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Photochemotherapeutic strategy against *Acanthamoeba* infections

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Abstract

Acanthamoeba is a protist pathogen that can cause serious human infections including a blinding keratitis and a granulomatous amoebic encephalitis that almost always results in death. Current treatment includes a mixture of drugs and even then infection recurrence can occur. Photochemotherapy has shown promise in the treatment of *Acanthamoeba* infections, however selective targeting of pathogenic *Acanthamoeba* has remained a major concern. The mannose-binding protein is an important adhesin expressed on the surface membranes of pathogenic *Acanthamoeba*. To specifically target *Acanthamoeba*, the overall aim of this study was to synthesize photosensitising compound (porphyrin)-conjugated with mannose and test its efficacy *in vitro*. The synthesis of mannose-conjugated porphyrin was achieved by mixing benzaldehyde and pyrrole yielding tetra-phenyl porphyrin. Tetra-phenyl porphyrin was then converted into mono-nitro phenyl porphyrin by selectively nitrating the para position of phenyl rings as confirmed by NMR spectroscopy. The mono-nitro phenyl porphyrin was reduced to mono-amino phenyl porphyrin in the presence of tin dichloride and confirmed by peak at 629 m/z. Finally, mono-amino porphyrin was conjugated with mannose resulting in the formation of imine bond. Mannose-conjugated porphyrin was confirmed through spectroscopic analysis and showed that it absorbed light of wavelength ranging from 425-475nm. To determine antiacanthamoebic effects of the derived product, amoebae were incubated with mannose-conjugated porphyrin for 1 h, and washed 3X to remove extracellular compound. Next, amoebae were exposed to light of the appropriate wavelength for 1 h. The results revealed that mannose-conjugated porphyrin produced potent trophicidal effects and blocked excystation. In contrast, *A. castellanii* incubated with mannose alone and porphyrin alone did not exhibit anti-amoebic effect. Consistently, pre-treatment with mannose-conjugated porphyrin reduced *A. castellanii*-mediated host cell

cytotoxicity from 97% to 4.9%. In contrast, treatment with porphyrin, mannose or solvent alone had no protective effects on host cells. These data suggest that mannose-conjugated porphyrin has application for the targeted photodynamic therapy of *Acanthamoeba* infections and may serve as a model in the rationale development of therapeutic interventions against other eukaryotic infections.

Keywords: *Acanthamoeba*; mannose-conjugated porphyrin; photochemotherapy; Targeted therapy; *In vitro* assays.

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Introduction

Pathogenic *Acanthamoeba* are well known to produce serious infections, including a fatal granulomatous amebic encephalitis (GAE), and a painful, sight-threatening keratitis (reviewed in 1, 2). The most distressing aspect is that the mortality associated with GAE due to pathogenic *Acanthamoeba* has remained significant (more than 90%) despite our advances in antimicrobial chemotherapy and supportive care (1, 2). Similarly, current diagnosis of *Acanthamoeba* keratitis is challenging (1, 2), and the available treatments are lengthy and not fully effective against all strains (3), in part due to the ability of amoebae to transform into a resistant cyst form (4), resulting in infection recurrence. A complete understanding of the pathogenesis and pathophysiology will undoubtedly lead to the development of diagnostic advances and therapeutic interventions (5, 6). Recent studies have identified a mannose-binding protein (MBP) as an important adhesin expressed on the surface membranes of *Acanthamoeba*, as free exogenous mannose blocks the parasites' adhesion to primary human brain microvascular endothelial cells and corneal epithelial cells (7, 8). Notably, oral immunization with recombinant mannose-binding protein ameliorates *Acanthamoeba* keratitis in the Chinese hamster model (9). *Acanthamoeba mbp* contains 6 exons and 5 introns that spans 3.6kbp. The 2.5kbp cDNA codes for an 833amino acids precursor protein with a signal sequence (residues 1 – 21 aa), an *N*-terminal extracellular domain (residues 22 – 733 aa) with five *N*- and three *O*-glycosylation sites, a transmembrane domain (residues 734 – 755 aa), and a C-terminal intracellular domain (residues 756 – 833 aa) (7).

Photochemotherapy is a promising technology that can target pathogens and thus, has the potential for therapeutic interventions. The basis of this technology is that visible light (or light of appropriate wavelength) activates the photosensitising compound, resulting in production of

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singlet oxygen and other reactive oxygen species (ROS) to induce cell death in the target pathogen/tissue (10, 11). However, it is possible that the photosensitising compound may bind to host cells and subsequent irradiation could lead to their destruction. In this context, selective targeting of pathogenic *Acanthamoeba* has remained a major concern (12). To specifically target *Acanthamoeba*, the overall aim of this study was to synthesize the photosensitising compound (porphyrin)-conjugated with mannose and test its efficacy *in vitro*.

Materials and Methods

Acanthamoeba castellanii cultures

All chemicals were purchased from Sigma Laboratories (Poole, Dorset, England), unless otherwise stated. A clinical isolate of *A. castellanii* belonging to the T4 genotype was obtained from American Type Culture Collection (ATCC 50492). Amoebae were routinely grown in 10 mL PYG medium [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)] in T-75 cm^2 tissue culture flask at 37°C as previously described (12). Media were refreshed 15-20 h prior to experiments. Amoebae adherent to the flask represented the trophozoite form and were collected by placing the flask on ice for 20 min with gentle agitation and used in all subsequent experiments.

Human brain microvascular endothelial cell (HBMEC) cultures

Primary brain microvascular endothelial cells were of human origin and were isolated and cultured as previously described (8, 13). The endothelial cells were purified by fluorescent activated cell sorting, and their purity tested using endothelial markers such as expression of F-VIII, carbonic anhydrase IV and uptake of acetylated low density lipoprotein (DiI-AcLDL) (8, 13), resulting in >99% pure endothelial cultures. The human BMEC were routinely grown in rat

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tail collagen-coated tissue culture dishes in RPMI-1640 containing 10% heat inactivated fetal bovine serum, 10% Nu-serum, 2 mM glutamine, 1 mM Na-pyruvate, 100 U penicillin/mL, 100 µg streptomycin/mL, non-essential amino acids, and vitamins and incubated at 37°C in a 5% CO_2 incubator. For cytotoxicity assays, human BMEC were grown in 24-well plates by inoculating 10^6 cells/mL/well. At this cell density, confluent monolayers were formed within 24 h and used in experiments.

Synthesis of mannose-conjugated porphyrin

Mannose-conjugated porphyrin was synthesized as follows: Initially 40 mL of propionic acid were heated at 90°C. A solution was prepared by mixing 1.9 mL (18.85 mM) benzaldehyde in propionic acid (2.5 mL). Benzaldehyde was added very slowly in hot propionic acid. After continuous heating of the solution at reflux temperature, pyrrole 1.3 mL (18.85 mM) dissolved in propionic acid (2.5 mL) was added in above mixture in a drop-wise manner for 30 min. The reaction was refluxed and monitored by thin layer chromatography (TLC) (14). After completion (about 1.5 h), the reaction mixture was cooled, and crude tetra-phenyl porphyrin in ethyl acetate (3 x 50 mL) was extracted from 2.5 M (50 mL) NaOH aqueous solution. Solvent was evaporated and solid product was passed from silica column (ethyl acetate:hexane, 1:9) to get the pure product in 7% yield as described previously (14). Next, 160 mg (0.26 mM) of tetra-phenyl porphyrin was nitrated using 90 mg (1.3 mM) sodium nitrite ($NaNO_2$) in 10 mL trifluoroacetic acid (TFA), and the reaction mixture was stirred for an hour. By controlling the amount of TFA and reaction time, mono-nitrated phenyl porphyrin was obtained as a major product along with di-nitro phenyl porphyrin. The reaction was quenched with excess of water and was extracted in dichloromethane (DCM, 3 x 25 mL). Next, it was evaporated in vacuum, and mono-nitrated phenyl porphyrin was isolated by using column chromatography (DCM:hexane, 3:1 to 1:1 ratio)

to get 30% yield. After this, 100 mg (0.15 mM) of mono-nitro phenyl porphyrin together with 100 mg (0.44mM) tin dichloride dihydrate was added in 0.5 mL concentrated HCl and heated at 60°C for 2.5 h. The progress of reaction was monitored by TLC. After completion, ammonium hydroxide was added to neutralize the reaction mixture and was extracted wit DCM (3 x 25 mL) and purified with column chromatography (DCM:hexane, 1:3 to 3:1 ratio) to afford mono-amino phenyl porphyrin in 53% yield. Finally, 15 mg (0.079 mM) of mannose along with 50 mg (0.079 mM) of mono-amino phenyl porphyrin was dissolved in ethanol (8 mL). Three drops of glacial acetic acid were added and reaction was refluxed overnight. Precipitates formed were filtered and washed with DCM and methanol separately and purity confirmed through spectroscopic analysis. The residue was dissolved in dimethyl sulfoxide (DMSO) and was subjected to NMR to determine product purity. The synthesized product absorbed photons of 450 nm, which is within the range of blue light.

Amoebicidal assays

To determine cidal effects of mannose-conjugated porphyrin, amoebicidal assays were performed as described previously but with slight modifications (12). Briefly, *A. castellanii* were incubated with 10µM (4 µL volume) and 50µM (20 µL volume) concentrations of mannose-conjugated porphyrin, as well as porphyrin and mannose alone in RPMI (10^6 amoebae/mL/well) in 24-well plates for 1 h. Amoebae were collected by centrifugation at 1000 x g for 5 min. The supernatant was aspirated and pellet resuspended in 0.5 mL of RPMI-1640. This process was repeated 3X to remove any remaining residual mannose-conjugated prophyrin. Finally, amoebae pellet was resuspended in PYG. Next, the lids were removed and plates were exposed to blue light for 1 h in a biosafety hood at room temperature using blue round light-emitting diode (LED), that is lead-free and at 20mA emits minimum wavelength of 465nm and maximum

wavelength of 480nm, while typical dominant wavelength of 470nm at pulse width of ≤0.1msec by placing the 5-mm LED approximately 15 cm above the 24-well plate (Cree Inc., Durham, NC, USA). Following this, plates were incubated for 24 h at 37°C. Next day, amoebae counts were determined using a haemocytometer and images obtained using a phase contrast inverted microscope. For solvent controls, amoebae were incubated in same amounts of DMSO (4 µL and 20 µL volume), chloroform:Methanol, 50:50 (1.34 µL and 6.7 µL volume), and RPMI alone. For positive controls amoebae were incubated with antiacanthamoebic drug, chlorhexidine (20µM in RPMI as a solvent), which exhibited 100% kill.

Host cell cytotoxicity assays

To determine antiamoebic effects of mannose-conjugated porphyrin *in vitro*, assays were performed using human BMEC. *A. castellanii* were pre-treated with mannose-conjugated porphyrin and exposed to light as described above. Next, amoebae were collected by centrifugation at 1000 x g for 5min. The supernatant were aspirated and pellet resuspended in 0.5 mL of RPMI-1640. This process was repeated 3X to remove any remaining residual mannose-conjugated prophyrin. Finally, amoebae were inoculated on human BMEC monolayers, grown in 24-well plates. Plates were incubated at 37°C in a 5% CO_2 incubator. Next day, cell-free conditioned medium was collected and host cell cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release using Cytotoxicity Detection kit (Roche, Indianapolis, IN). The principle of this assay is that cell supernatant containing LDH catalyzes the conversion of lactate (solution from kit) to pyruvate, generating NADH and H^+. In the second step, the catalyst (diaphorase, solution from kit) transfers H and H^+ from NADH and H^+ to the tetrazolium salt *p*-iodo-nitrotetrazolium violet (INT), which is reduced to formazon (dye), and absorbance is read at 492 nm. The human BMEC incubated alone were used as negative controls, whereas monolayers

lysed with 1% Triton X-100 for 30 min at 37°C were used as 100% cell death (high value). Absorbance of samples was converted to percentage cytotoxicity as follows: (sample value – negative value) / (high value – negative value) × 100 = % cytotoxicity.

In addition to amoebae pre-treatment, antiamoebic effects of mannose-conjugated porphyrin were determined by co-incubating *A. castellanii* + human BMEC + mannose-conjugated porphyrin and exposed to light as described above. Next, plates were incubated at 37°C in a 5% CO_2 incubator for 24 h and cell-free conditioned medium was collected and host cell cytotoxicity was determined by measuring LDH release.

Excystation assays

For excystation assays, *A. castellanii* cysts were prepared using non-nutrient agar plates (3% Oxoid Agar technical using distilled water) as previously described (12). *A. castellanii* trophozoites (2 x 10^6 amoebae) were inoculated on non-nutrient agar plates and incubated at 37°C for up to 14 days to allow trophozoite differentiation into cysts. Following this incubation, no trophozoite was seen when observed under a phase contrast inverted microscope. Next, agar plates were flooded with 15 mL of PBS and placed on a shaker for 30 min at room temperature. After this incubation, cysts were gently scraped off the agar surface using a rubber scrapper. Cysts were collected in a 50 mL tube, followed by centrifugation at 1500 x g for 10 min. The supernatant was discarded and the pellet resuspended in 10 mL PBS. This process was repeated 3X to wash *A. castellanii* cysts and counted using a hemocytometer. To determine the effects of mannose-conjugated porphyrin on *A. castellanii* cysts, excystation assays were performed as described previously (15). Briefly, *A. castellanii* cysts (5 x 10^5 cysts/mL/well) were incubated with 10 µM and 50 µM mannose-conjugated porphyrin, as well as porphyrin and mannose alone for 1 h. Amoebae were collected, washed 3X with PBS and resuspended in RPMI in 24-well

plates. Next, lids were removed and plates were exposed to blue light for 1 h as described above. Finally, cysts were collected and resuspended in growth medium, PYG (0.5 mL PYG medium/well) in 24 well plates. Plates were incubated at 37°C for 72 h and the emergence of actively growing trophozoite was observed microscopically. The trophozoite counts were determined using a hemocytometer.

Results

Synthesis of mannose-conjugated porphyrin was confirmed using NMR spectroscopy

The synthesis of mannose-conjugated porphyrin was achieved in four linear steps (Fig. 1). Initially tetra-phenyl porphyrin was obtained by mixing benzaldehyde and pyrrole in the presence of propionic acid. Tetra-phenyl porphyrin was then converted into mono-nitro phenyl porphyrin by selectively nitrating the para position of phenyl rings. As a result, a mixture of mono and di-nitro phenyl porphyrins were obtained, which were separated by column chromatography. By controlling the equivalents of nitrating mixture, mono nitro product was obtained in higher yield and checked using NMR spectroscopy (Fig. 2). Next, mono-amino phenyl porphyrin was obtained by reducing mono-nitro phenyl porphyrin in the presence of tin dichloride in hydrochloric acid. The reaction yielded mono amino porphyrin that resulted in slight shifting of signals in NMR spectrum (Fig. 3A), however the mass spectra of the product revealed the disappearance of mono-nitro phenyl porphyrin mass peak and formation of desired mono-amino porphyrin was confirmed by peak at 629 m/z (Fig. 3B). Finally, mannose-conjugated porphyrin was obtained by conjugating mono-amino porphyrin with mannose resulting in the formation of imine bond. Mannose-conjugated porphyrin was obtained as solid product which was then washed with dichloromethane and methanol several times to remove

217 excess starting materials and confirmed through spectroscopic analysis. The purity of mannose-
218 conjugated porphyrin was 75% as determined by NMR analysis (Fig. 4A). The
219 spectrophotometric analysis of the obtained product showed that it absorbed light of wavelength
220 ranging from 425-475nm (Fig. 4B).

221

222 ***Mannose-conjugated porphyrin inhibited viability of A. castellanii***

223 To determine the effects of mannose-conjugated porphyrin on *A. castellanii* trophozoites,
224 amoebae were incubated with 10 µM and 50 µM of mannose-conjugated porphyrin and
225 amoebicidal effects were determined. The results revealed that 50 µM of mannose-conjugated
226 porphyrin exhibited significant amoebicidal effects ($P<0.01$ using 2 sample T-test; two-tailed
227 distribution) (Fig. 5). Additionally, amoebicidal effects were confirmed by observing under the
228 microscope (Fig. 6). In contrast, *A. castellanii* incubated with 50 µM mannose alone and
229 porphyrin alone did not exhibit any killing effects. Also, solvent controls such as RPMI, DMSO
230 and 50% chloroform:methanol had no effect on the viability of *A. castellanii* (Fig. 5 and 6).

231

232 ***Mannose-conjugated porphyrin inhibited A. castellanii mediated host cell cytotoxicity***

233 Pre-treatment of *A. castellanii* with mannose-conjugated porphyrin, followed by host cell
234 cytotoxicity assays were performed. Without mannose-conjugated porphyrin, *A. castellanii*
235 produced 95%±8.5 BMEC cell deaths within 24 h. Pre-treatment of *A. castellanii* with mannose-
236 conjugated porphyrin reduced host cell cytotoxicity significantly to 4.9%±2.5 ($P<0.01$, using 2
237 sample T-test; two-tailed distribution) compared to amoeba alone (Fig. 7A). In contrast,
238 incubation of *A. castellanii* with porphyrin, mannose or solvent alone had no protective effects

on host cells (Fig. 7A). Similarly, solvent alone had no effect on BMEC cell cytotoxicity (Fig. 7A).

In co-incubation assays, *A. castellanii* + human BMEC + 50 µM mannose-conjugated porphyrin also reduced host cell cytotoxicity but to a lesser extent of 58%±4 (Fig. 7B) ($P<0.01$, using 2 sample T-test; two-tailed distribution). In the absence of mannose-conjugated porphyrin, *A. castellanii* produced 97%±5 host cell cytotoxicity. Similarly, other compounds and solvents alone did not show any effect on *A. castellanii*-mediated host cell cytotoxicity (Fig. 7B).

Mannose-conjugated porphyrin blocked excystation in A. castellanii

Cysts were pre-treated with and without mannose-conjugated porphyrin and inoculated in fresh growth medium. The findings revealed that *A. castellanii* cysts incubated with PYG alone emerged as viable trophozoites (1.2×10^6 amoebae) (Fig. 8). However, 50 µM mannose-conjugated porphyrin significantly inhibited *A. castellanii* excystation within 72 h ($P<0.01$, using 2 sample T-test; two-tailed distribution), compared with cysts inoculated in PYG alone. In contrast, viable trophozoites emerged in wells containing *A. castellanii* cysts treated with mannose alone, porphyrin alone and solvents alone such as DMSO and 50% chloroform: methanol within 72 h (Fig. 8).

Discussion

The successful treatment of *Acanthamoeba* infections is challenging. For example, current treatment of *Acanthamoeba* keratitis is problematic and involves hourly topical application of a mixture of drugs including chlorhexidine, polyhexamethylene biguanide, propamidine isethionate, neomycin, lasting up to a year and even then recurrence can occur (3).

One intriguing report was made by Ficker et al. (16), which showed the development of propamidine resistance during the course of therapy for *Acanthamoeba* keratitis, which lead to recurrence of the infection. Of concern, several studies have shown the resistance of *Acanthamoeba* to antimicrobial chemotherapy (2, 4, 5). This is of particular concern in the absence of available alternative chemotherapeutic agents. Our recent studies have shown the potential of photochemotherapy, namely the application of photodynamic compounds followed by exposure to a suitable source of UV-Vis radiation (17), however selective targeting of *Acanthamoeba* has remained a major concern. By synthesizing photo-activated compound, porphyrin and its conjugation with mannose to selectively target *Acanthamoeba*, the potential of photochemotherapy against *Acanthamoeba* was investigated in the present study. The results showed that mannose-conjugated porphyrin exhibited potent amoebicidal effects and blocked excystation, *in vitro*. The basis of this technology involves the activation of photosensitizing molecule by exposure to visible light (light of certain wave length). This in turn releases the singlet oxygen or reactive oxygen species which causes cell death (10, 11, 16). However, a major challenge in the use of photochemotherapeutic compound in treating infections caused by eukaryotic pathogens is the specific delivery of photo-activated agent to the pathogen. Previous studies have shown that pathogenic *Acanthamoeba* specifically binds to mannose but not other sugars and have identified MBP as an important adhesin, expressed on the surface membranes of *Acanthamoeba* (7-9). In the present study, mannose sugar was attached to a porphyrin ring to synthesize mannose-conjugated porphyrin, for the targeted delivery of this photo-activated compound to *Acanthamoeba*, which proved highly effective in killing parasite and exhibited both trophicidal effects as well as abolished excystation against *A. castellanii*, *in vitro*.

Notably, pre-treatment and co-treatment of *A. castellanii* with mannose-conjugated porphyrin showed varied host cell cytotoxicity, 4% versus 58% host cell cytotoxicity respectively. In contrast, no treatment resulted in more than 95% host cell cytotoxicity. A likely explanation of higher cell cytotoxicity in co-treatment assays is due to the production of reactive oxygen species produced by photosensitizing compound in the presence of human cells that may have contributed to increased host cell cytotoxicity. It is also interesting to note that mannose-conjugated porphyrin blocked excystation. At 50μM of mannose-conjugated porphyrin, complete inhibition of excystation was observed. This is unexpected as previous studies show that cysts do not exhibit adhesion to the host cells (18), and it was presumed that MBP is not expressed on the external cell wall membranes, however the expression of MBP in *Acanthamoeba* cysts and if so, then its precise localization has not been investigated. The inhibition of excystation as observed in this study could be due to lipid soluble material or prolonged exposure to the photo-activated compound (19, 20), affecting trophozoite emergence from the dormant cyst stage, however the underlying molecular mechanisms of accumulation of photodynamic compound at the cyst wall, its possible traversal across the cell wall, and/or through the operculum membranes to target residing trophozoite inside the shell remains to be investigated, as well as its efficacy *in vivo* and these studies will be the subject of further studies. Future studies are also needed to conjugate porphyrin with *Acanthamoeba*-specific antibody and testing its potential for enhanced targeted therapy against *Acanthamoeba*. Additionally, there is a need to test the possibility of using various photosensitive molecules with high intensity light but shorter duration (pulsed interval treatments) that may prove to be effective, and more feasible for clinical application. If successful, this technology will be particularly useful in treating *Acanthamoeba* keratitis cases due to ease of visible light usage and the fact that the current mode of eye inspection is exposure

of visible light, in addition to disseminated/tissue infections. For latter, several lines of evidence suggest that photodynamic therapy has proven ability against a range of malignant and other diseased cells and tissues (21-26). With targeted killing of pathogenic *Acanthamoeba*, as proposed in this study, photodynamic therapy can be recognized as a treatment strategy which is both minimally invasive and minimally toxic and should be investigated further *in vivo*.

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Figure legends

Figure 1. Steps involved in the synthesis of mannose porphyrin, as described in "Materials and Methods". Briefly, benzaldehyde and pyrrole was mixed in propionic acid to yield tetra-phenyl porphyrin. It was then nitrated in the presence of sodium nitrite and mono-nitrated phenyl porphyrin was formed. Mono-nitrated phenyl porphyrin when neutralized in the presence of ammonium hydroxide yielded mono-amino phenyl porphyrin. Finally the product was mixed with mannose and yielded mannose-conjugated porphyrin. The purity of intermediate compounds synthesized at each step was checked using NMR and Mass spectrometry. For mannose-conjugated porphyrin, mass spectrometry was not performed due to its high polarity.

Figure 2. (A) NMR spectroscopy performed after the synthesis of mono-nitrated phenyl porphyrin as described in figure 1, and **(B)** mass spectrometry performed after the synthesis of mono-nitrated phenyl porphyrin.

Fiogure 3. (A) NMR spectroscopy performed after the synthesis of mono-amino phenyl porphyrin as described in figure 1, and **(B)** mass spectrometry performed after the synthesis of mono-amino phenyl porphyrin.

Figure 4. (A) NMR spectroscopy performed after the synthesis of mannose-conjugated porphyrin as described in figure 1. Mass spectrometry of mannose-conjugated porphyrin was not performed due to its high polarity. **(B)** UV-visible absorption spectrum of mannose-conjugated porphyrin showed that it absorbed light of wave length ranging from 425-450 nm.

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Figure 5. Mannose-conjugated porphyrin exhibited amoebicidal effects against *A. castellanii.* Briefly, amoebae (10^6) were incubated with and without 10 µM and 50 µM of mannose-conjugated porphyrin and exposed to blue light as described in "Materials and Methods". Following this, plates were incubated for 24 h and amoebae were enumerated using a hemocytometer. Treatment of *A. castellanii* with mannose-conjugated porphyrin exhibited significant amoebicidal effects ($P<0.01$, using 2 sample T-test; two-tailed distribution) compared with amoebae incubated alone. In contrast, controls did not show any effect. The data are presented as the mean ± standard error of at least three independent experiments performed in duplicate.

Figure 6. *A. castellanii* was incubated with and without 50 µM of mannose-conjugated porphyrin and representative images were obtained at 100X **(A)** and at 400X **(B)** magnification using a phase-contrast inverted microscope.

Figure 7. (A) *A. castellanii* (5×10^5) pre-incubated with and without 50 µM of mannose-conjugated porphyrin, exposed to light, and their cytotoxic potential was determined using human brain microvascular endothelial cells (BMEC) as described in "Materials and Methods". When pre-treated with mannose-conjugated porphyrin, *A. castellanii* showed limited host cell cytotoxicity ($P<0.01$, using 2 sample T-test; two-tailed distribution) compared with non-treated amoebae. *A. castellanii* incubated with porphyrin, mannose, DMSO and chloroform: methanol also induced host cell cytotoxicity. However, compounds and corresponding solvents alone had no effect on human BMEC cytotoxicity. The data are presented as the mean ± standard error of at least three independent experiments performed in duplicate. **(B)** *A. castellanii* (5×10^5) were

co-incubated with human BMEC and 50 µM of mannose-conjugated porphyrin and exposed to light as described in "Materials and Methods". Co-incubation of 50 µM mannose-conjugated porphyrin with amoeba + human BMEC reduced cell cytotoxicity, albeit at lower levels compared with pre-treatment assays. The results represent the mean ± standard error of three independent experiments performed in duplicate.

Figure 8. Mannose-conjugated porphyrin inhibited excystation of *A. castellanii* cysts. *A. castellanii* cysts were scraped from non-nutrient agar plates and incubated with 50µM mannose porphyrin and exposed to light as described in "Materials and Methods". Following this incubation, cysts were incubated in fresh growth medium, PYG for up to 72 h. After this incubation *A. castellanii* trophozoites were enumerated using a hemocytometer. The dotted line indicates the original inoculum. When treated with 50 µM mannose-conjugated porphyrin, *A. castellanii* cysts did not re-emerge as actively growing viable trophozoites. Viable *A. castellanii* trophozoites emerged in control wells, as well as with all other compound and solvents tested. The results are presented as the mean ± standard error of at least three independent experiments performed in duplicate.

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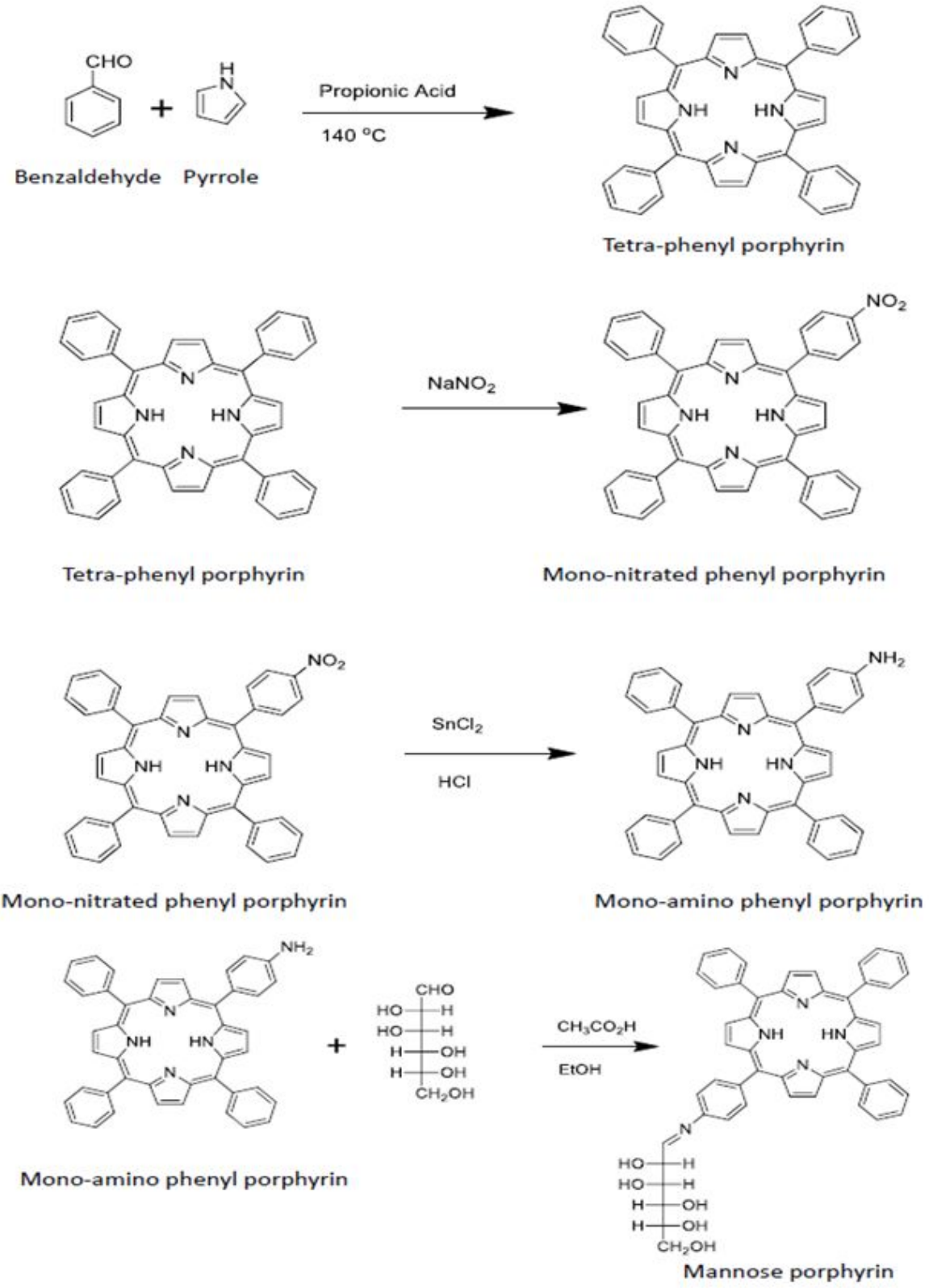
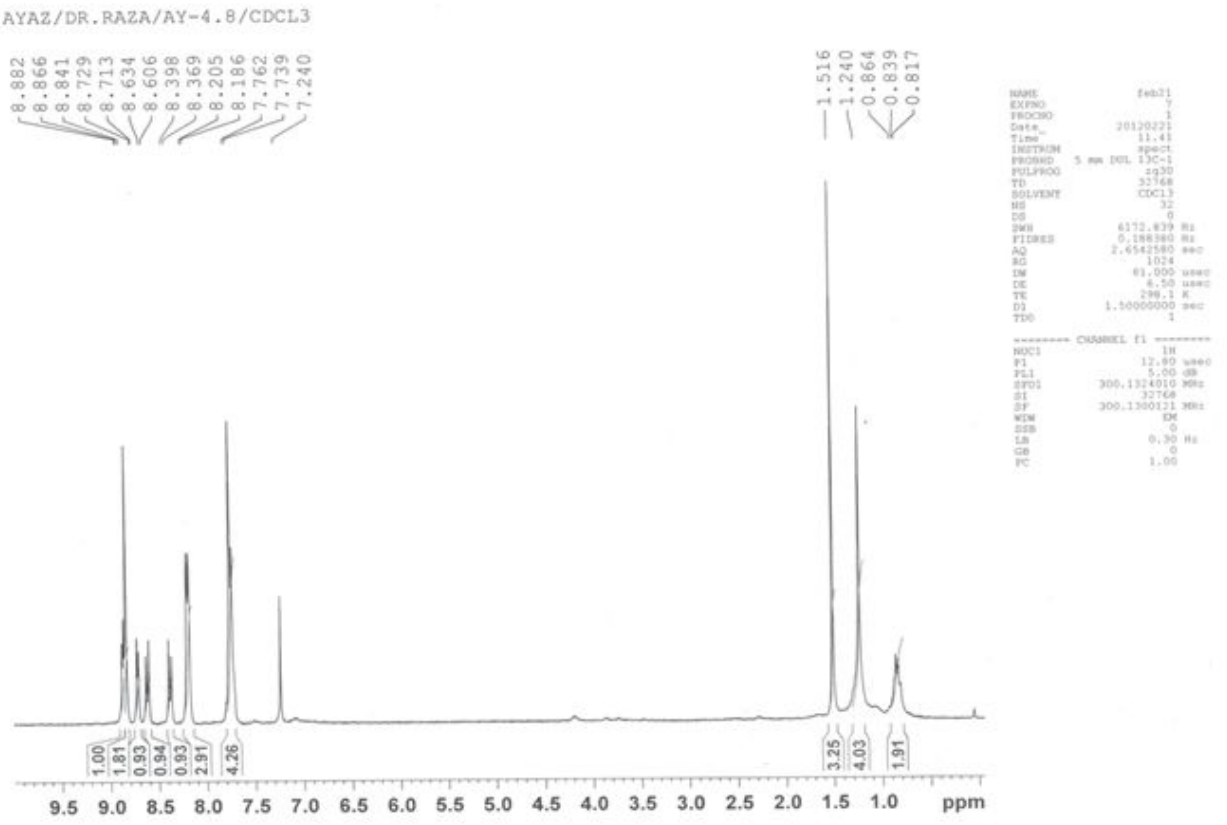


Figure 1. Steps involved in the synthesis of mannose porphyrin, as described in "Materials and Methods". Briefly, benzaldehyde and pyrrole was mixed in propionic acid to yield tetra-phenyl porphyrin. It was then nitrated in the presence of sodium nitrite and mono-nitrated phenyl porphyrin was formed. Mono-nitrated phenyl porphyrin when neutralized in the presence of ammonium hydroxide yielded mono-amino phenyl porphyrin. Finally the product was mixed with mannose and yielded mannose-conjugated porphyrin. The purity of intermediate compounds synthesized at each step was checked using NMR and Mass spectrometry. For mannose-conjugated porphyrin, mass spectrometry was not performed due to its high polarity.

(A)



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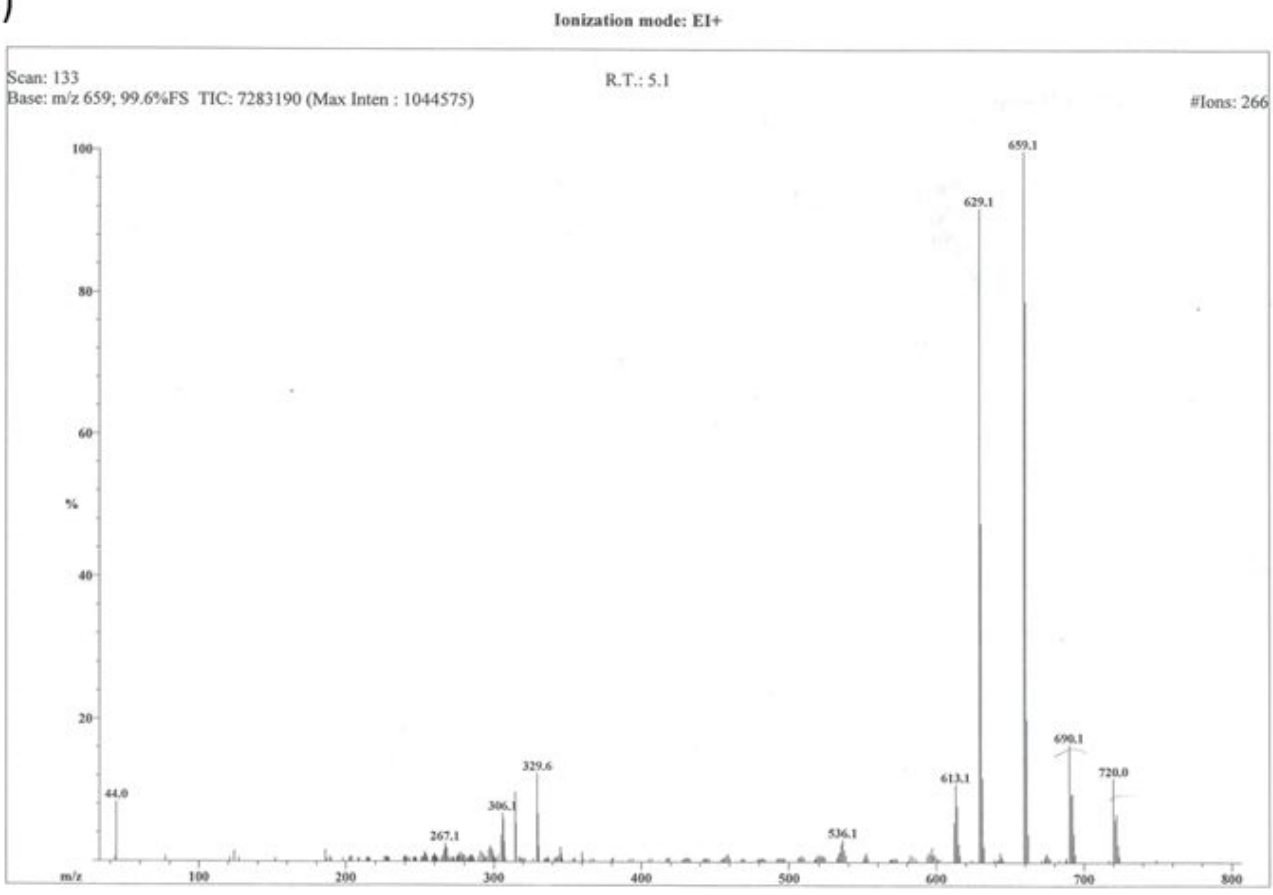
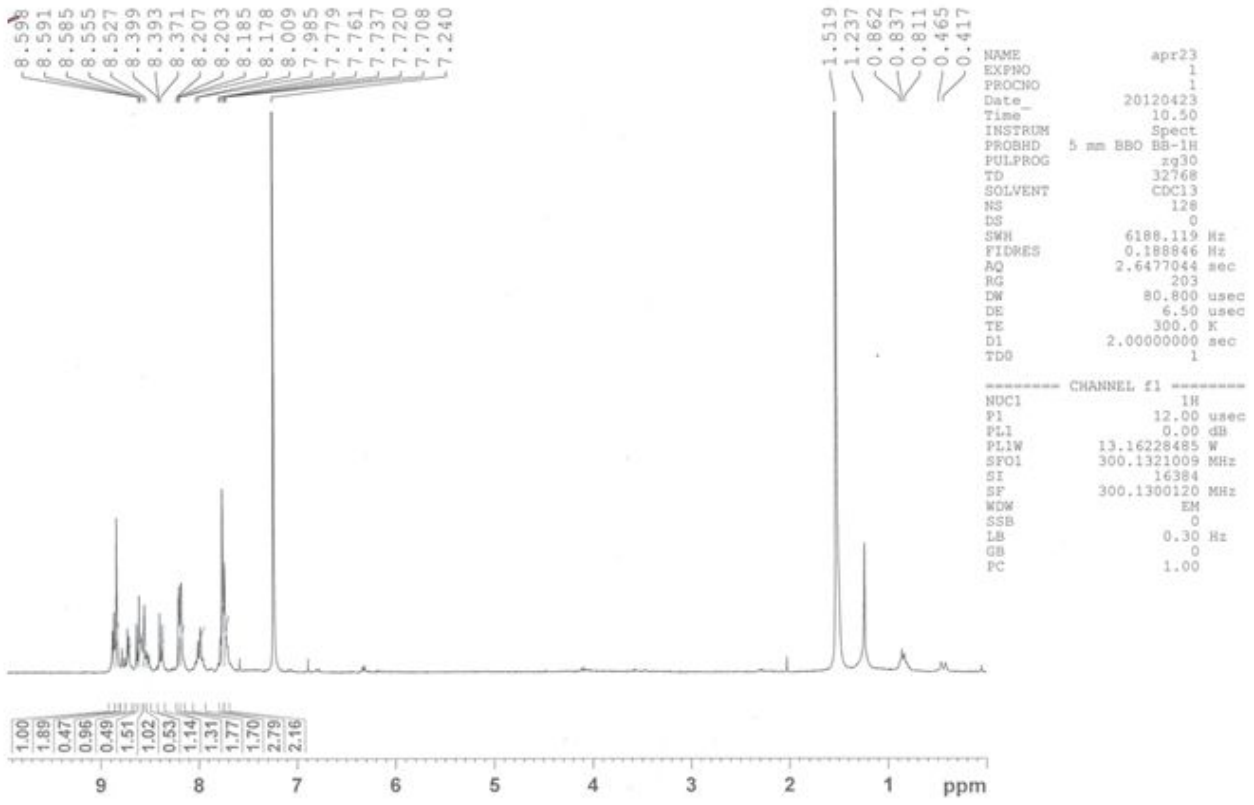


Figure 2. (A) NMR spectroscopy performed after the synthesis of mono-nitrated phenyl porphyrin and **(B)** mass spectrometry performed after the synthesis of mono-nitrated phenyl porphyrin.

(A)



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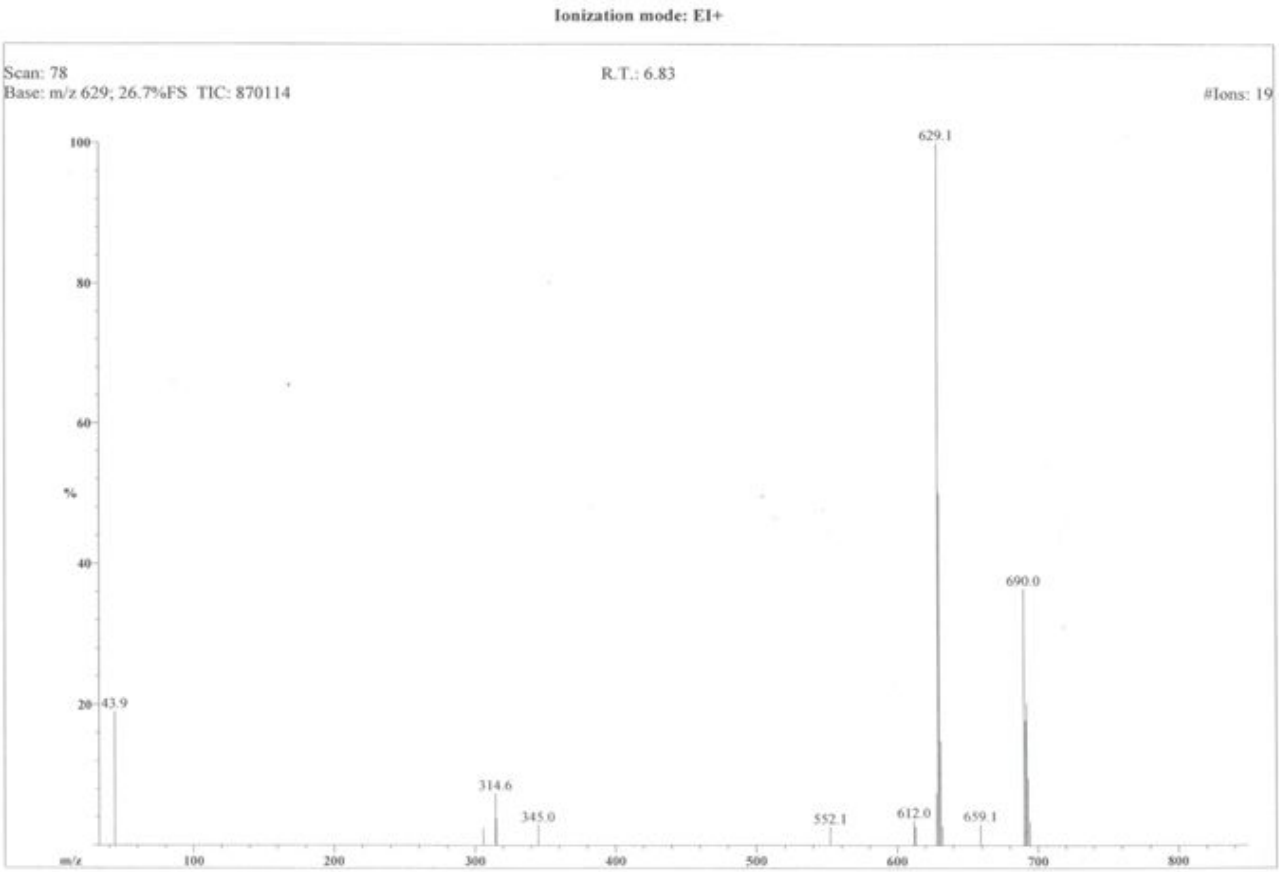
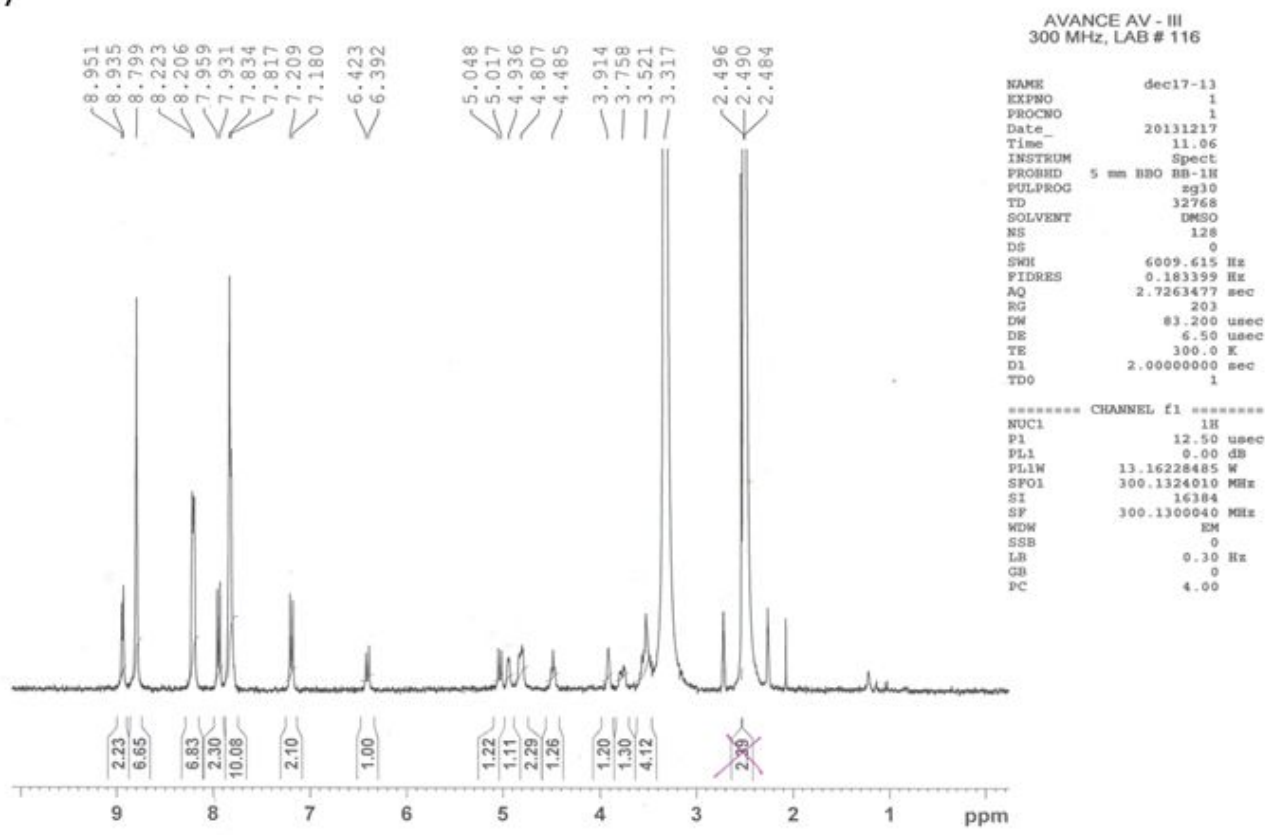
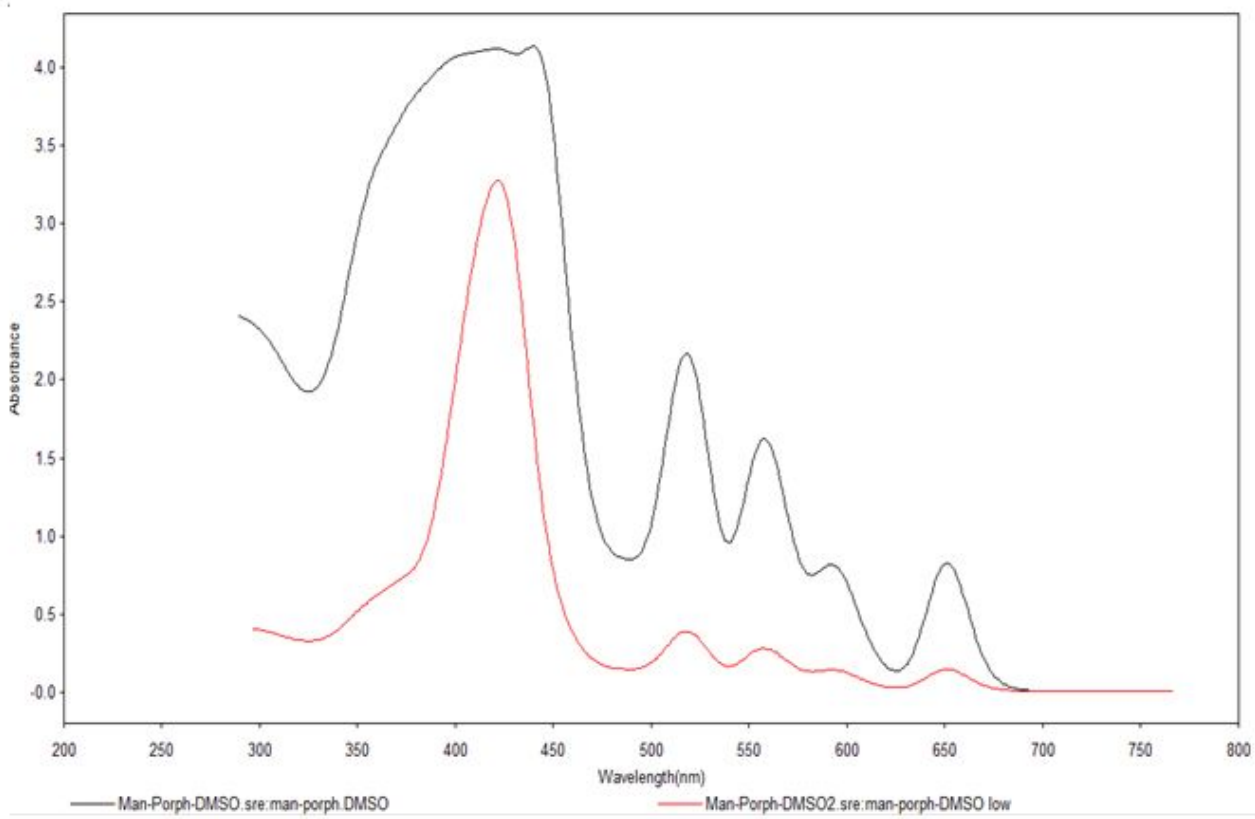


•**Fiogure 3. (A)** NMR spectroscopy performed after the synthesis of mono-amino phenyl porphyrin and **(B)** mass spectrometry performed after the synthesis of mono-amino phenyl porphyrin.

(A)



(B)



•**Figure 4. (A)** NMR spectroscopy performed after the synthesis of mannose-conjugated porphyrin. Mass spectrometry of mannose-conjugated porphyrin was not performed due to its high polarity. **(B)** UV-visible absorption spectrum of mannose-conjugated porphyrin showed that it absorbed light of wave length ranging from 425-450 nm.

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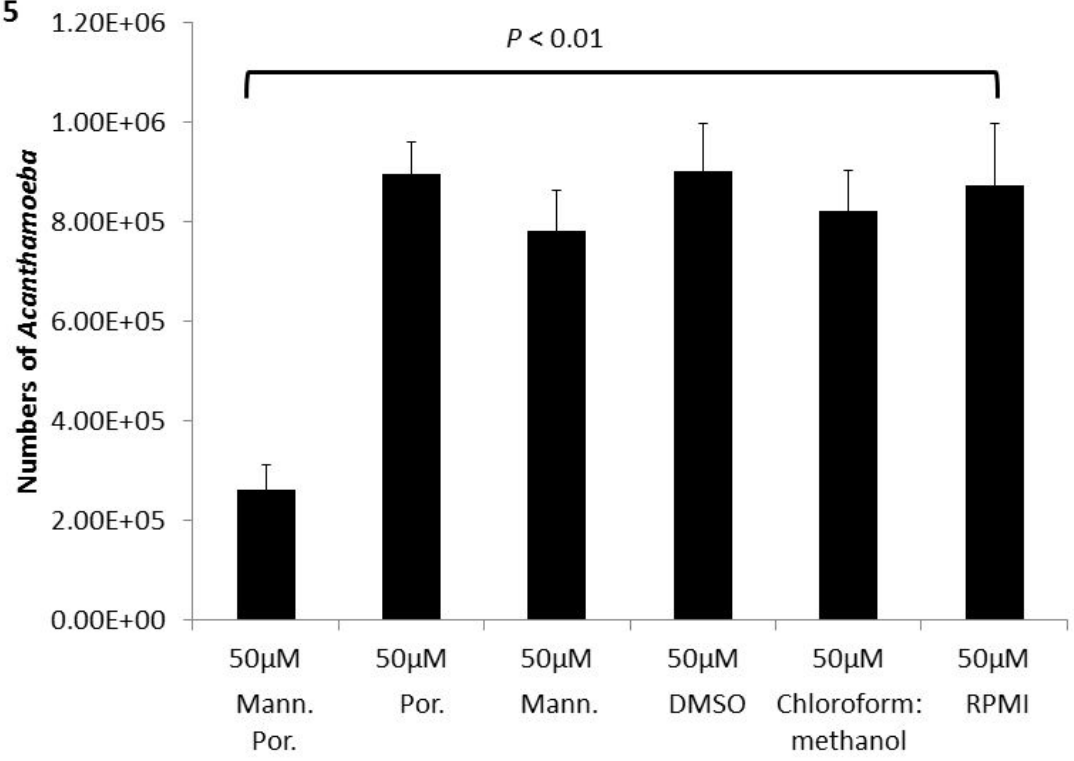
Figure 5



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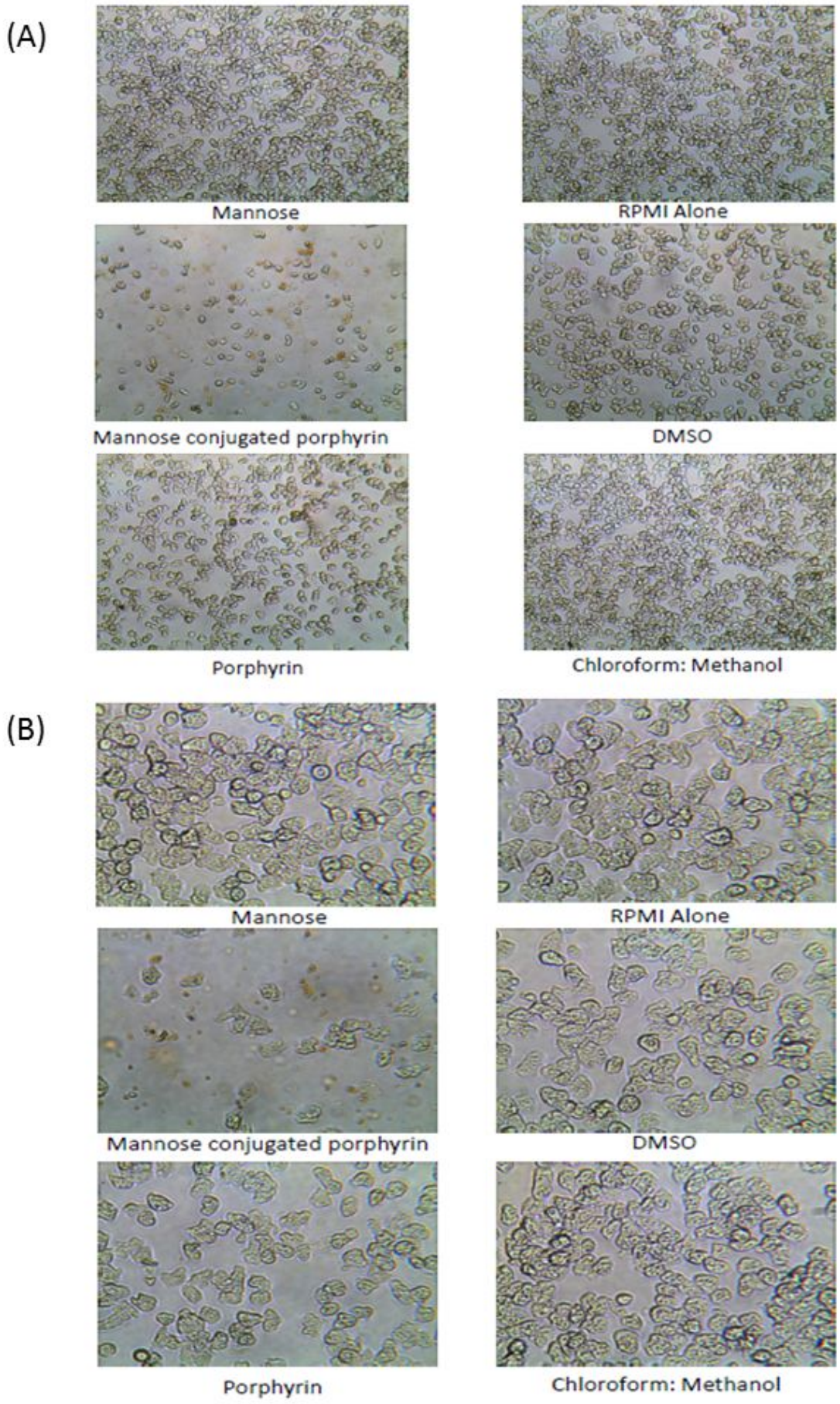

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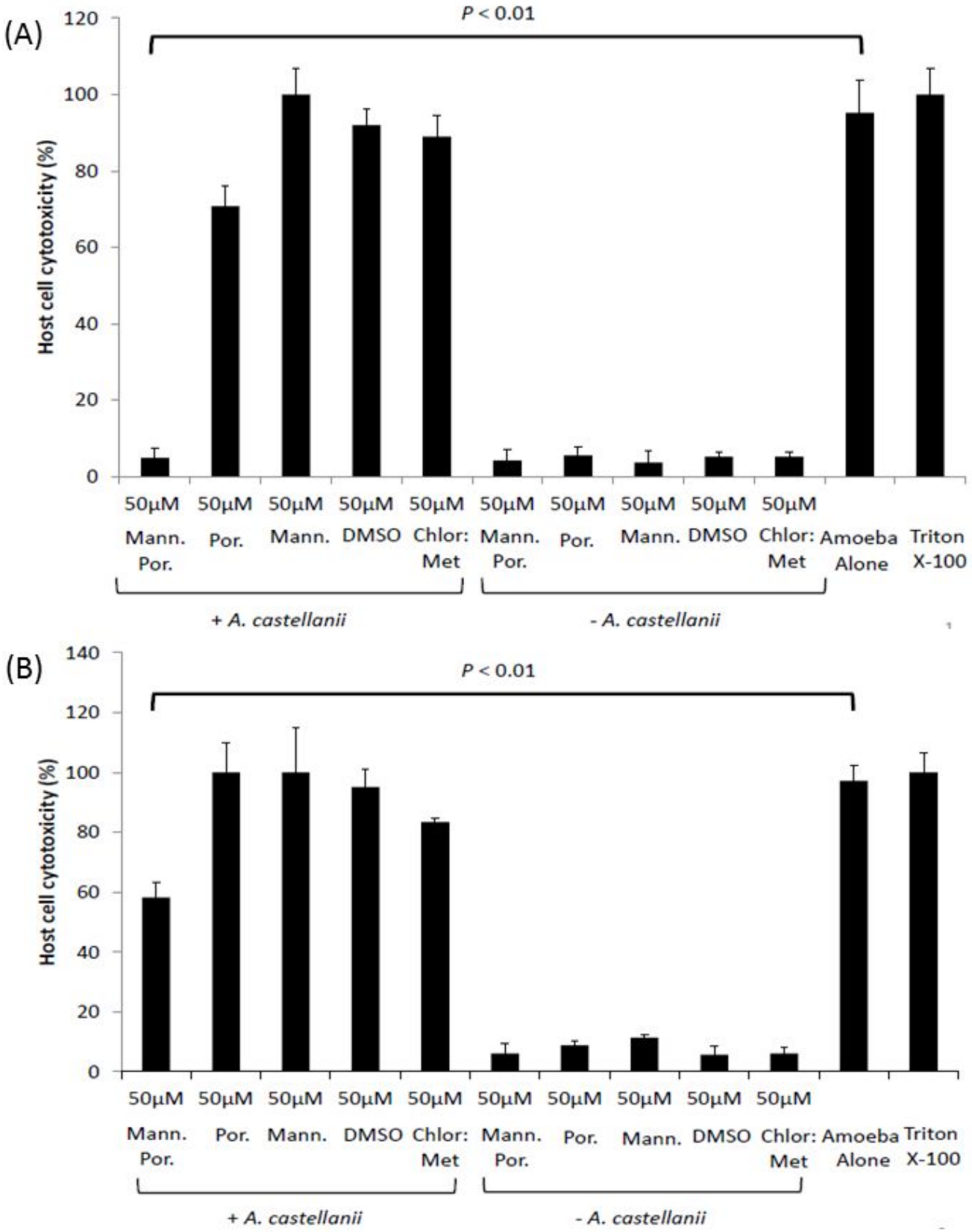

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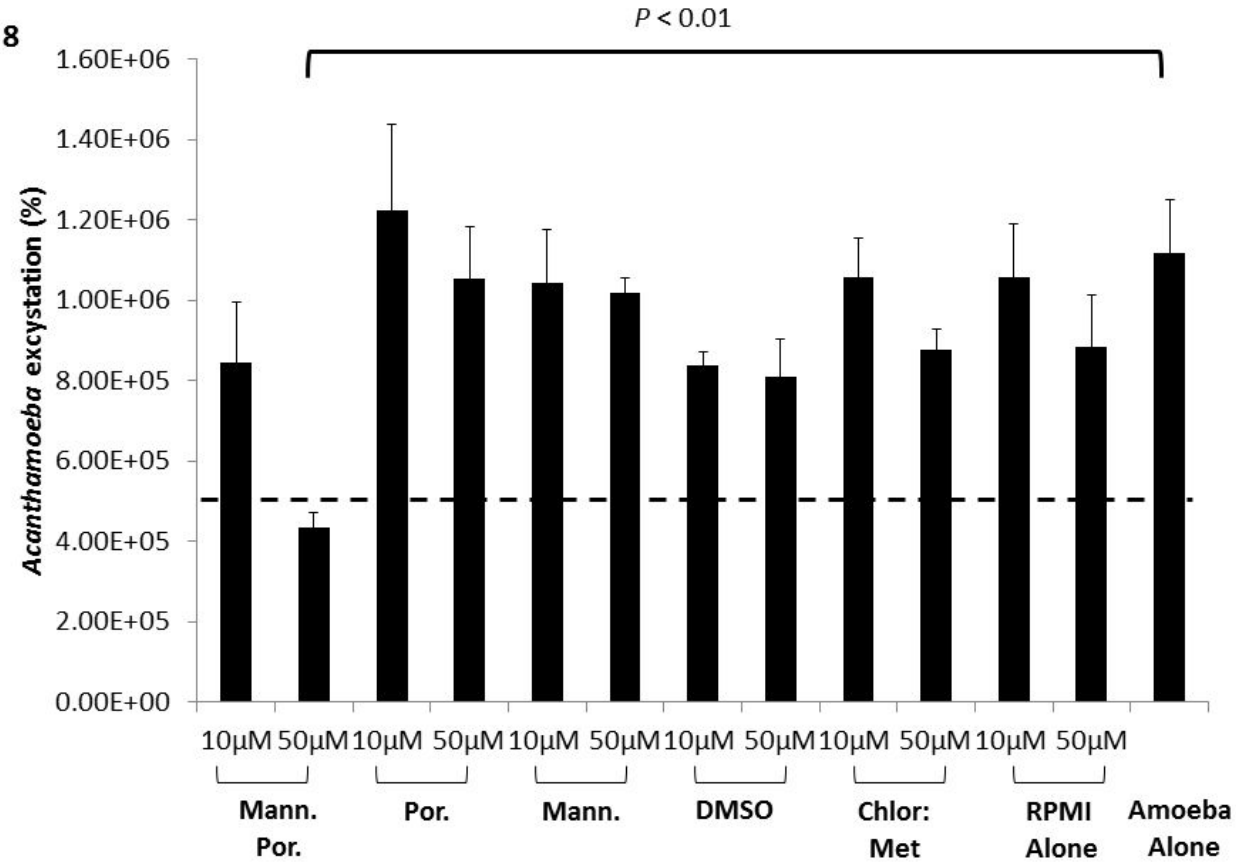
Fig. 8



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