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SHORT COMMUNICATION

Towards a new application of amaranth seed oil as an agent against *Candida albicans*

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ABSTRACT

Amaranthus spp. (Amaranthaceae family), known as amaranth, are plants native of Central America, today produced in many parts of the world. due to their popularity popular as a health food. Because of its composition, amaranth can be considered to be attractive not only as a food but also for pharmaceutical and cosmetics uses. To date, antifungal activity of amaranth extracts has not been totally investigated, therefore the scope of this study was to evaluate the antifungal effect of the apolar fraction from *Amaranthus cruentus* L. seeds extract, alone and in association with antifungal drugs terbinafine, a common antifungal agent, which itself has only fungistatic effect on *Candida albicans* strains without exerting fungicidal activity. Our results demonstrate that this amaranth oil in combination with terbinafine has synergic fungistatic and fungicidal activity, with FICI of 0.466 and 0.496, respectively. No fungistatic and fungicidal activity of terbinafine alone at concentrations up to 64 μg/mL and amaranth oil alone at concentrations up to 2000 μg/mL, against all tested *C. albicans* strains, were observed. does not show activity towards *Candida albicans* strains but it can effectively potentiate the antifungal activity of terbinafine, a common antifungal agent which itself This result suggests the possible application of amaranth oil in the preparation of formulations with terbinafine for topical use.

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KEYWORDS
Amaranthus cruentus L.; Amaranthaceae; supercritical fluid extraction; antifungal activity; *Candida albicans*; terbinafine

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Abbreviations: SQ: squalene; TAGs: triacylglycerols; FT-IR: Fourier-transform infrared; ^{1}H NMR: proton nuclear magnetic resonance; ^{13}C NMR: carbon nuclear magnetic resonance; HPLC: high performance liquid chromatography; ASE: amaranth seeds extract; UNSAP: unsaponifiable matter; MIC: minimum inhibitory concentration; GMMIC: MIC geometric mean; FICI: fractional inhibitory concentration (FIC) index; GMMFC: MFC geometric mean; MFC: minimum fungicidal concentration

1. Introduction

The genus *Amaranthus* spp. (Amaranthaceae family), better known with the common name of amaranth, is referred to tropical plants primarily that today are cultivated increasingly worldwide in Central America but that, today, are also produced by other countries such as China and United States (Coelho et al. 2018). Their seeds are known to biosynthesize big amount of lipids, proteins, carbohydrates, and dietary fibers as well as other important components such as squalene (SQ) (Venskutonis and Kraujalis 2013), a triterpenoid compound considered to be attractive for food, pharmaceutical and cosmetics uses (Narayan Bhilwade et al. 2010; Kim and Karadeniz 2012). SQ is an intermediate in the biosynthesis of ergosterol, a component of the fungal cytoplasmic membrane Most of the drugs currently used in antifungal therapy, as in the case of terbinafine, target the this pathway of fungal ergosterol, in whose biosynthesis SQ is an intermediate that inhibits the enzyme squalene epoxidase through a non-competitive mechanism, blocking ergosterol production and leading also in some cases to a toxic accumulation of SQ (Darkes et al. 2003). Fungicidal agents, such as terbinafine, are often preferred rather than fungistatic azoles since they may shorten up treatment time by topical application. However Among them, terbinafine has a narrow spectrum of activity and it is not very effective in infections caused by *Candida albicans* (Kyle and Dahl 2004). This opportunistic yeast is one of the major pathogens responsible for superficial (Kaushik et al. 2015) and systemic infections, forming biofilms on medical devices and host surfaces, severely limiting the available treatment options (Pandolfi et al. 2019). In previous works, we have already investigated on new anti-*Candida albicans* agents based on polar plants metabolites (De Vita et al. 2014, 2016). Here, we explored the antifungal properties of the apolar fraction of *A. cruentus* seeds, in order to evaluate the effect of an overload of SQ in *C. albicans* strains, alone and in combination with known antifungal drugs. The apolar fraction of *A. cruentus* has been extracted by supercritical CO_2 that provides various advantages. a technique suitable for extraction of non-polar compounds. Indeed, in addition to being non-toxic and

non-flammable, CO_2 provides the advantage to have has low critical parameters allowing to run the extraction in mild conditions, shortening the process time without decomposing compounds vulnerable to high temperatures.

2. Results and discussion

In this study, the oil extraction process from *A. cruentus* seeds has been optimized preparing the matrix by a pelletization followed by a grinding. As shown in Figure 1S (Supplementary material), the best kinetics and extraction yield were obtained when a pelletization was performed before the simple grinding process. The phytochemical analysis carried out on the crude extract showed the presence of triacylglycerols (TAGs) and SQ as main components. A FT-IR spectroscopic analysis (Supplementary material, Figure S5) highlighted the presence of the characteristic bands of C=O stretching (1744 cm^{-1}) and C–O–C stretching (1160 cm^{-1}) of TAGs ester groups. Additionally, free fatty acid carbonyl, strong alcohol C–O and O–H bands were missing, indicating the absence of free glycerol and acids. The presence of =C–H stretching due to unsaturations (3009 cm^{-1}) is also evident. The cis configuration could be easily proved by the C=C stretching (1656 cm^{-1}) and by the =C–H in-plane bending (1237 cm^{-1}). The structure of the major TAGs was elucidated by ^{1}H-NMR analysis that provided an average chain length of fatty acids of 18 carbons. Moreover, the chemical shifts values for the olefinic, allylic, and bis-allylic protons allowed to differentiate the nature of the C18 unsaturated components, that are oleic, linoleic and linolenic. Additionally, ^{13}C-NMR spectrum showed all the resonances belonging to TAGs where signals for esters (173 ppm), ethylenic carbons (129–131 ppm), carbons of glycerol (60–73 ppm) and aliphatic carbons (14–34 ppm) could be distinguished. Moreover, resonances of SQ were clearly observed (Supplementary material, Figure 4S). For what concerns SQ, a common way for its HPLC quantification consists of saponification and isolation of the unsaponifiable fraction where SQ is present. Here, analyses of amaranth seeds extract (ASE) were carried out by a simple and direct procedure, where no sample treatment was required. The content of SQ in ASE was 5% *w/w*.

After characterization, the oil was screened as a potential antifungal agent, alone and in combination with terbinafine, an antifungal drug used for topical treatment of superficial, skin and mucosal infections. Terbinafine is known to have not fungicidal activity against *C. albicans* strains (Petranyi et al. 1987). Indeed, the inhibition of 100% of *C. albicans* strains growth needs high concentrations of drug: Correa et al., for instance, reported that MIC_{100} values of terbinafine were $>$ 327.68 μg/mL against all the evaluated *Candida* ATCC strains (Correa Biancalana et al. 2008). Here, MIC_{100} of terbinafine was measured in combination with ASE at different concentrations against some strains of *C. albicans* (Table 1). While *C. albicans* strains are not susceptible to either terbinafine (MIC_{100} $>$ 64 μg/mL) or amaranth oil (MIC_{100} $>$ 2000μg/mL), terbinafine in combination with ASE showed antifungal synergistic activity. The antifungal activity, using a concentration of terbinafine $\leq$ 64 μg/mL, were obtained, with ASE concentration of 500 μg/mL against all strains tested, with $GMMIC_{100}$ values of 45.25 μg/mL. The MCF values of 45.25 μg/mL were obtained with ASE concentration of

Table 1. *In vitro* susceptibility of *Candida albicans* strains to ASE or UNSAP and terbinafine alone and in combination.

	MIC_{100}(μg/mL)					
	Alone		In combination			
Candida albicans strains	TRB	ASE	TRB	ASE	FICIs	Interpretation
ATCC10231	>64	>2000	32	500	0.375	SYN
3153A	>64	>2000	32	500	0.375	SYN
ATCC90028	>64	>2000	64	500	0.625	IND
PMC1024	>64	>2000	32	1000	0.500	SYN
PMC101IC	>64	>2000	64	250	0.562	IND
GMMIC	**>64**	**>2000**	**43.713**	**500**	**0.466**	**SYN**
	MIC_{100}(μg/mL)					
	Alone		In combination			
Candida albicans strains	TRB	UNSAP	TRB	ASE	FICIs	Interpretation
ATCC10231	>64	>2000	48	500	0.500	SYN
3153A	>64	>2000	64	500	0.625	IND
ATCC90028	>64	>2000	32	500	0.375	SYN
PMC1024	>64	>2000	32	500	0.625	IND
PMC101IC	>64	>2000	64	500	0.375	SYN
GMMIC	**>64**	**>2000**	**45.250**	**500**	**0.496**	**SYN**

TRB = terbinafine; ASE = amaranth seed extract; UNSAP = unsaponifiable matter. MIC_{100} is the lowest drug concentration that inhibited 100% of fungal growth. SYN indicates synergy (FICI $\leq$ 0.5) whereas IND indicates indifferent (FICI > 0.5–4). GMMIC = MIC geometric mean.

Table 2. Fungicidal activity of ASE or UNSAP and terbinafine alone and in combination on *Candida albicans* strains.

	MFC (μg/mL)					
	Alone		In combination			
Candida albicans strains	TRB	ASE	TRB	ASE	FICIs	Interpretation
ATCC10231	>64	>2000	32	1000	0.750	IND
3153A	>64	>2000	32	1000	0.750	IND
ATCC90028	>64	>2000	64	1000	1.000	IND
PMC1024	>64	>2000	64	250	0.562	IND
PMC101IC	>64	>2000	64	250	0.562	IND
GMMFC	**>64**	**>2000**	**46.851**	**574.350**	**0.496**	**SYN**
	MFC (μg/mL)					
	Alone		In combination			
Candida albicans strains	TRB	UNSAP	TRB	UNSAP	FICIs	Interpretation
ATCC10231	>64	>2000	48	500	0.500	SYN
3153A	>64	>2000	64	500	0.625	IND
ATCC90028	>64	>2000	32	500	0.375	SYN
PMC1024	>64	>2000	32	500	0.375	SYN
PMC101IC	>64	>2000	64	500	0.625	IND
GMMFC	**>64**	**>2000**	**45.250**	**500**	**0.496**	**SYN**

TRB = terbinafine; ASE = amaranth seed extract; UNSAP = unsaponifiable matter. MFC was defined as the concentration of drug that resulted in $\geq$3-log10 CFU/ml decrease (99.9% kill) after 24 h of incubation. SYN indicates synergy (FICI $\leq$ 0.5) whereas IND indicates indifferent (FICI > 0.5–4). GMMFC = MFC geometric mean.

1000 μg/mL. against all strains tested, using a terbinafine $GMMIC_{100}$ values of 43.713 μg/mL and ASE concentration of 500 μg/mL, showed a FICI of 0.466.

Moreover, the association showed also synergistic fungicidal activity using terbinafine GM values of 46.851 μg/mL and ASE concentration of 574.35 μg/mL (FICI of 0.496) as reported in Table 2.

These results demonstrated the fungicidal activity of the association. Trying to understand which component potentiates the activity of the drug, TAGs and SQ as main components of ASE were isolated and tested in combination with terbinafine. According to the results, TAGs and SQ did not increase the antifungal activity of terbinafine (data not shown). Finally, the unsaponifiable matter obtained by alkaline hydrolysis of the oil was combined at concentration ranging from 2000 to 250 μg/mL with terbinafine. The results show that the activity of the unsaponifiable fraction is comparable with the whole extract at the same concentration. Our data would suggest that, in the total extract, components with antifungal activity are present. In conclusion TAGs are not essential to potentiate terbinafine activity on *C. albicans*; SQ could be necessary for the activity, only when other oil components are present; indeed, SQ alone does not cause any significant improvement in the antifungal activity. Therefore, as happens with many extracts, the plant complex has more pronounced biological activity than the isolated components.

Finally, the effect of the ASE on the antifungal activity of fluconazole has been evaluated. According to the results, ASE did not increase the anti-*Candida* activity of fluconazole (data not shown).

3. Experimental

The experimental details are available as Supplementary material.

4. Conclusion

Our study was focused on the search for new applications of amaranth extract. To this end, we applied a green approach for the extraction of amaranth seed oil by using supercritical CO_2 as solvent. Testing its anti-*Candida albicans* activity, we found that the extract alone did not exert antifungal activity against the assayed strains. Interestingly, it increased the activity of terbinafine when a combination of oil and drug was tested. This result suggests the possible application of amaranth oil as a vehicle in the preparation of formulations with terbinafine for topical use.

Disclosure statement

The authors declare no conflict of interest.

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