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The Isolation of Anti-protozoal Compounds from Libyan Propolis

Short title: Anti-protozoal Compounds from Libyan Propolis

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Propolis is increasingly being explored as a source of biologically active compounds. Until now there has been no study of Libyan propolis. Two samples were collected in North East Libya and tested for their activity against *Trypanosoma brucei* . Extracts from both samples had quite high activity. One of the samples was fractionated and yielded a number of active fractions. Three of the active fractions contained single compounds which were found to be 13-epitorulosal, acetyl-13-epi-cupressic acid and 13-epi-cupressic acid **which have been described before in Mediterranean propolis. Two of the compounds had MIC valuesof 1.56 µg/ml against *T.brucei*. The active fractions were also tested against macrophages infected with *Leishmania donovani* and again moderate to strong activity was observed with the compounds having IC50 values in the range 5.1-21.9 µg/ml.**

INTRODUCTION

Propolis is harvested by honey bees in order to seal cracks of the hives and more importantly eliminate biological contamination in the colony. It has been reported to have various biological and pharmacological properties and is potentially a source of new medicines particularly for treating infective diseases (Sforcin and Bankova, 2011; Salatino *et al*,2011; Bankova *et al*, 2000) . Propolis has the advantage as a source of biologically active compounds since it has already been selected by bees for its biological activity and is collected from plants in a non-destructive way. The chemical and bioactive characteristics of propolis are highly dependent on its geographic origin (Seidel *et al*, 2008; Watson *et al* 2006; Salatino *et al*, 2005). Although it has never been proved, it would seem likely that the plants chosen by the bees in a particular location would exhibit activity against the environmental pressures encountered by the bees which include protozoal attack (Ruiz-Gonzales and Brown 2006). In a simulated experiment, artificial nectar containing the alkaloid gelsemine, which is also naturally contained in a source of nectar favoured by North American bumblebees, was

ingested by bumblebees and found to reduce the pathogen load of the protozoan parasite *Crithidia bombi* (Manson *et al* 2010). A number of previous studies have tested propolis against both *Trypansoma* and *Leishmania* species, these studies have largely focused on the use of extracts rather than isolated compounds. Extracts from two samples of Portuguese propolis were investigated for their activity against *Plasmodium falciparum*, *Leishmania infantum*, *Trypanosoma brucei* and *Trypanosoma cruzi* (Falcão *et al*, 2014). IC50 values were mainly < 10 µg/ml and the greatest activity of 1.8 µg/ml being against *T.brucei*. The anti-leishmanial activity of Turkish propolis was investigated (Duran *et al* 2011) and the two samples tested had IC50 values of 175 and 350 µg/ml. The effect of an extract of Brazilian propolis on *Trypanosoma evansi* was investigated (Gressler *et al* 2012) and *in vitro* the IC50 value for the extract was found to be 10µg/ml, but the propolis was ineffective in curing rats infected with *T.evansi*. Extracts of eighteen Cuban propolis extracts of different types were tested against *Leishmania amazonenis* (Fidalgo *et al* 2011). All of the extracts produced inhibition of *L.amazonenis* but they also displayed some toxicity against macrophages. Compounds isolated from *Baccharis dracunculifolia*, which is the major source of Brazilian green propolis, were investigated for antileishmanial and antiplasmodial activity (Da Silva *et al*, 2009). Ursolic acid and hautriwaic acid lactone had IC50 values of 3.7 and 7 µg/ml against *Leishmania donovani*. The activity of Brazilian green propolis against *T.cruzi* was investigated (Salomão *et al*, 2011). The extract was most effective against the intra-cellular amastigote stage of the parasite having an IC50 values of 8.5 µg/ml. Extracts of Brazilian red and Brazilian green propolis were tested against L. amazonensis (Ayres *et al* 2007) and an extract of red propolis was found to be the most effective having an MIC of 25 µg/ml. Bulgarian propolis extracts were found to have IC50 values of 36-40 µg/ml against T.cruzi (Dantas *et al* 2006). Thus it is apparent that anti-protozoal activity of propolis is common to propolis samples from many regions. Within this context, our study investigated the effect of Libyan propolis on the parasites *Trypanosoma brucei brucei*, which is the etiologic agent of sleeping sickness and

Leishmania donovani which causes visceral leishmaniasis. The biological properties and chemical

profile of Libyan propolis have not been investigated before.

MATERIALS AND METHODS

Materials

Absolute ethanol, HPLC grade acetonitrile, hexane, methanol, formic acid and Acrodisc syringe filters were obtained from (Fisher Scientific, Loughborough UK). Chloroform, DMSO, deuterated chloroform, D6 DMSO, silica gel 60, 0.04 -0.06mm mesh size and Wilmad NMR tubes were obtained from Sigma Aldrich, Dorset, UK. An ACE C_{18} column (3mm x 150mm, 3μm) was from Hichrom, Reading, UK. *T.brucei* (ATCC S427 blood stream form) was from Fisher Scientific, UK. HPLC grade Water was produced in house by a Milli Q system (Millipore, UK). RPMI 1640 medium, DMEM, penicillin-streptomycin, and L-glutamine were obtained from Gibco BRL, Paisley UK. D-luciferin potassium salt was obtained from Caliper Life Science, Massachusetts, USA. Amphotericin B was purchased from Sequoia Research Products (Berkshire, UK).

Two propolis samples were collected from Tokra or Tukra ,Al `Aquriyah, Libya, a small village in Eastern Libya, located about 70 km East of Benghazi city (LBA) and from Qaminis 53 km South of Benghazi (LBG). The beekeeper scraped the propolis sample off the top of the hive using a spatula and collected it in a clean tray. LBA possessed an intense orange like odour, was light brown and had a very sticky texture while LBG was darker brown, less sticky and had a less intense odour. A sample of the LBA propolis (23g) was extracted by sonication in 100 ml of absolute ethanol for 60 minutes then the extract was filtered and re-extracted twice more with 100 ml of ethanol, filtering each time after that and the extracts were combined and the solvent was evaporated. The crude ethanolic extract was tested against *T. brucei*.

Animals

Luciferase-expressing *L. donovani* promastigotes were derived from *Leishmania donovani* strain MHOM/ET/67:MHOM/ET/67: LV82 (XXX). In-house inbred male BALB/c mice (20-25 g) were used in studies. All animal studies were carried out in accordance with the Animals (Scientific Procedures)

Act 1986 and had UK Home Office approved and local ethical approval from the University of Strathclyde.

Open Column Chromatography and Medium Pressure Liquid Chromatography

A sample (2g) of the ethanolic extract of the propolis was dissolved in ethyl acetate and mixed with 6 g of silica gel in a beaker and the solvent was removed slowly under a stream of nitrogen. Then silica gel (50 g) was mixed with hexane (200ml) and used to pack a glass column. The sample mixed with silica gel was dry loaded onto the top of the column and elution was carried out as follows collecting fractions in 50 ml flasks: 200ml of hexane/ethyl acetate, (90:10) F1 , then 200 ml of hexane/ethyl acetate (60:40) F2 , then 200ml of hexane /ethyl acetate (40:60) F3, 200ml of ethyl acetate F4 and 200 ml of methanol F5 and finally 200ml of methanol/water (60:40) F6. All fractions obtained from open column chromatography were concentrated by rotary evaporation and weighed. Fraction F1-3 which had the highest weight was fractionated by medium pressure liquid chromatography (MPLC) on silica gel using a Grace Revelris Flash Chromatography system (Alltech Ltd. UK) with evaporative light scattering (ELSD) detection and UV detection. The sample was loaded onto celite (1.9g) and packed into a dry loading cartridge. The Revelris MPLC was set up with a 24 g silica gel column to run a stepwise gradient at 12 ml/min flow rate using linear gradients as follows: 100% hexane 0 min.; hexane ethyl acetate (80:20) 30 min.; 100% ethyl acetate 50 min. Fractions were collected automatically when triggered by the ELSD response. The fractions associated with the same peak according to the ELSD chromatogram were combined and the solvent was removed and they were weighed. The isolated fractions were profiled by reversed phase HPLC with ELSD; GC-MS, LC-MS and NMR.

Instrumental Methods

Fractions from the MPLC separation were profiled using an Agilent 1100 HPLC linked to a Shodex ELSD. An ACE C_{18} column (150 × 3 mm, 3 μm)) with a mobile phase of water (A) and acetonitrile (B)

with a flow rate of 0.5 ml/min and the following gradient: 0 min 70% B ;20 min. 100% B, 26 min. 100% B. The samples were prepared at 0.5 mg/ml by dissolving in acetonitrile and then adding water to give a solution in water/acetonitrile (30:70).

The crude sample, fractions obtained from chromatography and the purified compounds obtained from flash chromatography were dissolved in methanol and analysed by LC-MS in order to confirm their masses and molecular formulae. The high resolution mass spectra were obtained by using an LTQ Orbitrap mass spectrometer (ThermoFisher, Hemel Hempstead, UK) in negative ion mode with a needle voltage of -4.0 kV. Samples were dissolved in methanol to give 1mg/ml and the sample solution (20μl) was injected. The separation was performed on an ACE C_{18} column (150 × 3 mm, 3 μm) from HiChrom UK with 0.1% v/v formic acid in water as mobile phase A and 0.1% v/v formic acid in acetonitrile as B at flow rate of 0.300ml/min using the gradient described for HPLC-ELSD.

Nuclear Magnetic Resonance Spectroscopy (NMR)

Samples (5-10 mg) of the fractions obtained from the MPLC fractionation which exhibited good purity were dissolved in $CDCl_3$ and transferred to NMR tubes. ^{1}H NMR spectra were measured at a magnetic field strength of 400.13 MH_Z using a JEOL Delta GX 400 MHz FT nuclear magnetic resonance instrument. Proton spectra were referenced to the residual protons in $CDCl_3$. Broad band decoupled ^{13}C NMR was used to determine the number of carbons, their type and where necessary DEPT experiments were obtained in order to distinguish the carbons according to the extent of their proton attachments. Correlation spectroscopy (COSY), Heteronuclear Multiple-Bond Correlation Spectroscopy (HMBC) and Heteronuclear Multiple Quantum Coherence (HMQC) spectra were also obtained.

Optical rotation measurements were obtained by using a Perkin Elmer 341 polarimeter. The samples were dissolved in 2 ml of chloroform and measured using the sodium D line.

Antimicrobial assays

a) Trypanosome studies The *in vitro* anti-trypanosomal tests were carried out by using an AlamarBlue[TM] assay according to a standard protocol (Raz *et al* 1997; Igoli *et al* 2012).

b) *L. donovani* studies

Intraperitoneal macrophages were recovered from the peritoneal cavity of BALB/c mice 3 days after intraperitoneal injection with 1 ml 3% w/v aqueous sterile starch solution. The mice were then euthanized and 3 ml of incomplete medium (RPMI-1640, 100μg/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 x g for 5 minutes and then re-suspended in 10 mls complete medium (in complete RPMI-1640 supplemented with 10% heat inactivated FCS [v/v]). The cells were then used in antileishmanial assays. Bone marrow was then harvested from the femurs of each mice by flushing out the removed bone with 5 mls of bone marrow medium (DMEM, 20% heat-inactivated FCS [v/v], 30% L-Cell solution [v/v], 100 μg/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% CO_2:95% air. The medium was removed from the plate and 7 ml Tryple Express was added to detach the bone marrow derived macrophages. The resulting suspension of bone marrow derived macrophages was collected, pelleted by centrifugation and re-suspended in 10 ml of incomplete medium and then used in antileishmanial assays.

The number of live macrophages/ml was determined microscopically using a haemocytometer, by mixing a cell sample with 1:1 Trypan blue (20μl) and viewing at x10 magnification. In all cases cell viability was >95%. Cells (0.5×10^5 in 200μl complete medium) were

added to the appropriate wells of a 96 well tissue culture plate and incubated for 24 hours 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 hours. The medium was removed from each well and replaced with 200µl 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 (AMB, 4-0.02 µg/ml). The plate was incubated as before for 72 hours and then the medium was then removed and 150µl of luciferin solution (150µg/ml luciferin in complete RPMI-1640) was added to each well and the BLI emitted/well was determined using the IVIS® imaging system (Alsaadi *et al.,* 2012). The suppression in bioluminescent signal for each test sample was compared to the mean control value. The mean IC50 value was then calculated for each sample by Probit analysis (Vermeersch *et al.*, 2009).

Statistics

Data was 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 was normally distributed. Parametric data was analysed using a Student's unpaired t-test or by one-way analysis of variance (ANOVA) 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 <0.05.

RESULTS

The results for screening the crude extracts of two propolis samples and against *T.brucei* using an Almar blue assay are shown in table 1. Both extracts were quite active but since LBA was approximately two fold more active than LBG and it was selected for fractionation. First it was fractionated by open column chromatography and fraction 1, which was eluted with hexane/ethyl

acetate (90:10) and which had the highest weight, and fraction 3, which was eluted with hexane/ethyl acetate (40:60), were selected for further fractionation by MPLC. The fractions obtained from the Grace Revelris system were analysed by HPLC-ELSD and four of the fractions were found to be quite pure. The ^{1}H and ^{13}C NMR data for the pure fractions were compared to the literature and the data were found to closely match the NMR data reported previously for the labdane diterpenes 13-epitorulosal (1), acetyl-13-epi-cupressic acid (2) and 13-epi-cupressic acid (3) (Abdel-Sattar *et al* 2009; Wen-Chiung *et al* 1994) and for the lignin (+) sesamin (4) (Christov *et al* 1999) . In addition the following analytical information was obtained: (1) Eluted mainly in fraction 9 from the MPLC system which had a weight of 14 mg. It had an optical rotation of + 14° (lit +35°). (2) Eluted mainly in fraction 10 from the MPLC system which had a weight of 7 mg. It had an optical rotation of + 92°. (3) Eluted mainly in fraction 15 from the MPLC system which had a weight of 102 mg. It had an optical rotation of +55.5° (lit +57°).. (4) Eluted in fraction 7 when fractionation of open column fraction 3 was separated using the MPLC system and it had a weight of 7 mg. The optical rotation of the compound was measured in chloroform and gave [α] = +17°.

Biological Test Results

The results for the fractions obtained from MPLC are shown in table 1. Most of the fractions contained mixtures of diterpenes with fractions 9, 10 and 15 containing the diterpenes 1-3 described above. None of the fractions had greater activity than the open column fraction 1 itself suggesting that there might be synergisitic activity between the components within the fraction. Two of the diterpenes, (1) and (2) which elute in fractions 9 and 10 have activity equal to the unfractionated material. The results illustrate that relatively small structural changes can have an effect on activity with the diterpene in fraction 15 being considerably less active than the compounds in fractions 9 and 10. Thus the results point to a potential for carrying out semi-synthetic modifications of active

compounds in order to improve activity. In addition sesamin which was isolated from F3 by MPLC gave moderate anti-trypanosomal activity.

Anti-leishmanial activity

The MPLC fractions obtained for were also tested against *L. donovani*. The results are shown in table 2 and it can be seen that the IC50 values are somewhat variable which can be attributed to the difficulty in getting the compounds to dissolve in the aqueous test medium. Overall compound 3, which is in the most polar fraction tested, was the most consistent in terms of activity and the most active. The fractions were all active and overall their activity was greater in suppressing infection of peritoneal macrophages than in inhibiting infection of bone marrow macrophages. Most of the fractions were active at concentrations well below the levels at which the commonly used stibogluconate treatment is effective (Carter *et al* 2001). The levels of DMSO used in the samples were below the level where it had toxic effects on the leishmania.

DISCUSSION

The composition of the Libyan propolis LBA is typical of Mediterranean propolis. Profiling of Greek propolis was carried out by GC-MS (Popova *et al*, 2010) on six samples, three collected from the Greek mainland and three collected in Crete. All of the samples were rich in diterpenes with 37 diterpenes being characterised in the propolis extracts. It was proposed that the compounds were collected from conifer species in the Cupressaceae family. Some of these diterpenes, including compounds 1 and 3, were isolated from Cretan propolis (Popova *et al*, 2009) and tested against a range of bacteria against which they were found to have moderate activity. Compounds 2 and 3 were isolated from *Araucaria heterophylla* resin (Abdel-Sattar et al, 2009) and were tested against cancer cell lines. Compounds 2 and 3 were found to be quite cytotoxic having IC50 values of 2.7 and 9.8 μg/ml against MCF7 breast cancer cells. Thus if these compounds were to be effective anti-parasitic drugs the therapeutic window might not be that wide. Propolis proves to consistently have

potent biological activity and the activity is often specific to particular micro-organisms and in the current case earlier testing of the constituents of Cretan propolis, two of which were found in the Libyan propolis sample, revealed only moderate activity against bacteria. In the current case the acitivity against protozoa is considerably higher. Thus the propolis may reflect the particular environmental pressures that the bees are subject to within the region in which the hive is sited. Propolis remains a fascinating substance and if *in vivo* activity could be demonstrated to match its *in vitro* activity it is potentially a cheap readily available antibiotic.

Acknowledgements

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Figure 1 Structures of compounds isolated from Libyan propolis.
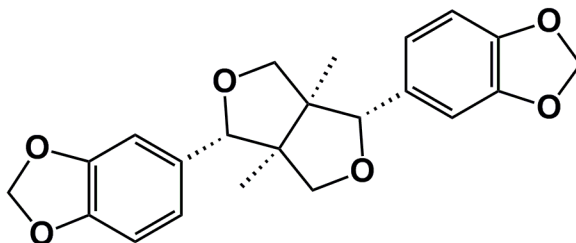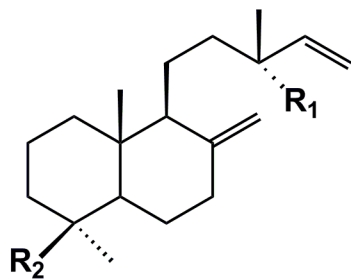
4 (+) sesamin

1 R$_1$=OH R$_2$= CHO
3 R$_1$= OCOCH$_3$ R$_2$= COOH
3 R$_1$ = OH R$_2$= COOH

Table 1 MIC values for crude LBA and LBG extracts and fractions of LBA tested against *T. brucei* blood

stream form S427.

Sample	MIC (μg/ml)
LBA1	3.12
LBG2	6.25
LBAF1	1.56
MPLC F1-2	10.00
MPLC F1-5	5.00
MPLC F1-7	10.00
MPLC F1-9 (1)	2.50
MPLC F1-10 (2)	1.56
MPLC F1-11	1.56
MPLC F1-14	5
MPLC F1-15 (3)	10
MPLC F1-16	2.5
MPLC F1-17	2.5
MPLC F1-18	5
MPLC F1-20	5
MPLC F3-7	6.5
Suramin	0.178

Table 2 MICs of MPLC fractions obtained from LBA1-4 tested against the amastigote stage of

L.donovani.

Fraction /compound	IC 50 µg/ml±SEM	
	Peritoneal infected macrophages	**bone marrow derived macrophages**
7	43±38 (n=2)	33±20 (n=3)
8	10.4 ± 1.6 (n=2)	22.8±7.8 (n=3)
9 (1)	6.9±3.7 (n=3)	7.4±5.0 (n=3)
10 (2)	7.0±4.0 (n=2)	21.9±12.3 (n=3)
11	2.2 (n=1)	32.2±2.4 (n=3)
15 (3)	5.1±2.1 (n=3)	6.3 ±3.7 (n=3)
Amphotericin b	0.01±0.0 (n=2)	0.024±0.06 (n=2)