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Journal of Medicinal Plants Research

Full Length Research Paper

Evaluation of antiplasmodial potential of *Aloe barbadensis* and *Allium sativum* on *plasmodium berghei*-infected mice

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A significant percentage of the developing countries are living in malaria-endemic communities and are prone to contacting the disease. The fight against malaria is faced with the occurrence of widespread resistance of *Plasmodium falciparum* to the available anti-malarial drugs. Thus, this study evaluates the anti-malarial activity of extracts of *Aloe barbadensis* and *Allium sativum* in *Plasmodium berghei* infected mice. To achieve this, crude extracts of *A. sativum* and *A. barbadensis* were prepared. These extracts were administered orally to *P. berghei* infected mice after 24 h of infection at the concentration of 400, 600 and 800 mg/kg/day respectively for three days following the modified Peter four- day suppressive test procedure. The clearance rates in *P. berghei* infected mice were determined. Following the internationally accepted principles for laboratory animal use, the blood sample of the experimental mice was collected through cardiac puncture and diluted with normal saline and the experimental mice were infected intravenously. Parasitaemia infection was confirmed using 10% Giemsa-stained thin film fixed with methanol. Clearance rate was evaluated using the same staining procedure after three days of treatment. *A. barbadensis* extract showed a clearance rate A. barbadensis extract showed a clearance rates (% parasitaemia) of 6.3, 18.3 and 32.0% respectively at 400 mg/kg concentration, 5.3 to 28.7% for 600 mg/kg concentration and 4.0 to 22.3% at 800 mg/kg concentration. Also, with Allium sativum, the clearance rates increased from 8.3% to 26.1%at 400mg/kg, 7.7%to 25.1%at 600mg/kg and 6.9% to 24.3%at 800mg/kg respectively. Each extract shows variable level of parasitaemia suppression in dose related manner, when compared with the control groups.

Key words: Antiplasmodial potential, *Aloa barabadensis*, *Allium sativum*, *Plasmodium berghei*.

INTRODUCTION

The incidence of malaria is increasing, and there is an urgent need to identify new drug targets for both prophylaxis and chemotherapy (WHO, 2014). The epidemic is on the increase due to increase in resistance of the parasites to the available drugs as well as unavailability of vaccines against the spread of malaria in developing countries. The biggest challenge yet to the effective control of malaria is combating drug resistance. The parasite responsible for malaria (*P. falciparum*) is becoming resistant even to Artemisinin-based combination therapy (ACT) (Obidike et al., 2013). Malaria is one of the most important infectious diseases in the

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world. Each year 300 to 500 million new cases are diagnosed and approximately 1.5 million people die of the disease. More than 40% of the world's population live in malaria-endemic areas and are at risk of contracting the disease (WHO, 2016). The traditional medicine is estimated to be used by 80% of the population of most developing countries (Abeku, 2007) and these herbal medicines are used for primary health care needs. Although plants are unique in their activities, it has also been found that a particular plant may be used by different tribes or countries for treatment of different ailments. This shows that plants possess a very wide range of biological activities which are attributed to their chemical composition (Edeoga et al., 2005). There is increase resistance to commonly used drugs for malaria treatment in developing countries (Kumar et al., 2009). However, not much has been done to project anti-malarial properties of indigenous plants most especially in Africa (Abosi and Raseroka, 2003). This study was aimed at evaluating the anti-malarial potential of *A. barbadensis* and *A. sativum* as medicinal plants and if found effective, may be useful for the production of tolerable, less toxic and cheap anti-malarial drugs.

MATERIALS AND METHODS

This study was carried out in the animal house, Ladoke Akintola University of Technology College of Health Science, Osogbo.

Sample collection and processing

The research was conducted in accordance with the internationally accepted principles for laboratory animal use and as found in the U.S. guidelines. The mice were purchased from Animal House Ladoke Akintola University of Technology (18g-22g body weight) and *P. berghei* was provided by INMRAT (Institute Of Nigeria Medical Research Advance Technology) in University College Hospital, Ibadan. The mice were infected with *P. berghei* in the same research institute.

The parasite and infection

For *in vivo* anti-malarial assays, chloroquine (CQ) resistances ANKA strain of *P. berghei* was used. The parasite was maintained by serial passage of blood from infected mice to non-infected ones on weekly basis. To infect the mice, blood sample was collected through cardiac puncture of an experimental mouse with a rising parasitaemia of about 24%. Then, the blood was diluted in normal saline; so that each mouse was passaged with 0.2 ml of the infected blood containing about$1x10^7$ *P. berghei* parasitized red blood cells via intraperitoneal route (IP). The mice were housed in plastic cages in the animal house, Pharmacology Laboratory LAUTECH, Osogbo. They were kept under standard twelve hours light and twelve hours dark schedule where room temperature, humidity and ventilation were controlled during the acclimatization period of seven days. The mice were randomly placed into five groups of seven mice each for each treatment including control groups (positive control and negative control), which made up of seven mice in each group. The positive control group was given 0.2 ml of 10 mg/kg/mouse chloroquine (CQ) as a standard drug control, while the negative control group was given 0.5 ml distilled water per mouse. The test extracts were prepared in three different doses (400, 600 and 800 mg/kg of body weight). They were administered daily via the oral route to mimic its use in the folklore. The mice were fed with commercially purchased pallet food. Water was given *ad libitum* while food was withdrawn eight hour prior to treatment to ensure effective absorption from the gastrointestinal tract after oral administration (Coppi et al., 2006). Food was re-introduced thirty minute after treatment and withheld for a further 3-4 h after administration of the extracts as described by Peter et al. (1976). Each extract was administered as a single dose per day for three days. All the treatments were administered orally by using oral cannula. Treatment was started after 24 h of infection on day 0 and was continued daily for three days (from day 0 to day 3). On the seventh day blood sample was collected from the tail of each mouse. Thin smears were prepared and stained with 10% Geimsa stain solution. This was repeated on days 10 and 14, respectively. Then each stained slide was examined under the microscope with an oil immersion objective of 100x magnification power to evaluate the percent suppression of each extract with respect to the control groups. The parasitaemia level was determined by counting the number of parasitized erythrocytes out of 200 erythrocytes in random fields of the microscope. Average percentage chemo-suppression was calculated using this formula:

Average % suppression = 100- average parasitemia in test group x 100 average parasitemia in negative control

Collection of plant materials and extract preparation

Clean garlic bulbs specimens were collected from Oja-Oba Market, Ibadan and sent to the Botany Department of Obafemi Awolowo University of Ile-Ife for identification. The cloves of *Allium sativum* were thoroughly ground and macerated using blender with warm water at 45°C for 15 min and were stirred continuously to form a liquidized material. It was sieved to remove the shaft. 100 ml of water was added to 100 g of cloves according to Koshimizu et al. (1994). The liquid extract was stored at –4°C pending the time it will be used. *Aloevera* leaves were purchased in the Botanical Garden of Obafemi Awolowo University Ile-Ife and identification was done in the same institution. 25 g of *Aloevera* leaves was collected from the base of the plant. The leaf's skin was peeled off with the use of knife. The translucent gel was scooped out and blended in a blender. The gels were blended and poured into clean, sterile Petri dishes and put in a hot air oven for 18 h at 40°C for evaporation of the fluid until it became dry. The powder was scraped out of the Petri dishes and blended again to get a desired texture which was kept in a safe, dry container that has a cap. The powder was afterwards reconstituted with distilled water making it ready for proper use. Healthy physically alright mice, weighing between 18 to 22 g were used. Unhealthy, physically deranged and those that weighed below 18 g and above 22 g were not used.

RESULTS

Results of the study were expressed as mean±standard error of mean (M±SEM). Comparison of parasitaemia between tests and control groups and their statistical significance were determined by student t-test. All data were analyzed at a 95% confidence interval (P=0.05). Percentage parasitaemia and percentage suppression were also calculated. On day one, the mice were infected intravenously with diluted blood containing *P. berghei* as

Table 1. Parasitaemia level within 24 h of infection.

Group	A	B	C	D	E	F	I	J
	%							
Mouse 1	4.5	11	15	9.5	15	8.5	12.5	7.5
Mouse 2	12.5	12.5	8	8.5	7.5	10.5	10	10
Mouse 3	12.5	11.5	8.5	16	9.5	13.5	9	13.5
Mouse 4	13.5	13.5	11.5	8	11	11.5	13	12.5
Mouse 5	15	7.5	12.5	10.5	13.5	8	8.5	9
Mouse 6	15	13.5	13	13.5	10	10.5	7.5	13
Weight (g)	20.4	19.1	20.3	19.3	20.6	20.4	18.8	18.5

Table 2. Distribution of test and control groups of experimental mice at different concentration of the extracts.

Test groups	Concentration (mg/kg)	% Parasitaemia (days)			SEM (days)			P value
		7	10	14	7	10	14	
Aloe vera test	400	6.3	18.3	32.0	6.3±0.73	18.3±1.87	32.0±2	<0.05
	600	5.3	17.4	28.7	5.3±0.63	17.4±1.23	28.7±1.5	<0.05
	80	4.0	12.5	22.3	4.0±1.07	12.5±0.89	22.3±1.3	<0.05
Garlic test groups	400	8.3	19.9	26.1	8.3±0.77	19.9±1.90	26.1±1.5	<0.05
	600	7.7	18.3	25.1	7.7±0.59	18.3±1.56	25.1±1.2	<0.05
	800	6.9	10.4	24.3	6.9±0.57	10.4±1.03	24.3±0.5	<0.05
Control groups	CQ 1	2.3	10.4	21.3	2.3±0.88	10.4±1.46	21.3±1.1	-
	D/W	33.8	36.1	45.2	33.8±1.77	36.1±3.31	45.2±1.7	-

CQ, chloroquine (positive control); D/W, distilled water (negative control).

Parasitaemia level was established in each mouse after 24 h as shown in Table 1. There was no mortality observed in the mice even after 24 h of infection. Table 3 shows the relationship between the different doses or concentration of *A. barbadensis* and *A. sativum* extracts, negative and positive controls in relation to the level of parasitaemia inhibition of the extract after three days of treatment with *A. barbadensis* and *Allium sativum* extracts. Table 1 shows the effect of *A. barbadensis* and *A. sativum* extracts on established malaria infection. The result of *in vivo* evaluation of the *A. barbadensis* and *A. sativum* extracts on established infection showed a reduced parasitaemia level when compared with the negative control. For groups treated with *A. barbadensis* extract, mean parasitaemia with *P. berghei* infected mice ranged from 4±1.07 to 6.3±0.73 on day 7, whereas the corresponding figure in the control groups; positive control treated with chloroquine was 2.3±0.88 and negative control treated with distilled water was 33.8±1.77 on day 7. The extract induced statistically significant inhibition of parasitaemia in all the doses tested compared to the negative control (P<0.05). For groups treated with *A. sativum* extract, mean parasitaemia with *P. berghei* infected mice ranged from 26.1±1.5 to 8.3±0.77 on day 14. The extracts induced statistically significant inhibition of parasitaemia in all the doses tested compared to the negative control (P<0.05). Values were presented as Mean±SEM, n=6. Days 10 and 14 results are also given in Tables 2 and 3, respectively. Also, the result of *in vivo* evaluation of the *A. barbadensis* and *A. sativum* extracts on established malaria infection showed a dose-dependent clearance or chemo-suppressive activity (Table 3). With the treatment of *A. barbadensis* extract at 400 mg/kg the suppression effect was 81%, at 600 mg/kg it was 84%, at 800 mg/kg it was 88% and the positive control CQ at 10 mg/kg it was 93% on day 7. Then, treatment with *A. sativum* at 400 mg/kg exerted 75%, at 600 mg/kg exerted 77%, at 800 mg/kg exerted 80% and positive control CQ at 10 mg/kg exerted 93% parasite clearance or suppression after seven days of treatment with *A. sativum* extract as shown in Table 3. There was reduction in the clearance rate of the parasite with the extracts and the standard drug used (CQ at 10 mg/kg). Also, after day 7, moderate suppressive effect was

Table 3. Effect of *Aloe barbadensis* and *Allium sativum* extracts on established malaria infection.

Test groups	Concentration (mg/kg)	% Suppression (days)		
		D7	D10	D14
Aloe vera test groups	400	81	49	30
	600	84	51	38
	800	88	65	52
Garlic test groups	400	75	41	43
	600	77	49	45
	800	80	71	47
Control groups	CQ 10	93	71	54
	D/W	0.5	0.5	0.5

observed both in the test and control groups.

DISCUSSION

In this study the extracts of *A. barbadensis* and *A. sativum* showed considerable anti-plasmodial properties. The anti-malaria activities of the extracts increased with increase in the concentration of the extracts. This is similar to the study carried out by Abu et al. (2014) and the study carried out on garlic (Coppi et al., 2006).

The parasitaemia suppressive effect of *A. barbadensis* can be hypothetically related with the presence of high concentration of anthraquinones and other quinoid compounds that are the characteristic constituents of the genus and in this species. The result of the present study shows that *A. barbadensis* probably has some intrinsic antimalarial activity from the percentage parasitaemia inhibition and suppression or clearance when compared to that of chloroquine which is the standard drug. This study also established a preliminary approach, the rationale for traditional use of this plant in Nigeria as a remedy for malaria infection.

The parasitaemia suppressive effect of *A. sativum* can be said to be due to the presence of high concentration of allicin (Edeoga et al., 2005) and thiosulfinates substance (Odugbemi et al., 2007) as well as other compounds that are the characteristic constituents of the genus *Allium* (Coppi et al., 2006).

The result of the present study (Table 3 and figure 4) showed that *A. sativum* has some intrinsic antimalarial activities by it percentage parasitaemia inhibition and suppression or clearance when compared to that of chloroquine which is the standard drug. It was observed that an average of one mouse died per test groups. In negative control group all the mice died before the end of the experiment because they were only given distilled water but none died in positive control group during the course of the experiment. Those that died in test groups were majorly those of lesser body weights. Possible causes may be as a result of competition for food, water and intrinsic individual resistance or susceptibility to the plasmodium. Also it was observed in those that died that they were feeling feverish, lacked appetite for food and water. They were sluggish in movement when excited. All these can be as a result of coming down with parasitaemia in those animals where probably extract utilization was slow.

The present study indicates that the extract possesses considerable in vitro antiplasmodial activity against plasmodium berghei infection relatively at high dose where low parasitemia was recorded.

In conclusion, This observation compares well with the advantages of polyherbal therapies over monotherapy (Madara et al., 2010). Similarly, (Odugbemi et al., 2007) reported that the two plants have been traditionally claimed to relieve fever and cure malaria. Crude extracts of *A. barbadensis* and *A. sativum* antimalaria activities against *P. berghei* infection probably indicated that these plants contain some antiplasmodial compounds (Sofowora et al., 2013). However, the crude extract should be further fractionated and tested for their activities against *P. falciparum* and *P. vivax* in order to consider them as potential sources for antimalarial agent for the treatment of human malaria. The fact that can be deduced from this study is that *A. barbadensis* and *A. sativum* possess antiplasmodium activities against *P. berghei.* It would therefore be worthwhile to purify the active compounds in these plants by fractionation and any other method of isolation.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Full Length Research Paper

Medicine bottled (*garrafada)*: Rescue of the popular knowledge

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Using plants to treat health problems is an ancient practice that is still practiced today. One way that plants are used to improve health is through medicine bottled (garrafada). A medicine bottled is a homemade mixture of medicinal plants added to a solvent. Medicines bottled are medicinal mixtures that have been widely used, especially by residents of rural areas. Therefore, the goal of this study was to determine the plant species and contents used to prepare medicine bottled by the Bananal Community in the municipality of Rondonópolis/MT/Brazil. Data was collected through semi-structured interviews with the residents of the community. Data analysis was descriptive. The study revealed a total of 12 medicine bottled types used by the community, with 27 plant species belonging to 24 genera and 14 families. The most frequently cited families were Fabaceae, Rutaceae, Moraceae, and Bignoniaceae. The most frequently cited species was *Brosimum gaudichaudii* Trécul, known popularly as a mama-cadela; was used in three cited medicine bottled. Some medicine bottled was produced with a single species of medicinal plant and others with three or more. Their therapeutic purposes were diverse, with some medicine bottled indicated to treat one disease and others to treat two or more diseases. It can be concluded that the Bananal community demonstrates knowledge about the plant species used and how to extract their active compounds. The strong historical and cultural context, in addition to the diversity and availability of native plant resources in Brazil, may have perpetuated the use of medicine bottled in the Bananal Community.

Key words: Medicinal plants, medicine bottled (*garrafada*), popular knowledge.

INTRODUCTION

The rural populations receive diverse understandings and classifications (Iglehart, 2018). In North America, in the United States, the Census Bureau's 2010, classifies by rural the entire population, housing and territory not included in an urban area; 59.5 million people (19.3% of the population) are rural (HRSA, 2017).

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In the countries of South America, specifically in Brazil, the rural population is characterized as people who have their livelihoods, production and social reproduction predominantly related to land (Brasil, 2013). Particularly noteworthy are family farmers, peasants, rural workers and settlers residing or not in the countryside. They are represented by 30 million people, about 15.64% of the total Brazilian population (IBGE, 2010). They are distributed in diverse races, ethnicities, peoples, religions, cultures and production systems (Brasil, 2013). It is by far the most important social group in the health area (Iglehart, 2018).

When we observe in a global context, we note that despite the differences between developed and developing countries, the health issue of the rural population is the same throughout the world (Strasser, 2003). In the national context, access to care is a major problem and it is a challenge to be overcome (Soares et al., 2015), either by the organizational aspect or the geographical aspect (Donabedian, 2002).

In the first instance, there is a waiting time for the service (Mendes et al., 2012), health professionals failure (Oliveira et al., 2017; Iglehart, 2018; Strasser, 2003), deficiency of sanitation and quality of health services (Soares et al., 2015). The second is the distance to the service unit, travel time, travel cost and access limitations (Mendes et al., 2012; Soares et al., 2015).

Given the difficulties that reduce the population's access to health services and the demand due to exposure to risk factors, the question is how do these rural communities treat their illnesses? From this question arises another important aspect, the traditional medicine that uses the popular knowledge usually acquired from the ancestors, for the preparation of medicine. Although modern medicine is growing from time to time, traditional medicine still plays a large role in the treatment of different diseases (Birhanu and Ayalew, 2018).

The use of medicinal plants prepared in different ways for treatment and cure of disease is the first choice of home remedies due to the availability of raw material at the site (Singh et al., 2017; Umair et al., 2017; Pasa, 2011) or, in some cases, because it is the only source of therapeutic resources (Messias et al., 2015).

A number of different forms of preparation of these medicinal products are available, such as decoction, extract, juice, powder, paste, infusion, tea (Umair et al., 2017; Aziz et al., 2018) and medicine bottled (Saric-Kundalic et al., 2010; Lima et al., 2016).

The term known as medicine bottled (*garrafada*) raises doubts about origin, may have been derived from the *Triaga Brasílica*, secret formulas known to physicists and priests (Camargo, 2011) which is composed of more than 60 substances (Santos, 2009), in particular for medicinal plants.

Probably, the term triaga was replaced in the popular way by medicine bottled, being supposed that the priests supplied their medicines in glasses and in bottles (Camargo, 2011). Carreira and Santos (2001) infers that the bottled medicine (*garrafada*) is a combination between wine and medicinal plants and the term appeared in Portugal between the sixteenth and eighteenth centuries.

In spite of the past decades, and with the advancement in the field of allopathic drugs in the present day, there are still practitioners of this folk medicine in Brazil. The use of medicine bottled, is a strong tradition that stays in the culture of Brazil. Usually, the apprentices are populations *quilombolas*, riverine, healers, herblists (*raizeiros*) people that live in the rural communities and other people that adhere to that habit.

Medicine bottled are homemade medicines that have as solvents wine or cane brandy, water, honey and addition of various types and parts of medicinal plants, such as barks, fruits, leaves, roots or flowers, dried or green, the which are macerated for three to several days (Dantas et al., 2008). Being a preparation of popular origin, there is no fixed relation between the quantity of plant material and the amount of liquid (Jorge, 2009).

The use of plants for the treatment of health is an important aspect for the rural population (Brasil et al., 2017) and although the use of medicine bottled in several various communities is current, researches on the subject are scarce in the literature (Santos and Silva, 2015). This fact boosted this research.On the stage and with the contribution of ethnobotany research and ethnopharmacology, the present study aimed to know the species of plants and the content used in the preparation of the medicine bottled for residents in the rural community of Bananal, Rondonópolis, MT, Brazil.

MATERIALS AND METHODS

Description of the study area

The research was performed in the Bananal Community, Rondonópolis, Mato Grosso, Brazil. Historically, the colonization of the Bananal region began around 1920 with the migration and occupation of landless workers in the Rondonópolis territory (Lima, 2010). The Bananal Community is a rural area located north the Rondonópolis municipality at 16°8'58.68 "S and 54°35'10.63"O (Figure 1). The climate of the region is of agreement Aw Köppen type tropical thermal mega with dry summers and temperatures reaching an annual average of 27ºC (INMET, 2017). The vegetation at the research site is dominated by savanna, with physiognomies of open and forested savannas.

Data collection

First, we visited the community to assess the location, meet the leaders, and present our objectives and the relevance of our study. The Community Health Agent (CHA) was assigned as the mediator during data collections/visits to residents' homes. The CHA has a Family Health Strategy Unit (FHS) in the Bananal Community, where residents receive monthly medical care and the CHA visits

Table 1. List of species registered in the production of bottled in the Bananal community, Rondonópolis, MT, Brazil.

Medicine bottled	Family	Scientific name	Popular name
Medicine bottled 1	Adoxaceae	*Sambucus australis* Cham. & Schltdl.	Sabugueiro
	Amaranthaceae	*Dysphania ambrosioides* (L.) Mosyakin & Clemants	Erva-de-santa-maria
	Asteraceae	*Mikania glomerata* Spreng.	Guaco
	Bignoniaceae	*Tabebuia aurea* (Silva manso) Benth. & Hook. F. ex. S. Moore	Paratudo
		Jacaranda rufa Silva Manso	Caroba
	Bromeliaceae	*Bromelia balansae* Mez	Gravatá
	Fabaceae	*Hymenaea stigonocarpa* Mart. Ex	Jatobá
		Dipteryx alata Vogel	Baru
	Lamiaceae	*Ocimum basilicum* L.	Alfavaca
	Moraceae	*Dorstenia cayapia* Vell.	Carapiá
	Rutaceae	*Citrus sinensis* (L.) Osbeck cv. *pera*	Laranjeira
		Citrus aurantifolia (Christm.) Swingle. cv *tahiti*	Limão
Medicine bottled 2	Malvaceae	*Waltheria americana* L.	Malva-branca
Medicine bottled 3	Rutaceae	*Spiranthera odoratissima* A. St.-Hil.	Manaca
Medicine bottled 4	Fabaceae	*Senna occidentalis* (L.) Link.	Fedegoso
		Amburana cearensis (Allemão) A.C.Sm.	Amburana
	Lauraceae	*Cinnamomum pseudoglaziovii* Lorea-Hern.	Canela
	Moraceae	*Morus nigra* L.	Amora
	Rutaceae	*Ruta graveolens* L.	Arruda
Medicine bottled 5	Lauraceae	*Persea americana* Mill.	Abacate
Medicine bottled 6	Euphorbiaceae	*Croton urucurana* Baill.	Sangra-dágua
	Moraceae	*Brosimum gaudichaudii* Trécul	Mama-cadela
	Euphorbiaceae	*Croton antisyphiliticus* Mart.	Pé-de-perdizes
Medicine bottled 7	Fabaceae	*Pterodon emarginatus* Vogel	Sucupira
Medicine bottled 8	Bignoniaceae	*Jacaranda rufa* Silva Manso	Caroba
Medicine bottled 9	Apocynaceae	*Mandevilla velame* (A.St.-Hil.) Pichon	Velame-branco
Medicine bottled 10	Apocynaceae	*Mandevilla velame* (A.St.-Hil.) Pichon	Velame-branco
	Moraceae	*Brosimum gaudichaudii* Trécul	Mama-cadela
	Rutaceae	*Spiranthera odoratissima* A. St.-Hil.	Manacá
Medicine bottled 11	Fabaceae	*Stryphnodendron adstringens* (Mart.) Coville	Barbatimão
Medicine bottled 12	Moraceae	*Brosimum gaudichaudii* Trécul	Mama-cadela

residents' homes every two weeks. Our goal was to gain confidence and establish a good relationship with the community. Subsequently, we carried out collections/semi-structured interviews from January to December 2016. The interviews were previously authorized by the participants, who signed a free and clear consent form. The following requirements for conducting research were established: prior consent of the participant, 18 years or older, resident in the rural community and registered in the FHS, family member(s) with knowledge of plant uses. Residents who were not found after three attempts during the data collection period were excluded from the survey. The medicinal plant species cited by the residents were visualized, photographed, and collected. The material was herbalized and identified by specialists and compared to exsiccate in the herbarium of the Federal University of Mato Grosso (UFMT), Cuiabá, MT. The scientific names of the species were confirmed using online databases of the Missouri Botanical Garden/Tropics (http:// www.tropicos.org) and the species list of the Flora of Brazil (Reflora, 2018). For taxonomic

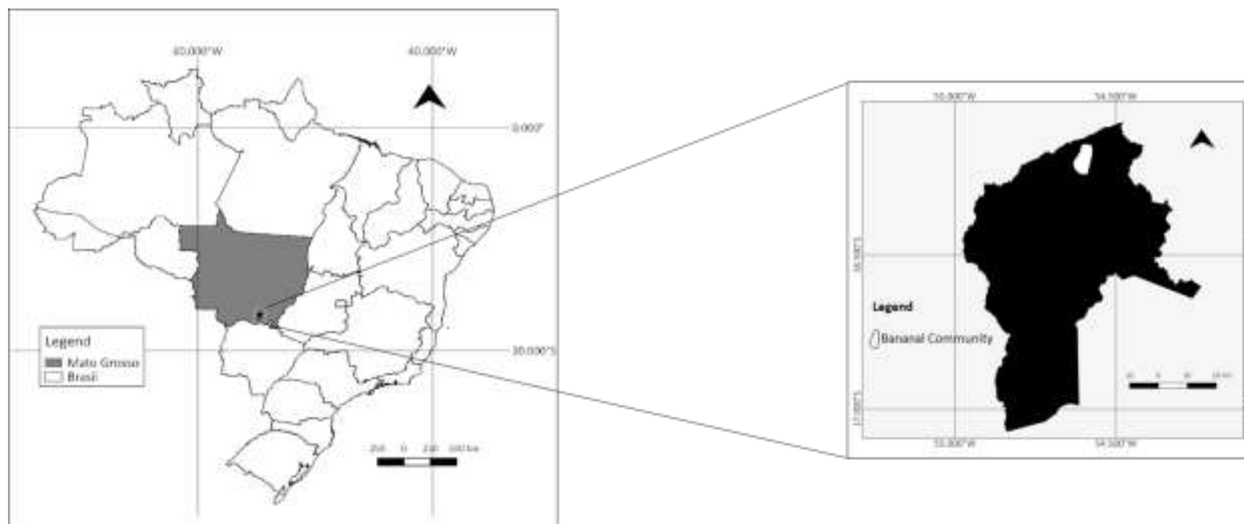

Figure 1. Location of the study site: Bananal Community, Rondonópolis, Mato Grosso, Brazil.

Figure 2. Medicine bottled (*garrafada*) preparation with *Mandevilla velame* (velame-branco) by inhabitant of the Bananal Community, Rondonópolis, Mato Grosso, Brazil.

classification, we used the Angiosperm Phylogeny Group IV (Stevens, 2017).

Data analysis

The information obtained from participants was transferred to Microsoft Excel ® 2013 program, using a general spreadsheet, and then descriptive analysis began. To calculate the similarity between the species used in medicine bottled, comparisons were made using a Jaccard similarity index (SJ), which expresses the similarity between environments based on the number of common species (Brower et al., 1998). The resulting floristic similarity matrix was used to generate a dendrogram.

Ethical aspects

Prior to data collection, the research was sent to the Comitê de Ética e Pesquisa do Hospital Universitário Julio Muller CEP/HUJM in compliance with resolution No. 466 of 12 December 2012 of the National Health Council and received favorable endorsement under CAAE number: 48675315.0.0000.5541. We state that the ethical aspects were fulfilled, and we informed participants about the objective of this study, anonymity of their identity, research consent, and their possibility of withdrawing at any time.

RESULTS AND DISCUSSION

Fifty residents from the Bananal Community were interviewed, who mentioned 12 types of medicine bottled (Table 1). Of these different medicine bottled (garrafadas), 27 plant species were used distributed in 24 genera and 14 families. The most frequently cited botanical families were: Fabaceae (19.35%), Rutaceae (16.13%), Moraceae (12.90%), and Bignoniaceae (9.68%).

Fabaceae also presented the highest number of cited species in other studies about medicinal plants (Birhanu and Ayalew, 2018; Lima et al., 2016; Morya et al., 2018; Alves et al., 2017; Santos and Silva, 2015; Agra et al., 2007). This can be attributed to the fact that this family is the third largest botanical family, with approximately

19,000 species distributed among more than 725 genera (Schwirkowski, 2015). Fabaceae has wide geographic distribution, found in all Brazilian biomes, and presents many life forms, from small herbs to tall trees (Zappi et al., 2015).

Brosimum gaudichaudii, Trécul, popularly known as mama-cadela was the most frequently cited species, which was mentioned in the preparation of three medicine bottled. Studies about this species refer action to treatment of vitiligo (Filho et al., 2015) and other studies support the morphoanatomical, immunohistochemical, physiochemical, phenological, pharmacological, and toxicological aspects of the species (Martins et al., 2015; Land et al., 2017; Filho et al., 2015; Faria et al., 2015; Barbosa et al., 2014; Jacomassi et al., 2007; Cunha et al., 2008; Pozetti, 2005).

The three species *Jacaranda rufa*, popularly known as caroba, *Spiranthera odoratissima* popularly called manacá, and *Mandevilla velame*, popularly known as velame-branco, were each cited in the preparation of two medicine bottled (Figure 2). Studies with *S. odoratissima* (manacá) shows this species ability to treat liver and kidney diseases, stomach aches, headaches, infection and rheumatism. Performed chemical and morfo-anatomical studies, as well as pharmacological research give the anxiolytic, anti-inflammatory, analgesic, anti-protozoan, and leishmanicidal activities of the plant and even isolation of Tiliroside as inhibitor of *Trypanosoma cruzi* (Souza et al., 2018; Cornelio et al., 2017; Galdino et al., 2012; Nascimento et al., 2012; Matos et al., 2004; Matos et al., 2014; Carneiro Albernaz et al., 2012; Santos et al., 2011).

Ethnobotanical studies support the use of *Jacaranda rufa* for stomach aches (Barbosa and Pinto, 2003). Furthermore, other ethnobotanical studies with *M. velame* refer to its use as a depurative, antibiotic, anxiolytic agent and for treatment of woman's health issues (Yazbek et al., 2016; Messias et al., 2015; Pinto et al., 2013). Other research with *M. velame* include catalogues (Morokawa et al., 2013), and microbiological analyses of medicinal preparations made by herbalist (Braz et al., 2015).

Plant parts used

Regarding the plant parts used, the root was the most frequently cited for medicine bottled, followed by peel and leaf, while the least frequently cited parts were seed, middle of peel, flower, and fruit (Figure 3). Similar results were found in another study about medicine bottled, where researchers identified the peel as the most frequently used plant part, representing 48.7%, followed by the root with 18.5% (Dantas et al., 2008). The main components of medicine bottled were the peels of stems and roots (Alves et al., 2017), which aligns with the findings of this study. In the Bananal Community, the plant parts which residents used to make medicine bottled may be related to their historical and cultural context. Historically, roots were used to prepare the brazilian triaga, where the roots were transformed into a powder and mixed with each other (Santos, 2009). Although widely used, Lagnika et al. (2016), states that the use of roots and bark can cause a devastating effect on biodiversity. In that sense, we advise that removal of these plant parts should be done consciously by community residents, in order to preserve the biodiversity of the local flora and ensure it for future generations.

Most used solvents

Three solvent types were cited for medicine bottled preparation, the most frequently used was wine with 58%, then water with 39% and alcohol 3% (Figure 4). Medicine bottled with alcohol solvents were for topical use only, with only one medicine bottled cited. All other medicine bottled must be ingested. The knowledge of the rural community corroborates with findings from the literature. Wine was used in the ancient *triagas* and some root extracts were added at the right time (Santos, 2009). A study about medicine bottled analysis (Dantas et al., 2008) found similar results as ours, citing white wine (56%) as the principal solvent, while water was one of the least cited solvents. Even though the main solvent is wine, other liquids can be used as solvents, as was found in this study. Water is the second most frequently used solvent in medicine bottled. Santos and Silva (2015), infers the liquid medium in which the plants are immersed depends on the community and the purpose of the medicine bottled. They affirm that some medicine bottled can only be produced with water and others with alcoholic beverages, due to the type of plant used. This study provides important data about the knowledge a community has about the preparation of medicinal plants in medicine bottled. Therefore, we infer that the community possesses knowledge about the plant species used, how they should be prepared, and the best solvents and methods to dilute and extract the active principles of the plant.

Preparation mode

Regarding the methods for medicine bottled preparation, the most frequently used method was mixing the plant (s) part (s) with the solvent, mostly wine, and allowing it to tan for a certain time period. It was found that the amount of time the plant remained in the solvent (tanned) was at the discretion of the producers, since the time varied from two to ten days.

Tanning is a method of extracting the active substances of a plant by soaking the plant in a liquid (Interviewed). This tanning method in wine has been reported by other researchers (Santos and Silva, 2015). One study

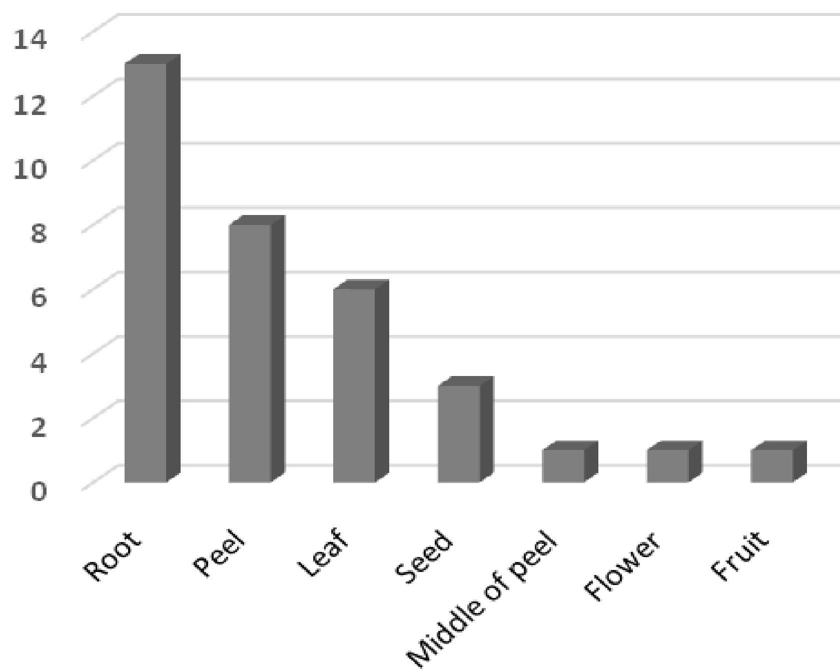

Figure 3. Plant parts used in medicine bottled (*garrafada*) preparations by inhabitants of the Bananal Community, Rondonópolis, Mato Grosso, Brazil.

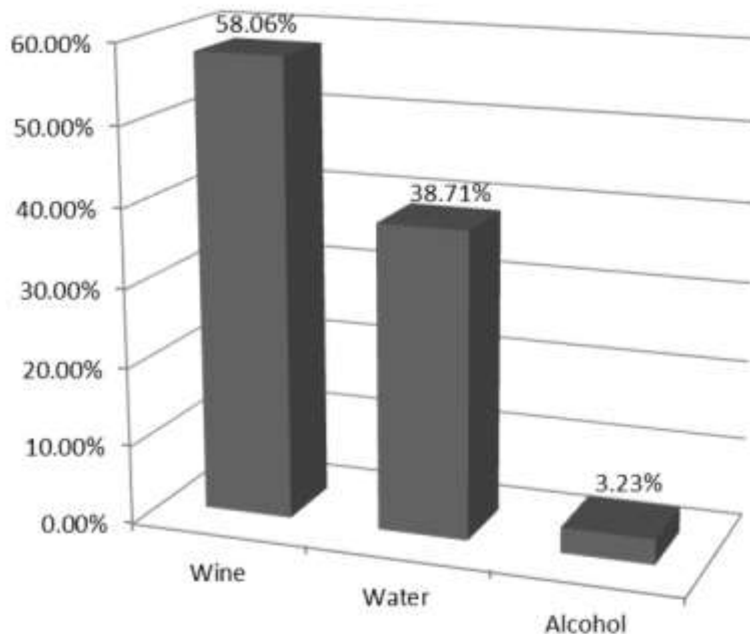

Figure 4. Solvents used to prepare medicine bottled (*garrafada*) by inhabitants of the Bananal Community, Rondonópolis, Mato Grosso, Brazil.

suggest that the medicine bottled should tan for seven days (Chaves and Barros, 2012). In general, (Jorge, 2009) states that medicine bottled can be made using dry or fresh plant material, and they macerate in a liquid,

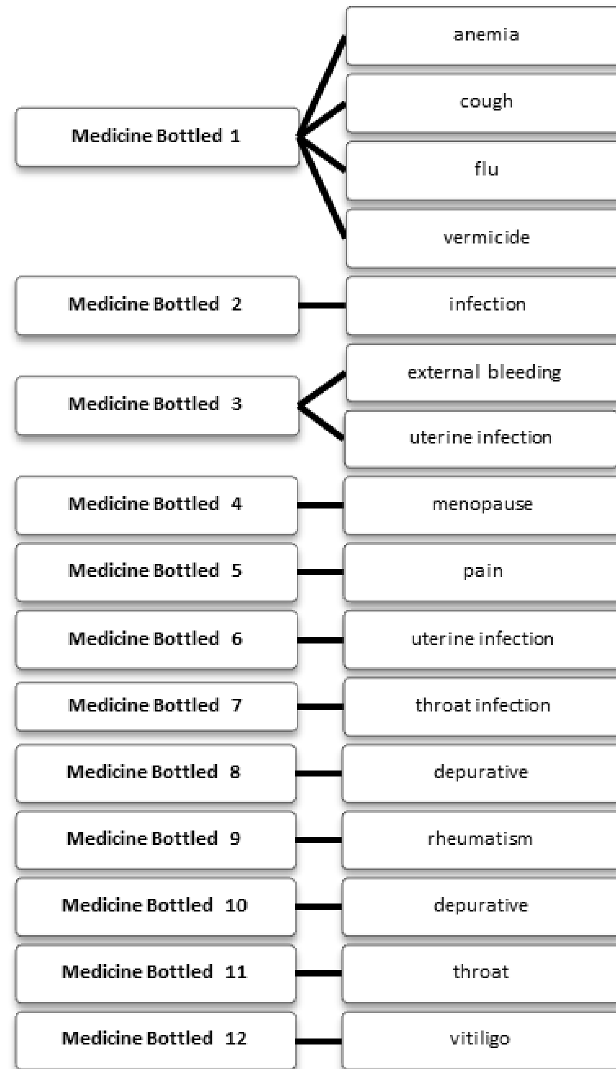

Figure 5. Types of diseases treated with medicine bottled (*garrafada*), Rondonópolis, Mato Grosso, Brazil.

usually cachaça, for a given period of time. Cane brandy has low alcohol grade (40 to 45°GL), which differs from most tinctures that have an alcohol grade between 50 to 700 GL. However, in terms of medicine bottled preparation, many residents did not mention the amount of time for tanning, stating that some plants should be placed in wine and ingested. This fact corroborates with (Santos, 2009), who refers to the divergence of preparation methods. Regarding the quantity of plant parts used in the medicine bottled (*garrafada*), in general, participants cited a small quantity as a "bunch" or a "hand full". In the study by Santos and Silva (2015), they also cited no exact measurement, with a "small piece" cited to make a drink. The authors confirm that the most experienced community members already knew the appropriate amount of plants for the medicine bottled and did not know the exact measurement used. The quantity is measured when they are in possession with the parts of the vegetable and carry out the measure with the hands.

Use purpose and composition of species

The plants used in the medicine bottled were indicated for a total of 13 use purposes (Figure 5). The most prominent uses were to treat throat infections, uterine infections, and as a depurative.

In this study, it was observed that most of the medicine bottled (10 medicine bottled -83.33≅ 83%) were indicated for one single use purpose. The other medicine bottled (2 medicine bottled -16.66≅ 17%) were indicated for two or more use purposes (Figure 5). Among the medicine bottled indicated with only one purpose, seven were

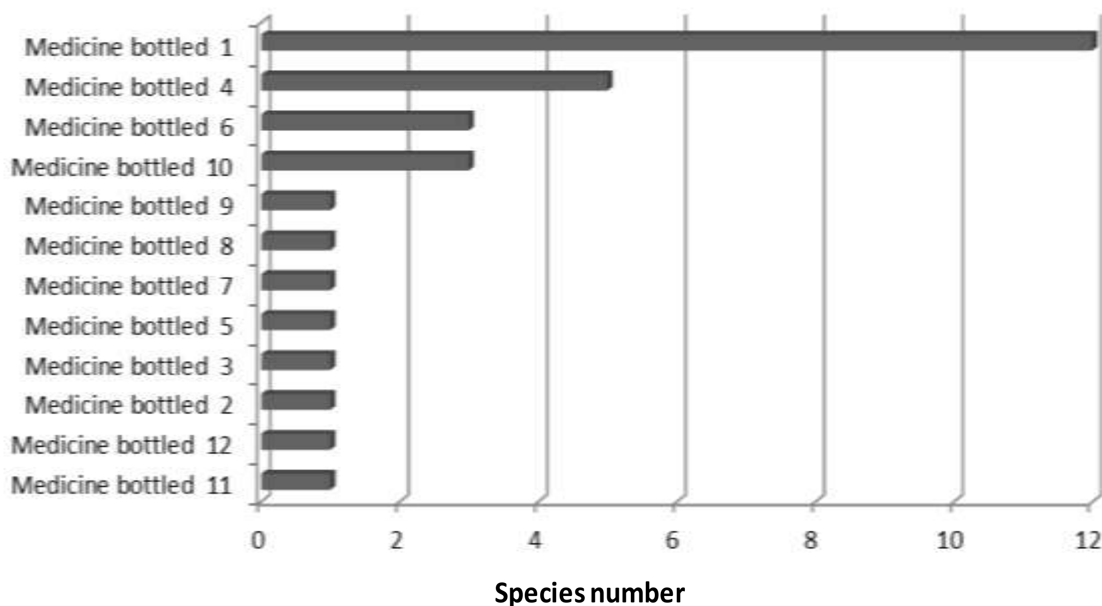

Figure 6. Number of plant species used to prepare medicine bottled (*garrafada*) in the Bananal Community, Rondonópolis, Mato Grosso, Brazil.

prepared with one plant species, these were medicine bottled 2, 5, 7, 8, 9, 11, 12. The other medicines bottled (4, 6 and 10) were prepared using associations of three or more plants species (Figure 6).

The medicine bottled indicated to treat more than one disease that is medicine bottled 3, is prepared with just a single kind of medicinal plant. The other medicine bottled 1, is made up of several species. This suggests that the popular knowledge residents have about medicinal plants and their uses is considerable and diverse. This finding is similar to the study by Agra et al. (2007), who reference a plant species with only one use purpose, as well as species used for more than five diseases. The authors support that the community members had considerable knowledge about plants and their uses, for a wide variety of diseases, which was due to the experience of previous generations.

The fact that some cited medicine bottled are produced with one medicinal plant species demonstrates that it is not the quantity of plants the community uses, but rather, their knowledge about how to use a plant. Furthermore, the drug potential and which solvent are used to prepare the medicine bottled to extract the plants active ingredient is the unique knowledge that the rural community holds. The preparation of medicine bottled using a single species was also reported by Chaves and Barros (2012), who mentioned that the medicine bottled are produced with the one or more plant part.

In daily practice, residents also used associate of different plants, that is three or more species. This method of composition of medicine bottled was also found in research conducted by Muniz and Ito (2016). In this sense, the medicine bottled has a special focus, because it uses the combination of two or more medicinal plants to obtain an effect synergic with the maximum therapeutic effect (Aziz et al., 2018). According to Camargo (2011), the bottle plays a double role, complementary, the symbolic and functional. The first refers to that subjectively constructed in the religious myth, the belief on the part of the patient, the healer. The second is functional role, which each plant performs from its active principles, pharmacological activities, scientifically proven.

Medicine bottled (*garrafada*) could be stored and consumed for a period of time. This action is only possible due to the presence of alcohol contained in the solvents that is acting as a preservative (Thompson and Davidow, 2016). This corroborates the results found in this work. We report that the determination of the number of species used in medicine bottled (*garrafada*) is based on the experience acquired, with no wrong or right number of species used. Therefore, this study corroborates with the results of Jorge (2009) that there is no fixed relationship between the amount of plant material and liquid used for medicine bottled.

Similarity

In relation to the similarity of plants used in the medicine bottled, we verified a division of two groups: G1 and G2 (Figure 7). The group 1 (G1) split into two sub groups: SG1 and SG2. The SG1 formed with 50% similarity, while the SG2 formed with 22% similarity. Group 2 (G2) represented the other samples of medicine bottled and

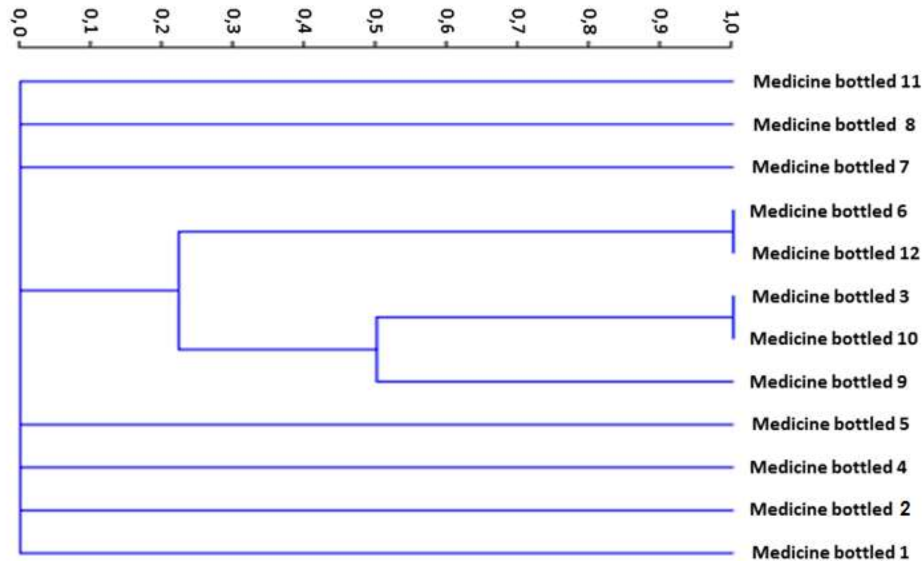

Figure 7. Dendrogram of Jaccard similarity between the medicine bottled (*garrafada*) produced by the Bananal community, Rondonópolis, MT.

showed similarity values = 0, which means that no species were shared in this group. In the SG1, the similarity was higher between the samples 3, 9, 10 who shared the species *S. odoratissim* (manacá) (3, 10) and *M. velame* (velame-branco) (9, 10). In the SG2, the similarity was greater among the samples 6 and 12, which shared the species *B. gaudichaudii* (mama-cadela). These results show that there is a selective choice of the species that make up each medicine bottled. This diversity of choice could be related to the knowledge acquired from the participants' ancestors and new information. Although our results do not present the entire medicinal flora used by the Bananal Community, it still represents a portion of it.

Considerations

The methods used by people for home-based extraction of active principles are important and should be used with caution, as the concentration of active substances could increase or decrease depending on the method used (Saraiva et al., 2015). Study microbiological analyses carried out (Braz et al., 2015) on medicine bottled, syrups, and capsules found contamination by molds and yeasts, which can present high fungal load. The authors highlighted that the high degree of contamination present in the preparations may alter the effectiveness of medicine bottled and pose health risks. It is stressed that although the study community has no intentions of manufacturing and selling their medicine bottled, we agree with Braz et al. (2015) and support maintaining quality of medicine bottled to ensure their efficacy and to keep users safe. The medicine bottled (*garrafada*) are produced by mixtures of several medicinal plants. They contain different active compositions that can potentiate or impede other physiological actions (Passos et al., 2018). In this way, we emphasize that special care should be taken to ensure the quality of products manufactured for internal or topical use. The appropriate manipulation, conservation, and identification of the product with the names of the plants, botanical component and used concentration are essential (Marques et al., 2015), as well as knowledge about associations between species that can increase effectiveness or inhibit the active compounds of the plant and cause adverse effects to human health (Muniz and Ito, 2016). The expressive use of medicinal plants by the population and popular knowledge needs studies that confirm the information about the actions of the plants. This is to minimize the side effects and toxicological effect, since its use must be reliable and safe (Firmo et al., 2012). The standardization of raw plant materials, including species selection, sowing, cultivation, harvesting, drying, storing, and quality evaluation is fundamental for plants with therapeutic purposes (Reis et al., 2011).

CONCLUSION

The community of Bananal uses the medicine bottled as alternative medicine for the treatment of their illnesses. Although differences exist in their preparation, the informers evidenced considerable knowledge about the medicinal plants and their action. The recognition of the

use of medicine bottled as a popular practice is important in the process of valorization of knowledge and rescue of tradition of a community. Medicine bottled research is still incipient and the present study contributes to the popularization of use of this resource as medicine in communities. The obtained result stimulates the accomplishment of researches of chemical, pharmacological, toxicological and clinical character. These can evaluate the interaction and the medicinal plants effectiveness in the treatment, as well, as discover bioactive potential that can generate new drugs. It is suggested that health professionals direct attention during the service to the rural community about the use of medicinal plants, once, which is part of the daily life of these people and public policies be implemented to strengthen popular knowledge and correct the rational use of plant resources.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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Comparison of several hydrophilic extracts of *Polypodium leucotomos* reveals different antioxidant moieties and photoprotective effects *in vitro*

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Oral photoprotection can be used to prevent photocarcinogenesis and photoaging. It is mainly based on the presence of antioxidant moieties in phytochemical extracts. The appearance of different botanical formulations endowed with apparently similar properties requires their side-by-side examination using an unbiased approach. The objectives of this study were to determine the antioxidant properties of several extracts of the fern *Polypodium leucotomos* and their ability to prevent ultraviolet (UV) damage *in vitro. In vitro* study with several extracts using cell-free and cellular assays was the design used. High performance liquid chromatography (HPLC), antioxidant assays, and cellular viability assays were the method used. Various extracts of the same plant species contain different amounts of antioxidant moieties. They bear different antioxidant and photoprotective capability at a cellular level, as determined by cellular viability assays and the appearance of DNA damage markers after UV exposure. Each botanical extract is endowed with different photoprotective properties. In these experiments, extracts from ferns' leaves are better photoprotectors than extracts from the rhizomes, but it is necessary to assess each formulation separately.

Key words: *Polypodium*, antioxidant, ultraviolet (UV), photoprotection

INTRODUCTION

Interest in photoprotection has grown recently. Photoprotection aims to prevent skin cancer in its various forms. Three main modes of photoprotection exist: (1) physical protection, which includes hats, sunglasses and clothes; (2) topical sunscreens; and (3) systemic photoprotectors. The three modes are not mutually exclusive; on the contrary, they all are necessary to prevent the deleterious effects of ultraviolet (UV) over-exposure. Of these, systemic photoprotectors are the most intriguing because, unlike the other two, they bear potential to revert some of the deleterious side-effects of previous unprotected exposure events, specifically photoaging and photo-induced carcinogenesis. This potential underlies the outburst of interest in this mode of photoprotection; however, it also provides ample opportunity for non-sanctioned, uncharacterized products

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under the promise of miraculous reversion of cancer, or aging.

Most systemic photoprotectors are phytochemicals endowed with potent antioxidant activities. Some of these can be obtained naturally from native sources, e.g. green Powerful antioxidants appear in phytochemicals obtained from non-edible, but non-toxic sources, e.g. ferns. For example, research on certain hydrophilic extracts obtained from the Central American fern *Polypodium leucotomos* (PLEs) has demonstrated their beneficial properties in terms of photoprotection (Berman et al., 2016).

Most *P. leucotomos* extracts studied to date are not toxic, even at high doses (Kuo and Yang, 2008; Nikitaki et al., 2015). *P. leucotomos* extracts inhibit UV-catalyzed formation of cyclobutane pyrimidine dimers (CPD) (Zattra et al., 2009) and prevent UV-mediated mitochondrial DNA damage (Villa et al., 2010). They also possess anti-inflammatory properties, decreasing sunburn and erythema (Gonzalez and Pathak, 1996). They also curb the expression of pro-inflammatory mediators such as tumor nuclear factor (TNF)-α, inducible nitric oxide synthases (iNOS) and cyclooxygenase (COX)-2 (Janczyk et al., 2007; Zattra et al., 2009). An interesting property of most *P. leucotomos* extracts studied is that they prevent UV-induced immunosuppression (Middelkamp-Hup et al., 2004; Siscovick et al., 2008), which is one of the hallmarks of photo-induced carcinogenesis (Cavallo et al., 2011). This is due to the inhibition of the isomerization of *trans*-urocanic acid. *P. leucotomos* extracts prevent the appearance of *cis*-urocanic acid (UCA) (Capote et al., 2006), which is immunosuppressive (Walterscheid et al., 2006). *P. leucotomos* extracts also preserve the viability and function of skin myeloid cells (Middelkamp-Hup et al., 2004).

Different *P. leucotomos* extracts are currently commercialized by several companies, including Fernblock® (Cantabria Labs, Madrid, Spain). At face value, all these formulations are endowed with anti-aging, anti-oxidant and photoprotective properties. However, the proprietary extraction methods of each company (use of different solvents, different biochemical and biophysical methods and different parts of the plant) and the different geographical origin and growing conditions of the plants suggest that these extracts may have vastly different properties.

In this study, some of these extracts have been compared in an unbiased, objective manner, analyzing their known antioxidant moieties by high performance liquid chromatography (HPLC), as well as their antioxidant capability in cell-free assays. Emphasis was made on specific antioxidant moieties previously described in these extracts (Garcia et al., 2006). Finally, their effect on cell survival and the appearance of DNA damage markers upon UV exposure have been assessed. These experiments were carried out in dermal fibroblasts and a keratinocyte cell line (HaCat cell line), which are widely accepted models to dissect the effect of irradiation *in vitro* (Fernandez et al., 2014). The results indicate that the different origins, extraction methods and additives together with the use of different parts of the plant result in very different antioxidant profiles, which underlie most, but not all, of the beneficial effects of these extracts.

MATERIALS AND METHODS

P. leucotomos extracts (PLEs)

Six different *P. leucotomos* extracts were included in the study: 1 to 3 are from aerial parts of the plant (leaves) and 4 to 6 are rhizome extracts. Specifically, Sample #1 consists of a buffered aqueous extract of *P. leucotomos* leaves at pH=7 (Fernblock®); Sample #2 is an aqueous extract of *P. leucotomos* leaves extracted at basic pH; Sample #3 is a hydro alcoholic, acidic extract of *P. leucotomos* leaves; (4) Sample #4, a granular hydro alcoholic extract of the rhizome of *P. leucotomos*; (5) Sample #5, a hydro alcoholic extract of the rhizome of *P. leucotomos*; (6) Sample #6, consisting of a hydro glycolic extract from the rhizome of *P. leucotomos*.

HPLC

Samples were prepared at 10 mg/mL by mixing 0.5 g of each dry extract with 50 mL double distilled water for 30 min at room temperature under constant stirring. Samples were filtered through a 0.45 µm-pore polyvinylidene difluoride (PVDF) membrane and 0.1 mL was loaded into a liquid HPLC device from Waters Alliance equipped with a Luna C18(2) column (5 µm, 100 A, 250 × 4.6 mm) from Phenomenex. Flux remained constant at 1 mL/min. Three mobile phases were used; 100% milliQ water (A); 10:90 (v/v) mixture of glacial acetic acid: milliQ water (B); 50:50 (v/v) mixture of acetonitrile: methanol. The elution gradient was carried out at 30°C.

Samples were monitored continuously at 260 and 290 nm, and represented at 260 nm from 0 to 65 min and 290 nm from 65 min to the end of the experiment (140 min). The reason for this switch is that 4-hydroxybenzoic and 4-hydroxy-3-methoxybenzoic acid are better detected at 260 nm, whereas the rest of the species analyzed are better detected at 290 nm.

HPLC standards

3,4-Dihydroxybenzoic (protocatechuic), 4-hydroxybenzoic, 4-hydroxy-3-methoxybenzoic acid (vanillic), 3,4-dihydroxycinnamic (caffeic), 4-hydroxycinnamic, and 4-Hydroxy-3-methoxycinnamic (ferulic) acid were from Sigma-Aldrich. Standards were diluted at 10 µg/mL in double distilled water and 0.1 mL was loaded into the column as described earlier.

ABTS antioxidant assay

ABTS antioxidant assay is a gold standard to study the antioxidant capability of a given substance, as reviewed previously (Dong et al., 2015). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), potassium persulphate (PPS), (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox C), and 3,3',4',5,7-pentahydroxyflavone were from Sigma-Aldrich and prepared at 7 mM in H_2O (ABTS), 2.5 mM in H_2O (PPS) and 0.2 mM in ethanol (Trolox C). ABTS+* (reactive) was prepared by mixing equal volumes of ABTS and PPS solutions, incubating the

mixture in the dark for 16 h at room temperature and adding PBS until 734 nm absorbance = 0.7-1.0. Extracts were prepared at 1 mg/mL in H_2O. The experiment was performed by mixing 0.3 mL of vehicle (H_2O), 0.3 mL of a 0.2 mM Trolox C solution or 0.3 mL of each extract with 2.7 mL of ABTS+*. The mixtures were incubated for 15 min in the dark and absorbance at 734 nm was measured immediately in a Biorad Smartspec 3000 spectrophotometer. Data were collected in triplicate and represented as the relative percentage of ABTS reduction compared to Trolox C, which is considered 100%.

FRAP antioxidant assay

FRAP is another classic method to assess the antioxidant capability of a substance (Amorati and Valgimigli, 2015). 2,4,6-Tripyridyl-s-triazine (TPTZ) and ferric chloride ($FeCl_3$) were from Sigma-Aldrich, and prepared at 10 mM in 0.04 M HCl (TPTZ) and 20 mM in 0.3 M acetate buffer pH 3.6 ($FeCl_3$). The protocol was as follows: the reactive solution (FRAP) was prepared by mixing TPTZ: $FeCl_3$: acetate buffer in a 1:1:10 proportion. Extracts were prepared as for the ABTS assay. The experiment was carried out by mixing 2.85 mL of FRAP solution with 150 µL of vehicle, each extract or Trolox C. Mixtures were incubated for 15 min in the dark and absorbance at 593 nm measured immediately. Data were collected in triplicate and represented as the relative percentage of FRAP reduction as compared to Trolox C, which is considered 100%.

Cellular assessment of the photoprotective activity of the *P. leucotomos* extracts

All the experiments involving primary cells were done in agreement with the Helsinki declaration, and the specific protocols were approved by the Research Ethics committee of the Universidad Autónoma de Madrid, Spain. Human retroauricular fibroblasts were obtained from healthy donors, who received thorough information regarding the use of their cells and signed an informed consent. HaCaT immortalized keratinocytes have been described previously (Boukamp et al., 1988). All the reagents were from Sigma-Aldrich unless otherwise specified. Both cell types were cultured in DMEM medium supplemented with 50 U/mL penicillin, 50 U/mL streptomycin, 2 mM L-glutamine and 10% fetal bovine serum and routinely passaged using trypsin-EDTA 0.25%. Fibroblasts were used between passages 12 and 15. To assess the photoprotective activity of the extracts, cells were cultured in 100 µL of DMEM in flat bottomed, 96-well dishes at 10^5 cells/well. After reaching confluence, medium was replaced with 100 µL of non-supplemented DMEM without phenol red containing the appropriate dilution of each extract (data shown at 1 mg/mL). Cells were immediately irradiated with an UV lamp (Micro-UV Irradiator UV-1047Xe from Frontier Laboratories) in the 280 to 400 nm range. Cells were irradiated at 5 cm for 7 min for a total energy of 2.2 J/cm^2. After irradiation, medium was replaced with supplemented DMEM and dishes were incubated for 2.5 h at 37°C, after which cells were fixed, stained with crystal violet, solubilized with 0.1% sodium dodecyl sulfate (SDS) and measured in a spectrophotometer at 543 nm. Data was relativized to the survival in the Fernblock condition, which was reported previously (Alonso-Lebrero et al., 2003). Each experiment was performed three times in triplicate.

Assessment of the appearance of DNA damage markers

HaCaT cells were cultured as described, treated and UV-irradiated as described earlier. Cells were allowed to recover for 4 h, fixed with 4% paraformaldehyde in PBS, permeabilized with 0.5% Triton X-100 and stained with a p139Ser-γH2AX antibody (Novus Bio) followed by AlexaFluor568-conjugated goat anti-rabbit antibody (Invitrogen). Alternatively, cells were stained with the CPD staining kit from Cell Biolabs Inc., according to the manufacturer's instructions. Flow cytometry was carried out in a FACS Canto machine (Becton-Dickinson) and immunofluorescence images were obtained with an epifluorescence microscope fitted with an Olympus DP50 digital camera and using the following excitation filters: UVA (360-370 nm, exciting filter UG-1) for DAPI or blue (450-490 nm, exciting filter BP 490) filers. Images were processed using the Adobe PhotoShop 7.0 software (Adobe Systems).

Statistical analysis

Data are given as mean ± standard deviation (SD) from three independent experiments. Statistical significance was evaluated using the Student's t-test, and differences were considered to be significant at a value of $p < 0.05$. Where indicated, *$p<0.05$; **$p<0.01$; n.s: not significant.

RESULTS

Antioxidant moieties present in different *P. leucotomos* extracts measured by HPLC

First, the different *P. leucotomos* extracts were analyzed by HPLC to determine their relative antioxidant content. Based on previous evidence gathered using some of these extracts (Garcia et al., 2006), these experiments focused on several specific antioxidant aromatic acids, including 3,4-dihydroxybenzoic (protocatechuic), 4-hydroxybenzoic, 4-hydroxy-3-methoxybenzoic acid (vanillic), 3,4-dihydroxycinnamic (caffeic), 4-hydroxycinnamic, and 4-Hydroxy-3-methoxycinnamic (ferulic). The first two were analyzed by absorbance at 260 nm and the rest at 290 nm to improve resolution. Samples were prepared as indicated in Material and Methods. Figure 1A shows the HPLC standards used at 10 µg/mL (0.1 mL). Samples #1 and #2 (Figure 1B to C) contained detectable amounts of these species. Sample #3 (Figure 1D) also contained most of these antioxidant moieties. Conversely, sample #4 contained very small amounts of 4-hydroxybenzoic and caffeic acids; whereas vanillic and cinnamic acids were almost undetectable (Figure 1E). Sample #5 contained smaller amounts of all these antioxidants, particularly ferulic acid, which was almost undetectable (Figure 1F). Sample #6 contained almost none of these antioxidant moieties, although it displayed a very prominent peak similar to ferulic acid in terms of retention time (98.56 vs. 97.386, Figure 1G and 1A, respectively).

Antioxidant activity of the different *P. leucotomos* extracts

To measure the actual antioxidant capability of the different *P. leucotomos* extracts, two gold-standard

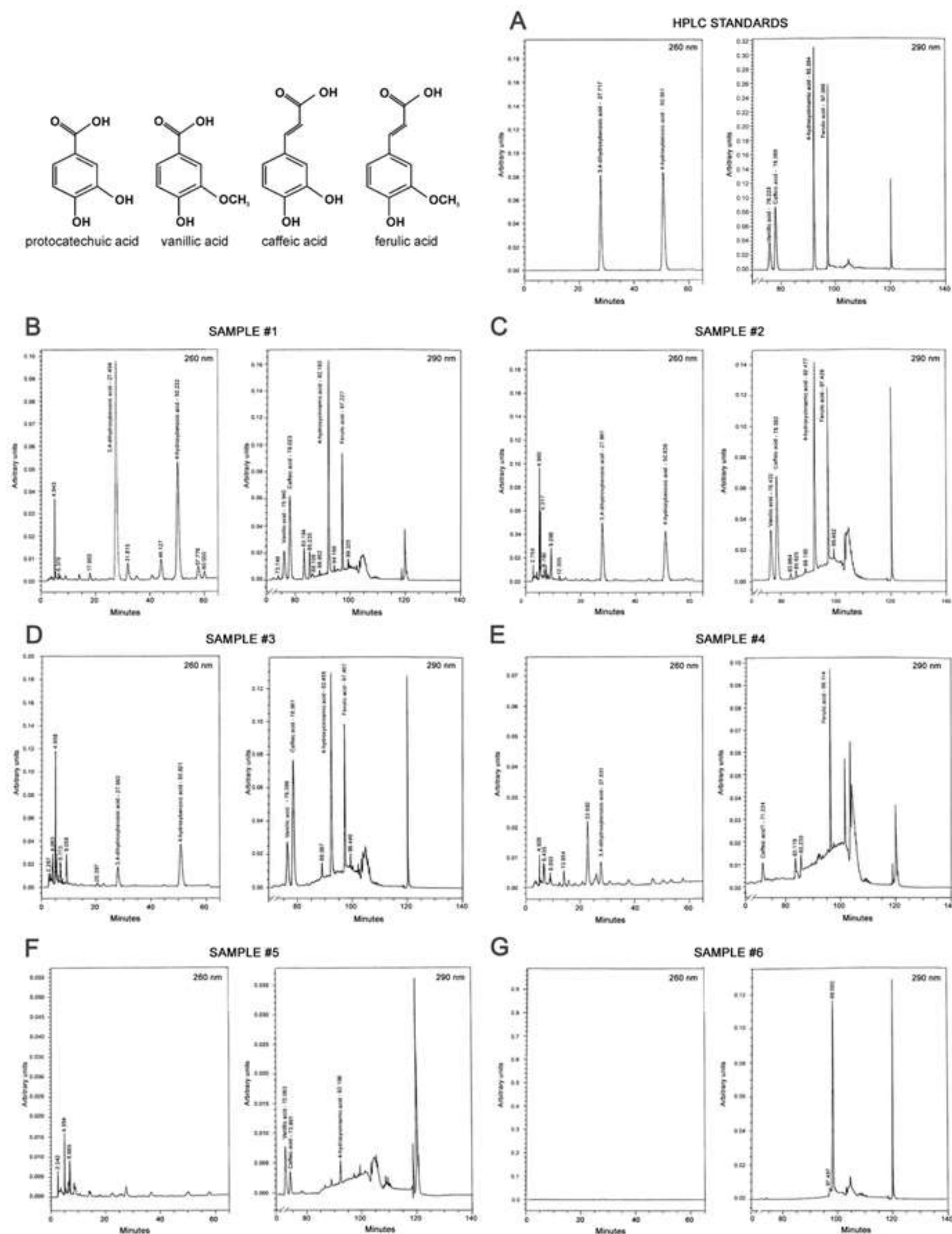

Figure 1. HPLC profiles of the *P. leucotomos* extracts reveal different levels of known antioxidant moieties. Top left, structures of some of the antioxidant moieties analyzed throughout. Panels depict HPLC profiles of control antioxidants (A) and the *P. leucotomos* extracts (B-G). In all cases, graphs represent arbitrary units of relative abundance in the Y axis and elution time in the X axis. Numbers in the images represent the elution time of each peak. (A) HPLC standards used in the experiment, 0.1 mL of each standard at 1 mg/mL. (B-G) HPLC curves of the extracts, labelled as samples #1-6. Identified moieties are indicated next to their retention times. Graphs are shown at 260 nm from times 0-65 min and at 290 nm from 70-140 min for clarity. A representative curve out of three performed is shown for each extract.

antioxidant assays were performed, FRAP and ABTS. Although both assays measure the antioxidant activity of the extracts, FRAP requires acidic pH, whereas ABTS requires a pH close to 7. Results are represented as the

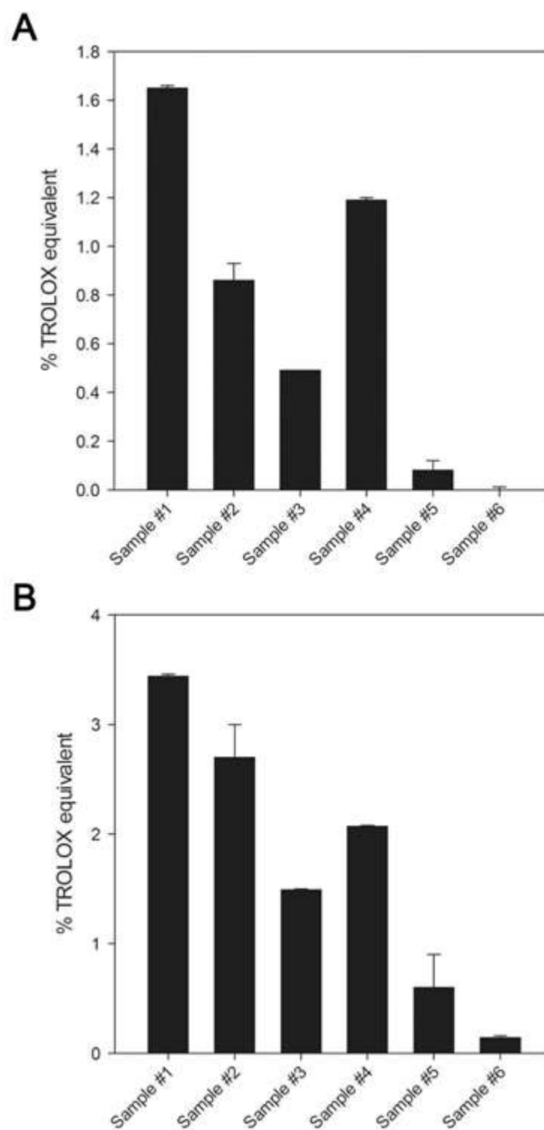

Figure 2. Antioxidant determinations in cell-free systems reveal the different antioxidant potential of the assayed *P. leucotomos* extracts. FRAP (A) and ABTS (B) antioxidant assays were performed as indicated in the Material and Methods section for each sample (#1-6). Vehicle was water and Trolox C was used as reference in each case to set up 100% on each curve. For FRAP, data represents the antioxidant potential of 0.15 mg of each extract (150 µL of 1 mg/mL solution) compared to 30 nmol of Trolox C (150 µL of a 0.2 mM solution of Trolox C), which was arbitrarily set up as 100%. For ABTS, data represents the antioxidant potential of 0.3 mg of each extract (300 µL of 1 mg/mL solution) compared to 60 nmol of Trolox C (300 µL of a 0.2 mM solution of Trolox C), which was arbitrarily set up as 100%. In both graphs, data is the mean percentage ± SD of three independent experiments performed in triplicates.

percentage of the antioxidant ability of a well-characterized antioxidant, Trolox. Data are as shown in Figure 2 (FRAP) and Figure 2B (ABTS). These results indicate that Samples #1 to 4 were endowed with significant antioxidant activity (a value ≥0.3% is considered an active antioxidant in these assays),

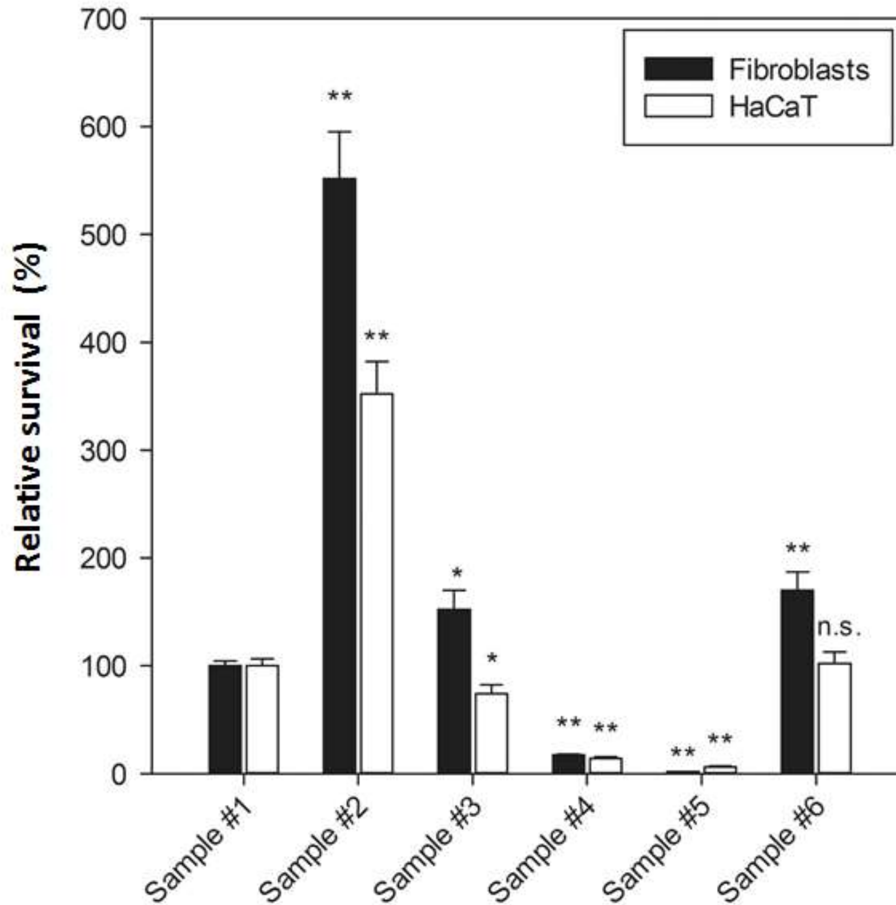

Figure 3. The assayed *P. leucotomos* extracts have a different effect on cell survival upon UV insult. Dermal fibroblasts (black bars) and HaCaT transformed keratinocytes (white bars) were treated with 1 mg/mL of each extract and irradiated. Cell survival was determined by staining with crystal violet by absorbance at 543 nm. Data was relativized to the survival induced by sample #1 (first pair of bars). Data is the mean ± SD of three independent experiments performed in triplicate. Significance was determined using Student's t test. * $p<0.05$; ** $p<0.01$; n.s., not significant.

whereas Samples #5 and #6 displayed much lower, almost negligible antioxidant capability.

In vitro photoprotective activity of the *P. leucotomos* extracts

The actual photoprotective activity of the different *P. leucotomos* extracts was then measured in *in vitro* assays using HaCaT cells (to represent skin keratinocytes) and dermal fibroblasts, which are the two most abundant cell populations in the skin (Fernandez et al., 2014). Cells were subjected to a dose of UV (280 to 400 nm) corresponding to 2.2 J/cm^2. Figure 3 shows the survival data relative to the survival induced by sample #2, as reported previously (Alonso-Lebrero et al., 2003). The data indicates that, compared to Sample #1, Sample #2 was much more active (>5 times on fibroblasts and >3 times on HaCaT). Samples #3 and #6 exerted a certain degree of photoprotection, but none of these reach a 2-fold threshold. Finally, Samples #4 and #5 were almost inactive in terms of UV-induced photoprotection.

Effect of the *P. leucotomos* extracts on the appearance of DNA damage markers upon UV irradiation

To gain further insight into the photoprotective mechanism of the extracts, the phosphorylation of H2AX (γH2AX) in Ser139 was assessed by flow cytometry and immunofluorescence. This is a non-exclusive marker of the appearance of double-strand breaks in DNA (Kuo and Yang, 2008). These experiments revealed that, upon UV

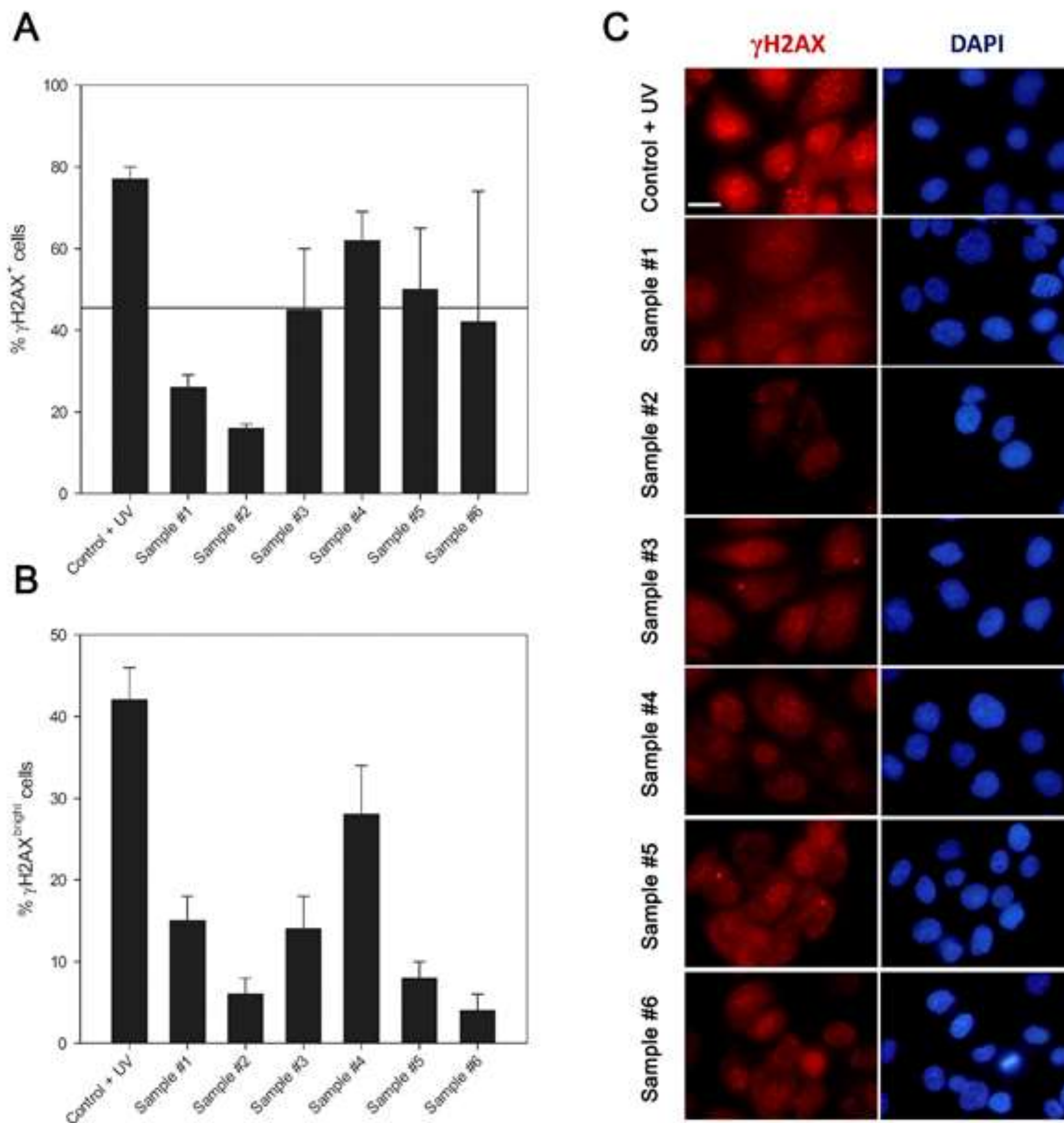

Figure 4. The assayed *P. leucotomos* extracts have a different effect on the phosphorylation of H2AX upon UV irradiation. (A-B) HaCaT transformed keratinocytes (white bars) were treated with 1mg/mL of each extract and irradiated as indicated in Material and Methods. Cells were then fixed, stained for gH2AX and measured by flow cytometry. Data in (A) represents the percentage of positive cells ($\gamma H2AX^{+}$), whereas data in (B) represents the percentage of very positive cells ($\gamma H2AX^{bright}$). (C) Representative immunofluorescence images of irradiated cells in the presence of the different extracts. Briefly, cells were allowed to adhere for 24 h to glass coverslips, medium was replaced with colorless medium containing the extracts at 1 mg/mL, irradiated, allowed to settle for 2.5 h, fixed and stained as in (A). Fields are representative of >40 examined per condition. Bar= 20μm.

irradiation, almost 80% of the cells became positive for γH2AX ($\gamma H2AX^{+}$, Figure 4A). Of these, over 40% contained high levels of γH2AX ($\gamma H2AX^{bright}$) as determined by setting an arbitrary threshold, MFI ≥ 30,000 (Figure 4B). As before, the extracts showed important differences in terms of numbers of $\gamma H2AX^{+}$ and $\gamma H2AX^{bright}$ cells. Sample #1 decreased the number of $\gamma H2AX^{+}$ cells to just over 20% and of $\gamma H2AX^{bright}$ cells to around 15%. Sample #2 was even more effective, decreasing the levels to less than 20% and 5%, respectively. On the other hand, samples #3-5 were less effective, although the latter decreased the number of $\gamma H2AX^{bright}$ cells than the other two. Finally, sample #6 was not very effective reducing the number of $\gamma H2AX^{+}$

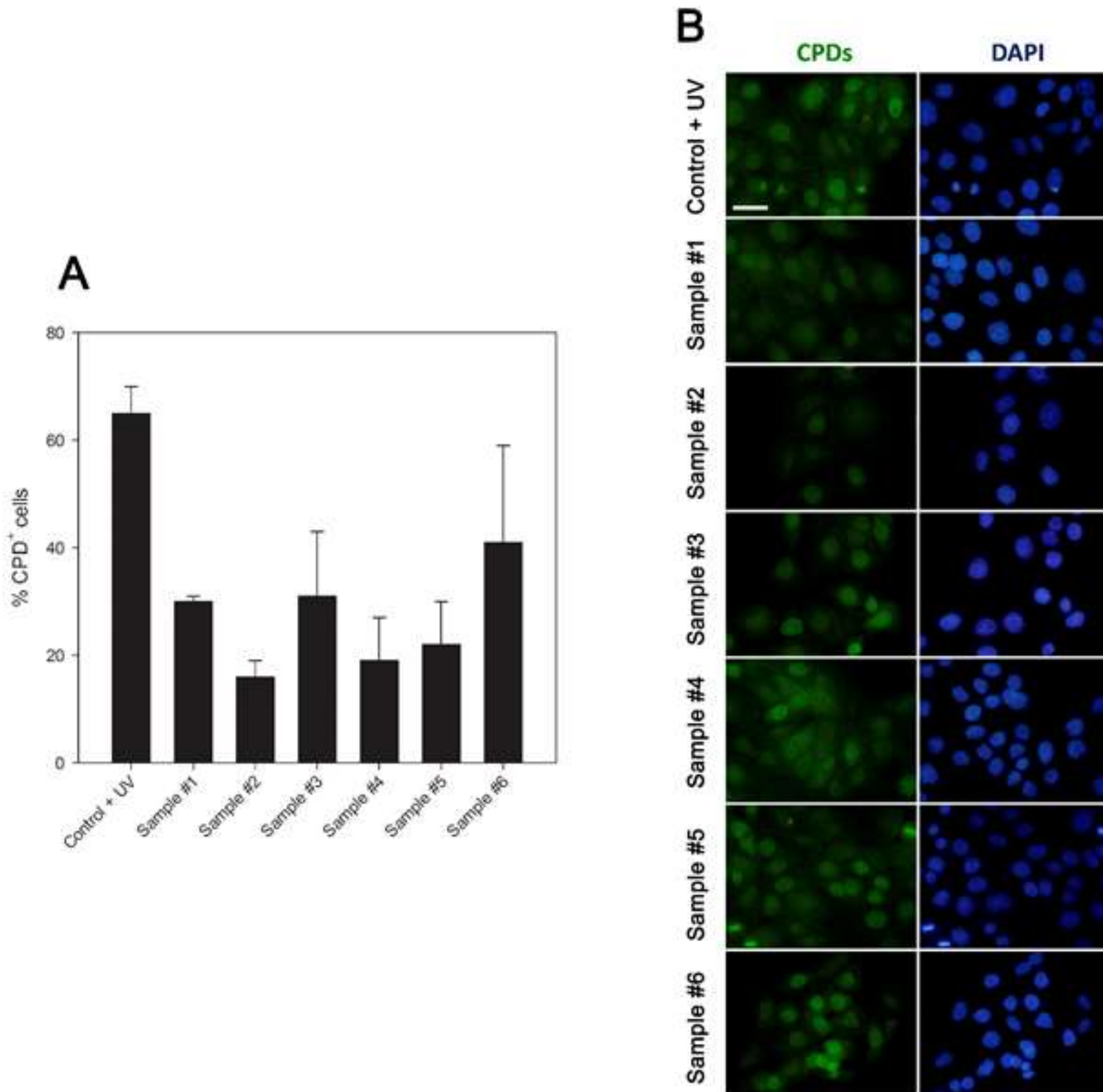

Figure 5. The assayed *P. leucotomos* extracts have a different effect on the appearance of CPDs. (A) HaCaT transformed keratinocytes (white bars) were treated with 1mg/mL of each extract and irradiated. Cells were then fixed, stained with the CPD detection kit as indicated in Material and Methods, and measured by flow cytometry. Data represents the percentage of CPD-positive cells. (C) Representative immunofluorescence images of irradiated cells in the presence of the different extracts. Briefly, cells were treated as in Fig. 4C, then stained with the kit as in (A). Fields are representative of >40 examined per condition. Bar= 20µm.

cells, but it reduced the number of γH2AX ^{bright} cells to levels similar to those elicited by Sample #2. These observations were consistent with the qualitative assessment of the cells by immunofluorescence, in which Sample #2 reduced the γH2AX signal most efficiently as compared to the other extracts (Figure 4C).

Also, formation of cyclobutane pyrimidine dimers was determined. CPDs constitute an additional marker of UV-induced DNA damage (Nikitaki et al., 2015). These experiments showed that UV irradiation induced the appearance of CPDs in almost 70% of the cells (Figure 5A). This phenomenon was quenched by all the extracts except Sample #6, but most efficiently by Sample #2 as determined by flow cytometry (Figure 5A) and immunofluorescence (Figure 5B).

DISCUSSION

In this study, a direct, unbiased comparison of the different extracts of the fern *P. leucotomos* has revealed

important molecular information regarding the reported beneficial effects of these extracts. HPLC analysis revealed different amounts of aromatic acids bearing antioxidant properties. In general, the three formulations made from the leaves of *P. leucotomos* are richer in antioxidant aromatic acids than the extracts obtained using the rhizome. This is particularly noticeable in the case of 3,4-dihydroxybenzoic and 4-hydroxybenzoic acids, which are absent from two of the rhizome formulations and present in a very small amount in the third (Sample #4). This is consistent with observations made in other plant species, e.g. *Drynaria* ferns. The fronds of *Drynaria* are endowed with higher anti-oxidant potential and tyrosinase activity than the rhizomes (Tan and Lim, 2015). Likewise, the leaves of ferns of selected highland ferns of Malaysia contain 10 to 20 higher levels of anti-oxidant moieties (flavonoids, hydroxycinnamic acid and proanthocyanidin) than the corresponding rhizomes of the same plants (Chai et al., 2013).

It is interesting to note that the overall antioxidant capability of the extracts seemed to depend directly on the presence of ferulic acid. Indeed, the only rhizome extract with significant antioxidant activity in the ABTS and FRAP assay (Sample #4) is also the only containing a significant amount of ferulic acid (Figure 1E to G).

Although caffeic acid is a more potent radical scavenger than ferulic acid (Kikuzaki et al., 2002), the peak of ferulic acid is much higher in Sample #4 than in the other two rhizome extracts. In addition, the small amount of caffeic acid present in Sample #5 could explain its low but significant antioxidant potential (Figure 1F). However, this does not seem to be the major factor in determining cellular photoprotection. Samples #4 and #5 do not protect cells from UV irradiation despite the presence of caffeic acid in both and ferulic acid in the former.

Conversely, Sample #6 does confer significant photoprotection. This is likely due to the presence of unidentified moieties in this extract, peptidic or otherwise. As for the leave extracts, the most obvious differences are the significant presence of 3,4-dihydroxybenzoic and 4-hydroxybenzoic acids as well as 4-hydroxycinnamic acid.

The concurrence of these three moieties could underlie the strong photoprotection conferred by the aerial extracts, although these experiments do not rule out the contribution of additional moieties of different molecular families, peptides, proteins or aromatic compounds.

It is interesting to note that survival correlates well with markers of DNA damage only at extreme positivity. Indeed, the basic leave extract (sample #2, Fernblock®) is the most efficient photoprotector considering all the metrics used in this study (survival, H2AX phosphorylation and CPD appearance). However, the rest of the extracts offered mixed effects. The acidic- and neutral pH-extracts (Samples #1 and #3) were very similar in all the photoprotection metrics, suggesting that the important moieties are those detected at 290 nm, e.g.4-hydroxycinnamic acid (the acidic extraction method decreases the recovery of 3,4-dihydroxybenzoic and 4-hydroxybenzoic acids, compare Figure 1B and D). Conversely, Sample #4 offered the worst results in terms of H2AX phosphorylation and CPD appearance. Sample #5 was slightly worse than Sample #2 in terms of overall numbers of H2AX-positive cells, but better in terms of H2AX-bright cells and appearance of CPDs. Finally, Sample #6 was worse in terms of overall numbers of H2AX-positive cells and appearance of CPDs, but much better in terms of H2AX-bright cells. There is no easy explanation for these results. It could be speculated that additional moieties present in Samples #4 and #6 could provide a higher threshold degree of resistance, preventing extensive damage (hence lower H2AX-bright cells). Such a mechanism could be explained by the ability of small amounts of undetermined moieties that could scavenge higher energy UV photons, likely responsible for deep damage. However, their lower levels of characterized antioxidants would not be sufficient to prevent the damage caused by the avalanche of lower energy UV photons. In this regard, it is worth noting that the bulk of UV illumination used here is UVA (315 to 400 nm), which contains lower energy than UVB, which is also used.

In conclusion, this work reveals profound differences among different extracts of *P. leucotomos*, which is likely linked to the part of the plant, the extraction method, and perhaps the geographical origin and growing conditions of the plants. In general, leaf extracts are more potent and yield results endowed with better significance.

However, this study also indicates that additional moieties, antioxidant or not, may also play fundamental roles in the function of these extracts as dietary supplements with antioxidant and antiaging properties.

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ABBREVIATIONS

UV, Ultraviolet; **PLE,** *Polypodium leucotomos* extract; **CPD,** cyclobutane pyrimidine dimers; **TNF,** tumor newcrosis factor; **iNOS,** inducible nitric oxide synthase; **HPLC,** high performance liquid chromatography; **HAX,** histone 2AX.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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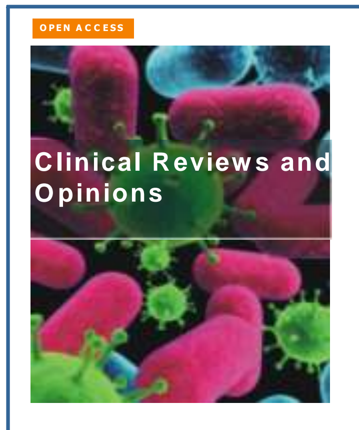

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