain NF-54 and cytotoxicity in cell lines U-937 and HepG2used the antimalarial potential of the *C. brasiliense*. The best extracts were stems with ethyl acetate and extraction after 4 hours, presented a yield of 4.154±0.301%, a promising antiplasmodial activity of $IC_{50}$ = 9.013 ± 1.288 µg/mL, a cytotoxicity of $CC_{50HepG2}$ =43.340±3.159 µg/mL, an IS = 4.808 and a content of marker substancesnamedsoulatrolide (1)of $C_1$ =0.257±0.021%. A new analytical method for the quantification of marker substances was established and the best extracts were obtained. The extracts presented promising pharmacological activities for studies that guarantee reproducibility and effectiveness in biological tests *in vivo* and framed in the transformation of a phytotherapeutic product.

**Keywords:** Antiplasmodialactivity, Cytotoxic activity, Phytomedicine, *Plasmodium falciparum*, Standardized extract.

## Introduction

Malaria is one of the most serious health problems in many parts of the world, particularly in Africa where the highest mortality occurs [1]; in Latin America presents high incidence rates [2]. It is estimated that malaria is present in 99 countries; between 203 and 262 million cases of malaria occurredin 2017 with 435,000 deaths, mostly children under 5 years of age [3]. At present, the situation is being further complicated by the spread of drug-resistant parasites, mainly where *Plasmodium falciparum* is endemic, therapeutic failure, in addition to limited access to medications, among other factors; that complicate their prevention and treatment [4]. There are few alternative medicines that are under development, and urgent measures are required to identify new classes of antimalarial agents, which makes the control of the disease one of the main public health challenges [5].Chemotherapy is one of the alternatives for the control of malaria morbidity and mortality, which has been possible thanks to the discovery of compounds from plants. An

[*]Corresponding author: Agrobiotechnology Research Group, Institute of Biology, Faculty of Exact and Natural Sciences, University of Antioquia, Medellín. Colombia
Email Address: amaría.mesa@udea.edu.co

example is quinine obtained from the plant of the genus *Cinchona*, the only antimalarial used for several centuries, a leading compound for the treatment of malaria [6]. Plants are a source of new medicines [7], however despite great efforts to conserve biodiversity, there is a serious deterioration and loss of useful species for life, so that it is necessary to carry out studies that allow exploring the potential of biodiversity towards the search for new natural chemical substances that have a high biological activity and that contribute to the control of a devastating disease such as malaria [8].

*Clusiaceae* is one of the families of plants in which compounds with antimalarial activity have been found, such as xanthones and derivatives of acylfloroglucinol. These compounds have been reported in plants of the genera *Hypericum, Vismia* and *Garcinia*. In the family Clusiaceae, the genus *Calophyllum* is composed of about 200 species, some of which present reports on compounds of the coumarin, xanthone and triterpene type with antimalarial, analgesic, antiviral, antiulcerogenic, anticancer, and antibacterial activities[9-10]. Thus, as the genus *Calophyllum* is a potential source of secondary metabolites, these species are little explored in the field of malaria and antioxidants, so their exploration is justified as a phytotherapeutic alternative in the treatment of malaria and likewise, contribute to the pharmacological knowledge of these plants.

In this work, the aim is to implement extractions and new analytical methodologies for the chemical standardization of the different extracts of the *C. brasiliense* specie and validate the antiplasmodial potential of the standardized extract *in vitro* on continuous cultures of *P. falciparum*chloroquine-sensitive strain NF-54 and cytotoxicity in cell lines U-937 and HepG2.

## Materials and methods

### Plant Materials

The *Calophyllum* specimens were collected in the municipality of Angelopolis. Three types of samples were collected: a sample for specimen for herbarium, samples as controls of the specimens collected and leaves and stems to obtain the extracts. The specimen for the University of Antioquia herbarium was processed, deposited, and taxonomically characterized as *Calophyllum brasiliense* Cambess. (Voucher 162,467). Leaves and stems were dried under cool and darkconditions for extraction purposes.

### Preparation of Extracts of Different Polarity and Isolation of Marker Substances

The extracts were obtained by Soxhlet extraction in triplicate with 20g of dry and ground plant material having a particle size of 5mm and extraction conditions in two times (6 hours and 4 hours); different solvents were used: ethyl acetate (EtoAc), dichloromethane (DCM), dichloromethane-methanol mixtures (DCM-MeOH 1:1), dichloromethane-ethyl acetate (DCM-EtoAc 1:1). Subsequently, the extracts were filtered and concentrated in a rotoevaporator, where the extraction yields were obtained. For the isolation of the major compounds, the serial fractionation of the dichloromethane extract was performed using the following separation techniques: Liquid-liquid extraction and evaporation, crystallization, flash column chromatography with stationary phase of silica-gel 60 $GF_{254}$ Merck®, chromatography on column with stationary phase of sephadex, different proportions of solvents with mixtures of petroleum ether, dichloromethane, ethyl acetate and methanol, previously distilled solvents before being used. In addition, a continuous monitoring by thin-layer chromatography Silica-gel 60 $GF_{254}$ Merck®. Chromatography was also performed on a CCP preparative layer with a stationary phase of Silica-gel 60 $GF_{254}$ Merck® to purify 30 mg of the major compound and follow-up of the purification by CCD thin layer chromatography [11-12].

Compound (1) was isolated from coded *C. brasiliense* called soulatrolide (1)was used as a reference standard (Fig. 1) [13]. Soulattrolide (1): Amorphous white solid with formula $C_{25}H_{24}O_5$ (1597.6 mg), IR $\nu_{max}$ 3431.71; 2975.62, 1714.41, 1639.2; 1587.13; 1145.51 $cm^{-1}$. $[\alpha]D$ = -33.48 (c = 0.09, $CHCl_3$). TOF MS ES+[M+Na]: (MeOH) m/z obsd 427.1529 (1,9 ppm) [M+Na]+, calculatedfor $C_{25}H_{24}O_5Na$ 427.1521. $^1$H-RMN-500MHz δ ($CHCl_3$ –d1) (ppm): 7.35-7.37 (m, 3H); 7.26-7.23 (m, 2H); 6.52 (d, 1H, J=10.27 Hz); 5.94 (s, 1H); 5.34 (d, 1H, J=10.23 Hz); 5.02 (d, 1H, J= 3.42 Hz); 4.28 (dq, 1H, J= 10.76, 6.36 Hz); 2.88 (bs, 1H); 1.76 (ddq, 1H, J= 10.27, 6.85, 2.94 Hz); 1.42 (d, 3H, J=6.36 Hz); 1.14(d, 3H, J=7.34 Hz); 0.93 (s, 3H); 0.92 (s, 3H). $^{13}$C-RMN-125MHz δ ($CHCl_3$ – d1) (ppm): 160.6; 156.3; 153.5; 153.7; 151.1; 139.9; 127.53; 127.3(CH x 2); 127.1(CH x 2);

115.9; 111.6; 106.1; 106.0; 103.0; 76.9; 73.0; 61.7; 61.3; 38.22; 26.84; 26.73; 18.80; 12.49.

Chromatographic Conditions HPLC

The standardization of the analytical method and the quantification was performed in a high efficiency liquid chromatography equipment coupled to a diode array detector (High-Performance Liquid Chromatography with Diode-Array Detection HPLC-DAD Agilent Technology 1200 series). The chromatography system consisted of a quaternary pump with degasser G1354A, autosampler G1313A, column compartment G1316A, detector UV / Visible with diode array and a ChemStation Software 32bit G2170BA HPLC 2D. The column used for the chromatographic separation was a Luna Phenomenex with particle size of 5µ, C18 100Å and dimensions of 250 x 4.60mm, series 564054-31. The solvents used for the separation were water as solvent (A) and acetonitrile as solvent (B) HPLC grade. The injection volume used was 20µL and a flow of 1.0 mL / min. To develop the quantification method, chromatographic separation parameters of the extracts were established and the retention times of the marker substances were determined (1). The precision of the method was calculated in triplicate and the percentage of marker substances in the extracts was quantified by percolation and Soxhlet extraction. For the quantification, standard curves of compound (1) were prepared in concentration ranges of 1000 - 0.1µg/mL. The extracts were prepared at a concentration of 3000 µg/mL. The standards and extracts were dissolved in an acetonitrile-water solution ($CH_3CN$ 50%: $H_2O$ 50%) and before the injection were filtered by Nylon membrane 0.45 µm (hydrophilic) DISMIC-13 NP; 0.39 MPa. ADVANTEC and injected in triplicate for HPLC analysis. A volume of20 µL of the standards to produce calibration curveand each of the extracts were injected in triplicate.The percentage of marker (1) was determined by an area ratio analysis and 3 successive injections of the standard solution are made and pure of peak is verified, the resolution: > 2.0 (peak of interest and adjacent peak), Tail Factor : < 2.0 (interest peak),% RSD: ≤ 3.5%.Based on retention times of peak1 and the calibration data,the concentration of compounds in the extract was determined. Identification of compoundswas confirmed by comparing the retention time ofstem extracts with that ofamarker (1) (Fig. 2)

Development of the Chromatographic Profile of Extracts of *Calophyllumbrasiliense*

The mobile phase consisted of water (A) and acetonitrile (B). The elution was programmed following the gradient of the solvents at a flow of 1.0 mL / min of 50% of A and 50% of B up to 0% of A and 100% of B in 30 min. The compound (1) were detected at wavelength of 286nm and $t_R$ =13.60. The marker substance was identified by comparing its retention time ($t_R$) under identical analytical conditions. The extracts of *C. brasiliense*were analyzed by percolation and those obtained by sothelx extractions for the quantification and differentiation of secondary metabolites.

Test of Biological Activity

*In vitro* determination of antiplasmodial activity: *In vitro* antiplasmodial activity assays were performed on the sensitive (NF-54) chloroquine strain. *P. falciparum* strains (NF-54) were cultured and maintained according to the method of Trager and Jensen (1976); using a suspension of 5% human A + erythrocytes in RPMI-1640 culture medium (Sigma R6504) dissolved in sterile water with 25 mM HEPES, 5.0% $NaHCO_3$, 10% fresh human A + serum (inactivated at 56 ° C for 30 minutes); incubated in an atmosphere of 5% $O_2$, 5% $CO_2$ and 90% $N_2$. Daily change was performed daily and fresh red blood cells were added twice a week. *Invitro*antiplasmodial activity by the SYBR Green I® method: In order to evaluate the *in vitro*antiplasmodial activity of the extracts and / or compounds, the standardized protocol was followed in the Malaria Group, which was adapted according to the methodology described by Smilkstein [14]. Assays were performed on Falcon® 96-well flat bottom plates. A suspension of parasitized red blood cells with a hematocrit of 2.5% and a parasitemia of 1% was prepared. Cultivation with treatments and positive control chloroquine (CQ) were incubated at 37 °C for 48 hours in an atmosphere of 5% $CO_2$, 5% $O_2$ and 90% of $N_2$. Subsequently, the contents of each well were transferred to Greiner Pro one dishes and the parasites were labeled with a solution of SYBR® Green I 2X in lysis buffer. The plates were incubated at room temperature for one hour in the dark and the relative fluorescence units (RFU) were read on a spectrofluorometer at an excitation wavelength of 485 nm and emission of 538 nm. Treatments from each crude extract were prepared

to a stock solution of 10 mg/mL in pure DMSO and sonicated to facilitate dissolution. From this solution, 50 µL were taken and adjusted to 1000 µL with complete RPMI-1640 medium, obtaining a final concentration of 0.5 mg/mL. The concentration of DMSO in the first dilution was 1% which has been shown to be non-toxic to the parasite. Seven concentrations of each extract were evaluated in a range between 100 -1.56 µg / mL, each concentration was evaluated in duplicate on the plate and three independent assays were performed. The CQ control was evaluated in a range between 150-4.7 nM and the control of Peruvian Quina extract (MeOH: $H_2O$; 70:30) was evaluated in the range of 0.01-10 µg / mL. Data from three trials were analyzed to find the inhibitory concentration 50 in µg / mL ($IC_{50}$). Inhibitory concentrations 50 ($IC_{50}$ ± SD) were calculated for each compound from a non-linear logistic regression model. A sigmoid concentration-response curve with slope of Hill (variable slope) was assumed. The data were analyzed and plotted using GraphPad Prism 4 for Macintosh version 4.0b which outputs the adjustment value (r) (GraphPad Software, San Diego, California, USA).To classify the antiplasmodial activity of an extract, the Malaria Group of the University of Antioquia established a consensus for the extracts evaluated: highly active <5 µg / mL, promising 6-15 µg / mL, moderate activity 16-30 µg / mL, low activity 31-50 µg / mL and non-active> 50 µg / mL [15-16].

*Cytotoxicity testing and calculation of the selectivity index (SI):* The method of 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyl tetrazolium bromide (MTT) according to Mosmann, 1983 was used to evaluate the cytotoxic activity of the extracts [17]which reveals cellular damage at the mitochondrial level.

*Cytotoxicity in the U-937 cell line and HepG2 cell line:* U-937 cells are human histiocytic lymphoma promonocitos, which were maintained in continuous cultures in the Malaria Group Laboratory. These cells were cultured at 37 °C and 5% $CO_2$ in RPMI medium supplemented with 10% inactivated Fetal Bovine Serum (FBS). The media changes were performed every 48 hours or according to pH changes of the medium, with centrifugation for 10 minutes at 1000 rpm, replacing with fresh medium. In Neubauer's chamber, U-937 cells were counted and plated in a 96-well flat bottom plate, 200,000 cells / mL in RPMI 1640 medium with 10% FBS. They were incubated at 37 °C with 5% $CO_2$ for 72 hours in the presence of each of the seven concentrations of each extract and / or compound evaluated in a range between 100 -1.56 µg/mL, each concentration was evaluated in duplicate in the dish and three independent trials were performed. Subsequently, mitochondrial dehydrogenase activity was measured by adding 20 µL / well of MTT to a concentration of 5 mg/mL and incubating for 3 hours at 37 °C and 5% $CO_2$[18]. To dissolve the formed crystals, 100 µL / well of a 50% solution of isopropanol and 10% SDS were added and the absorbance read at 595 nm in an ELISA reader (BioRad). Data from three independent trials were analyzed using the GraphPad Prism 5 program to find the toxic concentration in µg / mL ($CC_{50}$) using a non-linear logistic regression model[19]. For the assay, the HepG2 cells are counted in a Neubauer chamber and seeded in a 96-well flat bottom plate 2 x $10^5$ cells / well in 100 µl of RPMI-1640 medium with 10% fetal bovine serum. Subsequently, the cells are incubated for 30 hours at 37 °C in a 5% $CO_2$ environment, to allow the formation of the monolayer and then add 100 µL of each of the concentrations (100-1.54 µg/mL) of the extracts and / or compounds or 100 µL of the medium where the extracts and / or compounds were dissolved. Four wells are left, to which culture medium is added to evaluate the behavior of the cells under normal conditions (negative control). Each concentration of extracts and / or compounds and controls are evaluated in quadruplicate in 2 trials. Plates are incubated for 48 hours at 37 ° C. Then, 30 µL of MTT (Sigma Aldrich) is added at a concentration of 2 mg / mL and incubated again for 5 hours. After incubation, 130 µL of 96% DMSO were added and the dishes were incubated for 20 minutes at room temperature and gently mixed to allow the MTT crystals to dissolve. The production of Formazan crystals is measured at 550 nm in an ELISA reader (BioRad). The toxic effect is determined by comparing the absorbance obtained in the control with that of the treatments and thus determining whether or not there was a toxic effect. Data were analyzed with the GraphPad Prism 5 program to find $CC_{50}$ (cytotoxic concentration inhibiting 50% growth).

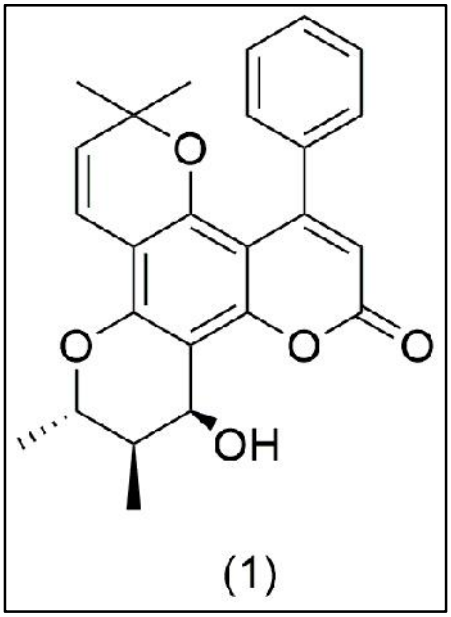


**Fig. 1**

## Results

To classify the cytotoxicity of the extract, the Malaria Group of the University of Antioquia established a consensus for the samples evaluated: highly toxic <10 µg/mL, cytotoxic 10-40 µg/mL, moderately cytotoxic 41-100 µg/mL and no cytotoxic>100 µg/mL. In addition, the selectivity index (IS) was calculated, which indicates selectivity towards the parasite and corresponds to the relationship between cytotoxic $CC_{50}$ activity and antiplasmodial activity $IC_{50}$. For the $HepG_2$ cell line, IS values above 5 were considered as promising extracts [20].*Data analysis:* Measurements were performed in triplicate and the results are presented as the mean and its standard deviation (DS). The data were analyzed and plotted using GraphPad Prism 4 for Macintosh version 4.0b which outputs the adjustment value (r) (GraphPad Software, San Diego, California, USA).
A new analytical method of chromatographic separation, where the compound (1) was quantified and developed and standardized. Compound (1) was quantified at a retention time of $t_R$ =13.597 min. The regression equations of the standard solutions (Y =19.314X-37.846. $R^2$ =0.9987). An example of the chromatographic profiles is observed in (Fig. 2), where the dichloromethane extract of the leaves and stems of *C. brasiliense* obtained by percolation extraction (Fig. 2 (A)), the dichloromethane extract of the stems for 4 hours (Fig. 2 (B)), dichloromethane extract from the stems for 6 hours (Fig. 2 (C)).The percentages of extractable material, the relative percentage of compound (1), the antiplasmodial activity *in vitro* in strain of *P. falciparum* sensitive to chloroquine NF-54, the cytotoxicity in human promonocytes U-938 and the determination of the selectivity index coming from The extracts by Soxhlet of *C. brasiliense*are presented in Table 1 and are plotted in Figure 3. All the extracts were classified according to antiplasmodial and cytotoxic potential. Table 1 includes the most active extracts obtained by percolation and are also plotted in Figure 3 to be able to compare them with the extracts obtained by soxhlet extraction. The percentages of extractable material with the different solvents showed higher yields for the leaf extract obtained by soxhlet extraction at a time of 4 hours in DCM: MeOH (1:1) with 10.75±0.385%, however it was not found a relationship with its activity and its extraction performance.

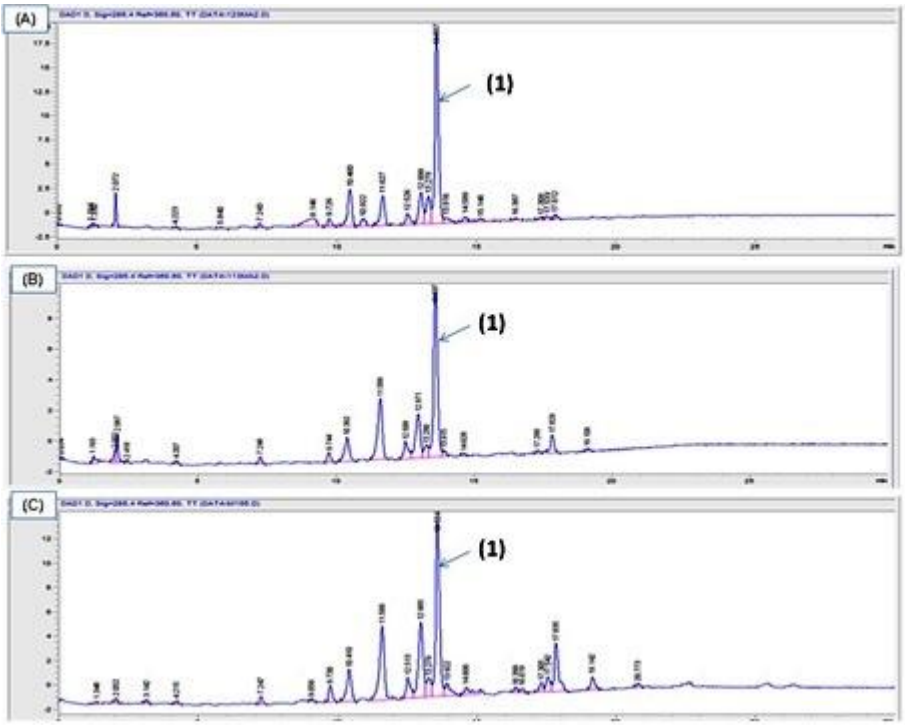


**Fig. 2**

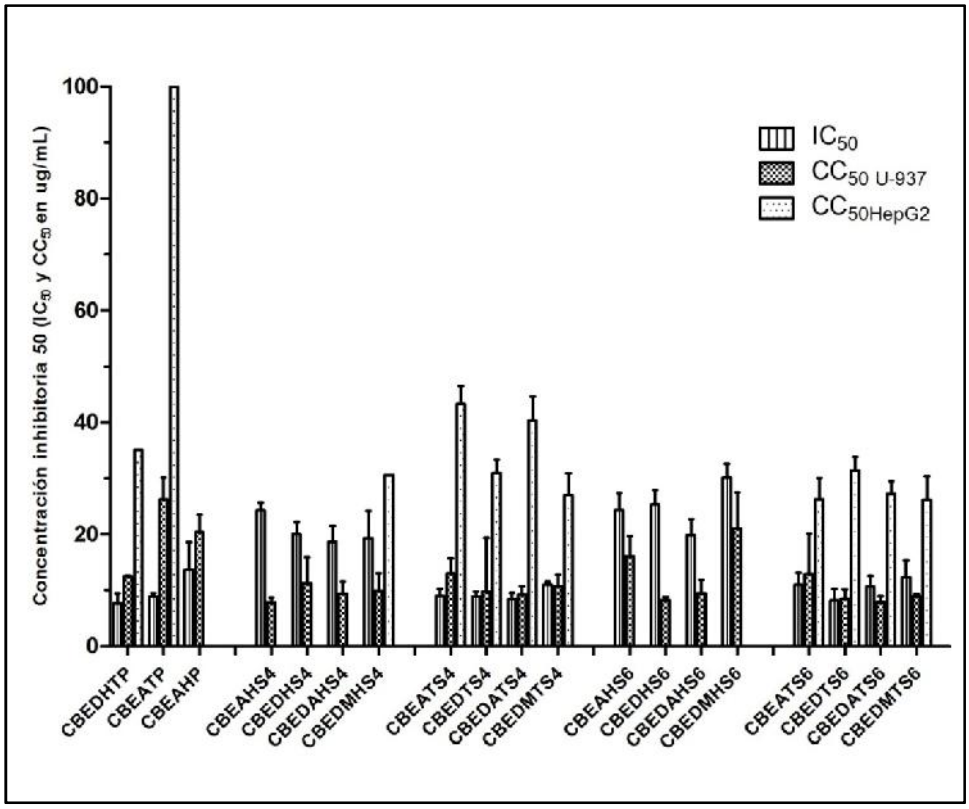


**Fig. 3**

The highest content of soulatrolide with 1.66± 0.169% was found in the DCM: EtoAc extract (1: 1) of the leaves of *Calophyllumbrasiliense*Cambess. obtained by soxhlet extraction at 4 hours. Analyzing all the variables together, the standardized extracts that presented the best biological responses were the extracts obtained in dichloromethane and ethyl acetate. The best extracts were, the extract from the stems with ethyl acetate and extraction after 4 hours, presented a yield of 4.15±0.301%, a promising antiplasmodial activity of $IC_{50}$ =9.01±

1.23 µg/mL, a cytotoxicity of $CC_{50HepG2}$ =43.34±3.16 µg/mL, an IS =4.808 and a content of marker substances of $C_1$ =0.257± 0.021%, and the extract from the stems with DCM: EtoAc (1:1) and extraction for 4 hours, presented a yield of 2.63±0.525%, a promising antiplasmodial activity of $IC_{50}$ = 8.31±1.19 µg / mL, a cytotoxicity of $CC_{50HepG2}$ =40.32 ±4.24 µg / mL, an IS =4.849 and a marker substance content of $C_1$=0.543± 0.100%. Both extracts presented interesting pharmacological activities for subsequent studies in which extracts are obtained under the same extraction conditions that guarantee reproducibility and effectiveness in other biological tests and framed in the transformation of a phytotherapeutic product. Likewise, a second step in the extraction can be used to reduce the cytotoxicity of the extracts by means of tannin precipitation reactions or to implement other forms of extraction such as extraction with $CO_2$ under supercritical conditions and in this way avoid the use of toxic solvents.

## Discussion

There are few studies where the antiplasmodial activity of genus Calophyllum. Albernaz *et al*. [21] demonstrated the antiplasmodial activity of dichloromethane extracts of the *C. brasiliense* plant. They found an $IC_{50}$ =6.7µg/mL and IS = 121.4 from the bark extracts and $IC_{50}$ =9.5 µg/mL and an IS =14.4, from the root parts. These values were determined by evaluating the cytotoxicity in NIH-3T3 cells, and are similar with the results described in this report. On the other hand, Brenzan *et al*. 2008reported the leishmanicidal potential of the leaves of the crude dichloromethane extract of *C. brasiliense*, demonstrating the importance of this species for its anti-leishmanicidal potential and the richness of its metabolites [22]. The positive results for these extracts mainly of dichloromethane extract and ethyl acetate of *C. brasieliense* direct the study towards the search for active substances by separating their main components. Specifically of the *C. brasiliense* species, a great variety of works have been reported in which a great variety of secondary metabolites has been characterized. Ito *et al*[23], Reported on the basis of chromatographic and spectroscopic tests, five phenolic compounds have been identified as hyperin (hyperoside), amentoflavone, quercetin, gallic acid and protocatechuicacid [24]. Fractions and some phenolic compounds have significant analgesic activity in formalin tests in mice, suggesting that this plant may be useful for the treatment of inflammatory processes [25]. Three analogues with a chromanone nucleus have also been determined, namely isoapetalic acid, white acid and an unknown acid homolog of isoapetalic acid from the hexane extract of *C. brasiliense*seeds [26].

Obtaining these parameters is fundamental and is useful for the development of new antimalarial drugs and in subsequent pharmacological studies to confirm the potential through biological tests *in vivo* in murine models of *P. berghei*, since they reveal new knowledge about the therapeutic effects of this medicinal plant [27]. Plant extracts either in the form of standardized extracts or as a source of pure compounds provide unlimited opportunities for the treatment of various diseases, but when marketed there are many variations in the quality and quantity of the bioactive components so these drawbacks they are the most important to consider in their therapeutic use [28-29]. The main components of an extract can be lost due to different extraction techniques and therefore an increase in toxicity, which limits its consumption for humans [30]. For this reason, the development of procedures to obtain standardized extracts that are more efficient, low cost, effective and safe, require a deeper investigation. On the other hand, the use of standardized extracts is justified for plants that have weaker or less specific pharmacological activities and when the active ingredients are not yet known. However, in order to guarantee constant therapeutic quality and efficacy [31]. It is important to standardize these extracts for their content of active ingredients or, if these are unknown, those compounds most representative of the chemical composition of the extract are selected. In this sense, the chemical standardization of extracts of the *C. brasiliense* species was carried out, in this case with a single marker substance type pyranocuomarin.

## Conclusions

The best extracts of the *C. brasiliense* species were, the extract from the stems with ethyl acetate and extraction after 4 hours, presented a yield of 4.154 ±0.301%, a promising antiplasmodial activity of $IC_{50}$ =9.013±1.288 µg/mL, a cytotoxicity of $CC_{50HepG2}$ =43.340±3.159 µg/mL, an IS=4.808 and a content of marker substances of C175MA2=

0.257±0.021%, and the extract from the stems with DCM: EtoAc (1:1) and extraction for 4 hours, presented a yield of 2.628±0.525%, a promising antiplasmodial activity of $IC_{50}$ =8.314±1.191 µg/mL, a cytotoxicity of $CC_{50HepG2}$ = 40,320±4,235 µg /mL, an IS =4,849 and a content of marker substance of $C_1$=0.543±0.100%. Both extracts presented interesting pharmacological activities for subsequent studies in which extracts are obtained under the same extraction conditions that guarantee reproducibility and effectiveness in other biological tests and framed in the transformation of a phytotherapeutic product.

## Acknowledgments

The authors are grateful for the financial support provided by the Colombian Ministry of Agriculture (No. 009-2007-V7552-38-07), Colciencias through the José de Caldas franchises and the University of Antioquia.

## References

1. WHO, 2015. World Malaria Report 2014. Geneva, World Health Organization. 2014. ( Internet: http://www.who.int/malaria/publications/world_malaria_report_2014/en/ ).
2. OMS, 2017. WorldTechnicalStrategyagainst Malaria 2016-2030. World Health Organization. 2016. http://www.who.int/malaria/publications/atoz/9789241564991/es/ consultado el 16 de Junio de. 2017.
3. World Malaria Report 2018. Geneva: WorldHealth Organization. 2018. Licence: CC BY-NC-SA 3.0 IGO (Internet: https://www.who.int/malaria/publications/world-malaria-report-2018/report/en/)
4. Menard D, Dondorp A. Antimalarial Drug Resistance: A Threat to Malaria Elimination. Cold Spring Harb Perspect Med July. 2017;7. doi:10.1101/cshperspect.a025619.
5. Grimberg BT, Mehlotra RK. Expanding the Antimalarial Drug Arsenal-Now, But How?.Pharmaceuticals (Basel). 2011;4:681-712.
6. Chin J. The control of communicable diseases 17th edition. Editor Officialreport of the American PublicHealthAssociationSeventeenthedition. Pan American HealthOrg.2001;748.
7. Olumese P. Guidelines for the treatment of malaria Third edition. World Health Organization. 313
8. Newman DJ, Cragg GM. Natural products as sources of new drugs over the 30 years from 1981 to 2010. J Natural Products. 2012;75:311-335.
9. Noldin VF, Isaias F, Cechinel V. Calophyllum Genus: Chemical and pharmacological importance. Quim. Nova. 2006;29:549-554.
10. Kimura S, Ito C, Jyoko N, Segawa H, Kuroda J, Okada M, Adachi S, Nakahata T, Yuasa T, Filho VC, Furukawa H. Maekawa T. Inhibition of leukemic cell growth by a novel anti-cancer drug (GUT-70) from *calophyllumbrasiliense* that acts by induction of apoptosis. Int J Cancer.2005;113:158-165.
11. Kouhestani F, Dayer MS, Kamali H. Reversed-phase Liquid Chromatographic Quantification of Pyrethrins in the Extract of Wild *Tanacetumparthenium* (Feverfew) from Northern Khorasan Province (Iran).2013
12. Hout S, Chea A, Bun SS, Elias R, Gasquet M, Timon-David P, Balansard G, Azas N. Screening of selected indigenous plants of Cambodia for antiplasmodial activity. J Ethnophar. 2006;107:12-18.
13. Mesa Vanegas, AM. Estudio fitoquímico de algunas especies de género Piper y Calophyllum con actividad antiplasmodial, citotóxica y antioxidante. estandarización química y estudios de relación estructura-actividad (SAR). Tesis doctoral, Universidad de Antioquia, Medellín, Colombia.2013.
14. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. Simple and Inexpensive Fluorescence-Based Techniquefor High-Throughput Antimalarial Drug Screening. Antimicrobial Agents and Chemotherapy. 2004;48:1803-1806.
15. Malebo MH, Tanja W, Cal M, Swaleh SAM, Omolo MO, Hassanali A. Séquin U, Hamburger M, Brun R, NdiegeIO. Antiplasmodial, anti-trypanosomal, anti-leishmanial and cytotoxicity activity of selected Tanzanian medicinal plants. Tanzania J HealthRes. 2009;11:47-56.
16. Deharo E. A search for natural bioactive compounds in Bolivia through a multidisciplinary approach Part II. Antimalarial activity of some plants used by Mosetene Indians. J Ethnopharm. 2000;69:139-155.
17. Mosmann, T. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunological Methods. 1983;65:55-63.
18. Moore GE, Gerner RE, Franklin HA. Culture of normal human leukocytes. Jama. 1967;199:519-524.
19. Reed GF, Lynn F, Meade BD. Use of coefficient of variation in assessing variability of quantitative assays. Clin Diagn Lab Immunol. 2002;9:1235-1239.
20. Kaou AM, Mahiou-Leddet V, Hutter S, Ainouddine S, Hassani S, Yahaya I. Antimalarial activity of crude extracts fromnine African medicinal plants. J Ethnopharmacol. 2008;116:74-83.
21. Albernaz LC, De Paula JE, Romero GA, SilvaMdo R,Grellier P, Mambu L, Espindola LS. Investigation of plant extracts in traditional medicine of the Brazilian Cerrado against protozoans and yeasts. J Ethnopharm. 2010;131:116-121.
22. Brenzan MA, Piloto Ferreira IC, Campana Lonardoni, MV, Honda PA, Filho ER, Nakamura CV, Garcia Cortez

DA. Activity of extracts and coumarins from the leaves of *Calophyllumbrasiliense*. Onleishmaniabraziliensis. Pharmace Biology. 2008;46:380-386.

23. Ito C, Itoigawa M, Mishina Y, Cechinel-filho V, Enjo F, Tokuda H, Nishino H, Furukawa H. Chemical constituents of *Calophyllum brasiliense*. 2: Structure of three new coumarins and cáncerchemopreventive activity of 4-substituted coumarins. J NatProd. 2003;66:368-375.
24. Kashman Y, Gustafson KR, Fuller RW, Cardellina JH, Mcmahon JB, Currens MJ, Buckheit RW, Jr., Hughes SH, Cragg GM, Boyd MR. HIV inhibitory natural products. Part 7. The calanolides, a novel HIV-inhibitory class of coumarin derivatives from the tropical rainforest tree, *Calophyllum lanigerum*. J Med Chem. 1992;35:2735-2743.
25. Itoigawa M, Ito C, Tan HTW, Kuchide M, Tokuda H, Nishino H, Furukawa H. Cancer chemopreventive activity of naphthoquinones and their analogs from Avicennia plants. CancerLett. 2001;169:15-20.
26. Kijjoa A, Gonzalez MJ, Pinto MM, Anantachoke C, Herz W. Xanthones from *Calophyllum teysmannii* var. inophylloide. Phytochem. 2000;55:833-837.
27. Barth A, Müller D, Dürrling K. In vitro investigation of a standardized dried extract of *Citrullus Colocynthis* Onlivertoxicity in adult rats. Exp Toxic Pathol. 2002;54:223-230.
28. Novak N, Haberstok J, Kraft S, Siekmann L, Allam JP, Bieber T. Standardized extracts from Chinese herbs induce IL-10 production in human monocyte-derived dendritic cells and alter their differentiation in vitro. J Allergy ClinImmunol. 2001;108:588-593.
29. Fidock DA, Rosenthal PJ, Croft SL, Brun R, Nwaka S. Antimalarial Drug Discovery: efficacy models for compound screening. Nature Reviews. Drug Discovery. 2004;3:509-520.
30. Jachak SM, Gautam R, Selvam C, Madhan H, Srivastava A, Khan T. Anti-inflammatory, cyclooxygenase inhibitory and antioxidant activities of standardized extracts of *Tridaxprocumbens* L. Fitoterapia. 2011;82:173-177.
31. Tanaea MM, Lima-Landman MTR, De Lima TCM, Souccar C, Lapa AJ. Chemical Standardization the aqueous extract Cecropiaglaziovii Snethendowed with antiacidsecretion like activities. Phytomedicine. 2007;14: 309-313.