Chemical and Antimicrobial Profiling of Propolis from Different Regions within Libya.

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Libyan propolis

Abstract

Extracts from twelve samples of propolis collected from different regions of Libya were tested for their activity against *Trypanosoma brucei*, *Leishmania donovani*, *Plasmodium falciparum, Crithidia fasciculata* and *Mycobacterium marinum* and the cytotoxicity of the extracts was tested against mammalian cells. All the extracts were active to some degree against all of the protozoa and the mycobacterium, exhibiting a range of EC_{50} values between 1.65 and 53.6 µg/ml. The toxicity against mammalian cell lines was only moderate; the most active extract against the protozoan species, P2, displayed an IC_{50} value of 53.2 µg/ml. The extracts were profiled by using liquid chromatography coupled to high resolution mass spectrometry. The data sets were extracted using m/z Mine and the accurate masses of the features extracted were searched against the Dictionary of Natural Products (DNP). A principal component analysis (PCA) model was constructed which, in combination with hierarchical cluster analysis (HCA), divided the samples into five groups. The outlying groups had different sets of dominant compounds in the extracts, which could be characterised by their elemental composition. Orthogonal partial least squares (OPLS) analysis was used to link the activity of each extract against the different micro-organisms to particular components in the extracts.

Keywords: Libyan propolis, *Trypanosoma brucei, Leishmania donovani, Plasmodium falciparum, Crithidia fasciculata, Mycobacterium marinum*.

Introduction

Bees collect propolis from plants and use it to coat the inside surfaces of the hive in order to maintain a sterile environment. A wide variety of plant species are used by bees as a source for propolis production, leading to a wide chemical diversity [1]. Even within a fairly limited geographical region such as the UK propolis composition varies substantially [2]. Bees are subject to infection by a range of micro-organisms and these include the protozoal *Crithidia* species, and the *Nosema* species that were originally classified as protozoa but have now been reclassified as fungi. It has been found that *N. ceranae* and *N. apis* infections are widespread in Scottish beehives [3]. The best-characterised *Crithidia* parasite that infects bees is *Crithidia bombi*, which infects bumble bees [4]. In a recent publication it was found that *Crithidia mellificae* and *Nosema ceranae* infections are associated with winter mortality in European bees [5]. Thus it would seem logical that selection pressure would drive bees to collect phytochemicals that are effective against protozoa and other micro-organisms that could infect the hive [6, 7]. *Crithidia*, which are classified as lower Trypanosomatidae and are very prevalent in the infection of invertebrates, are closely related to the human pathogens of the genera *Leishmania* and *Trypanosoma* [8]. Since propolis is collected by bees for the specific purpose of providing phytochemical protection against pathogens, there is a strong likelihood of finding highly active antimicrobials in it [9]. Moreover, the fact that propolis permeates the environment of the beehive makes it likely that it would not be particularly toxic to other multicellular organisms. Libya covers an area of over 1,759,540 km^2 and the Libyan Desert, which constitutes approximately 90% of Libya, is one of the most arid places on earth. Oases can be found scattered throughout Libya, the most important of which are Ghadames and El-Kufra. The northern regions enjoy a milder Mediterranean climate. Most of the commercial beekeepers are located in an agricultural belt that extends to about 30 km from the coast [10, 11]. Table S1 in File S1 summarises the main plants in Libya from which bees are known to collect nectar. The current work follows from our earlier work on a sample of propolis collected from the East of Libya, from which

four known compounds with activity against *T. brucei* and *L. donovani* were isolated [12]. The samples studied in this paper represent a larger variety of habitats and climates. The aim of the study was to continue our chemical mapping of the composition of African propolis and carry out anti-microbial screens in search of high activity samples.

Materials and Methods

Materials

Absolute ethanol, HPLC grade acetonitrile, methanol, formic acid and Acrodisc syringe filters were obtained from Fisher Scientific (Loughborough, UK). Chloroform and dimethyl sulphoxide (DMSO) were obtained from Sigma Aldrich, Dorset, UK. HPLC grade Water was produced in-house using a Milli Q system (Millipore, UK).

Propolis samples

Twelve propolis samples were collected from different Libyan localities: **Tukra** (Al`Aquriyah) 70 km East of Benghazi, Libya (P1); **Qaminis** 53km South of Benghazi (P2); **Bayda** East of Benghazi (P3); **Quba** East of Benghazi (P4); three samples from **Kufra** in South East Libya (P5, P6 and P7); **Ghadames** South West Libya (P8); **Tripoli** North West Libya (P9); **Khaser Khiar** 80 km East of Tripoli (P10) and two samples from **Khumas** 120 km East of Tripoli (P11, P12) (Fig 1). The samples were collected between December 2012 and March 2014. The physical properties of the samples are summarised in Table S2 in S1 File. The samples were collected by scraping the propolis sample off the top of the hive using a spatula and collecting in a clean tray.

Sample Extraction

A sample of approximately 20 g of each propolis sample was extracted by sonication in 100 mL of absolute ethanol for 60 min, after which the extract was filtered and re-extracted twice more with 100 mL of ethanol, filtering each time. The extracts were combined, and the solvent was evaporated using a rotary evaporator, and the residue weighed.

Anti-microbial Assays

Anti-trypanosomal Assay

Testing was carried out against a standard drug-sensitive *T. b. brucei* clone, Lister strain 427 (s427) [13,14], and the results were expressed as EC_{50} values based on three replicates at each concentration. The assay is based on viable cells metabolizing the blue non-fluorescent dye resazurin to resorufin, which is pink and fluorescent. The assays were performed using serial dilutions in white opaque plastic 96-well plates (F Cell Star, Greiner Bio-one GmbH, Frickenhausen, Germany), with each compound or mixture double diluted over 2 rows of the plate (i.e. 23 double dilutions and a no-drug control well), facilitating an optimally-defined EC_{50} value after plotting of the reading to a sigmoid curve with variable slope (GraphPad Prism 5.0). The seeding density at the start of the assay was 2×10^4 cells/well, and the cells were exposed for 48 h to the test compounds, at 37 °C/5% CO_2, before the addition of the resazurin dye and a further incubation of 24 h under the same conditions. Fluorescence was determined in a FLUOstar Optima (BMG Labtech) at wavelengths of 544 nm and 620 nm for excitation and emission, respectively.

Anti-leishmanial Assay

Intraperitoneal macrophages were recovered from the peritoneal cavity of BALB/c mice 3 days after intraperitoneal injection with 1mL 3% w/v aqueous sterile starch solution. The mice were then euthanized, and 3mL of incomplete medium (RPMI-1640, 100 µg/mL penicillin–streptomycin and L-glutamine) was injected into the peritoneal cavity. The macrophage-containing medium was then removed and collected, and the resulting cell suspension centrifuged at 3000 × g for 5 min and then re-suspended in 10mL complete medium (in complete RPMI-1640 supplemented with 10% heat inactivated fetal calf serum (FCS) [v/v]). The cells were then used in antileishmanial assays. Bone marrow was then harvested from the femurs of each mouse by flushing out the removed bone with 5ml of bone marrow medium (Dulbecco's modified Eagle's medium, 20% heat-inactivated fetal calf serum (FCS) [v/v], 30% L-Cell solution [v/v], 100µg/mL penicillin–streptomycin and L-glutamine). The cell suspension was added to sterile petri dishes (one petri dish/mouse) and incubated for 7 days at

37 °C in an atmosphere of 5% CO2:95%air. The medium was removed from the plate, and 7mL TrypLE Express was added to detach the bone marrow-derived macrophages. The resulting suspension of bonemarrow-derived macrophages was collected, pelleted by centrifugation and re-suspended in 10mL of incomplete medium and then used in antileishmanial assays. The number of live macrophages per millilitre was determined microscopically using a haemocytometer, by mixing a cell sample with 1:1 Trypan blue (20 µL) and viewing at ×10 magnification. In all cases, cell viability was >95%. Cells (0.5 × 105 in 200 µL complete medium) were added to the appropriate wells of a 96-well tissue culture plate and incubated for 24 h at 37 °C in an atmosphere of 5% CO2:95% air. Cells were then infected with *L. donovani* luciferase-expressing parasite using a 20:1 parasite/host cell ratio. The plate was incubated as before for 24 h. The medium was removed from each well and replaced with 200 µL complete medium (control, n = 6) or various concentrations of the one of the extracts (diluted in 4% DSMO v/v in complete medium, n = 3) or amphotericin B solution (4–0.02 µg/mL). The plate was incubated as before for 72 h, the medium was then removed, and 150 µL of luciferin solution (150 µg/mL luciferin in complete RPMI-1640) was added to each well. The bioluminescence intensity (BLI) emitted per well was determined using the IVIS® imaging system[12,15]. The suppression in bioluminescent signal for each test sample was compared with the mean control value. The mean IC50 value was then calculated for each sample by Probit analysis. Data were analysed using MINITAB® software version 16.1.1 supplied by Minitab Ltd. Coventry, UK, and an Anderson–Darling test was used to establish if the data were normally distributed. Parametric data were analysed using a Student's unpaired t-test or by one-way analysis of variance dependent on the number of treatments/experiments, and significance was confirmed by a Fisher test. A Mann–Whitney or Kruskal– Wallis test was used to analyse data that did not have a normal distribution. Results were considered statistically significant at a p-value of <0.05.

Anti Mycobacterium marinum Assay.

The anti-bacterial bioassays against *M. marinum* were performed in 96-well microtitre plates using a modification of the well-established AlamarBlueTM method [16,17]. The samples were tested in

duplicate over a concentration range of 100–0.19 mg/ml and negative and positive controls were included containing 1–0.0019% DMSO and 100–0.78 mg/ml gentamycin respectively. The turbidity of a suspension of *M. marinum* was matched to that of a 0.5 McFarland standard (_1 _ 108 FUs/ml) and diluted with MHB to give a final concentration of _0.5 _ 107 FUs/ml in the assay microplate. The assay microplate was incubated at 31 8C for 6 days, after which 10% AlamarBlueTM was added and the incubation continued for a further 24 h.

Anti-Plasmodium falciparum Assays

Activity against *P. falciparum* was determined as described previously [18, 19]. Synchronous ring stage parasites were seeded and incubated in triplicate into 96 well plates at 0.5 % parasitemia and 2.5 % haematocrit and increasing concentrations of each compound (0.1 to 200 µg/mL and no drug control) for 48 h, using hypoxanthine free RPMI 1640 medium containing 0.5 % Albumax. 5 µCi/mL [^{3}H]-hypoxanthine was then added to each well and parasites were incubated for an additional 24 h before being frozen at -20 °C. After thawing, plates were harvested onto filter mats with a Harvester 96^{TM} Mach III (TomTec) and [^{3}H]-hypoxanthine incorporation determined by scintillation counting using a Wallac 1450 MicroBeta Trilux counter.

Anti-Crithidia fasciculata Assays

C. fasciculata (ATCC50083) was grown in RPMI 1640 medium supplemented with L-glutamine and 10% v/v heat inactivated foetal bovine serum for 24 h with shaking prior to use [20]. These cells were then used to inoculate wells of a 96 well plate with 1 x 10^5 cells per well in 100µl of medium. Stock extracts were prepared in DMSO for each concentration so that there was a constant percentage of DMSO per well (< 5% v/v). The absorbance of plates was determined at 620nm (T_0) and these were then incubated for 48 h at 25°C. The absorbance of the wells was then determined again at 620nm (T_{48}). For compounds showing no change in absorbance (T_{48}-T_0) terminal subculture

was performed and growth determined by abs @620nm and by microscopy. Pentamidine was included as a control drug in all assays but it shows variable activity against *C. fasciculata* [21] and thus menadione was used as an additional control drug.

Cell Toxicity Assay

The U937 cells were grown until approximately 70-80% confluence before plating at $1x10^5$cells/ml in a 96 well plate. The cell plates were then incubated overnight at 37°C, 5% CO_2. Samples were prepared on a dilution plate in normal cell culture media respective to the cell line used. For initial testing, samples were added to the cells at a range of different concentrations to determine the IC50 value for each sample. Samples were serially diluted 1 in 2 from 200µg/ml to 1.56µg/ml. Following the addition of the extracts, the cell plates were incubated for 24 hours at 37°C and then resazurin solution was added to a final concentration of 10% (v/v). The cell plates were incubated at 37°C in the dark for 4 hours and 24 hours before the fluorescence reading (560nm excitation, 590nm emission) was recorded on a Spectramax Plate Reader. Each sample was tested in triplicate and the results are expressed as cell viability as a percentage of the cell only control. Extracts were considered to be toxic if they caused a reduction in cell viability by at least 50% or more.

Liquid Chromatography High Resolution Mass Spectroscopy LC-HRMS

A sample of the ethanolic extract of each crude sample (1 mg), was dissolved in methanol (1 mL) and analysed by LC–MS. The separation was performed on an ACE C_{18} column (150 × 3mm, 3 µm) from HiChrom, Reading, UK with 0.1% v/v formic acid in water as mobile phase A and 0.1% (v/v) formic acid in acetonitrile as mobile phase B, at a flow rate of 0.300 mL/min using a gradient as follows: 0-15 min linear gradient from 30% to 50% of B, 15-25 min 50% of B, 25-40 min linear gradient from 50% to 80% of B, 40-50 min 80% of B, 50-51 min increasing to 100% B, 51-59 min at 100% of B with the flow rate increasing to 500 µl/min, 60-70 min 30% of B. Data dependent MS^n fragmentation [19] was carried out by using collision induced dissociation (CID) at 35 V on a LTQ-Orbitrap mass spectrometer combined with a Surveyor HPLC system.

Software and Data processing

MZMine 2.10 [22] was used for LC-HRMS data processing. The procedure and the settings were the same as described in our previous study [23]. The generated peak lists from both ESI positive and negative modes were imported separately into SIMCA-P 14 (Umetrics, Sweden) for Principal Component Analysis (PCA). The first 500 LC-HRMS features from each sample were selected based on the mean peak area and putatively identified by searching for the accurate masses against the Dictionary of Natural Products (DNP 2013 version) [24].

Results

Propolis Samples Cluster Partly According to Geographic Origin

Fig 2 shows a PCA based on the 300 features with the highest mean peak areas across the 12 samples selected by m/z mine from the negative ion data which included 30020 features. The data was pareto scaled and log transformed prior to PCA modelling. HCA was used to divide the samples into 5 groups. Only samples P5, P6 and P7 from the SE of the country gave a distinct group and they were grouped close to the sample from the SW (P8). The samples from the coast did not divide according to longitude and the two groups P3, P4, P9, P10 and P11, P12 are composed of samples from the E and W. Although P10 was collected from a site close to P11 and P12 it seems to be quite different in composition. Table 1 lists the ten most important variables (VIPs) used in the PCA classification of the samples for each group [25]. Samples P1 and P2 were similar in composition and three diterpenes and a lignan were previously isolated from sample P2 in our earlier study [12]. However, in the PCA model shown in Fig 2 the most important variables for the classification of the samples are not the diterpenes isolated previously but unknown compounds with m/z values in negative ion mode at m/z 325.145 and m/z 341.140. All masses deviated by < 2 ppm from the proposed elemental composition but, as can be seen in table 3 the DNP often has many isomeric

possibilities matching the elemental compositions of the VIPS. A compound with m/z 373.27 in negative ion mode has the highest importance for locating P5, P6 and P7 and is present in smaller amounts in the other samples. Samples P11 and P12 from the West also have clear marker compounds whereas the weightings of the VIPs in samples P3, P4, P9 and P10 are weak, indicating that these samples have an average composition. Data extraction of the positive ion data yielded 6363 features of which the top 500 by mean intensity were selected for PCA. The groupings obtained

Table 1 The top 10 VIPs, composed of negative ion masses measured to within 2 ppm of that of the proposed elemental compositions responsible locating the groups shown in Fig 2.

m/z	**Rt (min)**	**Molecular formula**	**Isomers in DNP**	**VIP**
P1/P2				
325.145	24.9	$C_{20}H_{22}O_4$	109	10.1
341.140	21.4	$C_{20}H_{22}O_5$	188	8.2
595.168	3.3	$C_{27}H_{32}O_{15}$	52	3.5
329.067	11.1	$C_{17}H_{14}O_7$	163	3.5
325.145	10.1	$C_{20}H_{22}O_4$	109	2.8
331.155	17.7	$C_{19}H_{24}O_5$	106	2.7
341.140	13.6	$C_{20}H_{22}O_5$	188	2.6
341.103	10.5	$C_{19}H_{18}O_6$	127	2.5
421.093	14.2	$C_{23}H_{18}O_8$	16	2.4
357.135	29.0	$C_{20}H_{22}O_6$	236	2.2
301.217	43.6	$C_{20}H_{30}O_2$	598	2.0
381.192	8.2	$C_{20}H_{30}O_7$	184	2.0
P5/P6/P7				
373.275	52.6	$C_{24}H_{38}O_3$	45	13.0
401.306	56.4	$C_{26}H_{42}O_3$	27	10.1
375.291	57.4	$C_{24}H_{40}O_3$	27	9.3
369.244	48.8	$C_{24}H_{34}O_3$	11	7.1
385.239	36.8	$C_{24}H_{34}O_4$	45	5.7
345.244	50.0	$C_{22}H_{34}O_3$	127	5.0
387.254	49.1	$C_{24}H_{36}O_4$	51	4.8
347.259	52.9	$C_{22}H_{36}O_3$	114	4.6
361.275	54.9	$C_{23}H_{38}O_3$	24	4.2
371.260	50.3	$C_{24}H_{36}O_3$	21	3.6
P11/P12				
289.108	10.6	$C_{16}H_{18}O_5$	81	13.5
333.171	7.4	$C_{19}H_{26}O_5$	94	12.7
247.098	6.0	$C_{14}H_{16}O_4$	108	8.6
333.171	8.1	$C_{19}H_{26}O_5$	81	8.2

587.339	32.4	$C_{37}H_{48}O_6$	3	7.7
645.308	19.5	$C_{38}H_{46}O_9$	8	7.7
373.166	15.3	$C_{21}H_{26}O_6$	107	7.7
331.155	8.6	$C_{19}H_{24}O_5$	93	7.2
313.145	15.2	$C_{19}H_{22}O_4$	117	6.4
349.166	6.6	$C_{19}H_{26}O_6$	102	6.1
P3/P4/P9/P10				
619.438	47.9	$C_{40}H_{60}O_5$	1	1.5
347.187	19.5	$C_{20}H_{28}O_5$	531	1.2
763.551	57.9	$C_{48}H_{76}O_7$	1	1.0
707.474	9.1	$C_{40}H_{68}O_{10}$	5	0.9
763.551	53.6	$C_{48}H_{76}O_7$	1	0.8
369.301	47.9	$C_{22}H_{42}O_4$	8	0.7
397.223	12.4	$C_{21}H_{34}O_7$	26	0.7
333.207	14.0	$C_{20}H_{30}O_4$	776	0.6
379.213	20.0	$C_{21}H_{32}O_6$	52	0.6
187.098	6.0	$C_9H_{16}O_4$	31	0.5
P8				
401.306	56.4	$C_{26}H_{42}O_3$	27	4.2
345.244	50.0	$C_{22}H_{34}O_3$	127	4.2
371.26	50.3	$C_{24}H_{36}O_3$	21	4.1
375.291	57.4	$C_{24}H_{40}O_3$	27	3.7
369.244	48.8	$C_{24}H_{34}O_3$	11	3.4
255.066	15.6	$C_{15}H_{12}O_4$	145	3.2
347.259	52.9	$C_{22}H_{36}O_3$	114	3.1
373.275	52.6	$C_{24}H_{38}O_3$	45	2.9
375.291	55.6	$C_{24}H_{40}O_3$	27	2.6
397.275	50.8	$C_{26}H_{38}O_3$	23	2.1

were similar to those obtained with the negative ion data (Fig S1 File S1). The twelve propolis sample extracts were tested for their activity against *P. falciparum*, *T. brucei*, *L. donovani*, *C. fasciculata* and *M. marinum*. In addition cellular toxicity assays were carried out using mammalian cells.

Anti-microbial Activity

Activity of propolis extracts against *P. falciparum*

Fig 3 shows an OPLS plot for the observed activity of the extracts against *P. falciparum* shown in Table 2 constructed using 5 of the 300 variables used to produce Fig 1 by systematically discarding the variables with less impact on the model. The correlation between observed and predicted activity is very good with all the samples falling on the line. Table 3 shows the five most important variables contributing to the high activity of sample P2. From the loadings plot the greatest activity was associated with compound D which is abundant in samples P1 and P2. As can be seen from Fig S2 in File S1, the more active samples have a greater abundance of compound D. However, sample P11 is more active than would be predicted from levels of compound D and the activity appears to be based on a combination of the five marker compounds. Compound A seems to be associated with lower activity but not always since it is high in P7 which has relatively high activity. MS^2 and MS^3 spectra were obtained for the marker compounds and are described below. The MS^2 and MS^3 spectra for these compounds are shown in Figs S7-S16.

Compound A $C_{24}H_{38}O_3$, 45 isomers in DNP.

MS^2 m/z 329.2850 (100) ($C_{23}H_{37}O$). MS^3 (329.2850) No fragmentation at the energy used. Not much information can be derived from the mass spectra since the base peak formed in MS^2 does not fragment.

Compound B $C_{22}H_{36}O_3$, 114 isomers in DNP

MS^2 m/z 303.2689 (100) ($C_{21}H_{35}O$). MS^3 (303.2689) No fragmentation. Not much information can be derived from the mass spectra since the base peak formed in MS^2 does not fragment.

Compound C $C_{22}H_{34}O_3$, 127 isomers in DNP

MS^2 m/z 301.2550 (100) ($C_{21}H_{33}O$). MS^3 (301.2550) No fragmentation. Not much information can be derived from the mass spectra since the base peak formed in MS^2 does not fragment.

Compound D $C_{20}H_{22}O_5$, 189 isomers in DNP

MS^2 323.1284 (100) ($C_{20}H_{19}O_4$) 313.1287 ($C_{19}H_{21}O_4$) 311.1287 ($C_{19}H_{19}O_4$) 242.0584 ($C_{14}H_{10}O_4$)

MS^3 (311.1287) 216.0429 ($C_{12}H_8O_4$) 188.0479 ($C_{11}H_8O_3$) 144.0581 ($C_{10}H_8O$)

The ion at m/z 144.0581 is an important diagnostic fragment since it corresponds to naphthol and the ion at 188.0479 corresponds to a hydroxylated naphthoic acid. The ion at m/z 216.0429 has an additional CO suggesting a carbonyl is also substituted onto a hydroxynaphthoic acid and this fragment would arise from the molecular ion via the loss of a hydroxylated C_8H_{13} hydrocarbon chain. It was not possible to correlate this information to any structure in the literature.

Compound E $C_{20}H_{30}O_2$, 598 isomers in DNP

MS^2 220.1470 (100) ($C_{14}H_{20}O_2$), 205.1235 ($C_{13}H_{17}O_2$)

MS^3 (220.1470) 205.1235 (100) ($C_{13}H_{17}O_2$)

Not much structural information is revealed from the fragments produced.

Table 2 Activity of samples P1-P12 against *P.falciparum* (n=3).

	EC_{50} (ug/mL)				
Compound	1	2	3	Mean	SEM
P1	5.9	6.0	6.2	6.1	0.10
P2	5.3	2.3	2.6	3.4	0.96
P3	7.8	9.6	8.4	8.6	0.52
P4	14.5	13.7	15.4	14.5	0.48
P5	26.8	32.2	27.2	28.7	1.8
P6	40.7	44.1	43.6	42.8	1.0
P7	6.6	13.3	12.1	10.6	2.1
P8	46.7	50.3	63.8	53.6	5.22
P9	7.0	9.8	9.2	8.7	0.84
P10	23.1	20.0	24.9	22.7	1.43
P11	14.9	14.9	14.2	14.7	0.23
P12	14.6	14.9	13.1	14.2	0.57
Chloroquine (nM)	7.4	7.6	7.5	7.5	0.07

Activity of propolis extracts against *T. brucei*

Fig S3 in File S1 shows an OPLS model based on four compounds correlating strongly with activity against *T. brucei* (Table S3 in File S1). Two of these were compounds A and E which were also important in the activity against *P. falciparum*. Compounds F and G are discussed below.

Compound F $C_{17}H_{14}O_7$, 163 isomers in DNP

MS^2 m/z 314.0660(100) ($C_{16}H_{10}O_4$) m/z 299.0196 (14.3) ($C_{15}H_7O_7$)

MS^3 (299.0196) m/z 271.0246 (100) ($C_{14}H_7O_6$) m/z 255.0299 (6.3) ($C_{14}H_7O_5$)

The structure could be related to dimethylquercetin which occurs in temperate propolis. However, the diagnostic fragments which usually arise from cleavage of the C ring in flavonoids were not identified [26].

Compound G $C_{20}H_{22}O_4$, 109 isomers in the DNP

MS^2 m/z 242.0584 (6.1) ($C_{14}H_{10}O_4$) m/z 216.0427 (44.8) ($C_{12}H_8O_4$) m/z 188.0477 (65.4) ($C_{11}H_8O_3$) m/z 144.0581 (5) ($C_{10}H_8O$)

MS^3 (188.0477) m/z 144.0581 (100) ($C_{10}H_8O$)

This compound is related to compound D but lacks the hydroxyl group in the side chain and thus appears to be a substituted hydroxy naphthoic acid.

Activity of propolis extracts against *L. donovani*

Only 9 out of 12 propolis samples could be fitted into and OPLS model (Fig 4 and table 4 in File S1). Compounds A and D were important to the model and two additional compounds H and I were also important and are discussed below.

Compound H $C_{20}H_{22}O_5$, 189 isomers in DNP

MS^2 m/z 271.0973 (100) ($C_{16}H_{15}O_4$) m/z 242.0584 (12.0) ($C_{14}H_{10}O_4$) m/z 216.0429 (10.8) ($C_{12}H_8O_4$) m/z 188.0479 (14.2) ($C_{11}H_8O_3$) m/z 144.0581 (0.8) ($C_{10}H_8O$)

MS^3 (271.0973) 242.0584 (100) ($C_{14}H_{10}O_4$) 216.0429 (30.0) ($C_{12}H_8O_4$) 188.0479 (46.0) ($C_{11}H_8O_3$) 144.0581 (1.8) ($C_{10}H_8O$)

Compound H is an isomer of compound D and has very similar mass spectrum, and thus is clearly structurally related to compound D.

Compound I $C_{19}H_{18}O_6$ Isomers in DNP 128

MS^2 m/z 323.0923 (19.6) ($C_{19}H_{15}O_5$) m/z 311.0921 (52.8) ($C_{14}H_{10}O_4$) m/z 293.0818 (36.4) ($C_{18}H_{13}O_4$) m/z 265.0479 (10.7) ($C_{17}H_{13}O_3$) m/z 176.0478 (84.2) ($C_{10}H_8O_3$)

MS^3 (m/z 176.0478) m/z 147.0452 (100) ($C_9H_7O_2$)

Compound I is most probably closely related to the lignan sesamin previously characterised in Libyan propolis [12] but lacks one of the methylene groups, having a catechol structure in one of the aromatic rings rather than a methylene dioxy group.

Table 3 Most important variables determining the activity of P2 in anti-protozoal and anti-microbial tests and important variables determining cellular toxicity based on sample P8 which was the most cytotoxic sample.

	$[M-H]^-$	**Rt (min)**	**Molecular formula**	**Compound**
P.falciparum				
	373.275	52.6	$C_{24}H_{38}O_3$	Compound A
	347.259	52.9	$C_{22}H_{36}O_3$	Compound B
	345.244	50.0	$C_{22}H_{34}O_3$	Compound C
	341.14	21.4	$C_{20}H_{22}O_5$	Compound D
	301.217	43.6	$C_{20}H_{30}O_2$	Compound E
T. brucei				
	373.275	52.6	$C_{24}H_{38}O_3$	Compound A
	329.067	13.1	$C_{17}H_{14}O_7$	Compound F
	325.145	25.0	$C_{20}H_{22}O_4$	Compound G
	301.217	43.6	$C_{20}H_{30}O_2$	Compound E
L.donovani				
	373.275	54.6	$C_{24}H_{38}O_3$	Compound A
	325.145	25.0	$C_{20}H_{22}O_4$	Compound D
	341.14	13.6	$C_{20}H_{22}O_5$	Compound H
	341.103	10.5	$C_{19}H_{18}O_6$	Compound I

C. fasciculata				
	329.067	13.1	$C_{17}H_{14}O_7$	Compound F
	325.145	25.0	$C_{20}H_{22}O_4$	Compound G
	369.301	47.9	$C_{22}H_{42}O_4$	Compound J
M. marinum				
	341.14	21.4	$C_{20}H_{22}O_5$	Compound D
	325.145	25.0	$C_{20}H_{22}O_4$	Compound G
	289.108	10.6	$C_{16}H_{18}O_5$	Compound K
	369.301	47.9	$C_{22}H_{42}O_4$	Compound J
U937 Cells				
	373.275	52.6	$C_{24}H_{38}O_3$	Compound A
	341.14	21.4	$C_{20}H_{22}O_5$	Compound D
	325.145	25.0	$C_{20}H_{22}O_4$	Compound G
	397.275	50.8	$C_{26}H_{38}O_3$	Compound L

Activity of propolis extracts against *C. fasciculata*

The activity against *C. fasciculata* (Table 5 in File S1) correlated strongly with three compounds in an OPLS model (Fig 5 in File S1). Compounds F and G, which were important in other models of activity, also correlated with high activity; compound J correlated with low activity. Compound J is a relatively minor peak and did not afford a clear MS^2 spectrum.

Activity of propolis against *M. marinum*

An OPLS model based on four components (Fig 6 in File S1) gave a good fit to the activity against *M. marinum* (Table 6 in File S1). Again compounds D and G were responsible for high activity while compounds J and K correlated with low activity.

Toxicity of propolis against mammalian cells (U937)

The toxicity of the propolis extracts was tested against mammalian cells (Table 7 in File S1). For three of the samples, P9, P11, P12 there was no significant toxicity up to 100 µg/ml and thus they were excluded from the OPLS model (Fig 7 in File S1). The most toxic sample was P8 which gave an IC_{50} value of 34.1 µg/ml. Of the samples showing toxicity below 100 µg/ml P2 was the least toxic. The main compounds responsible for the toxicity of the samples were compound A and compound L. From the similar elemental compositions it seemed possible that compound A and compound L might be related. The mass spectrum of compound L is discussed below.

Compound L $C_{26}H_{38}O_3$, 23 isomers in DNP.

MS^2 m/z 353.2867 (100) ($C_{25}H_{37}O$). MS^3 (m/z 353.2867) 351.2715 (100) ($C_{25}H_{35}O$) m/z 337.2557 (15.7) ($C_{24}H_{33}O_3$), m/z 323.2400 (2.9) ($C_{23}H_{31}O$), m/z 309.2243 (5.9) ($C_{22}H_{29}O$), m/z 295.2084 (7.3) ($C_{21}H_{27}O$), m/z 281.1929 (6.3) ($C_{20}H_{25}O$), m/z 267.1771 (5.9) ($C_{19}H_{23}O$), m/z 253.1613 (5.6) ($C_{18}H_{21}O$), m/z 239.1451 (5.5) ($C_{17}H_{19}O$), m/z 225.1299 (3.4) ($C_{16}H_{17}O$) m/z 133.0667 (0.8) (C_9H_9O), 119.0511 (2.3) (C_8H_7O), 107.0509 (2.2) (C_7H_7O).

MS^3 suggested a phenol substituted with a 17 carbon chain containing four units of unsaturation. The compound also contains a carboxylic acid shown by the loss of CO_2 in the MS^2 spectrum. The structure is consistent with an anacardic acid, these compounds are found in cashew oil [27]. On closer examination of the MS^3 spectrum of compound A it was also observed that very small ions corresponding at m/z 119.0511 and 107. 0509 could be observed. Thus it seems likely that compound A is also an anacardic acid with substituted with a 17 carbon chain with two units of unsaturation. Looking at the marker compounds in table 1 all but one of the top 10 VIPs for sample 8, the most toxic sample, have elemental compositions that would fit anacardic acids substituted with varying alkyl chains. Sample P8 is from the SW of the country from an oasis area with a very dry climate thus there is nothing to suggest that cashew trees might grow in this area, however, pistachio trees (*Pistacia vera*) are cultivated in Libya and these contain anacardic acids [28]. A closely related series of alkylated phenols was recently observed in Cameroonian propolis [29] and were

thought to originate from the Anacardiaceae family of plants. Anacardic acids have also been observed in propolis from Oman and Brazil [30, 31]. Anacardic acids have been shown to exhibit cytotoxicity [32] and their high levels in P8 would explain why it is the most cytotoxic sample. The samples from the other oasis area in the SE of the country P5/6/7 also contain anacardic acids and are relatively cytotoxic.

Concluding Remarks

Evidence is mounting that propolis protects bee hives against microbial infection [6, 7, 33-36]. With the problems of colony collapse affecting bee hives in many parts of the world a better understanding of propolis is of great importance. The chemical composition of propolis could potentially reveal a great deal about the interaction between the bee and its environment. What is not known is whether or not bees through selection pressure have targeted plants producing resins with the desired biological properties or it just happens that the plant resins which are suitable the coating of hives just happen to have antimicrobial properties. Strong anti-microbial properties are not universal and in a survey of anti-bacterial activity of propolis from various parts of the world it was found that many samples from Sub-Saharan Africa did not have anti-bacterial properties [37] against the eight types of bacteria studied. In the current case the samples from the South of Libya were less active against protozoa but did exhibit more cytotoxicity. Is this variation just random because the plant sources are varied or is it that the bees face different environmental pressures in different regions? Considering protozoa specifically it is known that these infect insects [8] and it is also know that trypanosomatids occur in plant latexes and in fruits [38]. Thus plants also have an interest in defence against infection against protozoa and it might be expected that some plant resins would have anti-protozoal properties but obviously not all as judged from the current survey. Again a question which arises regarding whether or not plants from certain environments are more likely to face pressure from protozoal infection? The same might be true of bacterial infection and we concluded in our earlier study that propolis from tropical areas with high rain fall and warm

temperatures has the highest anti-microbial activity [37]. Thus since nature is so interconnected it might be that bees for instance in an environment where plants do not face pressure from protozoal attack also are not susceptible to this pressure. Protozoal infection might not occur in the dry areas in the South of Libya. However, propolis is still collected by bees in these areas and this might simply be for its properties as a mechanical barrier rather to ward off infection. The propolis from the South of Libya is more cytotoxic and from the plant's point of view this might be simply to make it unpalatable to animals. Finally there is little doubt the discovery of new anti-protozoal compounds is particularly important. There has been little development of new anti-protozoal drugs for many decades, resistance to the existing treatments has become a problem and the treatments that are used are quite toxic and often have poor bioavailability and have to be given by injection [39,40]. Although there is a resistance to the notion of using extracts as treatments bees appear to exert a degree of quality control as judged similar activity for samples P1 and P2. Thus could propolis extracts have a role in treating these diseases at low cost and in the process encourage bee keeping?

Author Contributions

Conceived and designed the profiling experiments: TZ DGW WS REE. Carried out he experiments: TZ WS MB MYH TP CJC NW GUE. Data analysis: DGW TZ REE MB MYH TP CJC NW GUE. Provided materials and facilities: JF VF HPD SM KCC CJC. Contributed to writing of paper: DGW WS VF HPD MB KCC JF. Collected propolis samples: WS.

Supplementary Material

Fig S1 PCA separation of propolis samples according to positive ion MS data.

Fig S2 Abundance of compound D according to chromatographic peak area in the 12 Libyan propolis samples.

Fig S3 OPLS model of the activity of Libyan propolis samples against *T.brucei* based on four compounds. P3 was omitted in order to improve the fit of the model.

Fig S4 OPLS plot of observed against predicted activity of propolis samples against *L.donovani*. Samples P3, P6 and P11 were omitted in order to improve the fit of the model.

Fig S5 OPLS plot of observed against predicted activity of propolis samples against *C. fasciculata*. Sample P3 was omitted in order to improve the fit of the model.

Fig S6 OPLS plot of observed against predicted activity of propolis samples against *M.marinum*.

Fig S7 OPLS plot of observed against predicted activity of propolis samples against cells. Samples P3 and P12 were omitted in order to improve the fit of the model.

Table S1 Main plants visited by bees in Libya and their flowering period

Table S2 The physical properties of the Libyan propolis samples.

Table S3 Anti-trypanosomal activity of samples P1-P12 against *T.brucei* (s427) (n=3).

Table S4 IC values obtained for P1-12 against *L. donovani* amastigotes (n=3).

Table S5 EC_{50} and EC_{90} values µg/ml (n=4) obtained for propolis extracts against *C. fasciculata*.

Table S6 MIC values for P1-P12 tested against against *M. marinum* (n=2, values identical for the replicates).

Table S7 Cytotoxicity for P1-9 and P11 measured against U937 cells.

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Fig 1 Libyan map showing the collection points Libyan Propolis samples **P1** (Alagoria), **P2** (Gaminis), **P3** (Byda), **P4** (Quba), **P5,P6, P7** (Kufra), **P8**(Ghadames), **P9** (Tripoli), **P10** (Khasr Khiar), **P11, P12** (Khumas).

Fig 2 PCA with HCA based on the 300 most intense features obtained in negative ion mode for the 12 propolis samples.

Fig 3 OPLS plot of observed against predicted activity against *P. falciparum* based on five compounds (A-E).