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Anti-yeast potential of some Annonaceae species from Cameroonian biodiversity

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ABSTRACT

An increased incidence of candidiasis and cryptococcosis has been recorded within the last two decades, mainly due to the increase in number of immunocompromised patients. Moreover, the emergence of resistant pathogenic yeast strains coupled with the shortcomings of the available antifungal drugs have emphasized the need of new drugs. Within this framework, this study aimed at investigating extracts from fourteen Annonaceae plants species *in vitro* against the major causative agents of mycoses, namely *Candida albicans*, *Candida parapsilosis* and *Cryptococcus neoformans*. Plant extracts and partitioned fractions were screened by broth dilution method. Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of extracts were determined by microdilution method and subculture. Also, the effect of combined active hexane fraction (UAst H) of *Uvaria angolensis* stem extract with the reference ketoconazole was studied. MIC values ranged from 0.625 mg/mL (for the leaf extract of *Uvaria banmanni*- UBl) to ≥10 mg/mL. Fraction UAst H showed the broadest spectrum activity with MIC value of 2.5 mg/mL against all the tested yeasts. Moreover, UAst H exhibited synergistic interaction on *C. albicans*, *Cr. neoformans* and *C. parapsilosis* when combined with ketoconazole. Overall, the results achieved in this study are promising and indicate that plants species from Annonaceae family are possible sources of potent and synergistic anti-yeast extracts.

Keywords: Annonaceae, Extract, Antifungal, Combination, Synergism.

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INTRODUCTION

The HIV pandemic has greatly increased the incidence of fungal infections over the last two decades (Khan et al., 2012; Okonkwo et al., 2013). As the most implicated, Candida species are responsible for a variety of infections from superficial to systemic (Nucci and Marr, 2005; Romeo et al., 2013). *Candida albicans* is the most frequently isolated (Ruhnke, 2006), and is responsible for over 50% of candidemias (Hachem et al., 2008), followed by *C. parapsilosis* (10%-20%), *C. glabrata* (20%-30%), *C. tropicalis* (17.3%) and *C. krusei* (1%-5 %) (Horn et al., 2009). In addition, HIV infection has been associated with more than 80% of cryptococcosis cases worldwide (Mirza et al. 2003; Perfect et al., 2010) with 100% mortality rate (Corbett et al., 2002) when untreated. The treatment of candidiosis and cryptococcosis is limited to drugs from few classes such as polyenes (amphotericin B, AMB), pyrimidine analogues (fluorocytosine), azoles and 1,3-betaglucan synthase inhibitors (echinocandins). However, the development of resistant pathogens along with high toxicity of these drugs have significantly impaired the control of fungal infections worldwide, stimulating the search for new, safe and more efficient drug candidates.

Plant-derived products for drugs discovery program have gained attention of scientific community since decades. In fact, medicinal plants have been the sources of variety of biologically active compounds for centuries and are used extensively as crude materials or as pure compounds for treating various conditions (Dias et al., 2012). Within this context, Cameroonian medicinal plants have been investigated against a wide range of microorganisms (Fokou et al., 2014; Sidjui et al., 2014; Kammalac et al., 2015; Kamdem et al 2015; Menkem et al., 2015; Menkem et al., 2016). However, Annonaceae species although widely investigated for their antiprotozoal activities, have gained less interest as potential sources of antifungal drugs. To fill this gap in, this study was designed to assess the anti-yeast activity of extracts from *Annona muricata*, *Anonidium mannii*, *Monodora myristica*, *Polyalthia oliveri*, *Polyalthia suaveolens*, *Uvaria angolensis*, *Uvaria banmanni*, *Uvaria muricata*, *Uvariastrum zenkeri*, *Uvariodendron calophyllum*, *Uvariodendron molundense*, *Xylopia aethiopica*, *Xylopia parviflora* and *Xylopia africana*, using *Candida albicans*, *Candida parasilosis* and *Cryptococcus neoformans* isolates as targets.

MATERIALS AND METHODS

Plant collection

Plant materials from Annonaceae family were harvested and identified as *Annona muricata* Linn, *Anonidium mannii* Gaertm (D. Oliver) Engl. & Diels, *Monodora myristica* (Gaertn.) Dunal, *Polyalthia oliveri* Engl., *Polyalthia suaveolens* Engl. & Diels, *Uvaria angolensis* Engl. & Diels, *Uvaria muricata* Engl. & Diels, *Uvaria banmanni* Engl. & Diels, *Uvariastrum zenkeri* Engl. & Diels, *Uvariodendron calophyllum* R.E Fries, *Uvariodendron molundense* (Engl. & Diels), *Xylopia aethiopica* (Dunal) A Rich, *Xylopia africana* (Benth) Oliv, *Xylopia parviflora* A Rich. Voucher specimens were deposited at the National Herbarium of Cameroon, Yaoundé under specific reference numbers. Plants reference numbers, traditional uses, collection site, previous scientific studies and parts used are summarized in Table 1.

Yeasts isolates

Yeast isolates were provided by the Laboratory of Clinical Biology of the Yaoundé Central Hospital (Yaoundé, Cameroon), and consisted of clinical isolates of *C. albicans*, *C. parapsilosis* and *Cr. neoformans*. These yeasts were maintained at room temperature (25-27 °C) and cultured at

37 °C for 24 h on Sabouraud Dextrose Agar (Oxoid) slants prior to use.

Extracts/fractions preparation and preliminary anti-yeast screening

One hundred grams of powder from each plant part were macerated in 1500 mL of ethanol (Merck) for 72 h at room temperature (25-27 °C). Filtrates obtained using filter papers (Whatman no.1) were evaporated to dryness using a rotary evaporator at 80 °C (Büchi 011, Flawil Switzerland). Stock solution for each extract was prepared at 100mg/mL using 10% DMSO. As positive control, ketoconazole (Janssen-cilag) was prepared at 200 mg/mL in sterile distilled water. Each solution was sterilized by filtration through 0.22 µm sterile filter (Acrodisc Syringe Filter).

All extracts were screened by broth dilution method (CLSI, 2008) at 10mg/mL in 96-well microtiter plates. To achieve this, 10 µL of each stock solution of extract were added in triplicate wells followed by 90 µL of yeast inoculum prepared at 2.5×10^3 CFU/mL in sabouraud dextrose broth (Difco) medium. Wells containing 10% DMSO without extracts were used as negative control while ketoconazole was tested as positive control. Plates were incubated at 37 °C for 48 h and extracts showing 100% inhibition of fungal growth against the three tested yeasts ascertained by macroscopic observation were selected and progressed.

Fifteen grams of each selected extract were added to 150 mL of water and further fractionated by liquid-liquid partition between water and hexane (*v/v*) to yield hexane and aqueous fractions. Hexane fractions were dried using rotavapor while aqueous fractions were freeze dried to obtained powders. In some few cases, interface precipitates were obtained and also tested for biological activity. Stock solution for each fraction was prepared at 100 mg/mL using 10% DMSO.

Determination of Minimal Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

MIC values for the selected extracts and fractions were determined according to the CLSI M27-A3 protocol (CLSI, 2008) with little modifications. Briefly, a serial two-fold dilution of each extract using sabouraud dextrose broth medium (Difco) supplemented with 2% glucose was done in 96-wells microtiter plates starting with 10 mg/mL down to 0.04 mg/mL. One hundred microliters of fungal inocula at the final concentrations of 2.5×10^3 CFU/mL were then added into each well of the plate to achieve a final volume of 200 µL. Plates were incubated at 37 °C for 48 h. Ketoconazole was tested as positive control at three concentrations (10, 5, and 2.5 mg/mL). MIC values were determined as the lowest extract concentration exerting complete visible inhibition of the yeasts based on turbidity in plate wells through macroscopic observation. MFC values were determined by transferring 50 µL aliquots of the clear wells (MIC wells) into 150 µL of freshly prepared broth medium and incubated at 37 °C for 48 hours. The MFC was defined as the lowest concentration of test sample which did not produce turbidity, indicating no microbial growth. All tests were performed in triplicates. The MIC cutoff value for fraction progression was $\leq$ 2.5 mg/mL against all the tested yeasts.

Phytochemical screening of the selected fraction

The most active fraction was subjected to phytochemical screening to detect the presence of secondary metabolites with potential antifungal activity, namely saponins, tannins, flavonoids, phenols, alkaloids, triterpenes, anthocyanins, essential oils and steroids as described by (Odebeyi and Sofowora, 1978).

Table 1: Ethnobotanical and scientific information on the investigated plants.

Plants and reference numbers	Local/common name	Collection site	Traditional indications	Scientific findings	Organs used
Annona muricata 32879/HNC	Graviola or soursop,	Yaoundé	Malaria, sedative, digestive, antipyretic, Boil, cough, diarrhea, dermatosis, hypertension, rheumatism and styptic, worms and parasites. headache, fever, toothache, asthma	anthelmintic, antiplasmodial, antiparasitic, antimicrobial, antipyretic, sedative, antispasmodic, hypotensive, anticonvulsant, digestive, antitumor and anticancer (Tchokouaha et al., 2015; Pinto et al., 2005)	Leaves, twigs, flower, pericarp, pulp of fruit, seeds
Anonidium mannii Gaertm 45582HNC	/	Mount Kalla	Male infertility, complication of pregnancy and childbirth, treatment of abscess	Antimycobacterial (Donfack et al., 2014)	Leaves, twigs
Monodora miristica 1927/SRFK	Medjeng, pébé, ding, ikoma, ozek	Yaoundé	Witchcraft, stimulant, stomachic, headaches, sores, stomach-aches, febrile, pains, eye diseases and haemorrhoids	Antimalarial (16), Cytotoxic (Bakarnga-Via et al., 2014), Antibacterial and antifungal activities (Tatsadjieu et al., 2003)	Leaves, twigs, stem bark
Polyalthia oliveri 19416SRF/Cam	/	Mount Kalla	Malaria	Antiplasmodial (Boyom et al., 2009)	Leaves, twigs, stem bark
Polyalthia suaveolens 1227/SRF/CAM	Afoumengen	Mount Kalla	Dysmenorrhea	Antimalarial activity (Cushnie and Lamb, 2005]	Leaves, twigs, stem bark
Uvaria angolensis 16797/SRF/CAM	/	Mount Kalla	Abdominal pains, dysentery, digestive	Antiplasmodial, antimicrobial (Hufford et al., 1987)	Leaves, twigs, stem
Uvaria banmanni 6427/SRF/Cam	/	Mount Kalla	Fatigue, abscess	Antimycobacterial (Donfack et al., 2014)	Leaves, twigs
Uvaria muricata HNC 6415/SRF/CAM	/	Mount Kalla	Abdominal pains, dysentery, digestive		Leaves, twigs, stem

Uvariastrum zenkeri 57355HNC	/	Mount Kalla	Gun-stock	Antimycobacterial (Donfack et al., 2014)	Leaves, twigs
Uvariodendron calophyllum 28734/SFR/CAM	Obom ossoé	Mount Kalla	Gun, malaria	Antifungal and Antioxidant activity (Horiuchi et al., 2007) Antiplasmodial activities (Hufford et al., 1987)	Leaves, Twigs Stem bark
Uvariodendron molundense 41685HNC	Ojobilobe	Mount Kalla	Gun, malaria	Antifungal and Antioxidant activities (Horiuchi et al., 2007)	Leaves, twigs
Xylopia aethiopica 28725/SFR/Cam,	Netsham, ikoli, ekur, ebongo mbonji, kembare, akwi	Kribi	Buruli ulcer, Cough, carminative, and as a postpartum tonic. Stomach ache, treatment of bronchitis, biliousness and dysentery	Antiplasmodial (Boyom et al., 2004), Cytotoxic, antiproliferative (Bakarnga-Via et al, 2014), Antibacterial and antifungal activities (Tatsadjieu et al., 2003)	Leaves, twigs
Xylopia africana 38322/HNC,	kojeup	Kribi	Bronchitis, dysentery and febrile pains, Asthma, stomach aches and rheumatism	Cytotoxic, antiproliferative activities (Bakarnga-Via et al., 2014)	Leaves, twigs, roots, stem bark
Xylopia parviflora 42351HNC	Mbatou'ou, odjobi	Kribi	Stomach disorders and barrenness, Headache, analgesic and antispasmodic purposes	Cytotoxic, antiproliferative activities (Bakarnga-Via et al., 2014)	Leaves, stem, seeds

Plant samples were identified and voucher specimens deposited at the National Herbarium of Cameroon, Yaoundé.

Study of combinations of the promising fraction with ketoconazole

The antifungal effect of combined fraction with ketoconazole was assessed by broth microdilution checkerboard technique. Checkerboard tests were used to determine fractional inhibitory concentration indexes (FICIs) of combinations against each test microorganism. The checkerboard broth microdilution method based on CLSI recommendations (CLSI, 2008) consisted of diluting the fraction and drug in the two directions of a 96-wells microplate. Mixed concentrations in wells ranged from 1/8 × MIC to 8 × MIC and 1/128 × MIC to 8 × MIC for the fraction and drug respectively. The fungal inoculum was added to give an ultimate concentration of 2.5×10^3 CFU/mL in a final volume of 100 µL. Plates were therefore incubated at 37°C for 48 h and each test performed in duplicate. Changes in the Fractional Inhibitory Concentration Indexes (FICIs) were calculated using the following formula, and the type of interaction was determined according to previously described criteria.

FICI(X1/X2) = (MICX1 in combination/MICX1 alone) + (MICX2 in combination/MICX2 alone) (Tobudic et al., 2010). According to the adopted criteria, a combination was considered synergistic when the FICI was ≤ 0.5, additive when it was > 0.5 to ≤ 1, indifferent when it was > 1.0 to ≤ 4.0, and antagonistic when the obtained FICI was > 4.0.

RESULTS

Yields of plant extraction and anti-yeast potential of fourteen Annonaceae plants

Plant samples were extracted by maceration using ethanol, and the yield of extraction calculated relative to the weight of the starting plant material. Extracts were screened for activity against yeasts. The data collected are summarized in Table 2.

The plants extraction yields varied from 0.10 to 13.94% and were highly dependent on the plants species and parts used. The preliminary antifungal screening also showed varying susceptibility of the tested yeasts. Amongst the 44 extracts prepared from 14 plants, only 13 (29.54%) inhibited the growth of all the tested yeasts, namely *C. albicans*, *C. parapsilosis* and *Cr. neoformans* at 10mg/ml, including AMfr, MMl, POl, POtw, POsb, PSl, UBl, UAl, UAtw, UAst, UMutw, UMust, and XPs. These promising extracts derived from different types of plant organ, including fruit, leaf, twig, stem, stem bark, and seed. They were selected and subjected to liquid-liquid partition coupled with MIC/MFC determination.

Determination of Anti-yeast activity parameters of promising extracts and their fractions

Table 3 below summarizes the activity parameters (MIC and MFC) of 29 fractions derived from the 13 selected crude extracts against three pathogenic yeasts, namely *C. albicans, C. parapsilosis,* and *Cr. neoformans.*

The activities displayed spanned from 0.625 mg/mL to >10 mg/mL depending on the plant extracts and yeast species. The highest potency was exhibited by the crude leaf extract of *Uvaria banmanni* (UBl) against *C. albicans* with an MIC value of 0.625 mg/mL and MFC of 1.25 mg/mL. Besides, UBl only showed moderate effect against *C. parapsilosis* and *Cr. neoformans* with MIC of 10 mg/mL and MFC > 10 mg/mL. The other few active crude extracts inhibited the yeasts with MIC values ranging from 2.5 mg/mL to >10 mg/mL, and MFC from 5 mg/mL to >10 mg/mL. Amongst

those, the more promising were MMl from the leaf of *M. myristica*, UAtw and UMutw from the twig of *U. angolensis* and *U. muricata* respectively and that showed MIC values of 5 mg/mL against the three yeasts. Extract POl from *P. oliveri* significantly inhibited *Cr. neoformans* with an MIC of 2.5 mg/mL and MFC of 5 mg/mL, but only showed moderate action against *C. albicans* and *C. parapsilosis* (MIC= 10 mg/mL).

As shown in Table 3, water/hexane partition of the 13 extracts (AMfr, MMl, POl, POtw, POsb, PSl, UBl, UAl, UAtw, UAst, UMtw, UMst, and XPs) yielded 29 fractions that were equally tested for biological activity, including 3 interface precipitates, MMl I, POl I, and UBl I respectively from *M. myristica* (leaf), *P. oliveri* (leaf), and *U. banmanni* (leaf). The MIC values of these fractions ranged from 2.5 to >10 mg/ml. The most active was the hexanic fraction from the stem of *U. angolensis* (UAst H) that exhibited potent fungicidal effects against *C. albicans* and *Cr. neoformans* (MIC= 2.5 mg/mL; MFC= 5 mg/mL), and to a certain extent against *C. parapsilosis* (MIC= 2.5 mg/mL; MFC > 10 mg/mL). In this particular case, the partition of the mother crude extract has delivered a two-fold more potent hexanic fraction and an aqueous fraction with mother extract-like activity (MIC of 5 mg/mL; MFC > 10 mg/mL against *C. parapsilosis* and *Cr. neoformans*, and MIC of 10 mg/mL; MFC > 10 mg/mL against *C. albicans*). Besides, it was noticed that fractionation did not show any overall activity improvement from the other partitioned extracts. Consequently, fraction UAst H from the stem of *U. angolensis* was selected for further phytochemical screening and combination studies.

Phytochemical screening of fraction UAst H

Qualitative phytochemical screening using standards methods has revealed the presence of a number of secondary metabolite classes in fraction UAst H that might sustain the observed activity against yeasts. These metabolites included phenols, flavonoids, triterpenes, steroids, saponins, anthocyanins, tannins, and essentials oils.

Fraction UAst H and ketoconazole combination studies

Fraction UAst H and ketoconazole were combined at their respective MIC values using the checkerboard format and tested against *C. albicans*, *C. parapsilosis,* and *Cr. neoformans.* Fractional inhibitory concentration indexes (FICI) were calculated and the type of interaction between the two reactants determined. The results obtained are summarized in the Table 4 below.

From the results presented in Table 4, the FICI values were found to range from 0.26 to 0.75 against *C. albicans*, from 0.28 to 0.75 against *Cr. neoformans,* and from 0.15 to 0.51 against *C. parapsilosis*. Overall, synergistic interactions could be achieved with most of the combinations, resulting in average FICI of 0.47±0.19, 0.38±0.21 and 0.29±0.15 on *C. albicans*, *Cr. neoformans,* and *C. parapsilosis* and significant reduction in MIC values of both UAst H fraction and ketoconazole. Besides, only three additive interactions were observed against *C. albicans*, and one each against *C. parapsilosis* and *Cr. neoformans*. Moreover, the reduction of ketoconazole's MIC ranged from 2 to 32-fold, and from 2 to 64 fold for UAst H.

Table 2: Extraction yield and results of anti-yeast screening of ethanolic extracts.

Plant species	Part	Codes	Extract Yield (%)	*C. albicans*	*C. parapsilosis*	*Cr. neoformans*
Annona muricata	Leaf	AnMl	4.85	-	-	-
	Twig	AnMtw	1.77	-	-	-
	Flower	AnMfl	2.32	-	-	-
	Pericarp	AnMp	2.29	+	-	-
	Pulp of fruit	AnMpf	1.09	-	-	-
	Seed	AnMs	3.49	-	-	-
Anonidium mannii	Leaf	AMl	1.65	+	-	-
	Twig	AMtw	0.55	+	-	-
	Fruit	AMfr	2.94	+	+	+
	Stem bark	AMsb	2.33	-	-	-
	root	AMr	1.80	-	-	-
Monodora myristica	Leaf	MMl	0.63	+	+	+
	Twig	MMtw	0.79	+	-	-
	Stem bark	MMsb	2.58	-	-	-
Polyalthia oliveri	Leaf	POl	2.57	+	+	+
	Twig	POtw	0.64	+	+	+
	Stem bark	POsb	1.88	+	+	+
Polyalthia suaveolens	Leaf	PSl	3.73	+	+	+
	Twig	PStw	0.73	-	-	-
	Stem bark	PSsb	1.70	+	-	-
Uvaria banmanni	Leaf	UBl	1.30	+	+	+
	Twig	UBtw	0.97	+	-	-
	Stem bark	UBsb	2.32	-	-	-
Uvariastrum zenkeri	Leaf	UZl	3.77	-	-	-
	Twig	UZtw	0.59	-	-	-
Uvariodendron calophyllum	Twig	UCtw	0.42	-	-	-
Uvariodendron molundense	Leaf	UMl	1.28	-	-	-
	Twig	UMtw	0.20	-	-	-
Uvaria	Leaf	UAl	9.07	+	+	+

angolensis	Twig	UAtw	8.83	+	+	+
	Stem	UAst	13.94	+	+	+
Uvaria muricata	Leaf	UMul	12.97	-	-	-
	Twig	UMutw	5.30	+	+	+
	Stem	UMust	4.84	+	+	+
Xylopia africana	Leaf	XAl	3.99	-	-	-
	Twig	XAtw	0.75	+	-	-
	Stem bark	XAsb	1.57	+	-	-
	root	XAr	0.49	+	-	-
Xylopia aethiopica	Leaf	XAEl	4.27	+	-	-
	Stem	XAEst	0.10	-	-	-
	seed	XAEs	1.98	-	-	-
Xylopia parviflora	Leaf	XPl	1.65	+	-	-
	Stem	XPst	0.67	-	-	-
	Seed	XPs	6.38	+	+	+
Ketoconazole				+	+	+

-: no inhibition at 10 mg/mL; +: 100% inhibition at 10mg/mL

Table 3: MIC and MFC of extracts and fractions (mg/mL).

Plant species	**Part**	**Extract/fraction**	***C. albican***		***C. parapsilosis***		***Cr. neoformans***	
			MIC	MFC	MIC	MFC	MIC	MFC
Anonidium mannii	Fruit	AMfr	10	>10	10	10	10	10
		AMfr H	>10	>10	>10	>10	>10	>10
		AMfr H_20	>10	>10	>10	>10	>10	>10
Monodora myristica	Leaf	MMl	5	>10	5	>10	5	>10
		MMl I	>10	>10	>10	>10	>10	>10
		MMl H	>10	>10	>10	>10	>10	>10
		MMl H_20	>10	>10	>10	>10	>10	>10
Polyalthia oliveri	Leaf	POl	10	>10	10	>10	2.5	5
		POl I	>10	>10	>10	>10	>10	>10
		POl H	10	>10	>10	>10	5	5
		POl H_20	>10	>10	>10	>10	>10	>10
	Twig	POtw	10	>10	10	>10	>10	>10
		POtw H	>10	>10	>10	>10	>10	>10

		POtw H_20	>10	>10	>10	>10	>10	>10
	Stem	POsb	10	>10	10	>10	10	>10
	bark	POsb H	>10	>10	>10	>10	>10	>10
		POsb H_20	>10	>10	>10	>10	>10	>10
Polyalthia	Leaf	PSl	10	10	10	>10	10	>10
suaveolens		PSl H	>10	>10	>10	>10	>10	>10
		PSl H_20	>10	>10	>10	>10	>10	>10
Uvaria	Leaf	UBl	0.625	1.25	10	>10	10	>10
banmanni		UBl I	>10	>10	>10	>10	>10	>10
		UBl H	>10	>10	>10	>10	>10	>10
		UBl H_20	>10	>10	>10	>10	>10	>10
Uvaria	Leaf	UAl	10	>10	5	>10	10	>10
angolensis		UAl H	10	>10	10	>10	10	>10
		UAl H_20	10	>10	10	>10	5	>10
	twig	UAtw	5	>10	5	>10	5	>10
		UAtw H	>10	>10	>10	>10	>10	>10
		UAtw H_20	5	>10	5	>10	5	>10
	stem	UAst	10	>10	5	>10	5	>10
		UAst H	2.5	5	2.5	>10	2.5	5
		UAst H_20	10	>10	5	>10	5	>10
Uvaria	twig	UMutw	5	>10	10	>10	5	>10
muricata		UMutw H	5	>10	5	>10	10	>10
		UMutw H_20	5	>10	5	>10	5	>10
	stem	UMust	10	>10	5	>10	5	>10
		UMust H	10	>10	5	>10	2.5	>10
		UMust H_20	5	>10	5	>10	5	>10
Xylopia	Seed	XPs	10	10	10	>10	10	>10
parviflora		XPs H	>10	>10	>10	>10	>10	>10
		XPs H_20	>10	>10	>10	>10	>10	>10
Ketoconazole			10	10	5	10	5	10

I: interface precipitate; H: hexane fraction; H_20: aqueous fraction

Table 4: Effect of combined UAst H fraction and ketoconazole on *C. albicans*, *C. parapsilosis* and *Cr. Neoformans.*

C. albicans		FICI (FIC Keto + FIC UAst H)	Int	*Cr. neoformans*		FICI (FIC Keto + FIC UAst H)	Int	*C. parapsilosis*		FICI (FIC Keto + FIC UAst H)	Int
FIC UAst H	FIC Keto			FIC UAst H	FIC Keto			FIC UAst H	FIC Keto		
0.25	0.01	0.26	S	0.25	0.03	0.28	S	0.13	0.03	0.15	S
0.50	0.03	0.53	A	0.25	0.06	0.31	S	0.13	0.06	0.18	S
0.50	0.06	0.56	A	0.13	0.06	0.18	S	0.13	0.13	0.25	S
0.13	0.13	0.25	S	0.13	0.25	0.37	S	0.13	0.25	0.37	S
0.25	0.25	0.50	S	0.25	0.50	0.75	A	0.01	0.50	0.51	A
0.25	0.50	0.75	A								
Average of FIC Index		0.47±0.19	S	Average of FIC Index		0.38±0.21	S	Average of FIC Index		0.29±0.15	S

FICI: Fractional Inhibitory Concentration Index; Int: interaction; A: additivity; S: synergy; keto: ketoconazole; UAst H: hexanic fraction of *Uvaria angolensis* stem extract.

DISCUSSION

Plants species from Annonaceae family are worldwide distributed and used all over the tropics in traditional medicine for the treatment of parasitic and microbial diseases, including fungal and bacterial infections. As reported by Tsabang et al (2012), Annonaceae plants are widely used by Cameroonian traditional healers to cure malaria and related symptoms, and many other infections. Moreover, phytochemical studies of this family have revealed chemical components which could offer new alternatives for the treatment and control of several infectious diseases (Frausin et al., 2014). Of note, secondary metabolites with antimicrobial activity from classes of alkaloids, flavonoids, tannins, phenolic compounds, anthocyanins, saponins, triterpenes, steroids, and acetogenins have been reported from many Annonaceae plant species (Leboeuf et al., 1980; Lewis and Ausubel, 2006; Taha et al 2013; Frausin et al., 2014; Liaw et al., 2016).

From the present investigation, fourteen different plants species from eight genera were investigated for their potential to inhibit the growth of *Candida albicans*, *Candida parapsilosis* and *Cryptococcus neoformans*. The results showed that few extracts and fractions could inhibit the tested yeasts. Of particular interest, the hexane fraction (UAst H) obtained from the water/hexane partition of the stem extract of *Uvaria angolensis* exhibited potent inhibitory effects against the three pathogens, more likely due to its content in secondary metabolites that have been shown to possess antifungal activity. This active fraction has also exhibited remarkable synergistic activity in combination with Ketoconazole (FICI from 0.29-0.47) against the three pathogens.

The qualitative phytochemical screening of fraction UAst H has revealed the presence of phenols, flavonoids, triterpenes, steroids, saponins, anthocyanins, tannins, and essentials oils. These compounds have the potential to exhibit potent activity against yeasts alone or in combination. For instance, phenolic compounds isolated from natural sources possess antifungal properties of interest. Particularly, phenolic acids have shown promising *in vitro* and *in vivo* activity against *Candida* species. However, studies on their mechanism of action alone or in synergism with known antifungals are still scarce (Teodoro et al., 2015). Also, flavonoids used in combination with fluconazole recently showed significant synergistic activity *in vitro* against *Candida tropicalis* strains resistant to fluconazole (Da Silva et al., 2014). Similarly, condensed tannins from *Stryphnodendron adstringens* showed *in vitro* and *in vivo* effect on *Candida tropicalis* growth and adhesion properties (Morey et al., 2016). Steroidal saponins have also been shown to exert significant activity against *Cr. neoformans* and *Aspergillus fumigatus* (Yang et al., 2006). Limited studies have indicated that phenols, saponins and flavonoids might exert their antimicrobial action through membrane perturbations (Tsuchiya et al 1996; Majorie, 1999; Cushnie and Lamb, 2005). As well, essential oils that are complex volatile compounds, synthesized naturally in different plant parts during the process of secondary metabolism have great potential in the field of biomedicine as they effectively destroy several bacterial, fungal, and viral pathogens. The presence of different types of aldehydes, phenolics, terpenes, and other antimicrobial compounds means that the essential oils are effective against a diverse range of pathogens.

A tentative explanation of the observed synergistic action of fraction UAst H in combination with ketoconazole might be the disruption of cell membrane coupled with the impairing effect of ketoconazole on the synthesis of membrane ergosterol (Ghannoum and Rice, 1999). Furthermore, these results are consistent with previous studies where

plant extracts were reported to increase *in vitro* efficacy of available antimicrobial drugs against bacteria and fungi (Betoni et al., 2006; Esimone et al., 2006; Horiuchi et al., 2007).

Opportunistic fungal infections elicited by *Candida*, *Cryptococcus*, and *Aspergillus* are life-threatening in immunocompromised patients (with AIDS, cancer, or organ transplant) (Liu and Lian, 2003). The currently prescribed antifungal drugs belong to five major drug classes, namely 1) polyenes (ex. Amphotericin B; interacting with fungal ergosterol, thereby disrupting the cytoplasmic membrane); 2) azoles (ex. Ketoconazole, fluconazole; inhibiting 14α-lanosterol demethylase in ergosterol biosynthesis pathway); 3) allylamines (ex. terbinafine, butenafine; inhibiting squalene epoxidase in ergosterol synthesis pathway), 4) echinocandins (ex. caspofungin, anidulafungin; inhibiting synthesis of β-1,3-glucan, which is a required component of the cell wall of many fungi), and 5) flucytosine (ex. flucytosine; incorporated into RNA and thus inhibiting DNA synthesis) (Ren, 2004). Unfortunately, each of these drug classes have at least one major shortcomings such as significant dose-limiting toxicities for Amphotericin B, rapid development of resistance for the azoles, limitation of the use to dermatophytoses for terbinafine, and lack of effectiveness in cryptococcosis for caspofungin) (Marr et al., 2001, 1998; Liang and Wang, 2004). This has emphasized the need to discover new antifungal agents, preferably with new modes of action. In fact, plant extracts can be used as synergistic enhancers even if they don't have any antimicrobial properties alone. They can enhance the effect of standard drugs when they are taken concurrently (Horiuchi et al., 2007). Many attempt to formulate such combinations either with commercially available antifungals, or potent phytochemicals have been described (Sibanda and Okoh, 2007; Adwan and Mhanna, 2008). In addition to the results described in this study, recent findings also support that plant extracts from Annonaceae have the potential to be further developed as drugs to control fungal diseases. For instance, methanol, chloroform, and aqueous extracts of *Annona squamosa* leaves recently showed activity against five different strains of fungi (*Alternaria alternata, Candida albicans, Fusarium solani, Microsporum canis,* and *Aspergillus niger*) (Kalidindi et al., 2015). Also, antifungal activity of ethanolic, methanolic, and aqueous extracts of *Annona muricata* was recently reported against *C. albicans* and *Cr. neoformans* (Vinothini and Growther, 2016). These previous findings further emphasize the potential of Annonaceae extracts as sources of potent antifungal agents.

Conclusion

The results from this investigation suggest that plants species from Annonaceae family can be used as promising sources for antifungal drug discovery. Moreover, the synergistic interaction of fraction UAst H from *Uvavia angolensis* with ketoconazole against the tested yeasts further suggest the possibility of development of novel synergistic antiyeast therapies from such extracts. However, in an optic to develop new drugs against opportunistic mycosis, further detailed studies of the selected fraction are required.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

This work was designed and supervised by FFB. Taffou, JBHF, EZM, LRYT, ANF, MSK, VN, IFKT collected and extracted the plants materials. They also performed the *in vitro* screening of plant

extracts. All authors contributed to manuscript drafting and revision.

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