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Malabaricones from the fruit of Myristica cinnamomea King as potential agents against *Acanthamoeba castellanii*

Usman Ahmed [a], Yasodha Sivasothy [b], Khalid Mohammed Khan [c], Naveed Ahmed Khan [d], Siti Mariam Abdul Wahab [e], Khalijah Awang [f g], Muhamad Aqmal Othman [f g] ✉, Ayaz Anwar [a] ✉

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Highlights

- *In-vitro* anti-amoebic activity of Myristica *cinnamomea* King fruit extract and its purified Malabaricones (A-C).
- Malabaricone C showed potent activity against *A. castellanii* with an IC_{50} of 49.95μM.
- Malabaricones (A-C) induced necrotic cell death in trophozoites.
- Malabaricones B and C are possible alternative therapeutic options against *A. castellanii* infections.

Abstract

Acanthamoeba castellanii is an opportunistic free-living amoeba (FLA) pathogen which can cause fatal central nervous system (CNS) infection, granulomatous amoebic encephalitis (GAE) and potentially blinding ocular infection, *Acanthamoeba* keratitis (AK). *Acanthamoeba* species remain a challenging protist to treat due to the unavailability of safe and effective therapeutic drugs and

their ability to protect themselves in the cyst stage. Natural products and their secondary metabolites play a pivotal role in drug discovery against various pathogenic microorganisms. In the present study, the ethyl acetate extract of *Myristica cinnamomea* King fruit was evaluated against *A. castellanii* (ATCC 50492), showing an IC_{50} of 45.102±4.62µg/mL. Previously, the bio-guided fractionation of the extract resulted in the identification of three active compounds, namely Malabaricones (A-C). The isolated and thoroughly characterized acylphenols were evaluated for their anti-amoebic activity against *A. castellanii* for the first time. Among tested compounds, Malabaricone B (IC_{50} of 101.31±17.41µM) and Malabaricone C (IC_{50} of 49.95±6.33µM) showed potent anti-amoebic activity against *A. castellanii* trophozoites and reduced their viability up-to 75 and 80%, respectively. Moreover, both extract and Malabaricones also significantly ($p < 0.05$) inhibit the encystation and excystation of *A. castellanii*, while showed minimal toxicity against human keratinocyte cells (HaCaT cells) at lower tested concentrations. Following that, the explanation of the possible mechanism of action of purified compounds were assessed by detection of the state of chromatin. Hoechst/PI 33342 double staining showed that necrotic cell death occurred in *A. castellanii* trophozoites after 8h treatment of Malabaricones (A-C). These findings demonstrate that Malabaricones B and C could serve as promising therapeutic options against *A. castellanii* infections.

Introduction

A. castellanii is an opportunistic free-living amoeba (FLA) and is ubiquitously present in the environment. Under different environmental conditions, *Acanthamoeba* species exist in two stages: an active trophozoite and a dormant cyst stage (Marciano-Cabral and Cabral, 2003). It is the causative agent of *Acanthamoeba* keratitis (AK), granulomatous amoebic encephalitis (GAE), nasopharyngeal, and cutaneous lesions (Damhorst et al., 2022; Siddiqui and Khan, 2012). There are two main factors that contribute to catastrophic outcomes of *Acanthamoeba* infection: delayed detection or misdiagnosis of the causative agent and the presence of a highly resistant cyst stage of *Acanthamoeba* (Juarez et al., 2018). The delayed misdiagnosis of *A. castellanii* infections is mostly due to misdiagnosis as herpetic keratitis, lack of clinical signs and symptoms such as absence of pain, harboring intracellular microbes, a lack of facilities and expertise, and a delay in recommendation to a care center (Rayamajhee et al., 2022; Shah et al., 2021). Therefore, the proper diagnosis of *A. castellanii* infections is important for effective therapy.

The incidences of *Acanthamoeba* infections are widely varied around the world. Previous studies indicated that the rate of AK in New South Wales, Australia, from 1997 to 2002 was 0.5 per million (Butler et al., 2005). In the USA, the rate of AK cases diagnosed between 2007 and 2009 was 0.8 per million (Yoder et al., 2012). Furthermore, in London, the prevalence from 2011 to 2016 was 5.8 cases per million, while in Coventry, the incidence from 2017 to 2018 was 22.1 per million for this period (Hassan et al., 2019; Carnt et al., 2018). However, to date the mode of action and the whole pathophysiology of *Acanthamoeba*'s infection remain unclear. The literature showed that *Acanthamoeba* has the ability to cross the blood-brain barrier (BBB) and cause GAE (Edwards-Smallbone et al., 2012; Khan and Siddiqui, 2009). The infections caused by *A. castellanii* are rare, but they present difficulties in the development of new therapeutic agents because cysts are resistant to most of the drugs and compounds (Anwar et al., 2018; Ahmed et al., 2022a). Miltefosine is a

repurposed drug that obtained FDA approval to treat AK. However, the drug is still in the development stage, and fewer details about its dosage, optimal timing, role, and route of administration are available (Somanietal., 2019, accessed at https://www.ncbi.nlm.nih.gov/books/NBK549863/ ↗; Tavassolietal., 2018). During the course of treatment, patients with keratitis have experienced severe side effects of the steroid-responsive inflammatory response (Thulasietal., 2021). Therefore, several synthetic and natural compounds have been employed against *A. castellanii* infections (Ahmedetal., 2023; Anwaretal., 2020). However, the majority of reported natural and synthetic compounds have shown potent activity against the trophozoite stage *in vitro* only though these compounds are associated with some limitations such as inability to cross the BBB, non-selectivity, unclear mode of action, and absence of *in vivo* studies (Ahmedetal., 2022a; Elsheikhaetal., 2020).

Natural products from various plants have offered several lead compounds with improved biological characteristics and have demonstrated promising results in the treatment of *Acanthamoeba* infection as reviewed (Niyyatietal., 2016; Weietal., 2019; Wehelieetal., 2022). Plant extracts and their secondary metabolites have been reported to exhibit the ability to limit the growth and survival of *A. castellanii in vitro*. Secondary metabolites from plants such as phenolic compounds, lignans, flavonoids, sesquiterpene lactones, and alkaloids have also shown potent anti-amoebic activity against *Acanthamoeba* species (García-Davisetal., 2018; Anwaretal., 2020; Rodriguez-Exposito etal., 2021; Siddiquietal., 2022).

Myristica cinnamomea King (Myristicaceae), commonly known as cinnamon nutmeg, is distributed in the Malayan Peninsula, Singapore, Borneo and the Philippines. *M. cinnamomea* is a tall tree. Its outer bark is dark brown, while the inner bark is pale brown. The fruit is yellow and globose to broadly globular oblong. Its seeds are red and used as spices (AbdulWahab etal., 2016). From 2016 to 2022, our group identified five acylphenols and four dimeric acylphenols in the bark and fruits of *M. cinnamomea*. These compounds exhibited alpha-glucosidase enzyme inhibitory activity (Sivasothyetal., 2022, 2016a), quorum sensing inhibitory activity (Sivasothyetal., 2016b), and NS2B/NS3 protease inhibitory activity (Sivasothyetal., 2021). Malabaricones A-C have demonstrated a variety of biological functions such as anti-inflammatory, antioxidant, anti-cancer, and anti-leishmanial (Patroetal., 2005; Senetal., 2007; Maityetal., 2012; Othmanetal., 2016; Sivasothyetal., 2022). Among the Malabaricones, Malabaricone C has shown a higher cytotoxic effect against cancer cell lines, which was accredited due to its B-ring catechol moiety, which demonstrates much higher Cu(II)-dependent nuclease activity (Patroetal., 2010).

In search of novel lead compounds against *A. castellanii*, we investigated the potential of *M. cinnamomea* extract and Malabaricones A–C, the major secondary metabolites from the fruits of *M. cinnamomea* in this study. Acylphenol class of secondary metabolites has been documented to possess significant antimicrobial and antiparasitic activities against various pathogens, including bacteria (*S. aureus* and *P. aeruginosa*), fungi (*Magnaporthe grisea, Rhizoctonia solani*, and *Botrytis cinerea*), and protozoan ptotists (*Leishmania donovani*) (Rajimoletal., 2022; Houdkovaetal., 2021; Choietal., 2008; Senetal., 2007). Their structures were characterized by means of NMR and MS spectral analyses. These acylphenols were subsequently evaluated for their anti-amoebic activity

against trophozoite and cyst stages of *A. castellanii*. Their cytotoxicity was evaluated against human cell lines and mode of action was determined using fluorescence microscopy.

Section snippets

Plant material

M. cinnamomea King was collected from Johor, Malaysia, in 2003. The plant was identified by Mr. Teo Leong Eng, and a voucher specimen (KL 5043) was deposited at the Universiti Malaya herbarium. …

Chemicals, extraction, isolation and characterization of Malabaricones (A-C)

The chemicals and reagents used in the present study were analytical grade and commercially available. Chlorhexidine (CHX) was procured from Sigma-Aldrich (San Francisco, USA). The other consumables, such as proteose peptones, D-glucose, yeast extract, phosphate-buffered saline tablets, trypan blue, …

Results

Preliminary screening of the ethyl acetate extract of the fruits of *M. cinnamomea* King at a concentration of 25–200µg/mL revealed that the extract exhibited significant amoebicidal activity against *A. castellanii* (Figs. 1A and 1B). The ethyl acetate extract was subsequently subjected to repeated silica gel column chromatography, Sephadex LH-20 column chromatography, and preparative thin layer chromatography to yield three acylphenols, identified as Malabaricone A, Malabaricone B, and …

Discussion

The treatments for *Acanthamoeba* infections involve a combination of different therapeutic agents such as biguanides, azoles, amidines, etc. However, most of the compounds are toxic to human cells at effective dosage and showed prominent activity against the trophozoites stage of *Acanthamoeba* only (Ahmed et al., 2022a). Therefore, there is an urgent need for the development of new drugs. Our group has assessed several synthetic, natural, and repurposed drugs and their nanoparticles effectively …