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Exploring the Anti-Infective Value of Inuloxin A Isolated from *Inula viscosa* against the Brain-Eating Amoeba (*Naegleria fowleri*) by Activation of Programmed Cell Death

Ikrame Zeouk,* Ines Sifaoui, Aitor Rizo-Liendo, Iñigo Arberas-Jiménez, María Reyes-Batlle, Isabel L. Bazzocchi, Khadija Bekhti, José E. Piñero,* Ignacio A. Jiménez, and Jacob Lorenzo-Morales*

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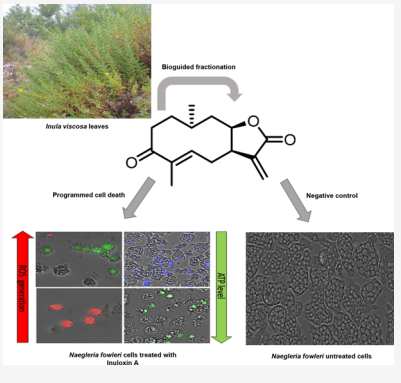
ABSTRACT: Primary amoebic meningoencephalitis (PAM), caused by the pathogenic free-living amoeba *Naegleria fowleri*, is a rare but fatal disease. Nowadays, no fully effective therapy is available to erradicate or prevent this disease. Natural products could constitute a promising source of useful bioactive compounds in drug discovery. The present study is a characterization of main active compounds from the ethanolic extract of *Inula viscosa* (Asteraceae) leaves against *N. fowleri* trophozoites. Four compounds (**1**−**4**) were successfully identified by spectroscopic techniques, but only inuloxin A displayed a potential antiamoebic activity with an IC_{50} of 21.27 μM. The specificity of this compound toward the studied strain leads us to analyze the insight into its mechanism of action by performing *in vitro* assays of programmed cell death markers and to discuss the structure−activity relationship (SAR). The obtained results demonstrated that inuloxin A interferes with various processes leading to membrane damage, mitochondria alteration, chromatin condensation, and ROS accumulation, which highlight features specific to apoptosis. The current findings could be a promising step for developing new effective drugs against PAM.



KEYWORDS: *Inula viscosa, bioguided fractionation, inuloxin A, brain-eating amoeba, Naegleria fowleri, programmed cell death*

1. INTRODUCTION

Primary amoebic meningoencephalitis (PAM) is a fatal disease caused by pathogenic free-living amoeba *Naegleria fowleri*, popularly known as the "brain-eating amoeba", and it has been classified as a category B priority pathogen by the National Institute of Allergy and Infectious Diseases (NIAID).[1] *N. fowleri* was discovered in 1899, and the first case of PAM was described in Australia in 1965 by Fowler and Carter.[2] The incidence of reported PAM cases caused by *N. fowleri* is becoming increasingly important around the world with high mortality rate.[3] Recently, more cases were recorded in Philippine, Southern Brazil, United States, Pakistan, and Asian countries.[4−8] In fact, *N. fowleri* is an environmental organism that can survive in water, soil, and host.[9] It has been primarily isolated from natural sources of water, namely, warm freshwater, tap water, and hot water spring.[10−12] When the contaminated water is inhaled or splashed into the nose, pathogenic species of *N. fowleri* develop acute PAM through the nasal cavity, which leads to a destruction of neurons.[13]

Studies on the pathogenicity of *N. fowleri* have figured more and more prominently and have focused on numerous aspects including the biology of this amoeba and its virulence factors such as the plasma membrane as an essential determinant in the pathogenicity of different microorganisms, but overall, the pathogenicity has been poorly investigated. Proteins such as 23-kDa protein (Nf23) and Mp2CL5 protein were detected specifically in pathogenic strains of *N. fowleri*.[14,15] In addition, the adhesion to fibronectin and fibrinogen is another important pathogenic mechanism of this amoeba, the overexpressed nf-actin gene has been demonstrated as contact-dependent that develop the cytotoxicity and phagocytosis by *N. fowleri*.[16]

The high pathogenicity of *N. fowleri* and high mortality rate of PAM are serious problems. Moreover, the drugs of choice, miltefosine and amphotericin B (Amp B), are associated with severe adverse effects when administrated alone or in combination with antibiotics such as rifampicin and miconazole.[17,18] Indeed, Amp B is known by its nephrotoxicity and acute-infusion related reactions.[19] Furthermore, among a total of 36 patients treated by Amp B, only 8% survived.[20] This critical situation makes the look of new amoebicidal agents an urgent need. In fact, natural products provide an unlimited resource for discovery of new drugs, many of the available medicines contain natural products or natural derivatives as

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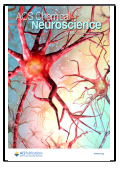

active compounds.[21] *Inula viscosa*, also known as *Dittrichia viscosa* belonging to asteraceae family, is endowed with multiple pharmacological activities.[22,23] Obviously, it stands as an unlimited resource for active compounds. Thus, we hypothesize that compounds from this species could allow the identification of bioactive compounds against *N. fowleri*. The results obtained in the present study showed that the sesquiterpenoid inuloxin A induces PCD via apoptosis in treated strain of *N. fowleri*.

2. RESULTS AND DISCUSSION

Primary amoebic meningoencephalitis (PAM) caused by *Naegleria fowleri* is a rare but deadly disease that requires more attention since this amoeba is opportunist. Nowadays, more cases of PAM caused by *N. fowleri* have been reported in different countries.[29] Furthermore, the current options to treat PAM have limted success. Natural products have been shown to exhibit amoebicidal activities.[30] Herein we isolated compounds from extract of *I. viscosa* and evaluated the *in vitro* activity and the engaged mechanism of action against *N. fowleri*. In fact, the phytochemical analysis of *I. viscosa* revealed the presence of different compounds belonging to flavonoids and sesquiterpenes corroborating previous studies.[31,32] In the current work, the ethanolic extract of *I. viscosa* leaves displayed an important inhibitory potent against trophozoites of *N. fowleri* (IC_{50} = 17.89 μg/mL) (Table 1). In order to identify the main compounds involved in the inhibitory potent, the purification procedure allowed us to characterize four pure compounds highlighting a highest inhibition exerted by compound **2** against *N. fowleri* (IC_{50} = 21.27 μM), while compound **3** showed moderate activity (IC_{50} = 74.83 μM) when compared to the crude extract and some fractions, subfraction, compounds **1** and **4** (Tables 1,2). However, when compared with the reference drugs, the results over the trophozoites of *N. fowleri* showed that compound **2** displayed important activity in the micromolar range, which is more potent than miltefosine (21.27 versus 38.74 μM) but less potent than amphotericin B (0.12 μM) ($p < 0.0001$). The cytoxicity on murine macrophages was also tested. Miltefosine showed a CC_{50} of 127.88 ± 8.85 μM, whereas amphotericin B showed a CC_{50}> 200 μM ($p < 0.0001$) (Table 2). Furthermore, compound **2** showed similar selectivity index (SI) to miltefosine, more selectivity toward *N. fowleri* than compound **3**, but less selectivity than amphotericin B (Table 2). Otherwise, the compounds were identified as 8-*epi*-xanthatin-1β,5β-epoxide (**1**),[24,25] inuloxin A (**2**),[26] sakuranetin (**3**),[27] and 3-*O*-acetyl-7-*O*-methylaromadendrin (**4**)[28] (Figure 1, Figure 2).

Table 1. Amoebicidal Activity against the Trophozoites of *Naegleria fowleri* of the Extract, Fractions, and Subfractions from Leaves of *I. viscosa*[a]

samples	*N. fowleri* IC_{50} (μg/mL)
crude extract	17.88 ± 1.59
F2	15.60 ± 2.34
F2/3	100 ± 0.5
F2/4	50 ± 0.5
F2/5	50 ± 0.5
F3	10.35 ± 1.43
F3/1	10.53 ± 3.01
F3/2	11.93 ± 3.69
F3/4-F3−6	100 ± 0.5
F4	14.88 ± 1.10
F4/9	22.74 ± 3.86
F4/11	50 ± 0.5
F5	100 ± 0.5
amphotericin B*	0.12 ± 0.03
miltefosine*	38.74 ± 4.23

[a]IC_{50}: inhibitory concentration that inhibits 50% of the growth of the tested parasite. IC_{50}: means ± standard deviation. Fractions, subfractions, and compounds that showed IC_{50} > 100 μg/mL were excluded. *Positive controls.

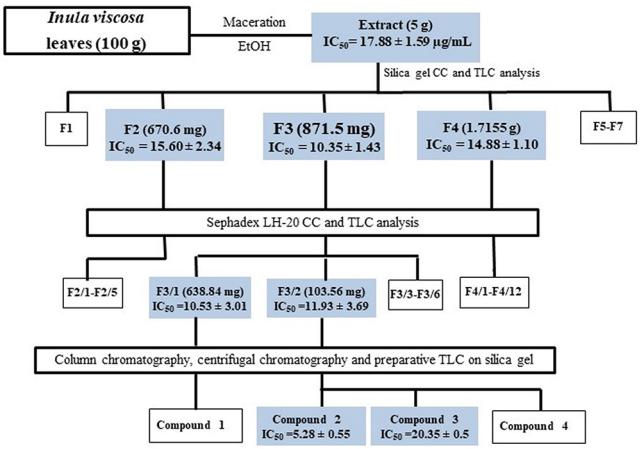

Figure 1. Flowchart of bioguided fractionation of Inula viscosa leaves against trophozoites of *Naegleria fowleri*. IC50: Inhibitory concentration that inhibits 50% of the growth of the tested parasite. IC50 values in μg/mL.

To examine the mechanism of cell death induced by inuloxin A, events indicating the type of PCD pathway were highlighted, and the experimental results are shown in Figures 3, 4, 5, and 6 (S1,2,3,4). It should be noted that apoptosis-specific markers are characterized in cells treated with the IC_{90} of inuloxin A with significant differences with the control. Data

Table 2. Amoebicidal, Cytotoxicity, and Selectivity Index against Trophozoites of *Naegleria fowleri* and Macrophage Cell Line (J774A.1) of Compounds 2 and 3 Isolated from *I. viscosa* by Bioguided Fractionation[a]

compounds	*N. fowleri* IC_{50} (μM)	*N. fowleri* IC_{90} (μM)	murine macrophages CC_{50} (μM)	SI
inuloxin A	21.27 ± 2.25[b]	70.47 ± 6.04	42.32 ± 0.07[a]	2.00
sakuranetin	74.83 ± 1.84[d]	-	95.93 ± 1.84[b]	1.28
amphotericin B*	0.12 ± 0.03[a]	-	>200[d]	≥1652.893
miltefosine*	38.74 ± 4.23[c]	-	127.88 ± 8.85[c]	3.30

[a]IC_{50}: inhibitory concentration that inhibits 50% of the growth of the tested parasite. IC_{50}: means ± standard deviation. CC_{50}: cytotoxic concentration that reduces 50% of the murine macrophage's viability. SI: selectivity index (CC_{50}/IC_{50}). *Positive controls; Data are presented as means ± SD (n = 3) and letters a−d reflect that means within compounds with different letters are significantly different ($p < 0.0001$).

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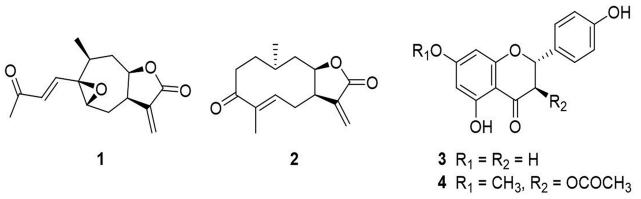

Figure 2. Chemical structure of natural compounds **1**−**4** isolated from leaves of *Inula viscosa*.

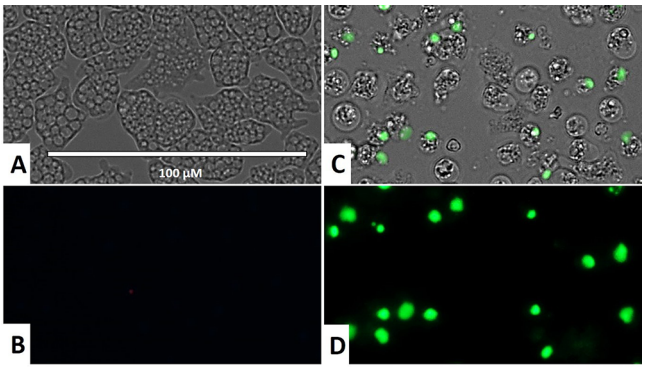

Figure 3. Images (40×) presenting the effects of IC90 concentration of inuloxin A (C, D) on the plasma membrane permeability of *Naegleria fowleri* trophozoites after 24 h incubation. Cells were labeled with Sytox Green. Images were obtained using an EVOS FL Cell Imaging System and untreated strains were used as negative control (A, B). (Images (100×) are attached in Supporting Information S1).

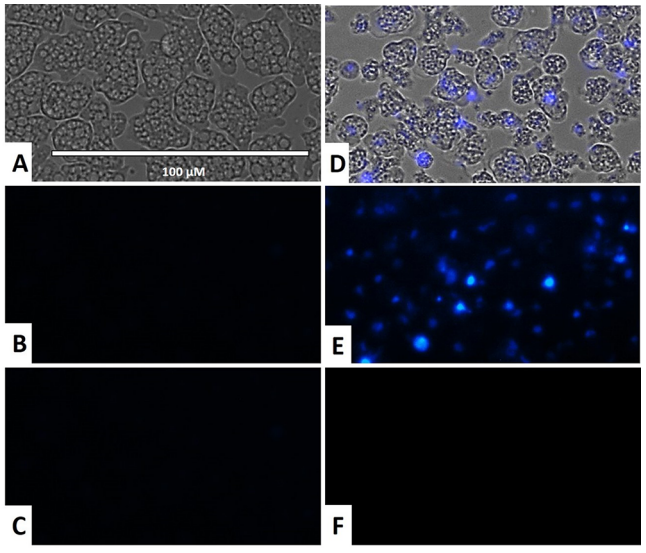

Figure 4. *Naegleria fowleri* trophozoites incubated with IC90 of Inuloxin A (D, E, F) for 24 h. Images (40×) are representative of the cell population observed in the performed experiments using an EVOS FL Cell Imaging System AMF4300, Life Technologies, U.S.A. Hoechst stain is different when comparing negative control (A, B, C) with treated cells where the nuclei are bright blue. Red fluorescence corresponds to the propidium iodide stain. Hoechst stain (B, E), Propidium iodide stain (C, F). (Images (100×) are attached in Supporting Information S2).

in Figure 3 (S1) showed that inuloxin A caused a remarkable membrane damage when compared with the untreated cells. Next, the chromatin condensation induced in the treated cells of *N. fowleri* was verified using the double stain Hoechst/PI.

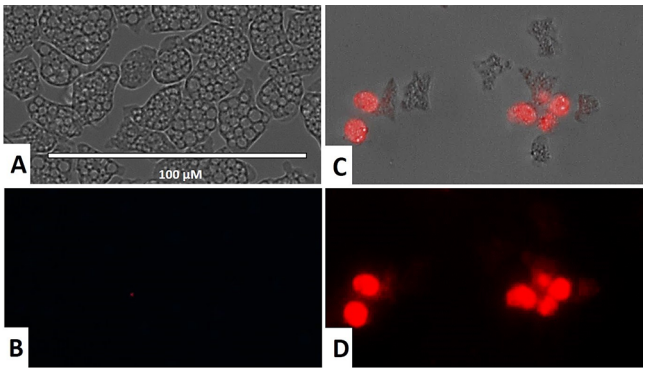

Figure 5. Representative images (40×) of CellROX Deep Red staining of *Naegleria fowleri* trophozoites after treatment with IC90 of inuloxin A (C, D) for 24 h. Images were obtained using an EVOS FL Cell Imaging system and compared to the negative control (A, B). (Images (100×) are attached in Supporting Information S3).

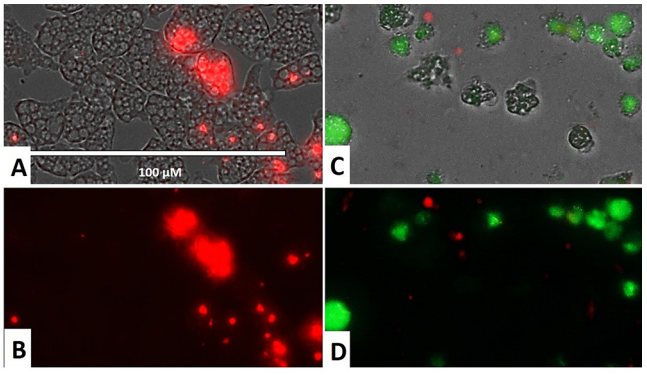

Figure 6. *Naegleria fowleri* (40×) stained with JC-1 kits after 24 h incubation of trophozoites with IC90 of inuloxin A (C, D). Images (40×) are representative of the effect on the mitochondrial potential of the parasites and observed using an EVOS FL Cell Imaging System. A control without treatment has been used (A, B). (Images (100×) are attached in Supporting Information S4).

Compared with the negative control, the treated cells indicated a strong blue fluorescence which highlighted the differences between normal and apoptotic parasites (Figures 4, S2). In addition, the treatment with inuloxin A enhanced the ROS accumulation into the treated parasites which is denoted by the high red fluorescence (Figures 5, S3). Regarding the energetic metabolism, a noticeable decreasing of mitochondrial membrane potential was recorded (Figures 6, S4) because JC-1 is a probe that can aggregate within mitochondria and record red fluorescence at higher $\Delta\Psi$m while in lower $\Delta\Psi$m, JC-1 is not able to accumulate within the mitochondria showing a green fluorescence because it stays in the cytosol area as monomers. To verify whether ATP production was affected by the loss of $\Delta\Psi$m, the ATP level generated in 24 h was quantified. The treatment of *N. fowleri* by inuloxin A caused a significant decrease in the total ATP level with a production of only 18% by the treated cells in comparison with the untreated cells that spontaneously produce 100% of ATP.

From the obtained data in the present work, we showed that inuloxin A was active againt *N. fowleri* trophozoites at low concentrations. The observed powerful biological activities of this compound has been linked to its chemical structure characterized by the furanone ring, which is an essential function for the biological activity.[33] Another investigation has

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confirmed the usefulness of this function to express the antiparasitic potency.[34] The important activity of inuloxin A deserved to be deeply studied in order to highlight the possible mode of action. Indeed, PCD signaling pathway has been widely reported in both unicellular and multicellular organisms and provides more information to well understand the pathogenesis and complexity of different parasites, which may help to elaborate new drugs able to eradicate infections caused by these microorganisms.[35] However, PCD has not yet been widely characterized in the genus *Naegleria*. In the present work, inuloxin A has combined different PCD features against treated cells of *N. fowleri* that are specific to apoptosis, ranging from the plasma membrane, the cell organelles, and the genetic material.

Indeed, the plasma membrane is involved in the pathogenicity of different microorganisms but poorly investigated in amoeba. Recently, Flores-Huerta et al. have conducted a comparative study where they have detected the expression of 23-kDa protein (Nf23) at a higher level in *N. fowleri* membrane than the nonpathogenic *N. lovaniensis* and *N. gruberi*.[14] Moreover, Reveiller et al. have identified Mp2CL5 protein that was highly present in the membrane of pathogenic trophozoites, but was absent in other species of *Naegleria* genus. This finding could characterize this protein as specific factors of *N. fowleri* pathogenicity.[15] In this context, it can be suggested that the nonpolar character of inuloxin A versus sakuranetin has facilitated the passage into the membrane of *N. fowleri* and probably the inhibition of the synthesis of these membrane proteins, since molecules such as this type of sesquiterpenoids, characterized by α,β unsaturated carbonyl, have been reported as inhibitors of proteins of multidrug resistance pathogens.[36] Subsequentaly, inuloxin A could constitute an attractive resource for new drugs design against *N. fowleri* targeting the plasma membrane.

In the literature, the mechanism of action of inuloxin A against amoeba is still unknown. However, it was reported that in fungi, sesquiterpenes could interfere with sterol biosynthesis[37] which is a key factor of *N. fowleri* growth.[38] However, in this study, inuloxin A enhanced the production of ROS which is normally expected to be inhibited by sterols.[39] Moreover, the interference between ROS generation, mitochondria dysfunction, decrease of ATP production, and DNA condensation in amoeba and especially in *N. fowleri* was previously demonstrated,[40] which could confirm the findings of the present study.

We have also shown that amphotericin B was more active and less toxic than inuloxin A and miltefosine. Moreover, several studies have reported the *in vitro* and *in vivo* potency of this drug reference.[41,42] However, treatment with amphotericin B has failed in a large clinical case series, among a total of 36 patients, only 8% survived[20] because of its inability to pass through the blood brain barrier (BBB).[43] Thus, the nonpolar character of inuloxin A could promote its future use. However, it is necessary to note that further studies are required since the structure of compounds is not the only factor influencing the permeability of BBB to antimicrobial agents, but several variables are involved such as cytokines, organism-associated molecules, and immunosuppressants.[44]

3. METHODS

3.1. General Procedure and Chemicals. Optical rotations were measured on a PerkinElmer 241 automatic polarimeter. NMR spectra, ROESY (spin lock field 2500 Hz), HSQC and HMBC (optimized for J = 7.7 Hz) were performed on a Bruker Avance 500 and 600 spectrometers at 300 K. Silica gel 60 (particle size 15−40 and 63−200 μm, Macherey-Nagel) was used for column chromatography, while silica gel 60 F254 (Macherey-Nagel) was used for analytical or preparative TLC. Sephadex LH-20 (Sigma-Aldrich) was used for the column chromatographic (CC) separation. Centrifugal preparative TLC was performed using a Chromatotron (Harrison Research Inc. model 7924T) on 4- or 1 mm silica gel 60 PF254 disks with flow rate 2−4 mL min^{-1}. The spots were visualized by UV light and heating silica gel plates sprayed with H_2O−H_2SO_4−AcOH (1:4:20). All the used solvents were analytical grade from Panreac. Reagents, deuterated solvents, and Amphotericin B were purchased from Sigma-Aldrich, alamarBlue reagent (Invitrogen, Life Technologies), EnSpire Multimode Plate Reader (PerkinElmer), Leika DMIL inverted microscope (Leika, Wetzlar, Germany), and an EVOS FL Cell Imaging System AMF4300 (Life Technologies, Madrid, Spain) were used.

3.2. Plant Material. Leaves of *Inula viscosa* were collected in the Atlas Mountains, Imouzzer region, Morocco, in July 2017 (33°55′ 37 N, 5°2′50 W). The plant material was authenticated by Prof. EL OUALIDI. J. and Pr IBN TATTOU. M (Scientific Institute, Rabat). A voucher specimen (RAB107342) was deposited in the Herbarium of the Scientific Institute, Rabat, Morocco.

3.3. Extraction and Fractionation. The extraction and chrolatographic separation were performed as previously described.[45] In this context, the clean leaves of of *I.viscosa* were shade dried at room temperature (25 °C, 1 week) and subsequently milled with a commercial electric grinder. Powdered material (100 g) was macerated in ethanol 96% (1:10 w/v) for 6 h at room temperature with continuous stirring at 500 rpm. The resulting mixture was filtered through Whatman filter no. 1, and the solvent was concentrated under vacuum in a rotary evaporator at 45 °C to afford a crude residue (5 g, 5%). Dried extracts were stored in a refrigerator at 4 °C. The obtained extract was tested against trophozoites of *N. fowleri*.

After the preliminary screening, the active ethanolic extract was subjected to a bioassay-guided fractionation protocol (Figure 1). In this way, the extract (5 g) was fractionated by column chromatograhic on silica gel eluted with mixtures of hexanes-EtOAc (100:0 to 0:100, 0.5 L each one) of increasing polarity, affording 12 fractions, which were combined on the basis their TLC in seven main fractions, coded from F1 to F7 and tested against *Naegleria fowleri*. Three fractions F2 (670.6 mg), F3 (871.5 mg), and F4 (1.7155 g) were active and were further chromatographed by Sephadex LH-20 column using a MeOH−$CHCl_3$ system (1:1, 2 L) as eluent, and subsequently TLC analysis allowed us to gather them in five (F2/1 to F2/5), six (F3/1 to F3/6), and 12 (F4/1 to F4/12) subfractions, respectively. F3/1 and F3/2 showed a promising amoebicidal activity. Subsequently, F3/1 (638.84 mg) and F3/2 (103.56 mg) were subjected to multiple chromatographic steps on silica gel, involving medium-pressure liquid chromatography, centrifugal planar chromatography and preparative TLC using mixtures of hexanes-EtOAc, hexanes-Et_2O, CH_2Cl_2-EtOAc, and CH_2Cl_2-acetone as eluent.

3.4. Characterization of Pure Compounds. Following purification and bioguided-isolation, four pure compounds were identified. Subfraction F3/1 (638.84 mg) yielded compound **1**, and subfraction F3/2 (103.56 mg) yielded compounds **2** (38.5 mg), **3** (3.6 mg), and **4** (1.95 mg). Therefore, structures were elucidated using means of spectrometric and spectroscopic studies (1D, 2D NMR) and comparison of these data with those previously reported in the literature.

3.5. Biological Assays. *3.5.1. Culture of Naegleria fowleri.* Phytochemical samples were tested against *Naegleria fowleri* (ATCC30808) received from American Type Culture Collection (LG Promochem, Barcelona, Spain). The strain was maintained under axenic conditions in 2% (w/v) Bactocasitone medium (ThermoFisher Scientific, Madrid, Spain) supplemented with 10% (v/v) fetal bovine serum (FBS), containing 0.5 mg/mL of streptomycin sulfate (Sigma-Aldrich, Madrid, Spain) and 0.3 μg/mL of Penicillin G Sodium Salt (Sigma-Aldrich, Madrid, Spain) at 37 °C. Because of the pathogenicity of the studied species and regarding to the Spanish

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biosafety guidelines, the strain was conserved in the biological security facilities level 3 in our institute.

3.5.2. Assessment of In Vitro Amoebicidal Activity. The *in vitro* assay was performed using the alamarBlue method as previously described.[46] Trophozoites in the logarithmic phase of growth were counted by a Countess II FL automatic cell counter (Thermo Fisher Scientific, Madrid, Spain) to get a concentration of 10^5 cells per well. From this prepared cell suspension, 50 μL was added per well in a 96-well plate (Thermo Fisher Scientific, Madrid, Spain). The experiment involves the incubation of the tested parasite with different concentrations of the evaluated phytochemicals. All samples were dissolved in 2% DMSO as maximum final dose without adverse effect on the parasite. First, serial dilutions of each sample were prepared in the culture medium and 50 μL were added to the plate. Amphotericin B and miltefosine were used as drug reference and negative control without samples was also prepared. As final step, the alamarBlue reagent was added in the entire plate (10% of medium volume), then plates were incubated with slight agitation for 48 h at 37 °C. After plates analysis in EnSpire Multimode Plate Reader (PerkinElmer, Madrid, Spain) using a wavelength of 570 nm and a reference wavelength of 630 nm. Amoebicidal activity was expressed as IC_{50} values (the concentration of a sample which caused a 50% reduction in parasite viability). Those values were calculated by nonlinear regression analysis with 95% confidence limits using SigmaPlot 12.0 software (Systat Software Inc., London, U.K.). All assays were carried out in triplicate. The results were defined as the mean values of three experiments.

3.5.3. Cytotoxicity Assay. Cytotoxicity test was performed as previously described;[47] therefore, different concentrations of the active compounds were incubated with the J774A.1 (ATCC TIB-67) murine macrophages cell line cultured in RPMI medium supplemented with 10% fetal bovine serum (10^5 cells/mL) for 24 h at 37 °C in a 5% CO_2 atmosphere. The viability of the macrophages was determined with the alamarBlue reagent. Dose−response curves were plotted, and the CC_{50} was obtained. The analyses were performed in triplicate.

3.6. Analysis of Programmed Cell Death. To analyze the type of cell death induced by inuloxin A in *N. fowleri*, four kits markers specific to apoptosis have been used following the manufacturer's protocol. After being treated with the inuloxin A at the IC_{90} value for 24 h, cells were incubated with reagents specific to each event marker as described below. For all assays, the fluorescence was observed using an EVOS FL Cell Imaging System AMF4300, Life Technologies, Madrid, Spain.

3.6.1. Chromatin Condensation. The double-stain apoptosis detection kit (Hoechst 33342/Propidium Iodide (PI)) (Life Technologies) was used to examine the state of chromatin in apoptotic cells. It is a kit of two dyes that differ in their spectral characteristics and their ability to enter the cell. Hoechst 33342 is a blue fluorescent dye, while propidium iodide (PI) is a red fluorescent dye. The properties of this test allow to distinguish between normal, apoptotic, and dead cells. After excitation, Hoechst 33342 stains condensed chromatin in apoptotic cells brighter than chromatin in normal cells, while propidium iodide, which is a DNA intercalator, is only permeable to dead cells coloring them red. To perform this assay, cells were washed with fresh medium, then Hoechst and PI stains at 5 μg/mL and 1, respectively, were added onto the cells before incubating at 26 °C for 15 min. After staining, the cells were observed red (Hoechst) and blue (PI) fluorescence.

3.6.2. Plasma Membrane Integrity. To evaluate if inuloxin A causes alterations in the membrane permeability, SYTOX Green assay was performed. It is a molecule with a very high affinity for nucleic acids, and its fluorescence intensity is multiplied by 500 times when it binds to DNA. SYTOX Green is a molecule impermeable to cells with intact membranes. Subsequently, the treated parasites have been incubated with SYTOX Green at a final concentration of 1 μM (Molecular Probes) for 15 min in the dark at room temperature. The increase in fluorescence due to binding of the fluorescent marker to the parasitic DNA was observed.

3.6.3. ROS Production. Under stress conditions, the parasite regenerates Reactive Oxygen Species (ROS) as a response to an antimicrobial agent. The ROS play an important role in apoptosis induction. The CellROX kit (Thermo Fisher Scientific), a fluorogenic probe, was used to measure oxidative stress in treated cells. It is a cell permeable dye nonfluorescent at reduced state. However, upon oxidation by reactive oxygen species (ROS), it exhibits intense bright fluorescence.To this end, treated parasites have been incubated with 5 μM of CellRox Reagent for 30 min at 26 °C. Hence, the signal for Deep Red is localized in the cytoplasm.

3.6.4. Mitochondria Potential Membrane. The JC-1 Mitochondrial Membrane Potential Assay Kit (Cayman Chemical) was used to measure the collapse of an electrochemical gradient across the mitochondrial membrane. In fact, the JC-1 is one of the most used methods for the analysis of mitochondrial membrane potential. It is a fluorochrome in the form of a voltage-sensitive lipophilic cation selectively accumulating in the mitochondria. Healthy cells have a high mitochondrial potential, and because of its positive charge, JC-1 accumulates, and forms J-aggregate complexes characterized by intense red fluorescence. However, in the case of low potential in apoptotic cells, JC-1 remains in the cytosol as a monomer showing green fluorescence. The fluorescence obtained is proportional to the potential of the mitochondrial membrane. Thus, the performed method involved the incubation of treated cells (after 24 h of incubation) with JC-1 reagent at 26 °C for 30 min. Depolarization of the mitochondrial membrane potential was confirmed by microscopic observation.

3.6.5. ATP Level. In order to confirm the pathway of mitochondria dysfunction, Cell Titer-Glo Luminescent Cell Viability Assay (Promega) was used to measure the ATP level. The effect of inuloxin A on the ATP production was evaluated after a 24 h incubation of the parasitic strains (10^6 cells/mL) with the previously calculated IC_{90}.

3.7. Statistical Analysis. All assays were carried out in triplicate. The results were defined as the mean values of three experiments. The obtained inhibition curves were performed using the Sigma Plot 12.0 software program (Systat Software Inc.). Statistical analyses were performed using the GraphPad Prism 8.0.2. Differences between the values were assessed using a one-way analysis of variance (ANOVA). Data are presented as means ± SD, and $p < 0.0001$ was considered statistically significant.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acschemneuro.0c00685.

Images presenting the effect of IC_{90} concentration of inuloxin A using different labels and cell staining methods, including SYTOX Green, Hoechst/PI double stain, CellROX Deep Red staining, and JC-1 kits (PDF)

■ AUTHOR INFORMATION

Corresponding Authors

Ikrame Zeouk − *Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, La Laguna, Tenerife, Islas Canarias 38203, Spain; Red de Investigación Colaborativa en Enfermedades Tropicales (RICET)*, https://www.ricet.es/; Departement of Biology, Sidi Mohamed Ben Abdellah University, Faculty of Sciences and Techniques, Laboratory of Microbial Biotechnology and Bioactive Molecules, PB 2202 Fez, Morocco; orcid.org/0000-0003-4481-0059; Phone: 212621290377; Email: ikramezeouk20@gmail.com

Jacob Lorenzo-Morales − *Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, La Laguna, Tenerife, Islas*

Canarias 38203, Spain; Red de Investigación Colaborativa en Enfermedades Tropicales (RICET), https://www.ricet.es/; Departamento de Obstetricia, Ginecología, Pediatría, Medicina Preventiva y Salud Pública, Toxicología, Medicina Legal y Forense y Parasitología, Universidad De La Laguna, La Laguna, Tenerife, Islas Canarias 38203, Spain; Email: jmlorenz@ull.edu.es

José E. Piñero − *Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, La Laguna, Tenerife, Islas Canarias 38203, Spain; Red de Investigación Colaborativa en Enfermedades Tropicales (RICET), https://www.ricet.es/; Departamento de Obstetricia, Ginecología, Pediatría, Medicina Preventiva y Salud Pública, Toxicología, Medicina Legal y Forense y Parasitología, Universidad De La Laguna, La Laguna, Tenerife, Islas Canarias 38203, Spain; Email: jpinero@ull.edu.es*

Authors

Ines Sifaoui − *Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, La Laguna, Tenerife, Islas Canarias 38203, Spain; Red de Investigación Colaborativa en Enfermedades Tropicales (RICET), https://www.ricet.es/; Departamento de Obstetricia, Ginecología, Pediatría, Medicina Preventiva y Salud Pública, Toxicología, Medicina Legal y Forense y Parasitología, Universidad De La Laguna, La Laguna, Tenerife, Islas Canarias 38203, Spain*

Aitor Rizo-Liendo − *Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, La Laguna, Tenerife, Islas Canarias 38203, Spain; Departamento de Obstetricia, Ginecología, Pediatría, Medicina Preventiva y Salud Pública, Toxicología, Medicina Legal y Forense y Parasitología, Universidad De La Laguna, La Laguna, Tenerife, Islas Canarias 38203, Spain*

Iñigo Arberas-Jiménez − *Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, La Laguna, Tenerife, Islas Canarias 38203, Spain; Departamento de Obstetricia, Ginecología, Pediatría, Medicina Preventiva y Salud Pública, Toxicología, Medicina Legal y Forense y Parasitología, Universidad De La Laguna, La Laguna, Tenerife, Islas Canarias 38203, Spain*

María Reyes-Batlle − *Instituto Universitario de Enfermedades Tropicales y Salud Pública de Canarias, Universidad de La Laguna, La Laguna, Tenerife, Islas Canarias 38203, Spain; Red de Investigación Colaborativa en Enfermedades Tropicales (RICET), https://www.ricet.es/; Departamento de Obstetricia, Ginecología, Pediatría, Medicina Preventiva y Salud Pública, Toxicología, Medicina Legal y Forense y Parasitología, Universidad De La Laguna, La Laguna, Tenerife, Islas Canarias 38203, Spain*

Isabel L. Bazzocchi − *Instituto Universitario de Bio-Orgánica Antonio González, Departamento de Química Orgánica, Universidad de La Laguna, 38206 La Laguna, Tenerife, Spain*

Khadija Bekhti − *Departement of Biology, Sidi Mohamed Ben Abdellah University, Faculty of Sciences and Techniques, Laboratory of Microbial Biotechnology and Bioactive Molecules, PB 2202 Fez, Morocco*

Ignacio A. Jiménez − *Instituto Universitario de Bio-Orgánica Antonio González, Departamento de Química Orgánica, Universidad de La Laguna, 38206 La Laguna, Tenerife, Spain*

Complete contact information is available at: https://pubs.acs.org/10.1021/acschemneuro.0c00685

Author Contributions

I.Z.: Data curation, Investigation, Methodology, Writing-original draft. I.S.: Data curation, Investigation, Writing-original draft. A.R.-L.: Investigation. I.A.-J.: Investigation. M.R.-B.: Investigation. I.L.B.: Funding acquisition, Supervision. K.B.: Supervision. J.E.P.: Funding acquisition, Supervision, Writing−review and editing. I.A.J.: Funding acquisition, Supervision, Writing−review and editing. J.L.-M.: Funding acquisition, Supervision, Writing - original draft, Writing - review and editing.

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Notes

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■ ABBREVIATIONS

PAM, primary amoebic meningoencephalitis; 1D, 2D-NMR, mono/bidimensional nuclear magnetic resonance; ROS, reactive oxygen species; NIAID, National Institute of Allergy and Infectious Diseases; Amp B, amphotericin B; ROESY, Rotating frame Overhause Effect SpectroscopY; HSQC, heteronuclear single quantum coherence spectroscopy; HMBC, heteronuclear multiple bond correlation; TLC, thin-layer chromatography; CC, column chromatographic; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; PI, propidium iodide; SI, selectivity index; PCD, programmed cell death; BBB, blood brain barrier; $\Delta\Psi m$, mitochondrial membrane potential

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