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Activity of ethanolic extracts of *Asparagopsis taxiformis* against the major molecular types of *Cryptococcus neoformans/C. gattii* complex

Giuseppa Genovese, Orazio Romeo, Marina Morabito*, Domenico Alessi, Giuseppe Criseo and Caterina Faggio

Department of Biological and Environmental Sciences, University of Messina, Viale F. Stagno d'Alcontres, 31, 98166 Messina, Italy.

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Infections due to *Cryptococcus neoformans/C. gattii* complex have been reported to afflict, not only humans but also other mammals including seabirds and cetaceans, proving that the actual animal exposure to these fungi in nature could be underestimated. In this study, antifungal activity of ethanolic extracts obtained from red alga *Asparagopsis taxiformis* was evaluated against eight major genotypes of the *C. neoformans/C. gattii* complex, using both disk diffusion and microdilution broth methods. The algal extracts were active against all fungal strains tested and were not cytotoxic to human red blood cells. This study suggests that *Asparagopsis taxiformis* extracts possess attractive antifungal properties which should encourage the search for new drugs derived from marine algae.

Key words: Cryptococcosis, *Cryptococcus neoformans*, *Cryptococcus gattii*, *Asparagopsis*, seaweeds, red algae, crude extracts, antifungal.

INTRODUCTION

Due to the great genetic diversity, marine algae produce a large amount of secondary metabolites, most of which have been isolated and are under investigation to develop new pharmaceuticals (Smit, 2004; Chanda et al., 2010; Dang et al., 2011). Moreover, seaweeds have been recognized as an important source of bioactive compounds having therapeutic potential to treat important human diseases like cancer, Acquired Immune-Deficiency Syndrome (AIDS) and a variety of bacterial and fungal infections (Mayer et al., 2011; Omar et al., 2012).

Some algal crude extracts showed significant *in vitro* activity against different fungal species which makes them interesting for screening natural antifungal products (Saidani et al., 2012). Therefore, it is important to study the activity of these extracts to identify promising candidates to combat fungal contamination including infections.

Currently, there are relatively few agents that can be used against pathogenic fungi and the continuous search for new natural molecules with potential antifungal activity is very important (Sudhir et al., 2010).

Fungi may be part of the normal flora in marine mammals and they are widely distributed in nature. Some are opportunistic, causing disease when the animal is in some way compromised. The incidence of life-threatening fungal infections has markedly increased in recent years and cryptococcosis is one of the most important opportunistic infections in mammals, including humans, afflicting not only immunocompromised individuals but also apparently immunocompetent subjects (Del Poeta and Casadevall, 2012).

Cryptococcosis is a systemic potentially fatal disease that is worldwide in distribution. It is caused by members of the genus *Cryptococcus*, namely *Cryptococcus neoformans*, which also includes the two biovarieties *C.*

*Corresponding author. E-mail: morabitom@unime.it. Tel: +39-0-9039-1940. Fax: +39-0-9039-2686.

neoformans var. *grubii* and *C. neoformans* var. *neoformans* and *Cryptococcus gattii* (Springer and Chaturvedi, 2010).

The development of molecular typing techniques have greatly extended our knowledge of the C. *neoformans*/*C. gattii* epidemiology and several genotyping methods are currently used to classify clinical and environmental strains into eight distinct molecular types: VNI and VNII (*C. neoformans* var. *grubii*, serotype A); VNIII (*C. neoformans* AD hybrid, serotype AD); VNIV (*C. neoformans* var. *neoformans*, serotype D); VGI, VGII, VGIII and VGIV (C. *gattii* serotypes B and C) (Meyer et al., 2003; Romeo et al., 2012). Furthermore, there is increasing evidence that these molecular types may represent cryptic species (Ngamskulrungroj et al., 2009; Bovers et al., 2008) with differences found in important traits, including virulence and susceptibility to antifungal agents (Chong et al., 2010). In this topic, the trend in drug discovery from natural resources emphasizes the research on marine environment in order to obtain various antifungal compounds.

Asparagopsis species, as well as other species of the family Bonnemaisoniaceae (Rhodophyta), are well known as sources of halogenated compounds (Mata et al., 2012) and are reported to have a strong antifungal, antibacterial and antiprotozoal activity (Salvador et al., 2007; Genovese et al., 2009, 2012).

Asparagopsis taxiformis (Delile) Trevisan de Saint-Léon is a tropical to warm temperate species, which exhibits a strong invasive behavior and is included in the list of the "Worst Invasives in the Mediterranean Sea" (Zenetos et al., 2010). For such species, as for many other marine algae, recent phylogeographic approaches have shown that it consists of a number of distinct lineages, probable cryptic species, biologically and genetically distinct but with similar morphology (Andreakis et al., 2007; Sherwood, 2008).

The aim of this study was to evaluate the activity of the crude extracts obtained from *Asparagopsis taxiformis* (AM020) collected from the Strait of Messina (Italy), against the major molecular types of *C. neoformans/C. gattii* complex. Moreover, in order to evaluate cytotoxicity, algal extracts were tested on human red blood cells *in vitro*.

MATERIALS AND METHODS

Sampling, identification and preparation of algal extract

Plants of *A. taxiformis* (AM020) were collected from the northeastern Sicilian coast of the Strait of Messina (Italy) in February 2011. Samples were identified by sequencing the 5' end of the mitochondrial cytochrome *c* oxidase gene (COI-5'), the official barcode proposed at the Consortium for the Barcode of Life (CBOL, www.barcodeoflife.org) for red algae (Le Gall and Saunders, 2010). COI-5' sequences were obtained according to the protocols described in the study of Manghisi et al. (2010) and contrasted by the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov). The population under investigation resulted *A. taxiformis* "lineage 2" [Genbank accession number JN642177 (Genovese et al., 2012)].

Fresh algae were washed in sterile seawater, manually cleaned of epiphytes, lyophilized (Laboratory freeze dryer LB4, Labco, Italy) and stored at -20°C. For crude extract preparation, 20 g of *A. taxiformis* lyophilized were soaked in ethanol at room temperature for 48 h. Extracts were dried with a Rotavapor® (Rotavapor-R, Buchi Labortechnik, Switzerland) at low temperature (35°C) to preserve volatile compounds from evaporation. Three distinct extractions were performed.

Cytotoxicity assay

Algal extracts were dissolved in saline solution (0.9% NaCl) at concentrations of 4 mg ml^{-1} and incubated with human red blood cells for 30, 60 and 90 min. A control test was performed with human red blood cells incubated in saline solution without algal extracts. Fresh human blood was collected from healthy adult donors (Bonaccorsi et al., 2013).

Cell viability was determined by trypan blue exclusion method. The percentage of unstained cells represents the percentage of viable cells in the suspension. The experiments were performed in triplicates and statistical analyses were performed using the Student's test (McDonald, 2009).

Antifungal activity

Disc diffusion method

Algal extract was assayed for antifungal activity against *C. neoformans/C. gattii* strains listed in Table 1. The identities of all strains were confirmed according to conventional phenotypic tests and URA5-RFLP analysis as described by Meyer et al. (2003).

The set of the yeast strains used here represents the eight major molecular types found so far for *C. neoformans* and *C. gattii* (Meyer et al., 2003). Isolates were long-term stored as water suspensions at 4°C. Before testing, each isolate was subcultured on Sabouraud dextrose agar (Oxoid, Italy) and Staib agar to ensure purity and viability (Romeo et al., 2012).

Antifungal activity of the algal extract was initially evaluated by the disk diffusion method according to the procedures reported in the National Committee for Clinical Laboratory Standards (NCCLS) document M44-A (NCCLS, 2004). Tests were carried out using RPMI-1640 agar (Gibco®, Invitrogen, Italy) supplemented with 2% of glucose and buffered to pH 7.0 with 0.165 M morpholinepropanesulphonic acid (MOPS) (Sigma-Aldrich, Italy). Sterile filter paper discs (7.0 mm diameter) were impregnated with 20 µl of the algal extract (10 mg/disc) dissolved in 100% DMSO (dimethylsulphoxide) (Sigma-Aldrich, Italy) and dried.

The inocula of the yeast strains were prepared from overnight Sabouraud broth cultures and suspensions were adjusted to 0.5 McFarland standard turbidity (approximately 5 x 10^6 cfu ml^{-1}).

RPMI-1640 agar plates were inoculated with 1 ml of standardized fungal suspensions and discs containing the algal extract were immediately placed on the surface. Discs impregnated with only 100% DMSO were used as negative control. All plates were incubated at 30°C for 48 h. At the end of the incubation period antifungal activity was evaluated by measuring the zones of inhibition. The experiments were performed in triplicates and the mean values of the diameter of inhibition zones and their standard deviations were calculated.

Minimal inhibitory concentration (MIC)

A microdilution broth method was used to determine the minimal

Table 1. Genetic background of the *Cryptococcus* strains examined and antifungal activity of *Asparagopsis taxiformis* extract obtained in this study.

Species	Strain/origin	Serotype/ genotype	IZD* (mm)	MIC (mg ml^{-1})
C. neoformans var. *neoformans*	ATCC[1] MYA-565 (JEC21)/USA	D/VNIV	30±0.15	1.56
C. neoformans var. *grubii*	ATCC[1] 202881 (H99)/USA	A/VNI	20±0.14	3.12
C. neoformans var. *grubii*	MAL[2] 042/Reggio Cal., Italy, 1995	A/VNI	20±0.32	3.12
C. neoformans var. *grubii*	MAL[2] 044/Reggio Cal., Italy, 1995	A/VNI	19±0.28	3.12
C. neoformans var. *grubii*	MAL[2] 048/Reggio Cal., Italy, 1995	A/VNI	20±0.32	3.12
C. neoformans var. *grubii*	CBS[3] 10084 (WM626)/Australia	A/VNII	28±0.16	1.56
C. neoformans AD hybrid	CBS[3] 132/Italy	AD/VNIII	32±0.24	1.56
C. gattii	CBS[3] 11860/Italy	B/VGI	22±0.18	3.12
C. gattii	WM163 /Australia	B/VGI	21±0.26	3.12
C. gattii	CBS[3] 6956 (NIH444)/USA	B/VGII	16±0.14	3.12
C. gattii	CBS[3] 6955 (NIH191)/USA	C/VGIII	16±0.16	3.12
C. gattii	CBS[3] 10101 (WM779)/South Africa	C/VGIV	21±0.36	3.12

*IZD (Inhibition zone diameter) represent the mean values of experiments done and respective standard deviation; [1]ATCC: www.atcc.org; [2]Culture collection housed at the Department of Life Sciences "M. Malpighi", University of Messina, Italy; [3]CBS: www.cbs.knaw.nl

inhibitory concentration (MIC) according to NCCLS reference document M27-A2 (NCCLS, 2002). The serial doubling dilutions of algal extract were prepared in RPMI-1640 medium supplemented with 2% of glucose and buffered to pH 7.0 with 0.165 M morpholinepropanesulphonic acid (MOPS) with concentrations ranging from 0.39 to 12.5 mg ml^{-1}. Extract-free and yeast-free controls were included.

Cultures of fungal strains in Sabouraud dextrose broth were adjusted to 0.5 McFarland standard turbidity (BD diagnostics, Italy) and 5 µl were used to inoculate each tube containing 1 ml of RPMI-1640 medium with algal extract. The tubes were incubated aerobically at 30°C for 48 to 72 h and MICs were determined. The MIC was calculated by two independent observers as the lowest extract concentration with no fungal growth. All experiments were performed in triplicate.

Statistical analysis

Statistical analysis were performed using Student's t-test. All calculations were carried out using Prism version 4.00 statistical software (GraphPad Software Inc., USA).

RESULTS AND DISCUSSION

Results of antifungal activity obtained using both disc diffusion and microdilution broth method are provided in Table 1 and Figure 1. The extracts of *A. taxiformis* (GenBank: JN642177) were active against all fungal strains tested without significant differences among the three extractions.

The best results in terms of sensitivity were obtained by disc diffusion method in which *C. neoformans* AD hybrid showed highest inhibition followed by *C. neoformans* var. *neoformans* and var. *grubii* (Table 1). Inhibition zone diameters observed for these *Cryptococcus* strains also showed a good correlation with MIC values obtained by microdilution broth method. In fact, using this method, highest inhibitory activity (MIC mg ml^{-1}) was seen against the VNIII, VNIV, and VNII genotypes which correspond to the three susceptible strains cited above. For all other tested strains the MIC value was 3.12 mg ml^{-1} and variability in zone diameters was observed (Table 1). Although these values are not still comparable with those obtained by commonly used antifungal agents (López-Jodra et al., 2000), it is reasonable to think that a chemical characterization and purification of the crude extract could contribute to the achievement of such objectives.

Algal extracts were not cytotoxic to human red blood cells in tested conditions. The trypan blue assay showed a viability of 92 to 100% in all experiments (data not shown). There were no statistically significant differences in the viability of cells exposed at different algal extracts or after various incubation times.

Currently, there are relatively few antifungal agents and the development of new therapies, especially with the use of natural molecules, is very important. Seaweeds are a valuable resource for this purpose and species of the family *Bonnemaisoniaceae*, including *A. taxiformis*, are well known as sources of metabolites with strong antifungal, antibiotic and antiprotozoal activity *in vitro* (Gonzalez del Val et al., 2001; Salvador et al., 2007; Genovese et al., 2009).

In recent years, members of the *C. neoformans/C. gattii* complex have attracted a considerable attention because cryptococcosis has become a significant public global health problem worldwide afflicting not only debilitated hosts but also apparently healthy humans and animals including marine mammals (Higgins, 2000; Springer and Chaturvedi, 2010).

Although, numerous studies have focused on virulence,

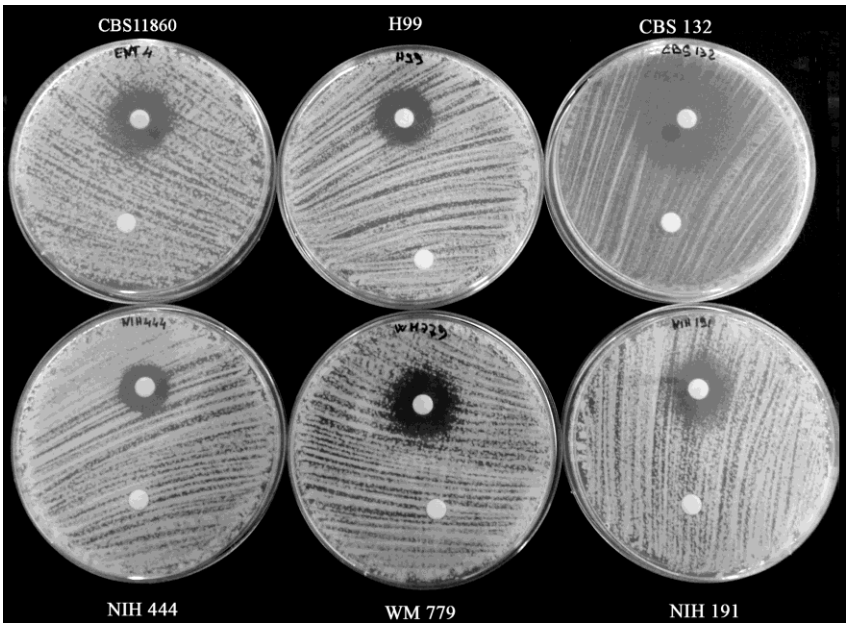

Figure 1. Antifungal activity of ethanolic extract from *Asparagopsis taxiformis* obtained by disk diffusion method (random selected strains are showed). Top: varying sizes of the inhibitory zone present around the disks impregnated with algal extract; Bottom: negative controls.

genotyping and epidemiology of these pathogenic fungi, still little is known about the susceptibility to antifungal compounds of the major molecular types of *C. neoformans/C. gattii* complex. However, a more recent study revealed a clear correlation between antifungal susceptibilities and genotypes (Trilles et al., 2012) emphasizing the importance of discovering new molecules active against all molecular types so far known.

It is well-known that *C. neoformans* and/or C. *gattii* are acquired from the environment by inhalation of spores or desiccated yeast cells and have been reported to be able to colonize trees, associated with pigeons and their habitat and possibly with seabird colonies. Reports of cryptococcal infections in dolphins speculated that seabirds might be the source of infections and that skin lesions due to oil and gas pollution might allow skin-associated microorganisms access to the blood flow (Miller et al., 2002; Mouton et al., 2009). In addition, although not a true halophile, *C. gattii* recently was shown to be able to survive for long periods of time in 35‰ sodium chloride solution, a concentration equivalent to that of seawater (Kidd et al., 2004). At the light of the previous, the actual exposure of marine mammals to *C. neoformans/C. gattii* complex could be underestimated in nature. Therefore, this pathogen merits more attention so its environmental occurrence and role in cryptococcosis can be accurately determined.

As demonstrated in this study, *A. taxiformis* extracts were active against all isolates of the *C. neoformans/C. gattii* complex examined here, indicating that this seaweed is a promising source of bioactive molecules to use against these clinically relevant pathogens.

At present, *Asparagopsis* species are cultivated for dermo-cosmetical and parapharmaceutical purposes in Atlantic Europe, especially in France, Portugal, Ireland, as well as in Hawaii, Indonesia, Philippines, and New Zealand (Kraan and Barrington, 2005; Mata et al., 2012).

Biomass yield proved to be high in nutrient rich water, such as the effluents of fish farm, where the alga acts as a biofilter in integrated multi-trophic aquaculture (IMTA) (Barrington et al., 2009). Therefore, in our opinion, the most preferable biomass supply for pharmaceutical uses should come from IMTA, rather than from the exploitation of natural resources, which could not give constant biomass yields.

Crude extracts of *A. taxiformis* conveys an amount and diversity of compounds with potential *in vitro* antimicrobial activity. In fact the extracts contains essential oils mainly composed of bromine- and iodine-containing haloforms with smaller quantities of other halogenated methanes and several halogenated ethanes, ethanols, formal-dehydes, acetaldehydes, acetones, 2-propanols, 2-acetoxypropanes, propenes, epoxypropanes, acroleins, butenones, halogenated acetic and acrylic acids (Kladi et al., 2004).

However, considering that our extracts were non-cytotoxic on mammalian red blood cells, the data reported here showed that it could be tested for the environmental control of these pathogens especially in

some geographic areas, such as Canada, where a particular hypervirulent VGII genotype of *C. gattii* continues to pose serious public health problems (Datta et al., 2009; Bartlett et al., 2012).

To our knowledge this is the first study reporting data on susceptibility of such important pathogenic fungi to algal extracts. In addition it also suggests that *A. taxiformis* possess attractive antifungal properties, which should encourage the search for new drugs derived from marine algae. Further investigations are in progress in our laboratory in order to test a large number of *C. neoformans*/*C. gattii* genotypes and to identify which of the various components present in crude extracts are responsible for the antifungal activity or if it is due to a synergistic action between the single chemical compounds.

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