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In Vitro Evaluation of Portuguese Propolis and Floral Sources for Antiprotozoal, Antibacterial and Antifungal Activity

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Propolis is a beehive product with a very complex chemical composition, used since ancient times in several therapeutic treatments. As a contribution to the improvement of drugs against several tropical diseases caused by protozoa, we screened Portuguese propolis and its potential floral sources *Populus x Canadensis* and *Cistus ladanifer* against *Plasmodium falciparum*, *Leishmania infantum*, *Trypanosoma brucei* and *Trypanosoma cruzi*. The toxicity against MRC-5 fibroblast cells was evaluated to assess selectivity. The *in vitro* assays were performed following the recommendations of WHO Special Programme for Research and Training in Tropical Diseases (TDR) and revealed moderate activity, with the propolis extracts presenting the relatively highest inhibitory effect against *T. brucei*. Additionally, the antimicrobial activity against *Staphylococcus aureus, Candida albicans, Trichophyton rubrum* and *Aspergillus fumigatus* was also verified with the better results observed against *T. rubrum*. The quality of the extracts was controlled by evaluating the phenolic content and antioxidant activity. The observed biological activity variations are associated with the variable chemical composition of the propolis and the potential floral sources under study. Copyright © 2013 John Wiley & Sons, Ltd.

Keywords: propolis; antiprotozoal activity; antimicrobial activity; phenolic compounds; antioxidant activity.

INTRODUCTION

Propolis is a resinous bee product collected by honeybees (*Apis melifera* L.) from parts of plants, buds and exudates on the vegetation around the hive and enriched with wax, salivary and enzymatic secretions (Marcucci, 1995). This natural resinous substance plays an important role as construction and defence material against infections in the hive (Bankova *et al*., 2000) and preserves some of the medicinal properties of certain plants. For instance, *Cistus* species secrete large amounts of a strong aromatic resin on the surface of leaves and stems, rich in polyphenols and used since ancient times to treat diarrhea, dysentery and menstruation problems (Robles *et al*., 2003). The *Populus* bud exudates have long been used in popular medicine for treating wounds and ulcers. Their antiseptic, anti-inflammatory and antimicrobial properties have been documented (Scaysbrook *et al*., 1992; Zhang *et al*., 2006).

During the last decades, propolis has become the subject of increased scientific interest for its wide variety of pharmacological and biological properties, such as antibacterial (Sforcin *et al*., 2000), antifungal (Millet-Clerc *et al*., 1987), antiviral (Amoros *et al*., 1992), antiprotozoal (Monzote *et al*., 2012), antioxidant, hepatoprotective, antitumor and anti-inflammatory (Banskota *et al*., 2001) activities. Approximately half of the propolis content corresponds to phenolic compounds, while beeswax, volatiles and pollen account for, respectively, 30%, 10% and 5% (Burdock, 1998). The chemical composition of propolis is highly variable and complex, depending strongly on the plant sources available at the site of collection and thus on the geographic and climatic characteristics of the apiary location. In regions of temperate climate, bees obtain resins from the buds of *Populus* spp., and derived propolis is mainly composed by flavonoids, phenolic acids and their esters. In tropical areas, species of *Baccharis* spp. in Brazil and *Clusia* spp. in Cuba and Venezuela are the main sources of propolis, with prenylated *p*-coumaric acids and polyisoprenylated benzophenones as the main compounds in *Baccharis* and *Clusia*-derived propolis, respectively (Bankova *et al*., 2000). Despite the compositional differences between propolis types, their biological properties are very similar, e.g. all possess antimicrobial activity.

Propolis from North-eastern Portugal was recently characterized, providing identification of 37 phenolic compounds, with pinocembrin, chrysin and pinobanksin-3-*O*-acetate being the most abundant (Falcão *et al*., 2010). The phenolic compounds show strong antioxidant activity and decrease erythrocyte membrane fragility in hereditary spherocytosis (Moreira *et al*., 2011). Propolis extracts also strongly suppress the proliferation of

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primary renal cancer cells in a concentration-dependent manner (Valente *et al*., 2011) and are able to exert moderate neuroprotection through the inhibition of caspase-3 activation (Cardoso *et al*., 2011). Portuguese propolis extracts were shown to have antimicrobial activity against *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Candida albicans* (Silva *et al*., 2012), with the Gram-negative bacteria being more sensitive to the bee glue. To the best of our knowledge, there are yet no studies on the activity of Portuguese propolis against pathogenic protozoa.

In this study, Portuguese propolis phenolic extracts and their potential plant sources were screened *in vitro* for their activity against the pathogenic protozoa *Plasmodium falciparum*, *Leishmania infantum*, *Trypanosoma cruzi* and *Trypanosoma brucei*. To assess selectivity of action, cytotoxicity against MRC-5 fibroblasts and antibacterial and antifungal activities were evaluated in parallel. The quality of the extracts was assessed by evaluating their phenolic content and antioxidant activity.

MATERIALS AND METHODS

Chemicals and reagents. Standard compounds such as galangin, pinocembrin, caffeic acid, chloroquine, melarsoprol, benznidazole, miltefosine, tamoxifen, as well as other chemicals, such as aluminium chloride, potassium ferricyanide, ferric chloride, trichloroacetic acid, 2,2-diphenyl-1-picrylhydrazyl (DPPH) and dimethyl sulfoxide (DMSO) were all purchased from Sigma Chemical Co (St. Louis, MO, USA). Analytical grade reagents like sodium carbonate, potassium hydroxide, Folin–Ciocalteau reagent, acetic acid, sulphuric acid, formic acid, ethanol and methanol were obtained from Panreac (Barcelona, Spain). 2,4-Dinitrophenylhydrazine (DNP), ampicillin and miconazole were from Fluka Chemical Co (St. Louis, MO, USA). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

Samples origin and preparation. The study was performed on propolis and plant samples present in the hive neighborhood that were reported (Bankova *et al*., 2000) as propolis floral sources. Two different propolis samples were collected from beekeepers after the honey harvesting season by scratching the hive walls and frames, followed by the removal of debris of wood and bees. Sample A1 was collected in North-eastern Portugal (Bragança county), while sample A2 was collected from the centre of Portugal (Leiria county). For the floral sources of the bee glue, the buds exudates and surface material present on the leaves and stems of *Populus x Canadensis* Moenchen (an hybrid species of *Populus*) male (PM) and female specimens (PF) and *Cistus ladanifer L*. (C) were collected during spring. All samples were stored at −20 °C until analysis. The voucher specimens are deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança with the reference number BRESA 5174, BRESA 5355 and BRESA 5356 for C, PF and PM, respectively.

The extraction was made according to the work previously described (Falcão *et al*., 2010). Prior to the extraction, 1 g of powdered propolis sample was homogenized and mixed with 10 mL of 80% of ethanol/water and kept at 70 °C for 1 h. The resulting mixtures were filtered, and the residues were re-extracted in the same conditions. After the second extraction, the filtrates were combined, concentrated and freeze-dried. For biological studies, stock solutions of the extracts were prepared in 13% DMSO/water (propolis) or 100% DMSO (floral sources) at 20 mg/mL.

Total phenolic content. The total phenolic content was determined with a modified Folin–Ciocalteu method (Singleton and Rossi, 1965). An ethanolic extract aliquot (0.5 mL) was mixed with 0.25 mL Folin–Ciocalteu's reagent. After 3 min, 1 mL of a saturated sodium carbonate solution was added to the mixture, and the volume adjusted to 5 mL with distilled water. The solution was then heated at 70 °C for 10 min, cooled in the dark for 30 min, and the optical density was measured at 760 nm (Analytikijena 200–2004 spectrophotometer, Analytik Jena, Jena, Germany). The ethanolic extracts were evaluated at the final concentration of 0.05 mg/L, and the total phenolic content was expressed in milligrams per gram of caffeic acid:galangin:pinocembrin (1:1:1) equivalents. For each extract, measurements were performed in three independent experiments.

Flavone and flavonol content. The content of flavone and flavonol was determined based on the method previously described (Cvek *et al*., 2007) with minor modifications. Briefly, 2 mL of the ethanolic extract was added to 0.2 mL of aluminium chloride solution (2% aluminium chloride in 5% acetic acid/methanol), and the volume was adjusted to 5 mL with 5% acetic acid/methanol. After 30 min at room temperature, the optical density was measured at 415 nm. The ethanolic extracts were evaluated in triplicate at a final concentration of 0.1 mg/L, and the flavone and flavonol contents were expressed as milligrams per gram of galangin equivalents.

Flavanone and dihydroflavonol content. Flavanones and dihydroflavonols were determined using a previously described method (Popova *et al*., 2004). Briefly, 1 mL of the test solution and 2 mL of DNP solution (1 g of DNP was dissolved in 2 mL of 96% sulphuric acid and the volume was adjusted to 100 mL with methanol) were heated at 50 °C for 50 min. After cooling to room temperature, the mixture was diluted to 10 mL with 10% potassium hydroxide in methanol (w/v). An aliquot (1 mL) of the resulting solution was added to 10 mL of methanol and diluted to 50 mL with methanol. Finally, the optical density was measured at 486 nm. The content in flavanones and dihydroflavonols was evaluated in triplicate and expressed as milligrams per gram of pinocembrin equivalents.

DPPH free radical-scavenging activity. The antioxidant effect on DPPH radical was measured according to the procedure described previously (Brand-Williams *et al*., 1995) with some modifications. The reaction was performed in a 96-well microplate where an aliquot of propolis extract (0.08 mL) in 80% ethanol containing

different extract concentrations (2.5–40 μg/mL) was added to 0.220 mL of DPPH (0.025 g/L in 80% ethanol, daily prepared). After 45 min at room temperature, optical density was measured at 515 nm using an ELX800 Microplate Reader (Bio-Tek Instruments, Inc.). A mixture of phenolic compounds (caffeic acid: galangin: pinocembrin; 1:1:1) was used as standard. The percentage of radical inhibition was calculated from the absorbance of the DPPH solution without sample (A_{DPPH}) and of the DPPH solution with sample (A_{sample}), using the following equation: % Inhibition = $[(A_{DPPH}-A_{sample})/A_{DPPH}] \times 100$.

This percentage was plotted against the extract concentration to obtain the amount of antioxidant needed to decrease the initial DPPH concentration by 50% (EC_{50}). The assay was performed in triplicate.

Reducing power. The reducing power of the propolis extracts was determined according to the method of Oyaizu (1986). 2.5 mL of the propolis ethanolic extract (10–200 μg/mL) was mixed with 2.5 mL phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. Then, 2.5 mL of 10% trichloroacetic acid was added to the mixture followed by centrifugation at 3000 rpm (Centurion K_2R series) for 10 min. The upper layer of the solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%). Finally, the optical density was measured at 700 nm. A mixture of caffeic acid: galangin: pinocembrin (1:1:1) was used as standard. An increase of the optical density for the reaction mixture indicates a higher reducing power. The complex with absorbance is the result of the phenolic compound reaction during the reduction of iron. The assay was run in triplicate in independent experiments.

Test plate production for antimicrobial evaluation. The experiments were performed in 96-well plates (Greiner) at four-fold dilutions in a dose-titration range of 64 μg/mL to 0.25 μg/mL. Dilutions were carried out by a programmable precision robotic station (BIOMEK 2000, Beckman, USA). Each plate also contained medium-controls (blanks: 0% growth), infected untreated controls (negative control: 100% growth) and reference controls (positive control). Tests were run in duplicate in two independent experiments.

Biological screening tests. The integrated panel of microbial screens for the present study and the standard screening methodologies were adopted as described before (Cos *et al*., 2006). Extracts with high cytotoxicity and/or non-selective activity against the different protozoa were not titrated down to their exact IC50.

Antiplasmodial activity. The chloroquine-susceptible *P. falciparum* GHA-strain was used. Parasites were cultured in human erythrocytes A^+ at 37 °C under a low oxygen atmosphere (3% O_2, 4% CO_2 and 93% N_2) in a modular incubation chamber (Trager and Jensen, 1976). The culture medium was RPMI-1640 supplemented with 10% human serum. Two hundred microliters of infected human red blood cells suspension (1% parasitemia, 2% hematocrit) were added to each well of the plates with test compounds and incubated for 72 h. After incubation, test plates were frozen at −20 °C. Parasite multiplication was measured by the Malstat method (Makler *et al*., 1993). One hundred microliters of Malstat reagent was transferred in a new plate and mixed with 20 μL of the hemolysed parasite suspension for 15 min at room temperature. After addition of 20 μL nitroblue tetrazolium (NBT)/phenazine ethosulphate (PES) solution (1.6 mg of NBT and 0.1 mg of PES) and 2 h incubation in the dark, the optical density was spectrophotometrically read at 655 nm (Biorad 3550-UV microplate reader). Percentage of growth inhibition was compared to the negative blanks. Chloroquine was used as reference drug.

Antitrypanosomal activity. Trypomastigotes of *T. brucei* Squib-427 strain (suramin-sensitive) were cultured at 37 °C and 5% CO_2 in Hirumi-9 medium (Hirumi and Hirumi, 1989), supplemented with 10% fetal calf serum (FCS). Assays were performed by adding 1.5×10^4 trypomastigotes/well. After 72 h incubation, parasite growth was assessed fluorimetrically by adding resazurin (Raz *et al*., 1997) for 24 h at 37 °C. Fluorescence was measured using a GENios Tecan fluorimeter (excitation 530 nm, emission 590 nm). Melarsoprol was used as reference drug.

Amastigotes of *T. cruzi* (Tulahuen CL2 strain, nifurtimox-sensitive) were maintained on MRC-5 cells in minimal essential medium (MEM) supplemented with 20 mM L-glutamine, 16.5 mM sodium hydrogen carbonate and 5% FCS at 37 °C and 5% CO_2. To determine *in vitro* activity, 4×10^3 MRC-5 cells and 4×10^4 parasites were added to each well of the test plate with compound. After incubation at 37 °C for 7 days, parasite growth was assessed by adding β-galactosidase substrate chlorophenol red β-D-galactopyranoside (Buckner *et al*., 1996) for 4 h at 37 °C. The colour reaction was read at 540 nm, and optical density values were expressed as a percentage of the blank controls. Benznidazole was used as reference drug.

Antileishmanial activity. Amastigotes of *L. infantum* (MHOM/ET 67), used to infect primary peritoneal mouse macrophages, were collected from the spleen of donor hamsters with an established *Leishmania* infection of 6 to 8 weeks. After aseptic removal of the spleen, a number of smear impressions were made on a microscope slide and stained with Giemsa stain to enumerate the amastigote spleen burden using the Stauber index (total number of amastigotes = weight of spleen (g) × number amastigotes/cell × 2 × 10^8). To purify the amastigotes, the spleen was grinded in 10 mL culture medium in a tissue grinder; the splenic cell suspension was transferred in a sterile 15 mL centrifugation tube and centrifuged for 10 min at 300 rpm and 4 °C to remove most of the cell debris. The supernatant was then transferred to a sterile 15 mL centrifugation tube and centrifuged for 10 min at 2200 rpm and 4 °C; the supernatant was discarded, and the pellet was re-suspended in 10 mL complete culture medium. After one additional washing cycle and appropriate dilution, this suspension was used as infection inoculum for the *in vitro* macrophage cultures.

To determine *in vitro* antileishmanial activity, 3×10^4 macrophages were seeded in each well of a 96-well plate. After 48 h outgrowth, 5×10^4 amastigotes/well were added and incubated for 2 h at 37 °C. Pre-diluted compounds were subsequently added, and the plates were further incubated for 120 h at 37 °C and 5% CO_2. Parasite burdens were determined microscopically after Giemsa staining and expressed as a percentage of the blank controls without propolis sample. Miltefosine was used as reference drug.

Cytotoxicity assay. MRC-5_{SV2} human foetal lung fibroblasts were cultivated in MEM, supplemented with L-glutamine (20 mM), 16.5 mM sodium hydrogen carbonate and 5% FCS at 37 °C and 5% CO_2. For the assay, 10^4 MRC-5 cells/well were seeded onto the test plates containing the pre-diluted compounds and incubated at 37 °C and 5% CO_2 for 72 h. Cell viability was determined after addition of resazurin. Tamoxifen was used as reference drug.

Antibacterial and antifungal assays. These assays have been also performed at the Laboratory of Microbiology, Parasitology and Hygiene (LMPH), Antwerp University, Belgium (Cos *et al*., 2006) against *Staphylococcus aureus*, *Candida albicans*, *Trichophyton rubrum* and *Aspergillus fumigatus*. IC_{50} values were determined from five 4-fold dilutions, starting from a maximum concentration of 64 μg/mL. Ampicillin was used as reference for *S. aureus*, while miconazole was used as reference for *Candida*, *Trichophyton* and *Aspergillus*. The impact of toxicity was determined by analyzing the selectivity index (SI), the ratios between the MRC-5_{SV2} cytotoxic and the antimicrobial IC50 values.

Statistical analysis. For the phenolic composition and antioxidant activity, the assays were carried out in triplicate and presented in the figures as mean values with standard deviation. The results were analyzed using one-way analysis of variance followed by Tukey's HSD test with $a = 0.05$. The analysis was performed using SPSS v. 18.0 program. The result of this analysis can be found in the figures: samples with the same letter are statistically no different.

For the antiprotozoal, antibacterial and antifungal activity, in each experiment, the 50% of microbial growth (IC_{50}) and human cell growth (CC_{50}) inhibition value was determined from the concentration–response curves, and the results were expressed as the mean ± standard deviation of two independent experiments.

RESULTS AND DISCUSSION

Propolis is a bee product with a complex chemical composition which is largely dependent on its plant origin. *Populus* bud exudates and *Cistus ladanifer L*. leaf exudates are documented to be potential resin sources for propolis in Europe (Bankova *et al*., 2000). In the present study, these plants were abundant in the hive neighborhood, and their phenolic content, antioxidant and antimicrobial activities were evaluated and compared to propolis.

Figure 1 shows the content of total phenolic compounds, flavones/flavonols and flavanones/dihydroflavonols in propolis and in the *Cistus* and *Populus* ethanolic extracts. All values are statistically different. The highest total phenolic content was found in the propolis samples, but there is no great difference between the two samples of distinct geographical origin. The total phenolic content of 329 mg/g for the central propolis (A2) is in line with other studies on Portuguese propolis (Moreira *et al*., 2008). For the plant extracts, the values ranged from 167 to 278 mg/g with the male *Populus* sample revealing a higher value than *Cistus ladanifer.*

For the flavones and flavonols quantified by the aluminium chloride method, a higher content was also found in propolis compared to the plant extracts. North-eastern and central propolis samples showed values ranging from 81 to 97 mg/g, while the plant extracts contained 39 to 47 mg/g (Fig. 1). The flavanones and dihydroflavonols quantified by the DNP method were present in smaller quantities in the *Cistus* extract (113 mg/g) compared with propolis and *Populus* ethanolic extracts (Fig. 1).

Phenolic compounds, due to their hydroxyl groups, are known to act as antioxidants (Rice-Evans *et al*., 1996). Therefore, the activity of the extracts was evaluated with the DPPH free radical scavenging method (Brand-Williams *et al*., 1995). The EC_{50} values are shown in Fig. 2A with all propolis and plant extracts possessing significant free radical scavenging activity. The extracts of male and female *Populus x canadensis* bud exudates exhibited the highest activity with EC_{50} values of 0.014 and 0.015 mg/mL, respectively. These values were close to the standard mixture of pure compounds used in this study (caffeic acid: galangin: pinocembrin, 1:1:1), pointing out the significant activity of these natural extracts. The propolis samples showed an EC_{50} value around 0.018 mg/mL, while *Cistus ladanifer* resin presented the lowest scavenging activity (Fig. 2A).

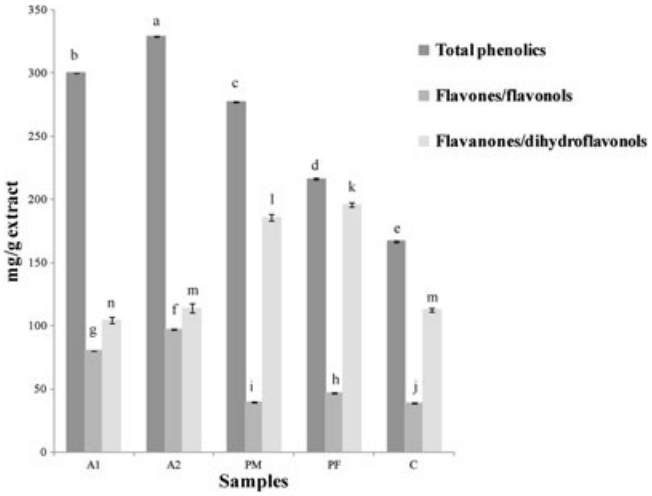

Figure 1. Phenolic composition of propolis and their ethanolic extracts. A1, North-eastern propolis; A2, Central propolis; PM, *Populus x canadensis* male; PF, *Populus x canadensis* female; C, *Cistus ladanifer*. Total phenolics were expressed as caffeic acid: galangin: pinocembrin (1:1:1) equivalents. Flavones/flavonols were expressed as galangin equivalents. Flavanones/dihydroflavonols were expressed as pinocembrin equivalents. In each column, different letters (a–n) mean significant differences between samples ($p < 0.05$)

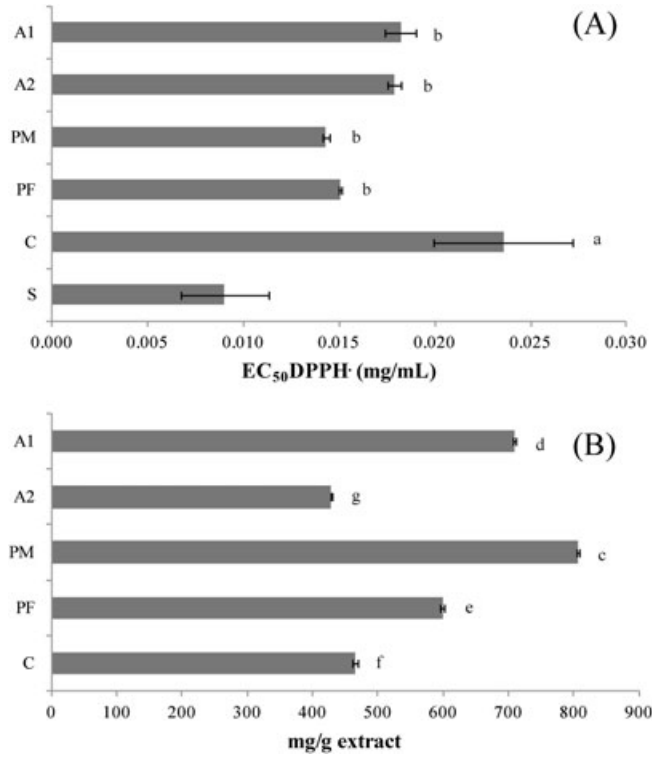

Figure 2. Antioxidant activity of propolis and ethanolic plant extracts (A) against DPPH and (B) reducing power. A1, North-eastern propolis; A2, Central propolis; PM, *Populus x canadensis* male; PF, *Populus x canadensis* female; C, *Cistus ladanifer*; S, caffeic acid: galangin: pinocembrin (1:1:1) used as standard. Reducing power was expressed as caffeic acid: galangin: pinocembrin (1:1:1) equivalents. In each column, different letters (a–g) mean significant differences between samples ($p < 0.05$)

Fe(III) reduction was used as an indicator of electron-donating activity, which is an important mechanism for phenolic antioxidant action (Yildirim *et al*., 2001). The reducing power was expressed as caffeic acid: galangin: pinocembrin (1:1:1) equivalents (Fig. 2B). Once again, the best activity was found for the male *Populus x canadensis* followed by the propolis sample A1 with values of 807 mg/g and 710 mg/g respectively. Sample A2 revealed a very low reduction power, even lower than the plant extracts.

Considering the above, the highest radical scavenging ability or reduction power was not found for the samples with the richer phenolic content, meaning that the observed bioactivity cannot be judged solely on the basis of the overall phenolic content. In fact, some of the individual phenolic compounds present in the extract can play a more important role in the activity than others.

Portuguese propolis phenolic extracts and their potential plant sources were evaluated for *in vitro* activity against the pathogenic protozoa *P. falciparum*, *L. infantum*, *T. cruzi* and *T. brucei* (Table 1). To assess selectivity of action, cytotoxicity against MRC-5 fibroblasts was included.

Activity against *P. falciparum* was found for the propolis sample from the central region of Portugal with an IC_{50} of 8.83 μg/mL, while the phenolic extract of male *Populus* showed the highest SI (SI = 4). Filho *et al*. (2009) reported an IC_{50} of 20 μg/mL (SI > 2.4) for Brazilian green propolis and of 25 μg/mL (SI > 1.9) for the extract of *Baccharis dracunculifolia*, the floral origin of green propolis. These values are actually in the same range as our results and in fact reveal that our samples A2 and PM show a marginally higher antiplasmodial potential, which may be associated with the richer composition in flavonoids for propolis from *Populus* (Machado *et al*., 2007). For an antimalarial 'hit', the WHO Special Programme for Research and Training in Tropical Diseases defines an activity criterion to be IC_{50} <0.2 μg/mL with SI >20 (TDR, 2007). Therefore, the relevance of the antimalarial action of our samples requires further research. Propolis toxicity was referred by Marcucci (1995) and Burdock (1998) to be associated with some of the propolis components, namely caffeic acid esters present in the European propolis with origin in the poplar buds. The synergistic effect of all compounds in the entire extract can be responsible for a real loss of activity, while the isolated compounds or fractions of it can indeed reveal a higher activity (Filho *et al*., 2009).

For *L. infantum*, propolis samples and male and female *Populus* showed a similar IC_{50} value of 8.11 μg/mL. *Cistus ladanifer* showed the lowest activity with an IC_{50} value of 32.46 μg/mL. Duran *et al*. (2011) reported antileishmanial activity on two different Turkish propolis samples with IC_{50} values ranging between 125 and 325 μg/mL. Filho *et al*. (2009) presented IC_{50} values of 49 and 45 μg/mL for a green propolis sample and its plant source *B. dracunculifolia* against *L. donovani*.

Our results for the activity against *T. cruzi* were very similar between all the samples tested, ranging from 6.16 to 8.59 μg/mL (Table 1). Prytzyk *et al*. (2003) and Cunha *et al*. (2004) tested the activity against *T. cruzi* in Bulgarian and Brazilian propolis whereby all the extracts showed a lower activity than the Portuguese propolis, with values

Table 1. Antiprotozoal activity against *P. falciparum*, *L. infantum*, *T. cruzi*, *T. brucei* and cytotoxicity in MRC-5 fibroblast cells

	IC_{50} μg/mL (mean ± sd) -- duplicate testing				
Sample[a]	***P. falciparum***	***L. infantum***	***T. cruzi***	***T. brucei***	**MRC-5**
A1	30.1 ± 4.1	8.1 ± 1.0	6.2 ± 1.9	1.7 ± 0.5	12.0 ± 4.3
A2	8.8 ± 1.8	8.1 ± 0.9	7.7 ± 2.1	3.8 ± 1.2	9.7 ± 3.5
PM	10.9 ± 2.1	8.1 ± 1.1	7.8 ± 1.8	5.7 ± 1.3	38.4 ± 5.8
PF	28.5 ± 3.8	8.1 ± 1.3	7.6 ± 1.6	5.3 ± 1.9	33.7 ± 6.2
C	17.0 ± 2.5	32.5 ± 2.7	8.6 ± 2.0	2.0 ± 0.4	32.2 ± 4.5
Chloroquine	0.04 ± 0.01	-	-	-	-
Miltefosine	-	2.4 ± 0.8	-	-	-
Benznidazole	-	-	2.5 ± 0.6	-	-
Melarsoprol	-	-	-	0.005 ± 0.001	-
Tamoxifen					10.5 ± 2.5

[a]Samples: A1: North-eastern propolis; A2: Central propolis; PM: *Populus x canadensis* male; PF: *Populus x canadensis* female; C: *Cistus ladanifer*.

ranging between 108.8 and 1065 μg/mL for Bulgarian propolis and between 421 and 1437 μg/mL for Brazilian propolis. These biological activity variations are likely associated with different chemical compositions presented by the different propolis types. No cytotoxicity tests were performed in that study.

For all antiprotozoal assays, the relatively highest activities were found against *T. brucei*. Sample A1 was the most active with an IC_{50} value of 1.70 μg/mL (Table 1) which is in the same range as the antitrypanosomal reference drug suramin (Otoguro *et al*., 2012). Isolation of phenolic compounds from propolis can significantly increase the activity: β-phenylethyl caffeate showed a high activity ($IC_{50} = 0.013$ μg/mL; SI = 150), while 2,2-dimethylallyl caffeate exhibited a reduced antitrypanosomal activity ($IC_{50} = 12.5$ μg/mL), demonstrating the potential loss of activity when testing the entire extract (Otoguro *et al*. (2012).

The activity of the propolis and plant ethanolic extracts was also evaluated against bacteria and fungi (Table 2). The results reveal that the plant extracts do no exhibit relevant antimicrobial activity compared to propolis ethanolic extracts, with exception of *T. rubrum*. In general, both propolis samples revealed a similar antimicrobial effect, with the highest activity found against *T. rubrum* and the lowest against *A. fumigatus*. Recently, Silva *et al*. (2012) verified that propolis from the North and Centre of Portugal was more active against *S. aureus* than against *C. albicans*, which agrees with our results. In fact, considering that the IC_{50} value for *T. rubrum* is even lower than for *S. aureus*, it can be considered a promising result. However, one should also consider the cytotoxicity, which was rather high in our study.

In summary, the present study focused on the screening of Portuguese propolis samples and two potential plant sources against pathogenic protozoa, revealing reduced activity and low selectivity. Since propolis has a complex chemical composition, an extract fractionation will be needed to identify putative active components. Also, these results were evaluated according to criteria set up by the WHO Special Programme for Research and Training in Tropical Diseases, what should be considered by future studies with others propolis samples to enable effective comparisons between scientific findings.

Table 2. Antimicrobial activity against *Staphylococcus aureus*, *Candida albicans*, *Trichophyton rubrum* and *Aspergillus fumigatus*

	IC_{50} μg/mL (mean ± sd) -- duplicate testing			
Sample[a]	***S. aureus***	***C. albicans***	***T. rubrum***	***A. fumigatus***
A1	24.6 ± 3.8	32.0 ± 3.2	14.5 ± 1.2	>64.0
A2	25.7 ± 2.9	43.1 ± 4.1	11.0 ± 0.9	>64.0
PM	>64.0	33.6 ± 3.5	38.9 ± 2.3	>64.0
PF	>64.0	>64.0	24.2 ± 2.1	>64.0
C	>64.0	>64.0	20.8 ± 1.8	>64.0
Ampicillin	1.1 ± 0.3			
Miconazole		4.0 ± 1.4	0.5 ± 0.1	1.5 ± 0.8

[a]Samples: A1: North-eastern propolis; A2: Central propolis; PM: *Populus x canadensis* male; PF: *Populus x canadensis* female; C: *Cistus ladanifer*

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Conflict of Interest

The authors report no conflict of interest.

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