RESEARCH ARTICLE

In vitro antifungal activity of *Artabotrys crassifolius* Hook.f. & Thomson against clinical isolates of *Candida* species

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ABSTRACT

The aim of the current study was to examine the antifungal activities of crude extracts of *Artabotrys crassifolius* on the growth of clinical isolates. The leaves and barks of *Artabotrys crassifolius* were extracted sequentially with hexane, chloroform and ethanol. The corresponding crude extracts obtained were then subjected to Kirby-Bauer disc diffusion assay. Among the extracts tested, hexane extract of barks demonstrated prominent inhibitory activity which warrants further isolation and characterisation of the bioactive compounds present in the respective extracts.

Keywords: Antifungal, *Artabotrys crassifolius*, *Candida*

INTRODUCTION

Candida is a genus of yeast-like fungi that reproduce by budding or fission [1]. Many species of this genus are harmless commensals that exist as part of the normal human microflora of the skin, oral cavity, respiratory, gastrointestinal and genitourinary tracts [2,3]. Nonetheless, certain *Candida* species are responsible for causing opportunistic mycoses ranging from superficial mucosal infections to life-threatening systemic diseases, predominantly in immunocompromised patients with cancer, human immunodeficiency virus (HIV) infection or organ transplantation [4].

In spite of the availability of wide array of antifungal agents, candidiasis remains as the fourth leading cause of nosocomial infections with an unacceptably high mortality rate [5,6]. More crucially, majority of the clinically used antifungal drugs are associated with various drawbacks including high toxicity [7], limited efficacy [8], narrow spectrum of activity [9] as well as poor tolerability [10]. Their widespread usage has also led to the rapid development of drug resistant strains during the course of therapy [11]. Consequently, it is imperative to search for alternative strategies for the effective management of *Candida* infections.

The tropical rainforest of Malaysia is considered as one of the most evolved and complex ecosystems in the world that serves a vast untapped biodiversity of natural resources [12]. This unique natural heritage has brought renewed interest in the screening of indigenous medicinal plants for bioactive compounds. Being as one of the largest genera of the family Annonaceae, *Artabotrys* comprises over 100 species of woody climbers and scandent shrubs distributed mainly in tropical and subtropical regions of the world, especially tropical Africa and Eastern Asia. Moreover, *Artabotrys* species have a long history of traditional use for the treatment of different human ailments, particularly malaria, scrofula and cholera [13]. In continuation of our earlier work on the pharmacological properties of medicinal plants [14], the present study was conducted to examine the antifungal activities of crude extracts of *Artabotrys crassifolius* against clinical isolates.

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MATERIALS AND METHODS

Collection and identification of plant material

The leaves and barks of *Artabotrys crassifolius*, with the local name of *akar mempisang*, were collected from Kuala Kangsar, Perak, Malaysia (4°46′N,

100°56′E) in March 2011. The plant was identified and authenticated by Mr. Kamarudin Saleh, Forest Biodiversity Division, Forest Research Institute Malaysia (FRIM). Voucher specimens were prepared and deposited in the Kepong Herbarium (KEP) of FRIM (PID 080311–05), and the School of Pharmacy, Faculty of Science, The University of Nottingham Malaysia Campus (UNMC 65) for future reference.

Preparation of plant material

After removal of extraneous matter, the freshly collected leaves and barks were air-dried in the shade at room temperature for at least 2 weeks. The dried leaves and barks were then finely pulverised by grinding prior to extraction. The pulverised leaves (1.30 kg) and barks (4.79 kg) were extracted sequentially with solvents of increasing polarity starting from hexane (Friendemann Schmidt, Australia), chloroform (Friendemann Schmidt, Australia) and 95% (v/v) of ethanol (John Kollin Chemicals, India). Each extraction was performed in triplicate at a solid-to-solvent ratio of 1:5 (w/v) in a 40°C water bath (Julabo, Germany) for three days. The respective extract was subsequently filtered through qualitative filter papers No. 1 (Whatman International Ltd., England) and the collected filtrate was concentrated to dryness under reduced pressure at 40°C using rotary evaporator (Buchi Labortechnik AG, Switzerland). Eventually, the dried extract obtained was weighed and stored at –20°C until further use. For stock solutions, each crude extract was dissolved in dimethyl sulphoxide (DMSO) (R & M Chemicals, UK) at a concentration of 100 mg/mL and stored at 4°C.

Microorganisms and culture media

The microorganisms used in the present study were clinical isolates as shown in Table 1. Four fungal strains were procured from the Mycology Unit, Department of Medical Microbiology and Immunology, Universiti Kebangsaan Malaysia Medical Centre (UKMMC). Table 1 also includes the types of culture media required for the growth of the respective fungi.

Table 1: Types of microorganisms and culture media

Microorganism	Fungal strain	Culture medium
Clinical isolate		
Yeasts	*Candida albicans*	Potato dextrose broth (PDB) (EMD Chemicals Inc., Germany)
	Candida glabrata	
	Candida parapsilosis	Potato dextrose agar (PDA) (Merck, Germany)
	Candida tropicalis	

Kirby-Bauer disc diffusion assay

The antifungal activities of crude extracts were examined against 4 clinical isolates using Kirby-Bauer disc diffusion method according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) [15], formerly known as National Committee for Clinical Laboratory Standards (NCCLS).

(a) Preparation of supplemented Mueller-Hinton agar

Mueller-Hinton agar (Difco Laboratories, USA) was prepared from a commercially available dehydrated base according to the manufacturer's instructions, and supplemented with 2% (w/v) of D(+)-glucose anhydrous (Systerm, Malaysia) and 0.5 µg/mL of methylene blue (R & M Chemicals, UK) (MH-GMB). Immediately after autoclaving, the agar medium was allowed to cool in a 45°C to 50°C water bath. The freshly prepared and cooled medium was poured into plastic, flat-bottomed Petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm, which corresponded to 25 mL to 30 mL of medium for plates with a diameter of 100 mm. The agar medium was allowed to cool further to room temperature, and unless the plates were used the same day, stored in a 2°C to 8°C refrigerator.

(b) Preparation of impregnated filter paper discs

Qualitative filter paper No. 1 was used to prepare discs approximately 6 mm in diameter, which were sterilised by autoclaving at 121°C for 15 min. Sterile filter paper discs were impregnated with 10 µL of each crude extract (100 mg/mL) to give a final

concentration of 1 mg/disc. Amphotericin B (1 µg/disc) (Sigma-Aldrich, USA) and DMSO were served as positive and negative controls respectively. Impregnated discs were left to dry under laminar flow cabinet overnight.

(c) Preparation of inoculum

Inoculum was prepared by picking five distinct colonies of approximately 1 mm in diameter from a 24 h-old culture grown on PDA. Colonies were suspended in 5 mL of sterile saline. The resulting suspension was vortexed for 15 s and the turbidity was adjusted either visually or with a UV/Vis spectrophotometer (Biochrom Libra, UK) by adding sufficient sterile saline or more colonies to adjust the transmittance to that produced by a 0.5 McFarland standard at 530 nm wavelength. The absorbance at 530 nm should be in the range of 0.12 to 0.15 for the 0.5 McFarland standard. This yielded a yeast stock suspension of 1×10^6 cells/mL to 5×10^6 cells/mL.

(d) Inoculation of test plates

Optimally, within 15 min after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab was rotated several times and pressed firmly against the inside wall of the universal bottle above the fluid level to remove excess inoculum from the swab. The dried surface of a MH-GMB agar plate was inoculated by evenly streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. As a final step, the rim of the agar was swabbed. The lid was left ajar for 3 min to 5 min, but no more than 15 min, to allow for any excess surface moisture to be absorbed before applying the impregnated discs.

(e) Application of discs to inoculated agar plates

The impregnated disc was placed individually using sterile forceps onto the surface of the inoculated agar plate. Each disc was pressed down to ensure complete contact with the agar surface. Eight discs were placed in each plate. The plates were inverted and placed in an incubator (Binder, Germany) set to 35°C within 15 min after the discs were applied.

(f) Reading plates and interpreting results

After 20 h to 24 h of incubation, each plate was examined. The diameters of the zones of complete inhibition (as judged by the unaided eye) were measured, including the diameter of the disc. Zones were measured to the nearest whole millimetre at the point at which there was a prominent reduction in growth, using sliding callipers (American Scientific LLC, USA) or a ruler, which was held on the back of the inverted Petri plate. The Petri plate was held a few inches above a black, non-reflecting background and illuminated with reflected light. Eventually, the sizes of the zones of inhibition were interpreted.

Statistical analysis

Statistical analysis of experimental data was performed using Microsoft Office Excel data analysis software. Data were presented by descriptive analysis as mean and standard deviation of three independent experiments performed in triplicate.

RESULTS AND DISCUSSION

Plants have received considerable research attention from the scientific community as potential candidates for the development of antifungal drugs [16]. The utilisation of medicinal plants appears to be an alternative to synthetic antibiotics in the prevention and treatment of *Candida* infections because they are relatively safe, easily accessible as well as inexpensive [17,18]. In the current study, Kirby-Bauer disc diffusion assay was performed to examine the antifungal activities of crude extracts against clinical isolates.

This qualitative method is widely employed for antibiotic susceptibility testing in which filter paper discs impregnated with antifungal agents are applied on the inoculated agar plate [19]. The efficacy of these agents can subsequently be determined by measuring the diameter of the zones of inhibition that resulting

from their diffusion into the agar medium around the discs [20].

The inhibitory effects of crude extracts on the growth of clinical fungal strains are illustrated in Fig. 1. All the crude extracts were found to be devoid of antifungal activity except for hexane extract of barks which was able to inhibit the growth of the tested *Candida* species with zones of inhibition ranging from 7.81±0.27 mm to 9.77±0.25 mm. This indicates that the non-polar active principles present in the barks may be responsible for the antifungal properties of this plant.

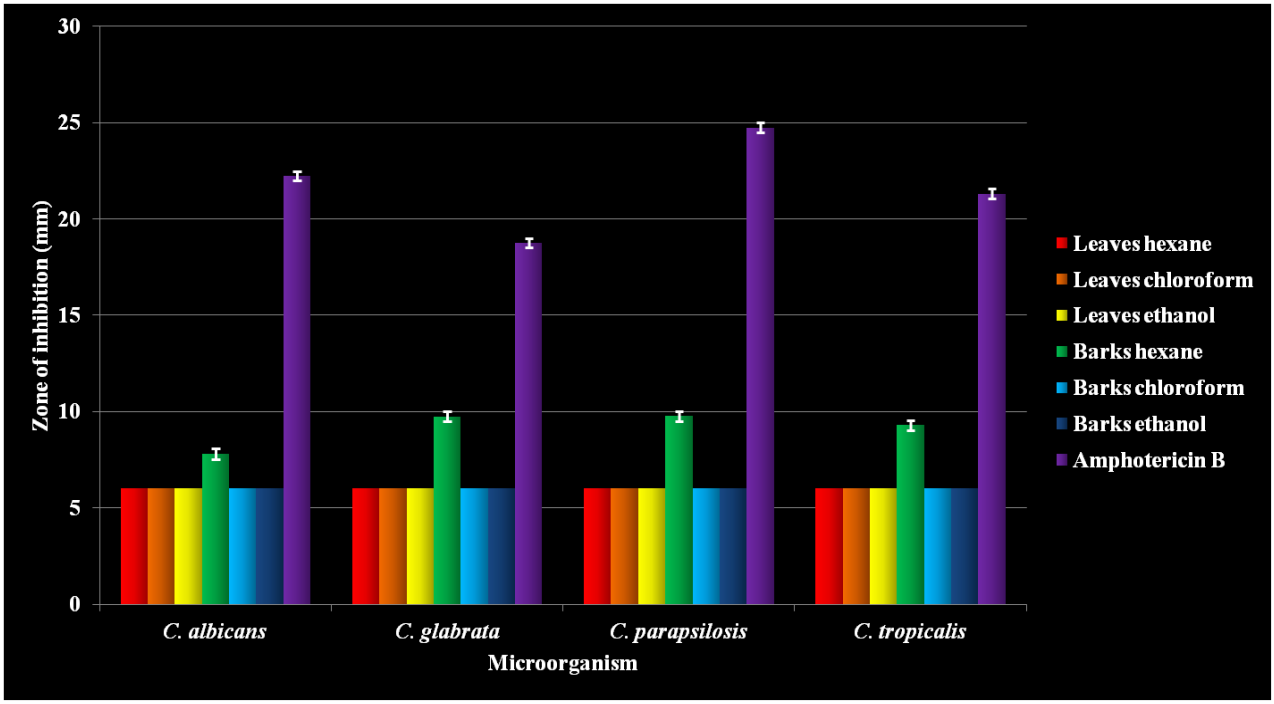

Fig. 1: Antifungal activities of crude extracts of *Artabotrys crassifolius* against clinical isolates. Results are expressed as mean ± standard deviation of three independent experiments performed in triplicate, n = 9.

Furthermore, the positive control, amphotericin B, produced zones of inhibition ranging from 18.76±0.25 mm to 24.74±0.25 mm against the tested *Candida* species. On the contrary, no inhibitory activity was detected in the negative control, DMSO. This implies that DMSO, the solvent used for the reconstitution of crude extracts, does not affect the susceptibility of the clinical fungal strains to the respective extracts.

Candida albicans is the most commonly isolated etiologic agent of candidiasis [21]. Nevertheless, there has been a significant epidemiological shift in the species of *Candida* causing nosocomial candidemia, with the emergence of non-*albicans Candida* species, particularly those exhibiting reduced susceptibility or intrinsic resistance to antifungal drugs [22,23]. The emerging species of clinical importance include *Candida glabrata*, *Candida krusei*, *Candida parapsilosis* and *Candida tropicalis* [24]. Considering the *Candida* species evaluated in the present study, *C. parapsilosis* was shown to be the most sensitive species, followed by *C. glabrata* and *C. tropicalis*, with *C. albicans* being the least susceptible to hexane extract of barks. This suggests that the corresponding extract may be more effective in inhibiting the growth of non-*albicans Candida* species.

With regard to the phytochemical analysis of crude extracts [12], the occurrence of alkaloids, cardiac glycosides and terpenoids in hexane extract of barks may explain its superior activity as compared to the other crude extracts tested. This warrants further isolation and characterisation of the potentially active principles from the respective crude extract.

CONCLUSION

Investigation of the *in vitro* antifungal activity of *Artabotrys crassifolius* revealed that hexane extract of

barks may be an important source of novel antifungal compounds in consideration of its prominent inhibitory activity predominantly against non-*albicans* *Candida* species. Therefore, further studies are required to isolate and characterise the bioactive compounds responsible for the observed antifungal properties of *Artabotrys crassifolius*.

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