The antileishmanial activity of xanthohumol is mediated by mitochondrial inhibition

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(*Received 14 July 2016; revised 10 October 2016; accepted 9 November 2016; first published online 12 December 2016*)

SUMMARY

Xanthohumol (Xan) is a natural constituent of human nutrition. Little is known about its actions on leishmanial parasites and their mitochondria as putative target. Therefore, we determined the antileishmanial activity of Xan and resveratrol (Res, as alternative compound with antileishmanial activity) with respect to mitochondria in *Leishmania amazonensis* promastigotes/amastigotes (LaP/LaA) in comparison with their activity in peritoneal macrophages from mouse (PMM) and macrophage cell line J774A.1 (J774). Mechanistic studies were conducted in *Leishmania tarentolae* promastigotes (LtP) and mitochondrial fractions isolated from LtP. Xan and Res demonstrated antileishmanial activity in LaA [half inhibitory concentration (IC_{50}): Xan 7 μM, Res 14 μM]; while they had less influence on the viability of PMM (IC_{50}: Xan 70 μM, Res >438 μM). In contrast to Res, Xan strongly inhibited oxygen consumption in *Leishmania* (LtP) but not in J774 cells. This was based on the inhibition of the mitochondrial electron transfer complex II/III by Xan, which was less pronounced with Res. Neither Xan nor Res increased mitochondrial superoxide release in LtP, while both decreased the mitochondrial membrane potential in LtP. Bioenergetic studies showed that LtP mitochondria have no spare respiratory capacity in contrast to mitochondria in J774 cells and can therefore much less adapt to stress by mitochondrial inhibitors, such as Xan. These data show that Xan may have antileishmanial activity, which is mediated by mitochondrial inhibition.

Key words: *Leishmania*, mitochondria, ESR spectroscopy, xanthohumol, resveratrol.

INTRODUCTION

Xanthohumol (Xan) and resveratrol (Res) (Fig. 1) are naturally occurring substances and constituents of human nutrition. Res is formed as a phytoalexin in plants, including grapes to defend against parasitic and fungal infections. Chemically, Res is a stilbene and belongs to the group of polyphenols and accumulates up to 14 mg L^{-1} (62 μM) in red wine (Stervbo *et al*. 2007). Likewise Xan belongs to the group of polyphenols but is structurally different and constitutes a prenylated chalcone. Xan is present as a hop extract in beers responsible for the bitter taste. In certain types of beer, Xan concentration is high and may exceed 1 mg L^{-1} (3 μM) (Walker *et al*. 2008). The oral bioavailability of Xan measured in rats is about 30% (Legette *et al*. 2012). In contrast, Res has an even lower bioavailability of <1% due to extensive metabolism in intestine and liver (Walle, 2011).

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For Res and related derivatives, several studies demonstrated that antioxidant, antiinflammatory and anticancer activities strongly depend on their chemical structure (Murias *et al*. 2005; Szekeres *et al*. 2010). Furthermore, it was suggested that Res also has a beneficial effect on glucose and lipid metabolism; however, clinical trials did not always confirm these findings (Cottart *et al*. 2014). For Xan, a broad-spectrum activity against Gram-positive bacteria, viruses, fungi and plasmodia was observed (Gerhäuser, 2005). In humans, the oestrogenic activity of hop extracts is well documented (Liu *et al*. 2001*a*) showing that this effect is not due to Xan but to related derivatives (Milligan *et al*. 1999; Zanoli and Zavatti, 2008). Furthermore, Xan also demonstrated antiproliferative and proapoptotic effects in various human cancer cell lines (Miranda *et al*. 1999) as well as chemopreventive properties [reviewed by (Zanoli and Zavatti, 2008)]. Xan and Res also interfere with mitochondrial functions in mammalian cancer cells thereby triggering apoptosis (Madan *et al*. 2008; Strathmann *et al*. 2010). Inhibition of individual mitochondrial complexes and transient mitochondrial superoxide production by Xan in mammalian

Parasitology (2017), **144**, 747–759.
doi:10.1017/S0031182016002389

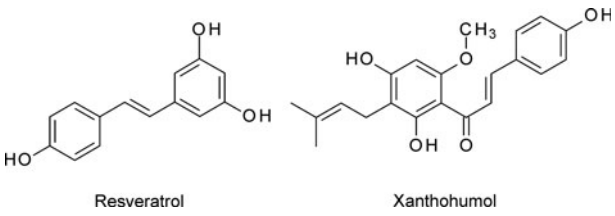

Fig. 1. Molecular structures of resveratrol (Res) and xanthohumol (Xan).

cells was suggested (Strathmann *et al*. 2010). In addition, inhibition of mitochondrial ATPase by targeting the F_1 subunit was reported for Res (Zheng and Ramirez, 1999).

In the present study, we were specifically interested in the role of mitochondria in the antileishmanial activity of Xan using Res as a reference compound since it was found to be effective against *Leishmania amazonensis* (Ferreira *et al*. 2014). For Res a study demonstrated that the antiamastigote effect against *Leishmania major* inside macrophages is more related to host cell toxicity than to direct amastigote killing (Lucas and Kolodziej, 2013). For Xan, an antiplasmodial effect was observed (Gerhäuser, 2005).

Although these publications provide some information about modes of action of Xan and Res on *Leishmania*, to our knowledge no studies exist directly comparing effects of Xan and Res in *Leishmania*.

MATERIAL AND METHODS

Chemicals

Diethylenetriaminepentaacetic acid (DTPA), ethylenediaminetetraacetic acid (EDTA), glucose, KCN, K_2HPO_4, KH_2PO_4, Na_2HPO_4, NaCl, KCl, HCl, NaN_3, succinate, sucrose, dinitrophenol (DNP) and tris(hydroxymethyl)aminomethane (TRIS) were obtained from Merck (Germany). Bovine serum albumin (BSA), cytochrome c^{3+} (cyt c^{3+}), decylubiquinone (dUQ), heat-inactivated fetal bovine serum (HFBS), Giemsa stain, haemin, 2,6-dichlorophenol–indophenol (DCPIP), 3-carboxy-proxyl (CP$^{\bullet}$), sorbitol, dithiothreitol (DTT), penicillin–streptomycin solution, resazurin, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium] bromide (MTT), pentamidine isethionate (Pen), antimycin A (AA), rotenone (Rot), valinomycin (Val), streptomycin, penicillin, carbonyl cyanide m-chlorophenyl hydrazone (CCCP), Brain–heart infusion (BHI) medium, RPMI 1640 and Schneider's medium were purchased from Sigma-Aldrich. Dubelco's Modified Eagle Medium (DMEM) was from Thermo Fisher Scientific and low-endotoxin FCS from Bio&Sell (Germany). Dimethyl sulfoxide (DMSO), desferal (DFO) and triethanolamine (TEA) were from Roth (Germany), Novartis Pharma (Germany) and Fluka (Germany), respectively; while 1-hydroxy-3-methoxycarbonyl-2,2,5,5-tetramethylpyrrolidine–HCl (CMH) was from Noxygen (Germany). Yeast extract powder was supplied by Amresco (USA). Idebenone (IQ) was obtained from Takeda (Japan). Oligomycin (Oligo) and 5,5′,6,6′-Tetrachloro-1,1′,3,3′-tetraethylimidacarbocyanine iodide (JC-1) was purchased from Calbiochem. Decylubiquinol ($dUQH_2$) was prepared from dUQ as described previously (Gille *et al*. 2001). Res and Xan were obtained from Sigma-Aldrich and their purity was >98%.

Macrophages

Peritoneal macrophages from mouse (PMM) were isolated immediately before use from adult female BALB/c mice in ice-cold RPMI 1640 medium supplemented with antibiotics (100 μg streptomycin mL^{-1} and 100 U penicillin $mL)^{-1}$.

The monocyte macrophage cell line J774A.1 (J774, Life Technologies cat. no. 12800-017) was grown in DMEM (high glucose, 1·5 g L^{-1} $NaHCO_3$) with 50 000 U L^{-1} penicillin and 50 mg L^{-1} streptomycin in 50 mL TubeSpin bioreactors using a roller culture apparatus (5 rpm) in an incubator at 37 °C and 5% CO_2 (Lam *et al*. 2009). J774 cells were passaged twice a week in a ratio of 1:10.

Leishmania

Leishmania amazonensis strain WHOM/77BR/LTB0016 provided by the Department of Immunology, Oswaldo Cruz Foundation (Brazil) was used. *Leishmania amazonensis* parasites were maintained in BALB/c mice's footpads (as amastigotes, LaA). All animal experiments were performed according to the 'Guideline on the Care and Use of Laboratory Animals' (National Research Council Washington, USA). The protocol for animal use was reviewed and approved by the Institutional Ethical Committee of the Institute of Tropical Medicine 'Pedro Kouri' (ID: CEI-IPK 14-12). Healthy animals were obtained from the National Centre of Laboratory Animals Production (CENPALAB), Havana, Cuba and maintained under standard conditions. LaA were isolated by aspiration with a needle and maintained as promastigote form [*Leishmania amazonensis* promastigotes (LaP)] in Schneider's medium containing 10% HFBS and antibiotics (100 μg streptomycin mL^{-1} and 100 U penicillin mL^{-1}) at 28 °C.

Leishmania tarentolae promastigotes (LtP) strain P10 from Jena Bioscience (Germany) were used. Parasites were cultivated at 26 °C in yeast extract medium (YEM) consisting of 20·7 g L^{-1} yeast extract powder, 1·2 g L^{-1} K_2HPO_4, 0·2 g L^{-1} KH_2PO_4, 2·9 g L^{-1} glucose or in BHI medium (37 g L^{-1}) supplemented each with 5 mg L^{-1} haemin

https://doi.org/10.1017/S0031182016002389 Published online by Cambridge University Press

and 50 000 U L^{-1} penicillin and 50 mg L^{-1} streptomycin in 50 mL Saarstedt tubes with gas-permeable caps and agitation in a tube shaker (0·05 s^{-1}).

Influence of Res and Xan on cell viability

To analyse the activity of Res and Xan in intracellular LaA, experiments were carried out to determine the viability of parasites after treatment. In a 24-well plate with cover slips, a monolayer of PMM was obtained. Afterwards, LaP in stationary-phase were added at a 4:1 parasite/macrophage ratio. The cultures were incubated for further 4 h and free parasites were removed. Then, 1995 μL of RPMI medium and 5 μL of compounds were added. Five 1:2 dilutions were carried out passing each time 1000 μL to the next well. The plate was incubated for 48 h and the experiment was carried out in triplicate (Torres-Santos *et al*. 1999). The parasites were then fixed with methanol, stained with Giemsa and examined using light microscopy at a magnification of 1000×, applying immersion oil. The number of intracellular amastigotes was determined by counting the amastigotes in 100 macrophages per sample. The results were expressed as percentage decrease in the infection rate compared with that of the controls. The infection rates were obtained by multiplying the percentage of infected macrophages by the mean number of amastigotes per infected macrophage (Torres-Santos *et al*. 1999; Delorenzi *et al*. 2001). In parallel, cytotoxicity of compounds was determined in PMM. Macrophages were harvested, distributed to 96-well plates ($1–6 \times 10^5$ PMM mL^{-1}) and incubated at 37 °C under an atmosphere of 5% CO_2 for 2 h. Non-adherent cells were removed and 2 μL of Res or Xan dissolved in DMSO were added to 98 μL of RPMI medium containing 10% HFBS and antibiotics (100 μg streptomycin mL^{-1} and 100 U penicillin mL^{-1}). Following, 1:2 serial dilutions were performed and the plate was incubated during 48 h at same conditions. Then, cytotoxicity was determined using the colorimetric assay based on MTT reduction. MTT solutions were prepared at 5 mg mL^{-1} in saline solution, sterile-filtered immediately before use, and 15 μL were added to each well. After incubation for additional 4 h, supernatant was eliminated and the accumulated formazan crystals were dissolved by addition of 100 μL DMSO per well. The optical density was determined using an EMS Reader MF Version 2.4–0 (Pharmacia, LKB, USA), at a wavelength of 560 nm and 630 nm as reference wavelength. The influence of the vehicle DMSO was negligible in used concentrations.

In addition, the viability of LaP was determined. Fifty microlitres of Schneider's medium with HFBS and antibiotics were distributed in each well of a 96-well plate. In the first one, additional 48 μL of medium were added and 2 μL of Res or Xan. Subsequently, five 1:2 dilutions were carried out. Afterwards, 50 μL of parasites at 2×10^5 promastigotes mL^{-1} in logarithmic phase were added to each well. Plates were sealed with parafilm and incubated at 26 °C during 72 h. Then, 20 μL of MTT was added to each well and cell viability was measured as described above.

In analogy, LtP in YEM/Phosphate-buffered saline (PBS) (1:1) were distributed in 96-well plates at 4×10^6 cells mL^{-1}. Compounds were added and five 1:3 serial dilutions were performed. Control rows with YEM/PBS (no activity) and with untreated (vehicle, DMSO) LtP (100% activity) were loaded. Then, 50 μL of resazurin stock solution was added in each well (20 μM final concentration) and the plate was incubated at 26 °C for 48 h. Finally, the absorption of resazurin was measured at 600 and 570 nm using a plate reader (Enspire, PerkinElmer, Waltham, MA, USA). From these data, the viability of cells in the absence and presence of drugs was calculated. In analogy to LtP resazurin assays, the J774 resazurin assays were performed with the exception that 8×10^4 cells mL^{-1} in DMEM were used. In all cases, Pen was used as reference drug and tested in parallel against LaA, LaP, LtP, J774 and PMM.

Influence of Res and Xan on oxygen consumption

To assay the effect of compounds on oxygen consumption of LtP, a Clark-type oxygen electrode (Hansatech, Germany) and software MCREC were used. LtP at 10^8 cells mL^{-1} in YEM (25 °C) were added and treated with increasing concentrations of test compounds between 10 and 200 μM. Each concentration was assayed in quadruplicates and the results were expressed as percentage of oxygen consumption in comparison with the untreated control LtP. The highest concentration of the vehicle (1% DMSO) caused only 2% inhibition.

OxoPlates are U-shaped 96-well plates (OP96U PreSens, Germany) with integrated fluorescence oxygen sensors. Oxygen concentrations were measured using a Perkin Elmer Enspire fluorescence plate reader using excitation wavelength 540 nm and two emission wavelengths (reference dye 590 nm, I_{Ref}, O_2-sensing dye 650 nm, I_{Ind}). The fluorescence intensity ratio $I_R = I_{Ind}/I_{Ref}$ was used in the following formula to calculate the oxygen concentration (μM O_2) according to the instructions of the manufacturer:

$$C_{O_2} = 2.48 \times 100 \times \frac{(k_0/I_R - 1)}{(k_0/k_{100} - 1)}.$$

OxoPlates were calibrated with air-saturated buffer ($I_R = k_{100}$) and sodium dithionite-treated buffer (1% w/v) ($I_R = k_0$). Measurements with LtP and J774 were performed in air-saturated PBS/YEM (1:1) and DMEM (without FCS), respectively. After calibration of the plates, they were loaded either with 200 μL medium (medium controls for drift corrections), 50 μL medium in wells for

https://doi.org/10.1017/S0031182016002389 Published online by Cambridge University Press

untreated control cells or 50 μL medium with test substances (Xan, Res, inhibitors/uncouplers of mitochondrial respiration) to wells for treated cells. Immediately before the measurement 150 μL of well-aerated cell suspensions were added to the respective wells to give final cell counts of 0·75–1 × 10^8 LtP mL^{-1} and 4–5 × 10^6 J774 cells mL^{-1}, respectively. Finally on the top of each well, 50 μL paraffin oil was layered. Two min after mixing the fluorescence measurements at 27 °C were started and eight measurements at 5 min intervals were performed. From the linear part of the O_2 decay the slopes were calculated and corrected for the medium drift for further statistic evaluation. All measurements were performed at least in triplicate. For inhibition studies the concentration ranges explored were: in LtP Xan, Res, Pen (0·41–100 μM) and in J774 cells Xan, Res (0·82–200 μM) and Pen (0·41–100 μM).

For bioenergetic characterization following inhibitor mixtures were used: 5 μM Oligo only, 5 μM Oligo + 100 μM DNP, 5 μM Oligo + 0·5 μM CCCP, 5 μM Oligo + 1 μM CCCP and 4 μM Rot + 4 μM AA. All concentrations given above are final concentrations in the OxoPlate wells. Derived bioenergetic parameters were calculated according to Brand and Nicholls (2011).

Influence of Res and Xan on mitochondrial complexes

Isolation of the mitochondrial fraction from LtP. To isolate the mitochondrial fraction from LtP (LtP-Mit), a culture of 2700 mL of LtP was centrifuged for 10 min at 1900 × ***g*** and 20 °C and the supernatant was discarded. The cell pellet was resuspended in buffer (10 mM TRIS–HCl, 0·3 M sucrose, 0·2 mM EDTA, 0·2% BSA, pH 7·4) and washed in two extra centrifugation steps for 10 min at 1900 × ***g*** and 20 °C. The washed cell pellet was incubated in lysis buffer (5 mM TRIS–HCl, pH 7·4) for 10 min at 20 °C and subsequently homogenized in a Dounce homogenizer. Cell debris was removed by centrifugation for 10 min at 1900 × ***g*** and 4 °C. The supernatant was centrifuged for 20 min at 13 200 × ***g*** and 4 °C to sediment the crude mitochondrial fraction. LtP-Mit were resuspended in 1 mL buffer (250 mM sucrose, 50 mM KH_2PO_4, 0·2 mM EDTA, pH 7·2) and stored in liquid nitrogen until use. Protein content was assayed by the Biuret method using BSA as standard (Gornall *et al*. 1949).

Isolation of mitochondrial fractions from other species. Bovine heart submitochondrial particles (BH-SMP) were obtained from bovine heart mitochondrial suspensions (Nohl and Hegner, 1978). Mitochondrial suspensions were sonicated ten times for 30 s with 1 min interruption at 4 °C in buffer (0·25 M sucrose, 10 mM TRIS, 1 mM EDTA, pH 9·0) using a Branson Sonifier (USA) set to 40 W energy output. Remaining mitochondria were removed by centrifugation at 6500 × ***g*** for 10 min at 4 °C and afterwards BH-SMP were sedimented from the supernatant by centrifugation at 95 000 × ***g*** for 30 min. Pellets were resuspended in buffer (0·25 M sucrose, 10 mM TRIS, 1 mM EDTA, pH 7·4) and stored in liquid nitrogen until use.

The preparation of the yeast mitochondria (YM) was performed according to the method of Daum *et al*. (1982). Yeast cells (W303 strain) were harvested by centrifugation at 1464 × ***g*** for 5 min at room temperature. The pellets were resuspended in 100 mL buffer I (10 mM TRIS, 10 mM DTT, pH 9·4) and transferred to centrifugation tubes. After 15 min incubation at 37 °C, cells were centrifuged again. Subsequently centrifugation tubes were tared and filled with cells resuspended in 60 mL buffer II (1·2 mM sorbitol, 20 mM potassium phosphate, pH 7·4). After a third centrifugation, the weight of the cell pellets was determined. To prepare spheroblasts the pellets were suspended in buffer II and 2 mg zymolyase were added per gram yeast cells. Then the suspension was incubated for 45 min at 28 °C. The following steps were performed at 4 °C. Spheroblasts were collected by centrifugation at 1464 × ***g*** for 5 min, resuspended in 60 mL buffer II, sedimented by another centrifugation and homogenized in as little buffer III (0·6 M sorbitol, 20 mM TRIS, pH 7·4) as possible. Then the suspension was homogenized by 20 strokes in a Wheaton 15 mL Dounce tissue grinder (tight-fitting pestle). Cells and cell debris were removed by two centrifugations at 1464 × ***g*** for 5 min. YM were collected from the supernatant by centrifugation at 11 952 × ***g*** for 10 min. Mitochondrial fractions from rat liver (RLM) of Sprague-Dawley rats were previously isolated by consecutive differential centrifugation steps as described in Staniek *et al*. (2005) and stored in liquid nitrogen.

Inhibition of mitochondrial electron transfer activities. Succinate:ubiquinone oxidoreductase (complex II) activities were measured using DCPIP as terminal electron acceptor. In a 1 mL cuvette buffer (250 mM sucrose, 20 mM TEA, 1 mM EDTA, pH 7·4), DCPIP (20 μM), KCN (1 mM), BSA (1·25 mg mL^{-1}), IQ (62·5 μM) and BH-SMP (0·159 mg mL^{-1}) or RLM (0·318 mg mL^{-1}) or YM (W303 strain, 0·0292 mg mL^{-1}) or LtP-Mit (0·08 mg protein mL^{-1}) were added. The reaction was started by adding succinate (4 mM). The reduction of DCPIP was followed photometrically at 600 nm using 780 nm as a reference wavelength with a Shimadzu Multispec 1501 diode array photometer in the presence of test compounds or the corresponding amount of vehicle (DMSO). DCPIP reduction rates were calculated using an extinction coefficient of 19 100 L mol^{-1} cm^{-1} at 600 nm. For each individual concentration triplicates were measured.

To measure the inhibition of ubiquinol:cytochrome c oxidoreductase (complex III) $dUQH_2$

https://doi.org/10.1017/S0031182016002389 Published online by Cambridge University Press

(75 μM) and cyt c^{3+} (100 μM) were used as substrates. The reduction of cyt c^{3+} in the presence of $dUQH_2$ was monitored at 550 nm using 540 nm as reference wavelength in a buffer consisting of 250 mM sucrose, 50 mM KH_2PO_4, 0·2 mM EDTA, 2 mM KCN, 4 mM NaN_3, pH 7·2 (Müllebner *et al*. 2010). Test compounds were added at different concentrations 120 s prior to addition of $dUQH_2$ starting the enzymatic reaction. In the presence of test compounds, the residual $dUQH_2$:cyt c^{3+} oxidoreductase activity of BH-SMP (0·0032 mg mL^{-1}), RLM (0·0425 mg mL^{-1}), YM (0·0291 mg mL^{-1}) and LtP-Mit (0·04 mg mL^{-1}) was obtained and expressed in percentage of the non-inhibited rates (100%). For all test compounds, the absence of significant reactions with $dUQH_2$ and cyt c^{3+} was verified in the absence of mitochondrial preparations. All compound concentrations were tested in triplicate and the reduction rates for cyt c^{3+} were calculated from the time trace of the absorption difference at 550 nm minus 540 nm using the extinction coefficient $\varepsilon_{550-540\,nm}$ = 19 000 L mol^{-1} cm^{-1}.

Influence of Res and Xan on superoxide radical production in LtP

Detection of superoxide radicals was performed using the cyclic hydroxyl amine CMH as reaction partner and measuring the formed stable nitroxyl radicals by electron spin resonance (ESR) spectroscopy. Measurements were performed in PBS buffer (136 mM NaCl, 1·15 mM KH_2PO_4, 14 mM Na_2HPO_4, 2·7 mM KCl, pH 7·4) containing 100 μM DFO and 25 μM DTPA. Before measurements LtP suspensions were washed twice with PBS to remove YEM. Samples contained 5×10^8 LtP mL^{-1} and were supplemented with 16 mM glucose. Simultaneously drug stock solutions in DMSO were added. Prior to the ESR measurement 400 μM CMH was added. For ESR measurements 17 μL of suspension was aspirated in a gas-permeable Teflon tube (ø 0·7 mm). This capillary tube was placed in a resonator (split ring, Bruker MD5) of the ESR instrument (Bruker EMX) and ten sequential measurements were performed. Following instrument settings were used: microwave frequency 9·682 GHz, modulation frequency 100 kHz, modulation amplitude 1 G, time constant 0·082 s, centre field 3446 G, scan rate 71 G min^{-1}, sweep width 100 G, scan time 84 s and attenuation $7{\cdot}96 \times 10^3$. From the ESR spectra, the middle peak intensity was retrieved and concentrations of oxidized CMH were obtained by comparison with a standard curve prepared from $CP^{\bullet}$ solutions with defined concentrations. In an additional experiment, the influence of Res (100 μM) and Xan (100 μM) on the $CP^{\bullet}$ (3 μM) signal was studied in PBS (pH 7·4) containing 100 μM DFO and 25 μM DTPA using same conditions as for CMH.

Influence of Res and Xan on mitochondrial membrane potential in LtP

JC-1 was used to investigate the changes in mitochondrial membrane potential of LtP. From a stock solution of JC-1 (6·1 mM) in DMSO a diluted solution (15 μM JC-1) in PBS (pH 7·4) and 15 mM glucose was prepared. The cell density of LtP was adjusted to approximately 5×10^7 LtP cells mL^{-1}. LtP cells cultured in YEM were centrifuged in a sterile 50 mL tube for 10 min at 2000 × ***g*** at 20–25 °C. After the centrifugation, the supernatant was discarded and the cell pellet was resuspended in a corresponding volume of the diluted JC-1 solution. To load cells with JC-1 they had to be incubated for 30 min under dark conditions in the incubator (26 °C). Then, a washing step with 10 min centrifugation at 2000 × ***g*** at 20–25 °C was included. The supernatant was removed and the pellet was resuspended in a PBS/glucose solution (without JC-1). Subsequently, 200 μL cell suspension loaded with JC-1 were placed in each well of a black 96-well culture plate. In the third row, 100 μL extra suspension were added and in the respective wells Res (200 μM), Xan (200 μM), Val (10 μM), CCCP (0·5 μM), oligomycin (Oligo, 10 μM) and AA (0·1 μM) were placed. Fluorescence measurements (Perkin Elmer Enspire) were started after 4 h of incubation using an excitation wavelength of 485 nm and an emission wavelength of 590 nm for JC-1 aggregates. The fluorescence intensity of control cells (including DMSO) was set to 100% and fluorescence intensities of other samples were expressed in relation to this value.

Statistical analyses

Percentages of residual activity in relation to control experiments (100%) were calculated at each concentration of compounds. Resulting activity-concentration plots were used to determine the half inhibitory concentration (IC_{50}) values by a non-linear regression according to a four-parameter logistic model (4PL, Hill-Slope model) (Müllebner *et al*. 2010) using Origin 6·1 (OriginLab Corporation). In case of PMM, LaP and LaA, the IC_{50} value was obtained by fitting a sigmoidal E_{max} model to dose–response curves (Bodley and Shapiro, 1995). Selectivity indices were calculated by dividing the IC_{50} values for PMM by the IC_{50} values for LaA. Statistically significant differences were identified using Student's *t*-test.

RESULTS

In this work, two natural products which are also present in certain beverages were compared for their effects on *Leishmania* parasites and their mitochondrial functions. Both, Res and Xan, are polyphenols but nevertheless structurally different

https://doi.org/10.1017/S0031182016002389 Published online by Cambridge University Press

Table 1. Influence of pentamidine isethionate (Pen, reference drug), resveratrol (Res) and xanthohumol (Xan) on the viability of *L. amazonensis* amastigotes (LaA) and peritoneal macrophages from mouse (PMM)

Compound	LaA $IC_{50} \pm$ S.D. (μM)	PMM $IC_{50} \pm$ S.D. (μM)	Selectivity index
Res	13·9 ± 1·7	>438	>31
Xan	6·9 ± 0·3	69·5 ± 9·8	10
Pen	3·4 ± 0·6	34·9 ± 2·8	9

IC_{50} values for LaA were obtained from a direct count in stained cultures by Giemsa using serial dilutions of the compounds. Cytotoxicity in PMM was determined by an MTT assay. Both experiments had an endpoint at 48 h. Data represent means ± S.D. from three independent cell batches.

Table 2. Influence of pentamidine isethionate (Pen, reference drug), resveratrol (Res) and xanthohumol (Xan) on the viability of *L. amazonensis* promastigotes (LaP), *L. tarentolae* promastigotes (LtP) and J774 macrophages

Compound	LaP $IC_{50} \pm$ S.D. (μM)	LtP $IC_{50} \pm$ S.D. (μM)	J774 $IC_{50} \pm$ S.D. (μM)
Res	97·9 ± 9·9	79·8 ± 2·7	162·0 ± 5·8
Xan	47·2 ± 5·0	0·78 ± 0·76	60·8 ± 8·9
Pen	1·21 ± 0·17	0·98 ± 0·02	23·8 ± 4·1

IC_{50} values for LaP were obtained from an MTT assay using serial dilutions of the compounds with an endpoint at 72 h using 10^5 cells mL^{-1}. IC_{50} values for LtP and J774 were obtained from a resazurin viability assay with an endpoint at 48 h using 4×10^6 cells mL^{-1} (LtP) or 1×10^5 cells mL^{-1} (J774). Data represent means ± S.D. from three independent cell batches.

(Fig. 1). While Res can be classified as stilbene derivative, Xan is a chalcone substituted by an isoprenyl residue. For the lipophilicity of Res and Xan, the $logP_{OW}$ values were predicted to 3·1 ± 0·8 and 5·0 ± 1·5, respectively. The water solubility (in cell free systems) of Res was calculated to be 15·8 ± 4·6 μM and that of Xan to be 3·9 ± 1·0 μM (ALogPS 3·0, Ochem Predictor) (Sushko *et al*. 2011).

In a first set of experiments, Res and Xan were tested for their antileishmanial activity. The pathologically relevant model systems consisted of PMM and pathogenic LaA. In the viability assay, the anti- leishmanial activity of Xan against amastigotes was confirmed with an IC_{50} value in the low micromolar range (Table 1), while Res required about 2-fold higher concentrations for amastigote killing. With respect to PMM viability Res was found to be less cytotoxic than Xan. In addition, the antileishmanial activity was tested in LaP and LtP (model for mechanistic studies). In both LaP and LtP (Table 2) Res showed the highest IC_{50} values, while Pen as reference compound was effective at 1 μM in both leishmanial species. Xan was more effective than Res in all systems, however, with different IC_{50} values. For mechanistic studies in mammalian cells the macrophage cell line J774A.1 was used. The IC_{50} values of J774 cells in the resazurin viability assay (Table 2) corresponded to the IC_{50} values of PMM (Table 1). Specifically an excellent agreement for Xan and Pen was observed, while the IC_{50} value of Res was lower than in PMM.

Since mitochondrial genome homology between different *Leishmania* species is high (Yatawara *et al*. 2008) we used the LtP model to evaluate the role of mitochondria in the leishmanicidal activity of Res and Xan.

For an overview, the influence of Res and Xan on LtP oxygen consumption using a Clark electrode was studied (Fig. 2). Neither Res nor the reference antileishmanial agent Pen inhibited LtP oxygen consumption strongly up to 200 μM. In contrast, Xan exhibited an IC_{50} value of 16 μM and therefore a strong inhibition of mitochondrial oxygen consumption in *Leishmania*. To extend these experiments oxygen consumption measurements were performed in the 96-well format using Oxoplates (Table 3). Again, in LtP Res and Pen did not show strong inhibition, while Xan inhibited oxygen consumption with an IC_{50} of about 7 μM. This confirms the measurement with the Clark electrode for LtP. Likewise, in J774 macrophages no strong inhibition of oxygen consumption by Res and Xan was observed which fits to the viability results in PMM and J774 cells (Tables 1 and 2).

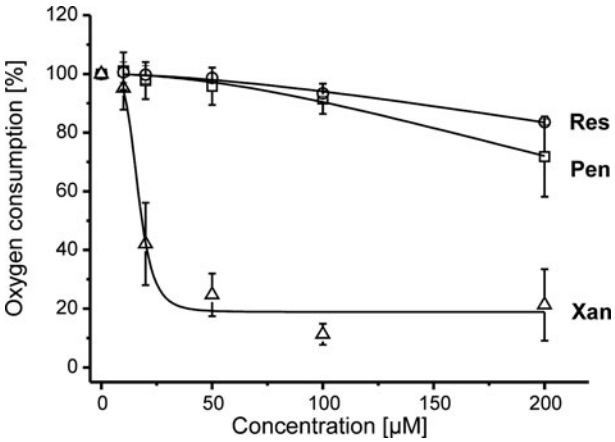

Fig. 2. Oxygen consumption of *L. tarentolae* promastigotes in yeast extract medium and its inhibition by resveratrol (Res), xanthohumol (Xan) and pentamidine (Pen). Xan exhibited an IC_{50} value of 16 ± 1 μM, while Res and Pen showed 16 and 28% inhibition at 200 μM. Non-inhibited oxygen consumption of cells was 9·3 nmol O min^{-1} $(10^8$ cells$)^{-1}$, which was set to 100%. Data represent mean ± S.E. from four independent experiments.

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Table 3. Influence of pentamidine isethionate (Pen, reference drug), resveratrol (Res) and xanthohumol (Xan) on the oxygen consumption of *L. tarentolae* promastigotes (LtP) and J774 macrophages

	Oxygen consumption	
Compound	LtP $IC_{50} \pm$ S.D. (μM)	J774 $IC_{50} \pm$ S.D. (μM)
Res	>100	>150
Xan	6·74 ± 0·27	>150
Pen	>100	38·7 ± 2·6

IC_{50} values for LtP and J774 were measured in 96-well Oxoplates using 1×10^8 and 8×10^4 cells mL^{-1}, respectively. Data represent means ± S.D. from three independent cell batches.

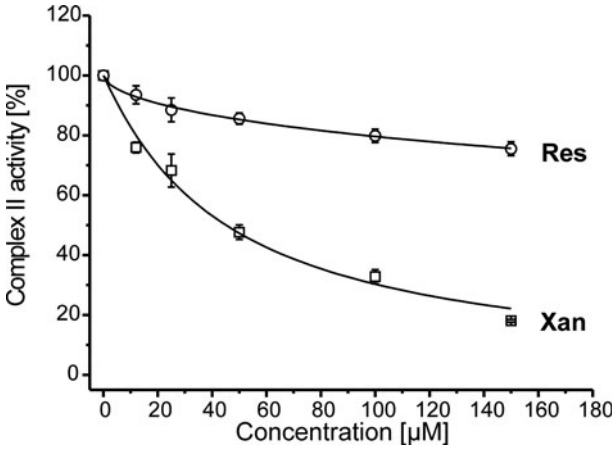

Fig. 3. Complex II activity of *L. tarentolae* promastigote mitochondria and its inhibition by resveratrol (Res) and xanthohumol (Xan). Xan exhibited an IC_{50} value of 45·1 ± 3·3 μM. Res showed an inhibition of 25% at 150 μM. Non-inhibited complex II activity of mitochondria (0·08 mg protein mL^{-1}) was 6·95 nmol min^{-1} mL^{-1}, which was set to 100%. Data represent mean ± S.D. from three independent experiments.

Table 4. Inhibition of complex II activities in mitochondrial fractions from different species by resveratrol (Res) and xanthohumol (Xan)

	Complex II activity	
Mitochondrial Fraction	Res inhibition at 50 μM (%)	Xan $IC_{50} \pm$ S.D. (μM)
BH-SMP	11 ± 6	10·4 ± 0·6
RLM	11 ± 6	55·6 ± 3·2
YM	6 ± 6	101·8 ± 5·5

Data represent means ± S.D. from three independent experiments. Protein concentrations were: submitochondrial particles from bovine heart (BH-SMP) 0·159 mg mL^{-1}, rat liver mitochondria (RLM), 0·318 mg mL^{-1}, yeast mitochondria, W303 strain (YM) 0·0292 mg mL^{-1}. Non-inhibited activities of mitochondrial fractions were: BH-SMP 4·5 nmol min^{-1} mL^{-1}, RLM 16·4 nmol min^{-1} mL^{-1} and YM 2·98 nmol min^{-1} mL^{-1}.

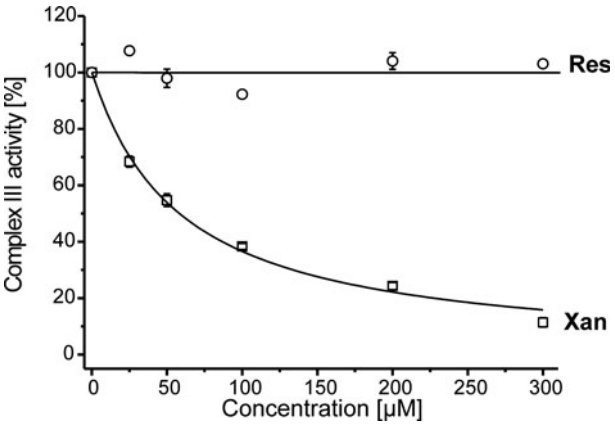

Fig. 4. Complex III activity of *L. tarentolae* promastigote mitochondria and its inhibition by resveratrol (Res) and xanthohumol (Xan). Xan exhibited an IC_{50} value of 58·2 ± 3·7 μM. Res showed no inhibition at 300 μM. Non-inhibited complex III activity of mitochondria (0·04 mg protein mL^{-1}) was 6·31 nmol min^{-1} mL^{-1}, which was set to 100%. Data represent mean ± S.D. from three independent experiments.

Next experiments addressed the question of the specific target of Xan in the electron transfer chain (ETC) of *Leishmania*. Therefore, we studied the influence of both compounds on the succinate:ubiquinone oxidoreductase (complex II) activity in mitochondria from LtP (Fig. 3). In agreement with results from oxygen consumption in LtP, we observed a strong inhibition with Xan, but only small effects with Res. Comparing the complex II inhibition in LtP-Mit with that in mitochondria from other species (Table 4) the inactivity of Res is observed in all mitochondria, while Xan inhibited mitochondria from mammalian cells in a similar range (RLM) or even stronger (BH-SMP). Only in YM Xan was less effective.

In analogy, the influence of both compounds on the ubiquinol:cytochrome c oxidoreductase (complex III) activity was tested. In these experiments, the difference between Xan and Res was even more pronounced (Fig. 4). While Res had almost no effect, Xan showed an IC_{50} value in the micromolar range. For both, complex II and III activities, the IC_{50} values of Xan with 45·1 ± 3·3 μM and 58·2 ± 3·7 μM, respectively, were rather similar. Comparison of complex III inhibition in LtP mitochondria to that in mitochondria from other species (Table 5), shows that also in these species Res had no strong effect, while Xan inhibited the complex III activity even stronger.

The influence of Res and Xan on mitochondrial superoxide radical formation was tested by ESR experiments with LtP cells using the conversion of CMH to 3-methoxycarbonyl-proxyl (CM$^{\bullet}$) for

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Table 5. Inhibition of complex III activities in mitochondrial fractions from different species by resveratrol (Res) and xanthohumol (Xan)

Mitochondrial Fraction	Complex III activity	
	Res inhibition at 25 μM (%)	Xan $IC_{50} \pm$ S.D. (μM)
BH-SMP	8 ± 3	7·10 ± 0·69
RLM	4 ± 2	8·68 ± 0·75
YM	4 ± 2	3·32 ± 0·19

Data represent means ± S.D. from three independent experiments. Protein concentrations were: submitochondrial particles from bovine heart (BH-SMP) 0·0032 mg mL^{-1}, rat liver mitochondria (RLM) 0·0425 mg mL^{-1}, yeast mitochondria, W303 strain (YM) 0·0291 mg mL^{-1}. Non-inhibited activities of mitochondrial fractions were: BH-SMP 15·7 nmol min^{-1} mL^{-1}, RLM 11·2 nmol min^{-1} mL^{-1} and YM 25·2 nmol min^{-1} mL^{-1}.

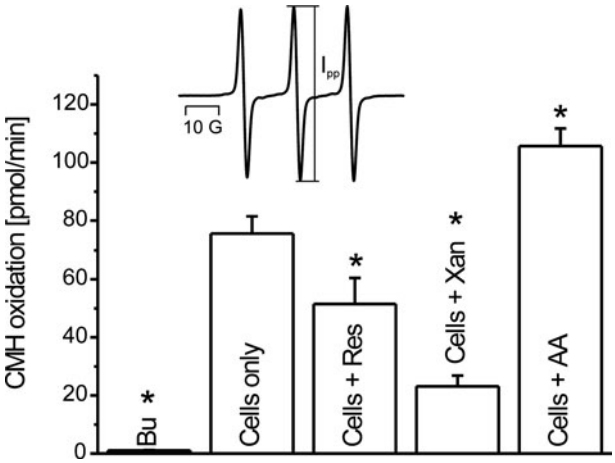

Fig. 5. CMH oxidation by *L. tarentolae* promastigotes (5×10^8 cells mL^{-1}, 17 μL) in the presence of resveratrol (Res, 100 μM), xanthohumol (Xan, 100 μM) and AA (5 μM) was measured by ESR spectroscopy in oxygen-permeable Teflon tubes. 'Cells only' samples contained the corresponding amount of vehicle (max. 2% DMSO) used for xenobiotics. Buffer (Bu) samples containing vehicle were prepared without cells. Relative changes of CMH oxidation in comparison with 'Cells only' samples (100%) were: Res (72 ± 5%), Xan (32 ± 2%) and AA (145 ± 27%). Data represent mean ± S.D. from quadruplicates and * indicates statistically significant differences on the level $P < 0{\cdot}05$ with respect to 'Cells only' samples.

superoxide radical detection (Fig. 5). While the negative (Bu, buffer) and the positive controls (+AA) yielded reasonable values in comparison with cells alone, Res and even stronger Xan diminished CMH oxidation in the presence of cells. Control experiments revealed that 100 μM Res or Xan did not directly diminish the slope of the ESR signal change of $CP^{\bullet}$ (an analogue of $CM^{\bullet}$, which is available as pure substance) over the measurement time for CMH-mediated superoxide radical detection.

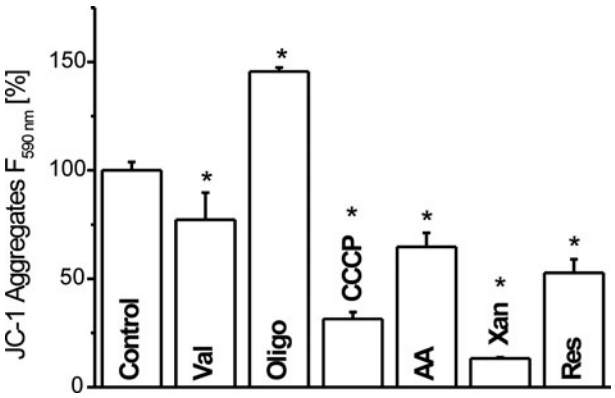

Fig. 6. Mitochondrial membrane potential in *L. tarentolae* promastigotes. 5×10^7 cells mL^{-1} were suspended in the presence of resveratrol (Res, 200 μM), xanthohumol (Xan, 200 μM), valinomycin (Val, 10 μM), carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 0·5 μM), oligomycin (Oligo, 10 μM) and antimycin A (AA, 0·1 μM). Aggregates of JC-1 (15 μM) were measured by fluorescence ($\lambda_{Ex} = 485$ nm, $\lambda_{Em} = 590$ nm). The fluorescence of the control cells was set to 100%. Data represent mean ± S.D. from quadruplicates and * indicates statistically significant differences on the level $P < 0{\cdot}05$ with respect to control.

A major mitochondrial function is the production of ATP, which requires the mitochondrial membrane potential to drive ATP synthase. Therefore, the influence of Xan and Res on the mitochondrial membrane potential was measured using the JC-1 method (Fig. 6). As expected the ionophore Val, the mitochondrial uncoupler CCCP and the complex III inhibitor AA strongly decreased the amount of JC-1 aggregates. In contrast, Oligo, an inhibitor of ATP synthesis, increased the concentration of JC-1 aggregates. Incubation with Res and Xan for 4 h resulted in a significant decline of the aggregates and therefore of the mitochondrial membrane potential.

To extend the understanding why Xan inhibits isolated mitochondria both in mammalian cells and *Leishmania* but nevertheless shows some selectivity for LtP in whole cell models, we compared the bioenergetic properties of mitochondria in LtP and J774 cells (Fig. 7, Table 6). Basal oxygen consumption was assessed in LtP and J774 cells within glucose-containing media in the absence of inhibitors/uncouplers. After addition of a mixture of potent mitochondrial inhibitors (AA and Rot) remaining oxygen consumption can be only of extra-mitochondrial origin. Surprisingly LtP exhibit virtually no extra-mitochondrial oxygen consumption. In contrast, in J774 cells about 30% of basal oxygen consumption corresponds to extra-mitochondrial respiration (possibly arising from NADPH oxidases). In the presence of Oligo, an inhibitor of the ATP synthase, oxygen consumption utilized for ATP synthesis was blocked. In J774 cells, about 60% of mitochondrial respiration is

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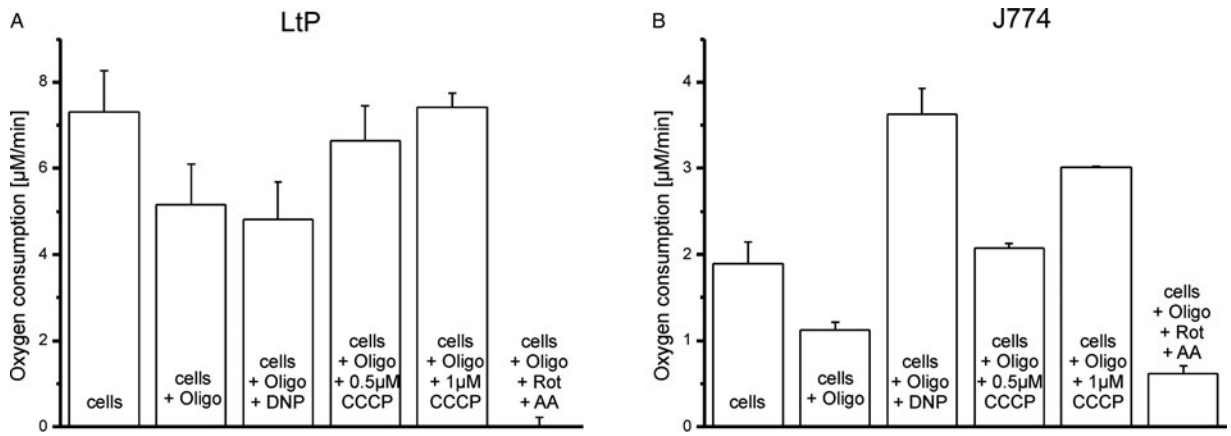

Fig. 7. Oxygen consumption of *L. tarentolae* promastigotes (LtP) and J774 macrophages in the absence and presence of inhibitors for bioenergetic characterization. Measurements were performed in Oxoplates with 1×10^8 cells mL^{-1} for LtP (A) or with 8×10^4 cells mL^{-1} for J774 (B). Basal oxygen consumption was assessed in the absence of inhibitors/uncouplers (cells). The proton leak rate of mitochondrial membranes was determined in the presence of the ATP synthase inhibitor oligomycin (Oligo, 5 μM). To obtain the maximally uncoupled respiration different Oligo/uncoupler mixtures were applied (5 μM Oligo + 100 μM DNP, 5 μM Oligo + 0·5 μM CCCP, 5 μM Oligo + 1 μM CCCP). Non-mitochondrial oxygen consumption was measured in the presence of 4 μM rotenone (Rot) + 4 μM antimycin A (AA). All measurements were performed in quadruplicates and data represent mean ± S.D.

Table 6. Bioenergetic parameters of *L. tarentolae* promastigotes (LtP) and J774 macrophages derived from experiments shown in Fig. 7 using the highest uncoupler-stimulated oxygen consumption rates (LtP: at 1 μM CCCP, J774: at 100 μM DNP) for calculation

Bioenergetic parameters	LtP	J774
Non-mitochondrial respiration (% of basal O_2 consumption)	0	33
Mitochondrial respiration (% of basal O_2 consumption)	100	67
Proton leak (% of mitochondrial respiration)	70	40
ATP turnover (% of mitochondrial respiration)	30	60
Maximum mitochondrial respiration (% of mitochondrial respiration)	101	236
Spare respiratory capacity (% of mitochondrial respiration)	+1	+136
Coupling efficiency	0·3	0·6
Respiratory control ratio	1·4	5·9

used for ATP production (ATP turnover). In LtP, this ATP turnover amounts only to about 30% of mitochondrial respiration. In the presence of the uncoupler DNP or CCCP, the maximal mitochondrial respiration was determined. In J774 cells, this uncoupler-accelerated respiration is more than 200% of mitochondrial respiration, while in LtP maximal uncoupled mitochondrial respiration is barely above normal mitochondrial respiration. The coupling efficiency (LtP: 0·3 *vs* J774: 0·6) and respiratory control ratios (LtP: 1·4 *vs* J774: 5·9) clearly indicate that LtP has a low efficiency in ATP production and cannot increase mitochondrial electron transfer under stress conditions as deduced from the lacking spare capacity (maximum uncoupler-stimulated mitochondrial respiration minus coupled mitochondrial respiration). This is a possible explanation why a comparable complex II and III inhibition by Xan in isolated mitochondria results in more severe consequences in LtP than in J774 cells.

DISCUSSION

Xan and Res are significant bioactive ingredients of widely consumed beverages. When ingested in moderate amounts (Liu *et al*. 2008) their polyphenol ingredients are considered to have beneficial effects on the cardiovascular and other organ systems (Magalhaes *et al*. 2009; Nabavi *et al*. 2014; Yang *et al*. 2014; Yao *et al*. 2015). Due to these findings beers with high Xan content were identified or even specifically produced (Wunderlich *et al*. 2005; Walker *et al*. 2008). Likewise methods for increasing the Res content of red wines were proposed (Sun *et al*. 2015). In parallel, the intake of food supplements of Res and Xan is on the rise. These compounds may also have a beneficial effect in the treatment of infectious protozoal diseases. Due to the climate change, endemic regions for leishmaniasis tend to spread globally. The fast growing beverage market in the affected areas with endemic leishmaniasis, such as in Brazil, generates an

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increased intake of Xan via beer consumption (de Araujo *et al*. 2013). Although there were a few studies on the *in vitro* antiprotozoal activity of Res and Xan derivatives, so far there was no study, which directly compared the activity of both compounds in the same model using the same methods. Therefore, we set up this study to compare the qualitative and quantitative effects of Res and Xan on *Leishmania* and their mitochondria as a major target.

Res was shown to be active against *L. amazonensis* parasites (Ferreira *et al*. 2014). However, another study demonstrated that the antiamastigote effect of Res against *L. major* inside macrophages is more related to host cell toxicity than to direct amastigote killing (Lucas and Kolodziej, 2013). Particularly, this polyphenol did not only target *Leishmania*, but also caused inhibition of arginase in macrophages, which provides ornithine for polyamine synthesis in *Leishmania* (Ferreira *et al*. 2014).

For Xan, antiplasmodial activity in the low micromolar range was described (Gerhäuser, 2005). Although the exact mechanism is still not clear, it was suggested that Xan could inhibit cysteine protease activity in plasmodia (Liu *et al*. 2001*b*). Alternatively, the interference of Xan derivatives in the GSH-catalysed haemin degradation, an important haeme detoxification pathway in plasmodia, was proposed (Frölich *et al*. 2005).

Viability tests for Xan and Res in pathogenic and non-pathogenic *Leishmania* in their promastigote and amastigote forms give evidence for an antileishmanial activity. Although IC_{50} values were different in the different *Leishmania* forms and species the ranking of the compounds was similar in LaA (Table 1), LtP and LaP (Table 2). In all cases, Xan was more effective as Res, while Pen as the reference drug was highly active in all *Leishmania*. Taking into account that Pen is a drug and Xan is a nutritional compound, this was rather unexpected. Xan shows a slightly higher toxicity against PMM (Table 1) and J774 cells (Table 2) than Res. Based on the high selectivity, both Xan and Res, possessing selectivity indices of 10 and 31, respectively, have potential as antileishmanial ingredients although higher concentrations compared to clinically used drugs may be required.

Major efforts in the present work were taken to identify mitochondrial effects of Res and Xan in *Leishmania* and mammalian cells. The single mitochondrion in *Leishmania* is an attractive target since it is essential for parasite survival. Many antileishmanial drugs were shown to affect this organelle (Fidalgo and Gille, 2011), which has several unique properties, partly different from mammalian mitochondria (Monzote and Gille, 2010). Furthermore, inhibition of mitochondria in *Leishmania* was shown to promote apoptotic cell death in these parasites (Mehta and Shaha, 2004). Clarification of the question whether a compound has a direct protein target is a prerequisite of structure-based drug modification as performed for other mitochondrial targets by *in silico* methods (Müllebner *et al*. 2010). Previously, we have shown that ascaridole has an effect on the mitochondrial membrane potential without strongly inhibiting the ETC as primary event (Monzote *et al*. 2014). For Res and Xan this has not yet been elucidated for *Leishmania*.

Res and other polyphenols were shown to inhibit the F_1-ATPase activity in rat liver mitochondria (Zheng and Ramirez, 1999). Furthermore, in carcinoma cells Res was shown to trigger apoptosis both via JAK/STAT pathway and the intrinsic pathway affecting mitochondria and resulting in cytochrome *c* release, an increase of Bax and a decrease of Bcl-2 (Madan *et al*. 2008). Additionally, in human cancer cell lines, a transient increase of superoxide radical production by mitochondria was triggered in low micromolar concentrations of Xan as deduced from the increased oxidation of dihydroethidium (Strathmann *et al*. 2010). These authors also studied the inhibition of ETC complexes by Xan in BH-SMP and observed significant inhibition of complex I–III only at concentrations >100 μM, while complex IV was not affected (Strathmann *et al*. 2010).

Effects of Xan on mammalian mitochondrial complex I in cancer cells (Zhang *et al*. 2015) could differ from those in *Leishmania* mitochondria due to strong structural differences of complex I in these organisms. In mammalian cell types, the increased reactive oxygen species formation was accompanied by a rapid decline of the mitochondrial membrane potential [measured by DiOC6(3) or JC-1] (Yang *et al*. 2007; Strathmann *et al*. 2010).

In this work for mechanistic studies on mitochondrial involvement in Xan actions LtP were used because *L. tarentolae* were successfully validated as non-pathogenic drug-screening model in the past (Taylor *et al*. 2010). A high-sequence homology between mitochondrial genomes of *Leishmania* exists (Yatawara *et al*. 2008) suggesting similar functions and drug sensitivity. In addition, LtP have the advantage that they can be cultivated in high cell densities and analytical techniques outside class II laboratories can be used.

Assaying individual complex II (Fig. 3) and III (Fig. 4) activities in LtP-Mit gives strong evidence for inhibition of complex II/III by Xan but not by Res. Whether this inhibition is based on covalent modification by the reaction with thiol groups of complex proteins [as shown for mammalian enzymes (Brodziak-Jarosz *et al*. 2016)] remains to be elucidated. Complex I activity assays in LtP-Mit using NADH as donor and DCPIP as acceptor resulted largely in a Rot-insensitive NADH consumption (possibly not linked to the respiratory chain) and was therefore not included in our study. Surprisingly, we observed also significant inhibition of complex II and III activities in mammalian

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mitochondria (Tables 4 and 5). These findings are qualitatively similar to findings of Strathmann *et al*. (Strathmann *et al*. 2010), but we observed for complex III in BH-SMP and RLM IC_{50} values in the low micromolar range (Table 5). A possible explanation for this discrepancy are different assay conditions, since absolute (not relative) IC_{50} values can strongly depend on the artificial substrate used for measurement of complex II/III activity. This raises the question why Xan in spite of inhibition of complex II/III in leishmanial (Figs 3 and 4) and mammalian mitochondria (Tables 4 and 5) shows higher selectivity for *Leishmania* in viability assays (Tables 1 and 2).

To assess this question, inhibition of respiration by Xan and Res in whole cells (LtP, J774) was studied. Oxygen consumption in LtP-Mit was not strongly stimulated by typical complex I substrates (data not shown) and is, therefore, unlikely to contribute to the activity in whole LtP (Fig. 2). Furthermore, it is known that the complex I equivalent in protozoa is different from mammalian complex I with respect to structure, inhibitor sensitivity and function (Chen *et al*. 2001; Vaidya, 2004). Measurement of oxygen consumption by glucose-consuming LtP gives a clear evidence that Xan [Clark electrode (Fig. 2): $IC_{50} = 16 \pm 1\ \mu$M, OxoPlate (Table 3): $IC_{50} = 6{\cdot}74 \pm 0{\cdot}27\ \mu$M], but neither Res nor Pen inhibited ETC in *Leishmania*. Unexpectedly for the respiration in J774 macrophages the IC_{50} values for Xan and Res were above 150 μM (Table 3). Only Pen inhibited respiration in J774 cells with an IC_{50} value of about 40 μM. The discrepancy between inhibition of mammalian complex II/III by Xan, but no strong effect on respiration of J774 cells may have several possible reasons: (i) Maximal rates of individual complex activities determined by artificial substrate concentrations exceeding Michaelis constant often exceed the respiration rates of the whole respiratory chain. (ii) Inhibition of individual complex activities measured by these assays may or may not result in inhibition of oxygen consumption depending on the flux control coefficient of the respective mitochondrial complex (Lenaz and Genova, 2007), which in turn is different in different cell types and between mitochondria from different species (Rossignol *et al*. 1999). (iii) At certain concentrations of test compounds possible uncoupling may reverse decreased oxygen consumption. Therefore, inhibition of oxygen consumption can be explained by inhibition of individual complex activities, but not necessarily the other way round.

To study the bioenergetic performance of LtP and J774 cells several cell respiratory control experiments were performed (Brand and Nicholls, 2011) (Fig. 7, Table 6). The major differences between both cell types are lower coupling efficiency, lower respiratory control ratios and the absence of any spare respiratory capacity in LtP in contrast to J774 macrophages. This gives a possible explanation why inhibition of complex II/III in LtP has more severe consequences than in J774 macrophages.

For many proapoptotic drugs the triggering of mitochondrial superoxide radical production is an essential step. Therefore, we determined the superoxide radical production by the ESR/CMH method (Dikalov *et al*. 2011) in LtP cells in the presence of xenobiotics. Although the positive control with AA (a complex III inhibitor) triggered additional superoxide radical production, neither Xan nor Res caused higher CMH oxidation rates. In contrast, for both substances and especially for Xan a decrease was observed (Fig. 5) indicating that Xan and Res can scavenge superoxide radicals. Indeed, Res was shown in a previous study to compete with the spin trap 5,5-dimethyl-1-pyrroline-N-oxide for superoxide radicals (Murias *et al*. 2005). For Xan, however, superoxide radical-scavenging was questioned (Schempp *et al*. 2010). An alternative explanation for Xan could be its strong inhibition of the ETC at complex II/III and if no oxygen is consumed also no superoxide radicals are produced at complex III. An interference of Xan and Res in the CMH-mediated superoxide radical detection was excluded.

Res and Xan had an effect on the membrane potential (Fig. 6). Both Res and Xan (200 μM) decreased the mitochondrial membrane potential detected by the fluorescence of JC-1 aggregates. Our detection system was verified by the positive controls Val, an ionophore, CCCP, a protonophore, and AA, a complex III inhibitor. All of them caused a breakdown of the membrane potential. As expected inhibition of ATP synthesis by Oligo increased the potential. The effect of Res and Xan on the membrane potential could have been mediated in part by protonophoric effects of phenolic OH groups in analogy to the classical uncoupler DNP. However, changes of the mitochondrial membrane potential by Res could be also related to indirect effects by apoptotic processes and do not necessarily imply direct mitochondrial targets for Res.

In summary, both natural products, Xan and Res, exhibited an antileishmanial potential in the lower micromolar range. However, only the effects of Xan are directly mediated by mitochondrial targets and the specific vulnerability of oxidative phosphorylation in *Leishmania*.

ACKNOWLEDGEMENTS

We are grateful for the support of Professor Veronika Sexl and BSc Matthias Tonner.

FINANCIAL SUPPORT

The award of an Ernst Mach scholarship to Dr Lianet Monzote by the Austrian Exchange Office is gratefully acknowledged. The work was supported by the FWF (grant no. P 27814-B22).

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https://doi.org/10.1017/S0031182016002389 Published online by Cambridge University Press

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