An ethnobotanical survey and antifungal activity of *Piper guineense* used for the treatment of fungal infections in West-African traditional medicine

Eunice Ego Mgbeahuruike[a]*, Yvonne Holm[a], Heikki Vuorela[a], Chinyere Amandikwa[b] and Pia Fyhrquist[a]

[a]*Division of Pharmaceutical Biosciences, Faculty of Pharmacy, P.O. Box 56, FI-00014 University of Helsinki, Finland.*

[b]*Department of Food Science and Technology, School of Engineering and Engineering Technology, Federal University of Technology Owerri, P.M. B 1526, Owerri, Imo state, Nigeria.*

Yvonne.holm@helsinki.fi, Heikki.vuorela@helsinki.fi, Amandikwa125@yahoo.com, Pia.fyhrquist@helsinki.fi

*Corresponding author's e-mail: eunice.mgbeahuruike@helsinki.fi +358442399653

Abstract

Ethnobotanical relevance: *Piper guineense* occurs commonly in West Africa where it is used for fungal infections instead of the costly and not always accessible conventional antifungals. Fungal, yeast-based diseases are common in West-Africa especially among those living with HIV/ AIDS, and thus this study was performed in Imo state, South-Eastern Nigeria, where *P. guineense* is predominantly used for the treatment of fungal diseases, such as skin rashes, oral thrush and vaginosis.

Aim of study: The scarce number of previous studies on the documentation of the traditional uses of *P. guineense* extracts for the treatment of fungal infections in Nigeria prompted this survey. The investigation focused on how traditional healers recognize and diagnose fungal infections, how *P. guineense* is collected, on the various parts used for the treatments, methods of preparations, administrations and treatments. In addition, an *in vitro* antifungal screening of *P. guineense* fruit and leaf extracts of various polarities, and piperine and piperlongumine, representing the main constituents in these extracts, were performed.

Methods: A house to house ethnobotanical survey was conducted using questionnaires. Twenty traditional medical practitioners (TMP) and herb sellers from ten villages were interviewed. Four human pathogenic strains of yeast and *Cryptococcus neoformans*, a yeast-like basidiomycete causing meningitis in immunocompromised individuals, were used for the antifungal screening.

Results: The traditional medical practitioners (TMP) and herb sellers explained that the leaves and fruits are the most commonly used plant parts for the treatments. The oral intake of the extracts in locally produced bamboo alcohol (Kai-kai) is the most common method of administration. In

accordance with these recorded traditional uses, we found that extracts of *P. guineense* were growth inhibitory against the fungal strains with MIC values ranging from 39 to 2500 µg/mL. The lowest MIC value of 39 µg/mL was recorded for a methanol fruit extract against *Candida albicans, C. glabrata* and *C. tropicalis*. In addition, ethanol and hexane fruit extracts were effective against the growth of *C. albicans* and *C. glabrata,* respectively, with a MIC of 78 µg/mL. Piperlongumine and piperine were active against *C. albicans* with MIC values of 39 and 78 µg/mL respectively. *Conclusion*: *P. guineense* fruit and leaf extracts, as well as their piperamide alkaloid constituents piperine and piperlongumine, have interesting antifungal properties and could have potential as new antifungal scaffolds. Our results warrant further in-depth investigations to isolate and characterize piperamide alkaloids and other compounds responsible for the antifungal activity in the extracts.

Key words: *Piper guineense*, antifungal uses, ethnobotanical survey, antifungal screening, piperine, piperlongumine

Introduction

Africa is richly endowed with medicinal plants that are utilized in the treatment of fungal diseases in traditional medicine and thus could be sources for standardized antifungal extracts and antifungal compounds. Examples of such plant species are *Jatropha curcas* L. (Euphorbiaceae), *Monodora myristica* Dunal. (Annonaceae), *Xylopia aethiopica* A. Rich. (Annonaceae) and *Piper guineense* Schumach &Thonn (Piperaceae) (Ngane et al., 2003; Abdelgadir and Van Staden, 2013; Dzoyem and Kuete, 2013). The mortality rate associated with fungal diseases is high in Africa especially among those living with HIV/ AIDS (Park et al., 2009). Due to the high cost of conventional medicine and accessibility of numerous medicinal plants in Sub-Saharan Africa, 70- 80% of the population use traditional medicine and plant-based preparations for the treatment of infectious diseases (Agyare et al., 2018). In most rural villages in Africa, the conventional health-care professionals are limited and there are no adequate health-care facilities, and thus, majority of the people patronize the traditional medical practitioners who use the readily available herbal formulations for the treatment of fungal infections (Osuchukwu et al., 2017).

African guinea pepper (*Piper guineense* Schumach &Thonn) is a medicinal plant and a spice that is highly valued in Africa because of its numerous ethno-medicinal uses (Besong et al., 2016; Ajibesin et al., 2011, Mgbeahuruike et al., 2017, 2018). The extracts of *P. guineense* are used to treat topical fungal diseases in West-African traditional medicine (Ngane et al., 2003). It is a vine that can grow up to 20 m in length with edible leaves and peppercorn fruits that have a strong pungent aroma

(Freiesleben et al., 2015). *P. guineense* is used for the preparation of herbal formulations for the treatment of numerous ailments and infectious diseases (Obodozie et al., 2010; Asase et al., 2012). Research has shown that fractions, natural compounds and extracts from *P. guineense* can be explored as antifungal preparations and agents for the prevention of skin infections (Ngane et al., 2003). In another study, it was established that *P. guineense* extracts could be used as botanical fungicides by local farmers because of the high cost and toxicity associated with synthetic fungicides (Asawalam, 2006). According to previous research, herbal formulations from medicinal plants are often more effective and safer than those from isolated active compounds (Fabricant and Farnsworth, 2001), and thus standardized extracts could be used in the combat against fungal infections to minimize the risk of the development of resistance.

Table 1. Summary of ethno-medicinal uses, pharmacology/antifungal screening of *Piper guineense.*

***P. guineense* plant part**	**Ethno-medicinal uses with reference source**	**Pharmacology/ Antifungal activity with reference source**	***In vivo/ In vitro***
Seeds/ Fruits	Seeds are used to prepare decoctions for the treatment of fungal infections (Ngane et al., 2003). Seeds and fruits in combination with other medicinal plants such as *Xylopia aethiopica* (Annonaceae) is boiled and administered to nursing mothers to aid the contraction of the uterus (Okigbo and Igwe, 2007).	Agar diffusion method with 90% ethanolic extract and fractions obtained by column chromatography. Amphotericin B, Griseofulvin and Clotrimazole at 10 µg/mL each were used as positive controls. MIC against *Candida albicans, Cryptococcus neoformans, Scopulariopsis brevicaulis, Aspergillus flavus,* and *Microsporum gypseum* were within the range of 50 -100 µg/mL for the extracts (Ngane et al., 2003).	*In vitro* and *in vivo* (Ngane et al., 2003).
	Seeds are used to prepare decoctions for the treatment of asthma and psychotic disorder (Gbekley et al., 2017; Oyemitan et al., 2015). The fruits are used for respiratory infections, female infertility, and aphrodisiac (Dzoyem et al., 2014). Fruits are used to prepare decoctions with other herbs to treat epilepsy (Abila et al. 1993).	The fruits were extracted with a mixture of methanol-dichloromethane (3:1 V/V) for 48 h at room temperature. Broth micro-dilution method was used, Nystatin was used as positive control, MIC was between 1.56 to 6.25 mg/mL against *Candida albicans, Candida parapsilosis, Candida tropicalis, Candida krusei*, *Candida glabrata* and *Cryptococcus neoformans* (Dzoyem et al., 2014)	*In vitro* (Dzoyem et al., 2014).
	Used for cough, bronchitis, rheumatism and syphilis (Konning et al., 2004; Sandberge et al., 2005). The fruits are used to prepare Yaji soup which is eaten as an aphrodisiac in West African countries (Ibrahim et al., 2010).	Agar diffusion method, Soxhlet 96% methanol extracts. Clotrimazole was used as a positive control, inhibition zones were between 7.4 to 9.8 mm against *Candida albicans* and *Aspergillus niger* (Konning et al., 2004).	*In vitro* (Konning et al., 2004).
	It is mixed with other herbs to produce Niprisan used in herbal medicine in West Africa for the treatment of sickle cell anemia (Obodozie et al., 2010, Freiesleben et al., 2015).	Petroleum ether extract obtained by Soxhlet extraction, aqueous extract and 95 % ethanolic extract. MIC was 200 mg/mL against *Aspergillus niger*, No positive control was mentioned (Ebana et al., 2016).	*In vitro* (Ebana et al., 2016).
Leaves	Leaves have anticonvulsive	Petroleum ether extract obtained by	*In vitro* (Ebana et al., 2016).

	activities and are used to make decoctions for the treatment of convulsion (Abila et al., 1993). Leaves are boiled with *Xylopia aethiopica* and taken for the treatment of malaria (Asase et al., 2012).	Soxhlet extraction, aqueous extract and 95 % ethanolic extract. MIC was 80 mg/mL against against *Aspergillus niger*, Significant antifungal activity was observed. No positive control was mentioned (Ebana et al., 2016).	
	Leaves are used to prepare soup for the treatment of sexually transmitted diseases (Ajibesin et al., 2011).	Hexane and ethanol extracts. The diameter of the inhibition zones were 11.0-34.0 mm against *Aspergillus niger and Aspergillus flavus* (Dada et al., 2013).	*In vitro* (Dada et al., 2013).
	Used for the treatment of dysentery and bronchitis (Ogunniran et al., 2009).	Water extracts obtained by maceration. 5, 10 and 15 % concentration of the water extract was used. The extracts were effective against *Fusarium oxysporum* at higher concentrations of 10 and 15 % (Abiala et al., 2015).	*In vitro* (Abiala et al., 2015).
Root	No references found	No references found	No references found

This present study is the first report resulting from an ethnobotanical survey on the traditional uses of *P. guineense* extracts for the treatment of fungal infections, including candidiasis, in South-Eastern Nigeria. Due to the frequent occurrence of fungal diseases in West Africa, this study was performed in Imo state, South-Eastern Nigeria, where *P. guineense* is predominantly used for the treatment of fungal diseases and for other various medicinal purposes. Imo state is one of the five states in South-Eastern Nigeria. The people in Imo state belong to the Igbo ethnic group and are predominantly Christians. This study area was selected because of the prevalence of fungal infections in the area, owing to the fact that it is a rain forest zone and the humid climate favours the occurrence of topical fungal infections. The area was also selected due to the fact that no previous investigations on the ethnopharmacology of *P. guineense* have been performed in this region. Moreover, the area is inhabited by over 4.8 million people, who largely cultivate *P. guineense* and depend on this species and other medicinal plants for the treatment of infectious diseases and among them fungal infections. Also, the people have a diverse cultural and historical background, and are well grounded with the knowledge of traditional medicine. However, this valuable traditional medicinal knowledge is usually not documented. Thus, the aim of the study was to conduct an ethnobotanical survey in order to document the ways in which traditional healers and herb sellers utilize *P. guineense* extracts and preparations for the treatment of fungal diseases. The investigation was focused on how fungal infections are recognized and diagnosed, how and which parts of *P. guineense* are collected, methods of preparations, administrations and treatments. To validate the ethnobotanical information, an *in vitro* screening of *P. guineese* extracts of the fruit and leaf was performed against selected human pathogenic *Candida* spp. and *Cryptococcus neoformans* in search for new antifungal extracts and agents.

2. **Materials and methods**

2.1 **Study area and survey**

Imo state is situated in South-Eastern Nigeria and share boundary with the South-South geo-political zone of Nigeria. It is a tropical rain forest zone located between 4°45 -7°15N and 6°50 - 7°25E, covering a total land area of 5,530 km^2 (Fig. 1). An ethnobotanical survey was conducted in Imo state, where *P. guineense* is frequently used as herbal remedy in the treatment of candidiasis and other fungal diseases. The field work was conducted in ten localities between November and December 2017. A total of 20 traditional healers and herb sellers were interviewed (two from each locality). The purpose of the work was to validate the use of *P. guineense* in the treatment of fungal diseases. The 10 localities visited during the interviews were chosen by random sampling technique and are not clustered, but are all distributed within the three senatorial districts in Imo state as seen in Figure 1. The two traditional healers interviewed from each locality were independent from each other.

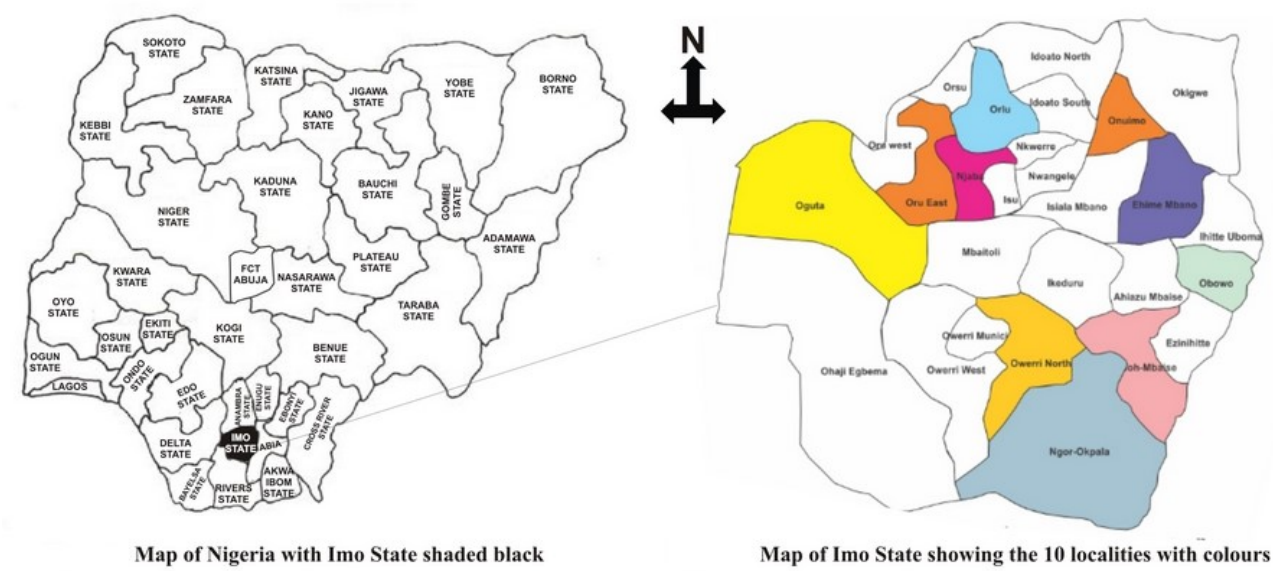

Fig 1. Map of Nigeria showing the study area (Imo state) shaded in black and Map of Imo state showing the 10 localities shaded in colours. Source, Eunice Ego Mgbeahuruike, University of Helsinki, Finland and Chijioke Chukwueke (Graphic designer).

2.2 **Data collection**

In the ethnobotanical survey, a house to house strategy was used with the permission of the village heads and traditional rulers. First, an ethical consideration of the project was done appropriately and approved by the University of Helsinki ethical committee, the survey plan was also approved by the head of the Division of Pharmaceutical Biosciences of the University of Helsinki, Finland. The village heads were notified prior to the investigation and the traditional healers were also well informed. The investigating team obtained an ethical approval from each of the village heads. The individual traditional healers gave an informed consent for the use of the data and pictures obtained during the interview. The consent was an oral agreement which was obtained from each of the traditional healers before the interviews began and permission to publish any photographs resulting from the interviews was obtained too. Detailed and validated questionnaires (Appendix A) were administered to twenty traditional healers (2 from each locality). This was followed by series of questions and conversations, so as to obtain the information needed for the work. The questionnaires were written in English, but the local language (Igbo) was used during the interviews and conversations because most of the participants could not interact well in English even though they had formal education. The traditional healers provided samples of the leaves, and fruits of *P. guineense* which was identified by a member of the investigating team who is a botanist. The traditional healers cooperated with our investigating team and some cash gifts were given to them at the end of the survey.

2.3 **Plant material for antifungal screening**

The fruits and leaves of *P. guineense* (African guinea pepper) used in this study were collected from a rural village in Imo State, South Eastern Nigeria. The plant materials were authenticated at the Department of Crop Science of the Federal University of Technology, Owerri, Nigeria. Voucher specimens are deposited in the herbarium of the Department of Crop Science of the same university with the specimen number FUTO/SAAT/NS/005A for the fruit and FUTO/SAAT/NS/005B for the leaf.

2.4 **Extraction**

The air dried plant materials were milled with a grinder to obtain finely ground powdery samples. Sequential extraction was carried out using solvents of varying polarities, starting with the least polar solvent. First, 40 g of the plant material was extracted with 300 mL of hexane, followed by extraction with 300 mL of chloroform, then 300 mL of ethanol and the residue was finally extracted and washed with 300 mL of methanol. Fruits and leaf plant materials were used for the extractions.

160 The extraction with each of the solvents was conducted in duplicates and each extract, except from
161 the water extracts, were filtered using filter paper (Whatman GE Healthcare, Chicago, IL, USA).
162 The water extracts were centrifuged at 689 g for 5 minutes. For each extract, the filtrate was
163 evaporated using a rotary evaporator (Rotavapor, Heidolph VV2000) combined with a water bath
164 not exceeding +40°C, thereafter the extracts were lyophilized for two days to dry completely.
165 Macerations and hot water decoctions were also prepared from the plant samples since these
166 preparations are used in traditional medicine. Macerations were prepared by weighing 10 g of the
167 fruits and leaf plant materials into Erlenmeyer flasks. 100 mL of water was added and extraction
168 was performed for 24 hours using a magnetic stirrer. The mixture was centrifuged at 689 g for 15
169 min (Eppendorf AG centrifuge 5810R, Germany). For the decoctions, 10 g of the plant material was
170 boiled with 100 mL of water and allowed to cool. The mixture was centrifuged for 15 min at 689 g
171 (Eppendorf AG centrifuge 5810R, Germany). Both the macerations and decoctions were carefully
172 filtered using filter paper (Schleicher & Schuell, ∅=150 mm, Germany), and freeze dried for two
173 days in a lyophilizer. Prior to the agar diffusion test, the freeze dried extracts were reconstituted and
174 re-dissolved in their corresponding solvents or in MeOH to a final concentration of 50 mg/mL for
175 the antifungal screening according to the method of Anyanwu and Nwosu, (2014) and Salih et al.,
176 (2017).
177
178 2.5 **Fungal strains**
179 The fungal strains used in this investigation were obtained from the Division of Pharmaceutical
180 Biosciences, Faculty of Pharmacy, University of Helsinki, Finland. In all, the growth inhibitory
181 activity of the extracts were investigated using five fungal strains (*Candida albicans* ATCC 10231,
182 *Candida glabrata* ATCC 2001, *Candida tropicalis* ATCC 750, *Candida parapsilosis* ATCC 7330,
183 and *Cryptococcus neoformans* ATCC 10226).
184
185 2.6 **Antibiotics and pure compounds**
186 Amphotericin-B (Sigma-Aldrich, St. Louis MO, USA) and itraconazole (Sigma-Aldrich, St. Louis
187 MO, USA), were used as standard antibiotics for the investigation. Analytical grade piperine and
188 piperlongumine standards (≥ 97.0% purity) purchased from TCI Europe N.V. (Zwijndrecht,
189 Belgium) were used as standard compounds.
190
191 2.6.1 **Agar disk diffusion method**
192 Four human pathogenic strains of yeast and *Cryptococcus neoformans*, a yeast-like basidiomycete,
193 were used to investigate the growth inhibitory effects of *P. guineense* extracts. A total of twelve

194 extracts from the fruits and leaf extracts of this plant were tested against the fungi. An agar disk
195 diffusion method was applied for the initial screening according to Salih et al., (2017). The freeze
196 dried extracts were re-dissolved in those solvents used originally for their extraction according to
197 the method of Anyanwu and Nwosu, (2014). Each of the extracts were prepared to a final
198 concentration of 50 mg/mL (stock solution). Amphotericin-B and itraconazole were used as positive
199 controls. The antibiotics were dissolved in methanol to a final concentration of 10 mg/mL used for
200 the test. Sterile petri dishes (Ø =15 cm, VWR Finland) were used for the screening. For the
201 screening, twenty-five mL of sterile base agar (Antibiotic agar No. 2, Difco, VWR Finland) was
202 applied as a bottom layer into the sterile petri dishes using a sterile, serological pipet (Falcon,
203 Becton Labware Europe) and allowed to solidify. Thereafter twenty-five mL of Saboraud agar
204 (OXOID, Thermo Fisher Scientific) was applied as the top layer. The petri dishes were all allowed
205 to solidify, and then stored in +4°C. The screening started with inoculation of the fungal strains
206 onto solid Saboraud agar slants which were incubated for 48 h at +37°C. The viable yeast cultures
207 from the agar slants were used to prepare an inoculum for the test. Fungi from the agar slants were
208 transferred into 2 mL of 0.9% (w/v) sodium chloride (NaCl) solution in a sterile glass tube using a
209 sterile inoculation loop. 1 mL of the suspension was transferred into another sterile glass tube, and
210 the absorbance was measured at 625 nm (UV–Visible Spectrophotometer, Pharmacia LKB-
211 Biochrom 4060). The other 1 mL of the suspension (sterile part) was diluted with the 0.9% NaCl
212 solution so that the absorbance at 625 nm becomes 0.1 (this suspension contains approximately 1.5
213 $\times 10^8$ CFU /mL). 200 µL of this diluted fungal suspension was spread evenly on each petri dish and
214 left to dry for some seconds with the lid open. A sterile cork borer (11mm diameter) was used to
215 make six holes equidistantly from each other on the agar surface of the petri dishes. 200 µL of the
216 50 mg/mL plant extracts and 200 µL of the 10 mg/mL antibiotics were carefully pipetted into the
217 holes respectively. Methanol, ethanol, hexane and chloroform, 200 µL of each, were used as solvent
218 controls respectively. The solvents were found not to inhibit the growth of the fungi. The petri
219 dishes were incubated at +4 °C for 1 h, and thereafter they were incubated for 48 h at +37 °C. The
220 diameters of the zones of inhibition were measured with a caliper under a petri dish magnifier and
221 expressed as the mean of the diameters of three replicates ± SEM.
222 The Activity index (AI) of the various extracts were measured in relation to the standard antibiotics
223 Amphotericin-B and itraconazole according to Fyhrquist et al., (2014).
224

225 Thus, AI = $\frac{\text{Inhibition zone of the plant extract}}{\text{Inhibition zone of standard antibiotic}}$

227

228 2.6.2 **Microdilution turbidimetric broth method for MIC and MFC estimation**

229 From the result obtained from the agar disk diffusion assay, minimum inhibitory concentration
230 (MIC) was estimated for some selected extracts based on their good antifungal activity. MIC is
231 considered to be the lowest concentration of an extract or compound resulting in the inhibition of at
232 least 90% of the growth of a fungal strain. MIC values were determined using a microdilution
233 turbidimetric broth method based on the guidelines of Clinical and Laboratory Standards Institute
234 (2012). Only extracts which expressed marked antifungal activity in the agar disk diffusion assay
235 were tested for MIC. For the MIC evaluation, two-fold serial dilutions of the extracts from 9.75-
236 2500µg/mL were prepared in sterile Saboraud broth. Commercial pure compounds, piperine
237 (1mg/mL concentration in methanol) and piperlongumine (1mg/mL concentration in methanol)
238 were also two-fold serially diluted in Saboraud broth. Itraconazole and amphotericin-B were each
239 two-fold serially diluted in Saboraud broth from 0.48-125 µg/mL respectively. 96 well microtiter
240 plates were used for the tests (Nunc, Nunclone, Denmark). The fungal cultures were inoculated on
241 Saboraud agar slants or in 5 mL Saboraud broth and grown for 48 hours at +37°C before the test.
242 The absorbance of 1 mL of the 48 h fungal culture was measured for turbidity at $\lambda = 625$ nm using a
243 UV–Visible Spectrophotometer type 1510 (Thermo Fisher Scientific Oy). The absorbance was
244 adjusted to 0.1 at 625 nm (approximately 1.0×10^8 CFU/mL). 100 µL of this suspension $A_{625}= 0.1$
245 was further diluted 100-fold to get a working suspension or inoculum containing 1.0×10^6
246 CFU/mL. 100 µL of this inoculum, and 100 µL of the plant extracts, pure compounds, antibiotics,
247 and solvent controls, were pipetted into the 96 well microtiter plates. Therefore, each well contained
248 5×10^5 CFU/mL. The solvent controls contained a maximum of 5 % (v/v) of each solvent to be
249 tested for toxicity. At this concentration the solvents were found not to be toxic. The growth control
250 (GC wells) contained only the fungal suspension, and the test wells (T wells) contained plant
251 extracts or pure compounds + fungal suspension. Moreover, sample controls wells were prepared
252 for each plant extract/compound to be tested, and these wells contained plant extract/pure
253 compound and the broth only. The microwell plates were incubated for 48 hours in an incubator
254 coupled to a shaker at +37°C. The turbidity of the wells at $\lambda = 620$ nm was recorded using a Victor
255 1420 spectrophotometer (Wallac, Finland). The tests were done in triplicate and the % growth was
256 expressed as the mean of these triplicates ± standard error of mean (SEM). The minimum fungicidal
257 concentration (MFC) was evaluated by pipetting 100 µL from those wells of the microtiter plate,
258 which contained the MIC concentration as well as 2 and 4 times higher concentrations than their
259 MIC values on petri dishes (∅ = 9 cm) containing Saboraud agar, and incubating the dishes for 48

hours at +37°C. The MFC was taken as the lowest concentration where no visible growth on the petri dish was observed after the incubation.

3. **Results and discussion**

3.1 **Ethnobotanical survey and field study**

An ethnobotanical survey was carried out in Imo state in South-Eastern Nigeria, as shown in Fig. 1. During the interviews and conversations, the various methods of preparations and administrations of the decoctions and concoctions from the roots, leaves, and fruits of *P. guineense* for the treatment of fungal diseases, were widely explained by the traditional healers. 20 traditional healers (14 male and 6 female) were interviewed with the cooperation of their village heads. The participants were between the ages of 40 to 70 years. In the communities visited, it was observed that most of the traditional healers are of older age and have long been practicing traditional medicine. It was also observed that there were more male than female traditional healers and this is in line with previous findings that there are more male than female traditional healers in Africa (Cheikhyoussef, et al., 2011; Ngarivhume et al., 2015). The socio-economic characteristics of the respondents are shown in Table 2.

Table 2. Demographic characteristics of the respondents (n=20)

Parameters	Specifications	Number of Respondents
Gender	Male	14
	Female	6
Occupation	Herbalist	7
	Traditional medical practitioners	5
	Traditional healers/ herb sellers	8
Age	20 - 35	0
	36 - 55	9
	56 - 75	11
Year of practice (experience)	1 – 15 years	6
	16 -30 years	9
	31 years and above	5
Learning/source of knowledge of traditional medicine	Inherited and learnt from parents	12
	Training from other herbalists	8
Level of Education	University education	3
	High school	8
	Primary education	6
	No formal education	3
Religion	Christianity	16
	Traditionalist	4
	Islam	0

Interestingly, most of the traditional healers have remarkable experience in traditional medicine and have inherited and learnt the practice from their parents. All the participants have practiced herbal

281 medicine for a long period of time, and 11 out of the 20 participants have practiced traditional
282 medicine for over 25 years as their only source of income. Notably, some of the participants also
283 had university education and thus some of the information on the preparation of *P. guineense* they
284 also had in written form. One of the traditional healers has a big herbal clinic where he displayed
285 various herbal preparations from *P. guineense* and he is referred to as "Doctor" in his community.
286 It was observed during the course of this study that most of the traditional healers could read and
287 write, that is to say that the trend in traditional medicine in Africa is gradually changing to become
288 a modern profession. It was also observed that some of the traditional healers have books where
289 they recorded the name of each patient and the treatments. From the survey, a variety of herbal
290 preparations from various parts of *P. guineense* were identified. The respondents explained the
291 sources from which they obtain the plant materials for their traditional healing practice. Most of
292 them, apart from traditional healing practice, are also farmers. They have farms within their yards
293 where they planted *P. guineense* and other medicinal plants to be used for the treatment of various
294 infectious diseases and for the purpose of making local profit. The participants explained that they
295 sometimes sell *P. guineense* fruits and leaves in local markets as spices and medicine to the general
296 population. This illustrates that the traditional healers do not have problems in getting the plant
297 materials used for the herbal treatments since they are sold in the local markets. All the respondents
298 gave similar explanations on the symptoms and methods of diagnosis of the fungal infections. The
299 traditional healers explained that they could differentiate between bacterial, viral and fungal
300 infections based on the symptoms they observe in their patients especially when it comes from
301 fungal skin infection and oral thrush. They argued that it was easy for them to identify and
302 differentiate between these infections owing to their long time practice of traditional medicine.
303 They gave some common symptoms for the various fungal infections as shown in Table 3. The
304 method of preparation ranges from hot infusion, decoction in combination with *Xylopia aethiopica*
305 and then the plant material is soaked in mild alcohol as shown Table 3.
306
307 The traditional healers understood the dangers of fungal infections and sometimes send their
308 patients to the government hospitals if the symptoms persist as a result of their failed treatment or
309 lack of proper understanding of the type of fungal infection. The participants explained widely the
310 methods of administration of *P. guineense* extracts. The oral intake is the most common method of
311 administration and these correlates with the findings of Maroyi, (2013) that herbal preparations are
312 mostly administered orally by traditional healers. The traditional healers argued that the decoctions
313 made from the fruits and leaves, prepared in mild alcohol is mostly effective when administered
314 orally for the treatment of fungal diseases expressed by thrush on the tongue or candidal vaginosis.

When asked about the dosage, they further explained that it is usually taken with a small glass tumbler which can be measured to be about 100 mL, 3 to 4 times in a day. It was observed that the leaves and fruits are the most frequently used plant parts for the treatments and this correlates with the findings of Rahmatullah et al., (2012), that the leaves of medicinal plants are used more often in traditional medicine than the other plant parts perhaps because leaves are easy to collect. The traditional healers cooperated with the investigating team and claimed that their methods were effective in the treatment of fungal infections. This study demonstrates that the traditional healers could easily identify *P. guineense* owing to their long practice of the use of extracts of this species in herbal medicine. Our results indicate that *P. guineense* is an important medicinal plant which is highly utilized in the treatment of fungal infections in Imo state, South- Eastern Nigeria and could be a source for the discovery of a new antifungal scaffolds for drug discovery.

Table 3. List of fungal diseases often treated with *P. guineense* herbal remedies in Imo state, South Eastern Nigeria and the symptoms used by the traditional healers to diagnose the fungal infections. Frequency means the number of healers that mentioned it.

Diseases	Local names in native Igbo language	Symptoms/ Method of diagnosis	Plant parts used	Method of preparation	Administration	Frequency
Skin ringworm	Nwanra, ọria ringoringo	Ring-like rashes, and fever	Fruits and leaves	Soaked in alcohol local gin (Kai-kai)	Taken orally	8
Vaginal candidiasis	Ọria nwanyi	Vagina itching, unpleasant vaginal discharge, fever	Leaves, fruits, roots	Soaked in alcohol local gin (Kai-kai)	Administered orally	6
Fungal eye infection	Ọria anya	Eye itching, swollen of the eye	Leaves	Cold and hot water infusion	Administered in drops in the eye	3
Skin diseases	Ọria enuahụ	Rashes, itching, reddish skin, blisters	Fruits and leaves	Cold maceration combined with *Zingiber officinal*e	Applied on the skin	8
Urinary infections	Ọria akpaamịrị	Painful urination, constant stooling and body weakness	Leaves, and fruits	Soaked in alcohol local gin (Kai-kai)	Administered orally	5
Mouth infections/ Thrush	Ọbu, Ọria Ọnuụ, nla	Blisters in the mouth, sour throat, and fever	Fruits and leaves	Decoction combined with *Xylopia aethiopica*	Used to wash the mouth regularly and taken orally	7
Nail infections/ athlete's foot	Ọgbaukwu	Reddish and swollen toes and nails, itchy small blisters	Leaves, and fruits	Soaked in mild alcohol local gin (Kai-kai)	Taken orally	8
Scalp ringworm	Ọria Isiakpụkpọ, Akpụkpọisi	Bald patches, rashes and painful blisters on the head, fever and headache	Fruits and leaves	Soaked in mild alcohol to produce a paste, Hot infusion	Applied as paste on the affected area, infusion taken orally	8

The leaf and fruit extracts of *P. guineense* were most frequently used for skin diseases, nail infections/ athlete's foot, scalp ringworm and skin ringworm (recorded 8 times by the 20 traditional healers). Mouth infections/ Thrush and vaginal candidiasis were recorded 7 and 6 times respectively.

From previous reports, *P. guineense* extracts of the seed, fruit and leaf contained piperamide compounds such as piperine, dihydropiperine, piperylin, piperlonguminine, dihydropiperlonguminine, wisanine and dihydrowisanine (Adesina et al, 2003; Scott et al., 2005). However, to the best of our knowledge, no reports are available on the antifungal effects of isolated phytochemicals from *P. guineense*. The chemical structures of piperine, 4, 5-dihydropiperine, piperlonguminine, 4, 5-dihydropiperlonguminine, and piperylin are shown in Figure 2. These bioactive compounds have been identified as the major bioactive compounds in *P. guineense* fruit extract (Scott et al., 2005).

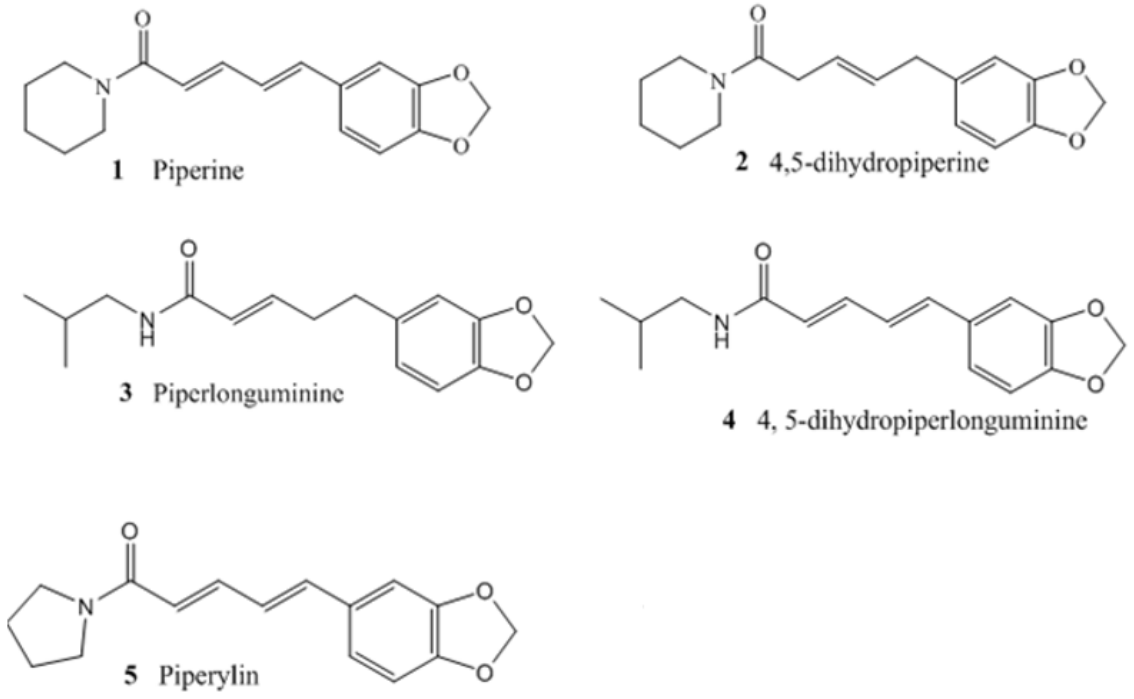

Fig. 2. Chemical structures of some piperamide compounds in *Piper guineense* fruits

3.2 Antifungal activity

In the present study, a total of 12 extracts of various polarities from the leaf and fruit of *P. guineense*, as well as hot water decoctions and cold water macerations were screened for antifungal activity against four yeast strains and *C. neoformans* (Table 4 and 5). Additionally, piperine and piperlongumine commercial piperamide compounds were also screened for antifungal activity. The screening was done based on our ethnobotanical results on the uses of *P. guineense* in West-African

351 traditional medicine for the treatment of fungal diseases (Table 3). For the investigation, some
352 potentially pathogenic fungal strains of *Candida* spp. and *Cryptococcus neoforamns*, which could
353 be responsible for some of the fungal diseases treated by the traditional healers were chosen.
354 Among all the fungal strains selected, *C. albicans* is known to be the most significant human
355 pathogenic species of yeast that can cause serious fungal diseases in humans (Brown et al., 2014).
356 The extracts were found to exhibit marked antifungal activity against the various fungi used in our
357 study and MIC values, as low as 39 µg/mL were recorded against some of the fungal strains (Table
358 5). Our results are in agreement with a previous report that fractions, natural compounds and
359 extracts from *P. guineense* can be explored as antifungal agents in the prevention of skin infection
360 (Ngane et al., 2003). Moreover, evaluation of the antifungal potentials of *P. guineense* extracts
361 along with other Cameroonian spices, revealed that the fruit extract of *P. guineense* is effective
362 against *C. albicans, C. parapsilosis, C. tropicalis, C. neoformans,* and *C. glabrata* with MIC values
363 ranging from 1560-6250 µg/mL (Dzoyem et al., 2014). Compared to this study, we recorded even
364 lower MIC and MFC values which could be due to different kinds of extraction methods, Dzoyem
365 et al. (2014) using only methanol: dichloromethane (3:1), but not sequential extraction.
366 Generally, from our study, we observed that the leaf extracts showed a different spectrum of
367 activity compared to the fruits. For example, the hexane leaf extract showed an inhibition zone of
368 21.7 mm against *C. albicans*, whereas the hexane fruit extract only gave an IZ of 14.3 mm against
369 this fungus. The result correlated well with the MIC values, which were recorded to be 78 µg/mL
370 for the leaf extract and 625 µg/mL for the fruit extract (Table 5). A similar trend was observed with
371 the leaf extract in ethanol which gave an inhibition zone of 23.8 mm, whereas the fruit extract gave
372 an IZ of 22.2 mm against *Cryptococcus neoformans.* The MIC values were 78 µg/mL and 156
373 µg/mL, respectively. However, this could be attributed to the fact that the concentration of the
374 antifungal compounds varies between different extracts, thus, this result demonstrates that the
375 leaves might contain a larger quantity of the bioactive compounds responsible for the antifungal
376 activity compared to the fruit extracts. This result could be supported by our ethnobotanical survey
377 results, where the traditional healers explained that the leaves of *P. guineense* are sometimes more
378 active in the treatment of some of the fungal infections and are therefore used more frequently than
379 the fruit for some specific symptoms of fungal infection. Moreover, our results are in accordance
380 with Rahmatullah et al., (2012), who argued that the leaf extracts of medicinal plants are used more
381 often in traditional medicine because they are usually more effective than extracts made from other
382 plant parts.
383

Table 4. Antifungal effects of extracts of *P. guineense*, piperine and piperlongumine against four potentially pathogenic strains of yeast and *Cryptococcus neoformans*. Results obtained with the agar diffusion method.

Plant extracts and antibiotics	*Candida glabrata*	*Candidaal bicans*	*Candida parapsilosis*	*Candida tropicalis*	*Cryptococcus neoformans*
PSMeOH	**24.3** ±0,33	**20,3**±0,33	18,0± 0,33	**21**,3± 0,67	**19,7**±0,33
AI. Itra.	1,49	0,80	0,61	0,82	0,66
AI. Amp.	0,71	0,57	0,41	0,73	0,52
PSCHCL$_3$	**23,3**± 0,33	17,7±0,33	14,7± 0,67	16,2±.0,17	18,2±0,17
AI. Itra.	1,43	0,70	0,49	0,62	0,61
AI. Amp.	0,68	0,50	0,34	0,55	0,48
PSHex	**20,3**±0,33	14,3± 0,33	15,3± 0,67	11,0±0,58	14,3±0,33
AI. Itra.	1,24	0,57	0,52	0,42	0,48
AI. Amp.	0,59	0,40	0,35	0,38	0,38
PSH$_2$o*	NA	NA	NA	NA	NA
PSHH$_2$o	NA	NA	NA	NA	NA
PSEthanol	NT	**21,8**± 0,17	NT	**19,8**± 0,17	**22,2**±0,17
AI. Itra.	NT	0,86	NT	0,76	0,75
AI. Amp.	NT	0,61	NT	0,68	0,58
PLMeOH	**20,7**± 0,31	**19,7**±0,33	**28**,7±0,67	17,3± 0,33	**21,3**±0,67
AI. Itra.	1,27	0,78	0,97	0,66	0,72
AI. Amp.	0,60	0,55	0,66	0,59	0,56
PLCHCL$_3$	11,7± 0,33	18,0±0,00	13,7±.0,17	15,5± 0,50	17,3±0,33
AI. Itra.	0,71	0,71	0,46	0,59	0,58
AI. Amp.	0,34	0,50	0,31	0,53	0,46
PLHex	13,7± 0,33	**21,7**± 0,33	17,8±0,17	12,0± 0,58	11,7± 0,33
AI. Itra.	0,84	0,86	0,60	0,46	0,39
AI. Amp.	NT	0,61	0,41	0,41	0,31
PLH$_2$o*	NA	NA	NA	NA	NA
PLHH$_2$o	NA	NA	NA	NA	NA
PLEthanol	NT	**19,8**± 0,17	NT	18,2±0,17	**23,8**±0,17
AI. Itra.	NT	0,78	NT	0,69	0,80
AI. Amp	NT	0,56	NT	0,62	0,63
Pure compounds					
Piperine	17,3± 0,67	18,3±.0,33	**23,3**± 0,33	16,7±0,67	11,0±.0,00
AI. Itra.	1,06	0,72	0,79	0,64	0,37
AI. Amp	0,50	0,51	0,53	0,57	0,29
Piperlongumine	14,7± 0,33	**19,3**±.0,33	16,3± 0,33	18,0±0,00	11,8±0,17
AI. Itra.	0,90	0,76	0,55	0,69	0,40
AI. Amp	0,43	0,54	0,37	0,61	0,31
Antifungal drugs					
Itraconazole	16,3± 0,33	**25,3**±.0,33	**29,7**± 0,67	**26,2**±0,17	**29,7**±0,33
AI. Amp	0,48	0,71	0,68	0,89	0,78
Amphotericin B	**34,3**± 0,33	**35,7**± 0,33	**43,7**± 0,67	**29,3**±.0,67	**38,0**± 0,00
AI. Itra.	2,10	1,41	1,47	1,12	1,28
Solvent controls					
Methanol	2.3	2,8	NA	NA	NA
Chloroform	NA	NA	NA	NA	NA
Hexane	3,8	2,3	NA	NA	NA
Ethanol	NT	3, 7	NT	2, 3	NA

The diameter of the zones of inhibition in mm as the mean of triplicates (n = 3) ± SEM (standard error of mean). 200µL of extracts and fractions (50 mg/mL), as well as pure compounds and antibiotics (10 mg/mL) were applied in the wells. PSMeOH, *P. guineense* fruit methanol extract; PSCHCL$_3$, *P. guineense* fruit chloroform extract; PSHex, *P. guineense* fruit hexane extract; PSH$_2$o*, *P. guineense* fruit cold water maceration; PSHH$_2$o, *P. guineense* fruit hot water decoction; PSEthanol, *P. guineense* fruit ethanol extract; PLMeOH, *P. guineense* leaf methanol extract; PLCHCL$_3$, *P. guineense* leaf chloroform extract; PLHex, *P. guineense* leaf hexane extract; PLH$_2$o*, *P. guineense* leaf cold water maceration; PLHH$_2$o, *P. guineense* leaf hot water decoction; PLEthanol, *P.*

393 *guineense* leaf ethanol extract; AI Itra., activity index in relation to itraconazole; AI Amp., activity index in relation to amphotericin
394 B; NA, not active and NT, not tested. The results marked with bold show promising antifungal activity.

395 Interestingly, the water extracts were not active against the fungal strains evaluated in our study.
396 Our results are consistent with a previous report that cold water macerations and hot water
397 decoctions of *P. guineense* are not active against *C. albicans* and other *Candida* spp. (Okigbo and
398 Igwe, 2007). Contrary to our results, water extracts of *P. guineense* are used in traditional medicine
399 for fungal infections, even though it is sometimes administered as herbal formulations in
400 combination with other medicinal plants, and often mixed with the local gin (Table 3). In our study,
401 we observed that some of the alcohol and n-hexane extracts have marked antifungal activity with
402 MIC values as low as 39 µg/mL, and the inhibitory activity recorded with these extracts could
403 result from their piperamide compounds, which are not well dissolved in water. It is possible that
404 the water extracts contain only small quantities of piperamide alkaloids, which might explain their
405 inactivity against the yeasts. The water extracts may contain other bioactive compounds than
406 alkaloids, and thus *P. guineense* water extracts might be better to use in combination with other
407 medicinal plants as herbal remedy for treatment of fungal infections in traditional medicine, which
408 could explain the uses of *P. guineense* water extracts as concoctions for fungal infections in West-
409 African traditional medicine.
410 Our results demonstrate that the ethanol fruit and leaf extracts of *P. guineense* give good growth
411 inhibition against *C. albicans* (IZ 21.8 mm and 19.8 mm, respectively) (Table 4), and accordingly
412 both extracts gave low MIC values of 78 µg/mL against *C. albicans*. *C. albicans* is an opportunistic
413 pathogenic fungus that can cause serious systemic infections in humans, affecting mostly
414 individuals with compromised immune defences, such as HIV/AIDS patients and elderly people
415 (Brown et al., 2014). *C. albicans* is the main causative agent of candidiasis and our results are in
416 agreement with the traditional uses of the fruits and leaves soaked in mild alcohol for the treatment
417 of vaginal candidiasis (Table 3). Our result is also consistent with a previous report by Ejele et al.,
418 (2012), that ethanol extracts of *P. guineense* have significant activity against *C. albicans*. We also
419 observed significant and promising growth inhibitory results of 20.3 mm and 19.7 mm with the
420 methanol fruit and leaf extracts against *C. albicans*. Moreover, the chloroform extracts were active
421 against *C. albicans* with a MIC value of 78 µg/mL for the leaf and fruit extracts. In our screenings,
422 piperlongumine was found to be very active against *C. albicans* with a MIC value of 39 µg/mL,
423 while piperine gave a MIC of 78 µg/mL. To the best of our knowledge, the inhibitory efficacy of
424 piperlongumine has not been tested against *C. albicans*, and little information is available on the
425 activity of piperine on *Candida* species. Since there is a report of multi-drug resistance in *C.*
426 *albicans* (Gulshan and Moye-Rowley, (2007), our result demonstrates that piperlongumine and

piperine could be scaffolds for new natural plant derived antifungal agents to combat multi-drug resistance in *Candida* strains. More research should be conducted to ascertain the antifungal mechanism of action of piperamide alkaloids as well as on producing extracts of *P. guineense* leaves or fruits which would be standardized to their piperamide alkaloid contents.

In comparison to our results with the plant extracts and alkaloids, our amphotericin-B standard drug showed a large inhibitory zone of 35.7 mm and a MIC of 0.48 µg/mL against *C. albicans*. The activity of *P. guineense* extracts and its piperamide alkaloids on *C. albicans* demonstrates that its extracts and alkaloids could be effectively utilized in combinations with conventional antifungals for the treatment of infections caused by antibiotic resistant *C. albicans* strains.

We also recorded promising growth inhibitory effects against *C. glabrata,* the second most significant human pathogenic species of yeast.The methanol, chloroform, and hexane extracts were all more active against *C. glabrata* than itraconazole (Table 4). Methanol and chloroform extracts gave the largest inhibition zones against *C. glabrata* (IZ ranging from 24.3 mm to 20.7 mm) (Table 4). The MIC value for the chloroform fruit extract was 78 µg/mL. Notably, itraconazole was only moderately active against *C. glabrata*, with an inhibition zone of 16.3mm and a MIC value of 15.6 µg/mL. Drug-resistance against itraconazole has been found to be associated with 5% of nosocomial isolates of *C. glabrata* (Walker et al., 2013), and our results demonstrate that extracts of *P. guineense* could be sources for new antifungal scaffolds and drug adjuvants against drug resistant *C. glabrata*. A very low MIC value of 0.48 µg/mL was recorded with amphotericin B against *C. glabrata*, and our result is in agreement with the more frequent use of amphotericin B for the treatment of severe infections caused by *C. glabrata* (Mario et al., 2012). Piperine was active against *C. glabrata* with a MIC of 78 µg/mL, while piperlongumine was moderately active with a MIC value of 156 µg/mL. Apart from *C. albicans, C. glabrata* is another important fungal pathogen responsible for systemic infections which are associated with high mortality rate (Pfaller et al., 2004; 2007). Our results demonstrate that the extracts and the piperamide compounds could be possible sources and antifungal drug scaffolds for the discovery of a new antifungal drugs and drug adjuvants for the treatment of systemic infections caused by *C. glabrata.*

Table 5. Minimum inhibitory concentration and minimum fungicidal concentrations (MIC and MFC) in µg/mL of fruit and leaf extracts of *Piper guineense* and the alkaloids piperine and piperlongumine

Plant extracts and antibiotics	*Candida glabrata*	*Candida albicans*	*Candida parapsilosis*	*Candida tropicalis*	*Cryptococcus neoformans*

PSMeOH					
MIC	**39**	**39**	625	**39**	156
MFC	78	78	1250	78	312
PSCHCL$_3$					
MIC	**78**	**78**	625	312	156
MFC	312	156	125	625	312
PSHex					
MIC	**78**	625	625	625	1250
MFC	1250	1250	1250	1250	2500
PSEthanol					
MIC	NT	**78**	NT	**78**	156
MFC	NT	156	NT	156	625
PLMeOH					
MIC	**78**	156	312	**78**	156
MFC	156	312	625	156	312
PLCHC$_3$					
MIC	**78**	**78**	625	312	156
MFC	156	156	1250	625	312
PLHex					
MIC	312	**78**	625	312	1250
MFC	625	625	1250	625	2500
PLEthanol					
MIC	NT	**78**	NT	156	**78**
MFC	NT	156	NT	312	156
Piperine					
MIC	**78**	**78**	**39**	**78**	312
MFC	156	156	78	156	625
Piperlongumine					
MIC	156	**39**	156	**78**	312
MFC	312	78	312	156	625
Amphotericin B					
MIC	**0.48**	**0.48**	**0.48**	**0.48**	**0.97**
Itraconazole					
MIC	**0.97**	**15.6**	**0.97**	**0.48**	**0.48**

MIC value represents mean of triplicates. MIC, minimum inhibitory concentration; MFC, minimum fungicidal concentration. For key to other abbreviations, see Table 4. Only extracts with promising antifungal effect in the agar disk diffusion assay were tested for MIC. The MFC was evaluated by taking 100 µL from the wells containing extracts of the MIC concentration and 2 times and 4 times the MIC concentrations and inoculating on a petri dish for 48 hours. The MFC was taken as the lowest concentration where no visible growth was observed after the incubation. The results marked with bold show promising MIC values.

For *Candida tropicalis*, the methanol fruit extract gave the largest inhibition zone (IZ 21.3 mm) and a MIC value of 39 µg/mL. The MIC value for piperine against this fungus was 78 µg/mL and thus was less effective than the methanol fruit extract. Piperine has been reported to be one of the main piperamide compounds found in *P. guineense* (Scott et al., 2005). Our result could indicate that some other piperamide compounds in the methanol fruit extract could be responsible for the good growth inhibitory effects of the extract, or then that these compounds act in synergy together with piperine to produce a lower MIC than that of pure piperine. The other extracts of the fruits were moderately active against *C. tropicalis* with inhibition zones ranging from 19.8 to 11.0 mm (Table

4). The methanol leaf extract was also active against the fungus with a MIC of 78 µg/mL.The MIC value of the ethanol fruit and leaf extracts was found to be 78 µg/mL for both. In contrast to the other extracts, the decoctions and macerations showed no growth inhibition against *C. tropicalis*. All extracts, except for the macerations and decoctions were active against *C. parapsilopsis* with MIC values ranging from 312 to 625 µg/mL. The largest inhibition zone of 28.7 mm and 18.0 mm were recorded for a methanol leaf and fruit extract, respectively. A mixture of methanol-dichloromethane (3:1 v/v) of the fruit extract of *P. guineense* has been previously found to be active against this fungus with a MIC value of 625 µg/mL (Dzoyem et al., 2014). Our result indicates that pure methanol is a better extractant for antifungals than methanol-dichloromethane (3:1), since we recorded a larger inhibition zone of 28.7 mm and a lower MIC value of 312 µg/mL with the methanol leaf extract compared to Dzoyem et al., (2014). The growth inhibitory effect of the ethanol extracts were not tested against *C. parapsilopsis* due to lack of material. Piperine and piperlongumine were active against *C. parapsilosis* with inhibition zones of 23.3 and 16.3 mm, respectively. The MIC values for piperine and piperlongumine were found to be 39 µg/mL and 156 µg/mL, respectively. Thus, piperine could be responsible for some of the good activity displayed by the methanol leaf extract.

In our study, *P. guineense* extracts were active against *Cryptococcus neoformans*. The largest inhibitory activity was observed with the ethanol leaf and fruit extracts (23.8 mm and 22.2 mm) (Table 4). The MIC values for the ethanol leaf and fruit extracts were in agreement with the agar diffusion result (78 µg/mL and 156 µg/mL respectively). The methanol leaf and fruit extracts recorded inhibition zones of 21.3 mm and 19.7 mm respectively, and their MIC values were recorded to be 156 µg/mL for both. It was observed that the leaf extracts of *P. guineense* were more active against this fungus than the fruits extracts. The chloroform fruit and leaf extracts were active against the fungus with inhibition zones of 18.2 mm and 17.3 mm. The hexane fruit and leaf extracts were moderately active also (IZ 14.3 mm and 11.7 mm). The MIC values for the chloroform and hexane extracts against this fungus were 156 µg/mL and 2500 µg/mL, respectively. Our result is consistent with one previous report that *P. guineense* fruit extract has growth inhibitory activity against *C. neoformans* with a MIC of 312 µg/mL (Dzoyem et al., 2014). Piperine and piperlongumine were active with a MIC of 312 µg/mL for both.

Conclusion

P. guineense extracts, piperine and piperlongumine have been found to possess interesting antifungal properties as recorded in this study. The antifungal efficacy of the methanol, ethanol,

chloroform and *n*-hexane extracts of the fruit and leaf of *P. guineense*, as well as piperine and piperlongumine on various *C. albicans* and non- albicans *Candida* strains supports the use of *P. guineense* in the treatment of fungal infections in traditional medicine and demonstrates that extracts from this plant could contain bioactive compounds that could be utilized as therapeutic agents or scaffolds for the production of new antifungal drugs to treat diseases associated with *Candida* species and *Cryptococcus neoformans*. The study demonstrated that the extracts and piperamide compounds exert promising antifungal properties against pathogenic *Candida albicans* and other non-albicans *Candida* strains. Moreover, various extracts, especially from *P. guineense* leaves, showed a promising growth inhibitory profile against *Cryptococcus neoformans* which is known to cause life-threatening meningitis in immunocompromised individuals. In view of this current knowledge on alkaloids of *P. guineense* as antifungal agents, we recommend that additional research should be done to evaluate the *in vivo* antifungal properties of the extracts and piperamide compounds from *P. guineense* with some animal models. The marked inhibitory activity recorded with piperine and piperlongumine against *C. albicans* revealed that they could be good antifungal agents. Further research is needed to evaluate the inhibitory activity of these piperamide compounds in combination with currently known standard antibiotics because piperine and piperlongumine could possibly enhance the effects of the currently available antifungal drugs for the treatment of fungal infections. This study has clarified and broadened the traditional knowledge on the uses of *P. guineense* as an antifungal remedy in African traditional medicine. Systematic studies are in progress and for further investigations, HPLC-DAD and UHPLC/Q-TOF MS analysis are currently being conducted by our research team to characterize and identify the compounds present in the extracts with promising antifungal activity.

Author's contributions

E.E.M, Y.H, P.F, H.V, and C.A designed the experiment.

E.E.M and C.A conducted the field work, ethnobotanical survey and documentations in Nigeria.

E.E.M, Y.H, H.V and P.F performed the *in vitro* antifungal screening and MIC estimations. The manuscript was first drafted by E.E.M, C.A, and P.F. The manuscript was further revised and corrected by H.V, Y.H and P.F. All the authors have read, agreed and approved the final version of the manuscript.

Conflict of interest

The authors indicate no potential conflicts of interest.

Ethical issues

The ethical consideration of the project was done appropriately according to the authors' institution and was approved by the institutional ethics committee. The ethnobotanical survey was done with the approval of the village heads and according to the national institutional rules of the indigenous people.

Acknowledgement

The authors are grateful to the Faculty of Pharmacy, Division of Pharmaceutical Biosciences, University of Helsinki and Department of Food Science and Technology, School of Engineering and Engineering Technology, Federal University of Technology Owerri, Imo state, Nigeria for supporting this work. The first and last authors acknowledge the financial support from Ekhaga foundation (project grant number 2017-7), Sweden. The authors are grateful to the traditional healers and herb sellers for giving their time.

Appendix A. Supplementary material

Questionnaire used for the ethnobotanical survey on the traditional use of *Piper guineense* for the treatment of fungal infections in Imo state, South Eastern Nigeria.

We are researchers from the University of Helsinki, Finland and Federal University of Technology Owerri, Imo state, Nigeria. We would like to know the various methods that you use for utilization of Uziza (*Piper guineense*) for the treatment of fungal infections.

1. Gender Male ☐ Female ☐
2. What is your name (optional)……..
3. What is your major occupation? : a). Herbalist ☐ b). Traditional medical practitioner ☐
   c). Traditional healer/ herb seller ☐ d). Other ☐
4. What is your age? : a). 20-35 ☐ 36 – 55 ☐ 56 – 75 ☐
5. For how long have you been practicing traditional medicine? : a) 1 – 15 years ☐
   16 -30 years ☐ 31 years and above ☐
6. What is your source of knowledge of traditional medicine? : a). Inherited/learnt from parents ☐
   Training from other herbalists ☐ Other ☐
7. What is your level of education? : a). University education ☐ High school ☐
   Primary education ☐ No formal education ☐

571 8. What is your religion? : a) Christianity ☐ Traditionalist ☐ Islam ☐ Other ☐

572 9. Can you name some of the fungal diseases that you treat with Uziza (*Piper guineense*)?

…….

……

……....

10. How often do you treat these diseases?

……

11. What are the symptoms and how do you diagnose this fungal infections?

Fungal infections	Local name	Method of diagnosis/ symptoms

12. Which part of the plant do you often use?: Fruit ☐ Leaves ☐ Roots ☐ Other ☐

13. How do you get the *Piper guineense* used for the treatment? : a) Cultivate them ☐ b) Buy from the market ☐ c) Collect from the wild forest and bushes ☐ d) Other ☐

14. What are the method of preparations and administrations?

Fungal diseases	Method of preparation	Mode of administration

573 15. What are the dosage of administration and how long does the treatment last?

574 ……

575 16. What do you do if you observe that the patient is not responding to your antifungal treatment?

576 …….

577 Do you have some general comment about the possible challenges you face in your daily treatment
578 of fungal infections using *Piper guineense* extracts?

579 ……..

580

581 Thank you for your cooperation.

582

583 **References**

584 Abiala, M.A., Ayandeko, F.M., Odebode, A.C., 2015. Antifungal effects of selected botanicals on
585 fungal pathogens of watermelon fruit. Arch. Phytopathol. Plant Prot. 48, 569-577.

586 Abila, B., Richens, A., Davies, J., 1993. Anticonvulsant effects of extracts of the West African
587 black pepper, *Piper guineense*. J. Ethnopharmacol. 39, 113-117.

588 Abdelgadir, H. A., and Van Staden, J., 2013. Ethnobotany, ethnopharmacology and toxicity of
589 Jatropha curcas L.(Euphorbiaceae): A review. S. Afr. J. Bot, 88, 204-218.

590 Adesina, S.K., Adebayo, A.S., Adesina, S.K., Groning, R., 2003. New constituents of *Piper*
591 *guineense* fruit and leaf. Die Pharmazie. 58, 423-425.

592 Agyare, C., Spiegler, V., Asase, A., Scholz, M., Hempel, G., Hensel, A., 2018. An
593 ethnopharmacological survey of medicinal plants traditionally used for cancer treatment in the
594 Ashanti region, Ghana. J. Ethnopharmacol. 212, 137-152.

595 Ajibesin, K., Bala, D.N., Umoh, U.F., 2011. The use of medicinal plants to treat sexually
596 transmitted diseases in Nigeria: Ethnomedicinal survey of Niger Delta Region. Int. J. Green Pharm.
597 (IJGP). 5, 3.

598 Anyanwu, C., Nwosu, G., 2014. Assessment of the antimicrobial activity of aqueous and ethanolic
599 extracts of *Piper guineense* leaves. J. Med. Plants Res. 8, 436-440.

600 Asase, A., Hesse, D.N., Simmonds, M.S., 2012. Uses of multiple plants prescriptions for treatment
601 of malaria by some communities in southern Ghana. J. Ethnopharmacol. 144, 448-452.

602 Asawalam, E., 2006. Insecticidal and repellent properties of *Piper guineense* seed oil extract for the
603 control of maize weevil, *Sitophilus zeamais*. Electron. J. Environ. Agric. Food Chem. 5, 1389-1394.

604 Besong, E.E., Balogun, M.E., Djobissie, S.F., Mbamalu, O.S., Obimma, J.N., 2016. A review of
605 *Piper guineense* (African Black Pepper). Int. J. Pharm. Pharm. Res. 6, 368-384.

606 Brown, A.J., Brown, G.D., Netea, M.G., Gow, N.A., 2014. Metabolism impacts upon *Candida*
607 immunogenicity and pathogenicity at multiple levels. Trends Microbiol. 22, 614-622.

608 Cheikhyoussef, A., Shapi, M., Matengu, K., Ashekele, H.M., 2011. Ethnobotanical study of
609 indigenous knowledge on medicinal plant use by traditional healers in Oshikoto region, Namibia. J.
610 Ethnobiol. Ethnomed. 7, 10.

611 Cockerill, F.R., Wikler, M., Bush, K., Dudley, M., Eliopoulos, G., Hardy, D., 2012. Clinical and
612 Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing:
613 twenty-second informational supplement. Approved Standard—Ninth Edition. 950 West Valley
614 Road, Suite 2500, Wayne, Pennsylvania 19087, USA.

615 Dada, A.A., Ifesan, B.O.T., Fashakin, J.F., 2013. Antimicrobial and antioxidant properties of
616 selected local spices used in "Kunun" beverage in Nigeria. Acta Sci. Pol. Technol. Aliment. 12,
617 373-378.

618 Dzoyem, J. P., Kuete, V., 2013. Review of the antifungal potential of African medicinal plants. In
619 Antifungal Metabolites from Plants pp. 79-153. Springer, Berlin, Heidelberg.

620 Dzoyem, J.P., Tchuenguem, R.T., Kuiate, J.R., Teke, G.N., Kechia, F.A., Kuete, V., 2014. *In vitro*
621 and *in vivo* antifungal activities of selected Cameroonian dietary spices. BMC Complement. Altern.
622 Med. 14, 58.

623 Ebana, R., Edet, U., Ekanemesang, U., Ikon, G., Etok, C., Edet, A., 2016. Antimicrobial activity,
624 phytochemical screening and nutrient analysis of *Tetrapleura tetraptera* and *Piper guineense*. Asian
625 J. Med. Health. 1, 1-8.

626 Ejele, A., Duru, I., Oze, R., Iwu, I., Ogukwe, C., 2012. Comparison of antimicrobial potential of
627 *Piper umbellatum, Piper guineense, Ocimum gratissimum* and *Newbouldia laevis* extracts. Int. Res.
628 J. Biochem. Bioinform. 2, 35-40.

629 Fabricant, D.S., Farnsworth, N.R., 2001. The value of plants used in traditional medicine for drug
630 discovery. Environ. Health Perspect. 109 Suppl 1, 69-75.

631 Freiesleben, S.H., Soelberg, J., Jäger, A.K., 2015. Medicinal plants used as excipients in the history
632 in Ghanaian herbal medicine. J. Ethnopharmacol. 174, 561-568.

633 Fyhrquist, P., Laakso, I., Marco, S.G., Julkunen-Tiitto, R., Hiltunen, R., 2014. Antimycobacterial
634 activity of ellagitannin and ellagic acid derivate rich crude extracts and fractions of five selected
635 species of *Terminalia* used for treatment of infectious diseases in African traditional medicine. S.
636 Afr. J. Bot. 90, 1-16.

637 Gbekley, H.E., Katawa, G., Karou, S.D., Anani, S., Tchadjobo, T., Ameyapoh, Y., Batawila, K.,
638 Simpore, J., 2016. Ethnobotanical study of plants used to treat asthma in the maritime region in
639 Togo. Afr. J. Trad. Complement. Altern. Med. 14, 196-212.

640 Gulshan, K., Moye-Rowley, W.S., 2007. Multidrug resistance in fungi. Eukaryot. Cell. 6, 1933-
641 1942.

642 Ibrahim, J., Muazzam, I., Jegede, I., Kunle, O., 2010. Medicinal plants and animals sold by the
643 Yan-Shimfidas of Sabo Wuse in Niger State, Nigeria. Afr. J. Pharm. Pharmacol. 4, 386-394.

644 Konning, G., Agyare, C., Ennison, B., 2004. Antimicrobial activity of some medicinal plants from
645 Ghana. Fitoterapia. 75, 65-67.

646 Mario, D.A.N., Denardi, L.B., Bandeira, L.A., Antunes, M.S., Santurio, J.M., Severo, L.C., Alves,
647 S.H., 2012. The activity of echinocandins, amphotericin B and voriconazole against fluconazole-
648 susceptible and fluconazole-resistant Brazilian *Candida glabrata* isolates. Mem. Inst. Oswaldo
649 Cruz. 107, 433-436.

650 Maroyi, A., 2013. Traditional use of medicinal plants in south-central Zimbabwe: review and
651 perspectives. J. Ethnobiol. Ethnomed. 9, 31.

652 Mgbeahuruike, E., Yrjönen, T., Vuorela, H., Holm, Y., 2017. Bioactive compounds from medicinal
653 plants: Focus on *Piper* species. S. Afr. J. Bot. 112, 54-69.

654 Mgbeahuruike, E.E., Vuorela, H., Yrjonen, T., Holm, Y., 2018. Optimization of thin-layer
655 chromatography and high-performance liquid chromatographic method for *Piper guineense*
656 extracts. Nat. Prod. Commun. 13, 25-28.

657 Ngane, A.N., Biyiti, L., Bouchet, P., Nkengfack, A., Zollo, P.A., 2003. Antifungal activity of *Piper*
658 *guineense* of Cameroon. Fitoterapia. 74, 464-468.

659 Ngarivhume, T., van't Klooster, C.I., de Jong, J.T., Van der Westhuizen, Jan H, 2015. Medicinal
660 plants used by traditional healers for the treatment of malaria in the Chipinge district in Zimbabwe.
661 J. Ethnopharmacol. 159, 224-237.

662 Obodozie, O.O., Ameh, S.J., Afolabi, E.K., Oyedele, E.O., Ache, T.A., Onanuga, C.E., Ibe, M.C.,
663 Inyang, U.S., 2010. A normative study of the components of niprisan—an herbal medicine for
664 sickle cell anaemia. J. Diet. Suppl. 7, 21-30.

665 Ogunniran, K.O., 2009. Antibacterial effects of extracts of *Ocimum gratissimum* and *piper*
666 *guineense* on *Escherichia coli* and *Staphylococcus aureus*. Afr. J. Food Sci. 3, 77-81.

667 Okigbo, R., Igwe, D., 2007. Antimicrobial effects of *Piper guineense* 'Uziza'and *Phyllantus*
668 *amarus* 'Ebe-benizo'on *Candida albicans* and *Streptococcus faecalis*. Acta Microbiol. Immunol.
669 Hung. 54, 353-366.

670 Osuchukwu, N.C., Eko, J.E., Abia, R.P., Ochei, K.C., 2017. Use of herbal medicine among adult
671 residents in Calabar metropolis, Cross River State, Nigeria. J. Complement. Altern. Med. Res. 2, 1-
672 14.

673 Oyemitan, I. A., Olayera, O. A., Alabi, A., Abass, L. A., Elusiyan, C. A., Oyedeji, A. O., &
674 Akanmu, M. A. 2015. Psychoneuropharmacological activities and chemical composition of
675 essential oil of fresh fruits of *Piper guineense* (Piperaceae) in mice. J. Ethnopharmacol, 166, 240-
676 249.

677 Park, B.J., Wannemuehler, K.A., Marston, B.J., Govender, N., Pappas, P.G., Chiller, T.M., 2009.
678 Estimation of the current global burden of *Cryptococcal meningitis* among persons living with
679 HIV/AIDS. AIDS (London, England). 23, 525-530.

680 Pfaller, M.A., Diekema, D.J., 2007. Epidemiology of invasive candidiasis: a persistent public health
681 problem. Clin. Microbiol. Rev. 20, 133-163.

682 Pfaller, M.A., Diekema, D.J., 2004. Rare and emerging opportunistic fungal pathogens: concern for
683 resistance beyond *Candida albicans* and *Aspergillus fumigatus*. J. Clin. Microbiol. 42, 4419-4431.

684 Rahmatullah, M., Hossan, S., Khatun, A., Seraj, S., Jahan, R., 2012. Medicinal plants used by
685 various tribes of Bangladesh for treatment of malaria. Malar. Res. Treat. 2012, 371798.

686 Salih, E., Kanninen, M., Sipi, M., Luukkanen, O., Hiltunen, R., Vuorela, H., Julkunen-Tiitto, R.,
687 Fyhrquist, P., 2017. Tannins, flavonoids and stilbenes in extracts of African savanna woodland trees
688 *Terminalia brownii, Terminalia laxiflora* and *Anogeissus leiocarpus* showing promising
689 antibacterial potential. S. Afr. J. Bot. 108, 370-386.

690 Sandberg, F., Perera-Ivarsson, P., El-Seedi, H.R., 2005. A Swedish collection of medicinal plants
691 from Cameroon. J. Ethnopharmacol. 102, 336-343.

692 Scott, I.M., Puniani, E., Jensen, H., Livesey, J.F., Poveda, L., Sánchez-Vindas, P., Durst, T.,
693 Arnason, J.T., 2005. Analysis of Piperaceae germplasm by HPLC and LCMS: a method for
694 isolating and identifying unsaturated amides from *Piper* spp extracts. J. Agric. Food Chem. 53,
695 1907-1913.

696 Walker, L.A., Gow, N.A., Munro, C.A., 2013. Elevated chitin content reduces the susceptibility of
697 *Candida* species to caspofungin. Antimicrob. Agents Chemother. 57, 146-154.

698