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Evaluation of the Medicinal Uses and Antimicrobial Activity of *Piper guineense* Schumach &Thonn

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ACADEMIC DISSERTATION

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AUTHOR'S CONTRIBUTION TO THE ORIGINAL PUBLICATIONS

I. The author collected the plant materials, participated in planning the experiment with the co-authors, performed the extraction, performed the thin-layer chromatography experiments, participated in the high-performance liquid chromatographic experiments, participated in analyzing the data and interpreting the results, the author wrote the article with the co-authors.

II. The author planned the experiment in collaboration with the co-authors, prepared the plant materials and performed the sequential extraction, performed the antimicrobial activity experiments, participated in performing the HPLC-DAD and UHPLC-QTOF-MS experiments, participated in analyzing the data and interpreting the results, the author wrote the article with the co-authors.

III. The author planned the experiment, conducted the ethnobotanical survey in collaboration with the co-authors, prepared the plant materials and performed the sequential extraction, performed the antifungal screening experiments, analyzed and interpreted the results with the co-authors and wrote the article.

IV. The author participated in planning the experiments with the co-authors, participated in performing the antimicrobial activity and synergistic evaluation, analyzed and interpreted the results with the co-authors and wrote the article.

ABBREVIATIONS

AI Activity Index

AIDS Acquired Immune Deficiency Syndrome

ATCC American Type Culture collection

CFU Colony Forming Units

CLSI Clinical and Laboratory Standards Institute

DAD Diode Array Detector

FICI Fractional Inhibitory Concentration Index

GC Growth Control

HIV Human Immune-deficiency Virus

HPLC High Performance Liquid Chromatography

IZ Inhibition Zone

LOD Limit of Detection

LOQ Limit of Quantitation

MBC Minimum Bactericidal Concentration

MDR Multidrug-Resistant

MeOH Methanol

MFC Minimum Fungicidal Concentration

MIC Minimum Inhibitory Concentration

MS Mass Spectrometry

NaCl Sodium Chloride

PS Selectivity Value

QTOF Quadrupole Time of Flight

RPM Revolutions per Minutes

RSD Relative Standard Deviation

S Slope

SD Standard Deviation

SEM Standard Error of Mean

sp. Species

ST Solvent Strength

TLC Thin Layer Chromatography

TMP Traditional Medical Practitioners

UHPLC Ultra-High Performance Liquid Chromatography

UV Ultraviolet

ABSTRACT

Piper guineense is a medicinal plant that has wide application in African traditional medicine where it is often used in the treatment of bacterial and fungal infections. It is an economic plant with numerous health benefits which is also consumed regularly as a functional food. The fruits, leaves and seeds are used as spices and flavouring agents in commercial food preparations in West Africa. The extracts are also used for the treatment of various diseases ranging from diarrhea, intestinal diseases, rheumatoid arthritis, bronchitis, cough, stomach ache, asthma to febrile convulsions, fever and mental disorders. There is also recent interest on the biological and pharmacological properties of its bioactive compounds such as piperine, the main alkaloid constituents of *P. guineense* which is responsible for its pungent aroma. Based on these numerous ethnobotanical, traditional and economic uses of this plant, it became interesting to evaluate the bioactive compounds present in the extracts and to further screen the extracts against some selected human pathogenic bacterial and fungal strains so as to ascertain the efficacy of the extracts and its compounds as potent antibacterial and antifungal lead compounds. Microbial resistance to the currently available antibiotics is a global problem that has resulted to a constant search for a new antimicrobial drug with strong efficacy and low cost. There is need to screen the extracts and bioactive compounds from *P. guineense* for possible lead compounds for antibacterial and antifungal drug discovery.

In this study, first, a method was developed for the chemical profiling, qualitative and quantitative analysis of *P. guineense* extracts and a good mobile phase composition was developed for the high performance liquid chromatography (HPLC) and thin layer chromatographic (TLC) analysis of the extracts. The effect of the chamber type on the separation was also evaluated using unsaturated horizontal chamber in sandwich configuration, horizontal chamber in non-sandwich configuration and twin-trough vertical chamber. Furthermore, the *in vitro* antibacterial activity of the extracts were evaluated using 8 pathogenic Gram-positive and Gram-negative bacterial strains. An ethnobotanical survey was also conducted on the use of *P. guineense* extracts in the treatment of fungal infections in West African traditional medicine. The study area was chosen to be Imo state, South Eastern Nigeria were *P. guineense* is mostly used by traditional healers for the treatment of fungal infections which is often common among those suffering from HIV and AIDS. The aim of the survey was to document the various methods of preparations and administrations of these extracts for the treatment of fungal diseases. From this ethnobotanical approach, the leaves and fruits extracts of the plant was further tested against 5 fungal strains including *Cryptococcus neoformans* which causes meningitis in immunocompromised individuals.

HPLC and TLC methods were developed for the analysis of *P. guineense* extracts with emphasis on the shortest analysis time and minimal solvent consumption, and the best mobile phase giving favourable resolution of bands was found to be toluene: ethyl acetate (P_S 6-4 corresponding to 60:40 % v/v). The result of the TLC analysis showed that the developing chamber conditions does not affect the TLC separation efficacy in the analysis of *P. guineense* extracts. From the HPLC-DAD and UHPLC/Q-TOF MS investigation, piperine, dihydropiperine, piperylin, dihydropiperylin or piperlonguminine, dihydropiperlonguminine, wisanine, dihydrowisanine, and derivatives of piperine, and piperidine were identified in the extract of *P. guineense*. From the ethnobotanical survey, it was recorded that the leaves and the fruits extracts of the plant is the predominant plant part used for the treatment of fungal diseases in West African traditional medicine. A total of twenty traditional medical practitioners (TMP) and herb sellers explained their methods of administration of *P. guineense* extracts for the treatment of fungal infections. According to these results, the oral intake of the extracts in locally produced bamboo alcohol (Kai-kai) is the most common method of administration of the extracts for effective treatment. For the dosage, the TMPs explained that they use a small glass tumbler which measures about 100 mL, administered 3 to 4 times in a day. The TMPs explained that they sometimes send their patients to the government hospitals if the symptoms persist as a result of their failed treatment in order to avoid the death of their patients. From the results obtained from this study, it could be seen that *P. guineense* fruit and leaf extracts have activity against human pathogenic bacterial and fungal strains including multidrug-resistant pathogens such as *Pseudomonas aeruginosa*. The extracts were active against the tested bacterial and fungal strains with minimum inhibitory concentration (MIC) values ranging from 19 to 2500 µg/mL. Significant antifungal and antibacterial activity were observed with the lowest MIC of 19 µg/mL against *Sarcina sp.* A low MIC value of 39µg/mL was recorded for a methanol fruit extract against *Enterobacter aerogenes, Candida albicans, Bacillus cereus, Candida glabrata* and *Candida tropicalis*. Ethanol and hexane fruit extracts were effective against the growth of *Candida albicans, Staphylococcus aureus, Proteus mirabilis* and *Candida glabrata,* respectively, with a MIC of 78 µg/mL. Piperlongumine and piperine were active against the bacterial and fungal strains with MIC values ranging from 19 and 156 µg/mL. The water extracts were not active against the tested bacterial and fungal strains. Also, an *in vitro* biological and synergistic effects of piperine and piperlongumine were tested in combination with conventional antimicrobials (rifampicin, tetracycline and itraconazole) at various ratios. From the results, both piperine and piperlongumine showed synergistic effect against *Staphylococcus aureus* when combined at various ratios with rifampicin. Synergistic interaction was also observed with piperine

in combination with tetracycline against *Staphylococcus aureus* while antagonistic interaction was recorded for piperlongumine and tetracycline against *Staphylococcus aureus.*

The result of the present study demonstrates that *P. guineense* extracts contain antibacterial and antifungal compounds that may be useful for the discovery of new antibiotics.

1. INTRODUCTION

Piper guineense Schumach &Thonn, is a medicinal plant growing in various parts of Africa and other parts of the world. It is commonly called Ashanti pepper or African black pepper, and has been shown to be one of the most valuable spices with numerous health benefits (Ene-Obong et al., 2018). Economically, *P. guineense* is a spice which is used in flavouring local dishes in West Africa and the fruits are sold in local markets as condiments. It is a medicinal plant which has been utilized traditionally in the treatment of various ailments and infectious diseases such as rheumatoid arthritis, diarrhea, bronchitis, cough, intestinal diseases, stomach ache and asthma (Konning et al., 2004; Ogunniran, 2009; Gbekley et al., 2017). It is also used in the treatment of mental disorders, febrile convulsions, fever and to enhance female fertility (Oyemitan et al., 2015). It is a rich source of antioxidants and has been found effective in the management of liver diseases (Oyinloye et al., 2017). The leaves, seeds and fruits of this plant is used to prepare postpartum soup for women after child birth as it is believed that it helps in the contraction of the uterus and cleansing of the womb of women of possible remaining placenta after child birth and also enhances breast milk production (Uhegbu et al., 2015, Ene-Obong et al., 2018). It has been shown to have effect in white blood cell and red blood cell counts, thereby increasing the hemoglobin level (Uhegbu et al., 2015).

Biological studies have revealed that *P. guineense* extracts possess antifungal and antibacterial properties (Okeke et al., 2001; Konning et al., 2004; Tekwu et al., 2012). *P. guineense* extracts have been reported to have antifungal efficacy against various fungal strains, and could be a lead to new antifungal agents (Ngane et al., 2003). It was found to be effective against *Mycobacterium tuberculosis* which is the causative agent of tuberculosis, a life-threatening disease that is common in the developing countries (Tekwu et al., 2012). The essential oils and the extracts possess anticonvulsant, hypothermic, sedative, muscle relaxant, antipsychotic and anticonvulsant properties (Oyemitan et al., 2015). The extracts and essential oils also have antioxidant and antidiabetic activities and can be used in the treatment of depression (Okon et al., 2013; Oboh et al., 2013; Oyemitan et al., 2015).

P. guineense contains valuable bioactive compounds which could serve as therapeutic agents for drug discovery (Parmer et al., 1997; Adesina et al., 2003; Scott et al., 2005; Mgbeahuruike et al., 2017), and these bioactive compounds such as alkaloids and amides have been reported to be present in various parts of the plant (Adesina et al., 2003; Scott et al, 2005). Apart from its antibacterial and antifungal properties, *P. guineense* extracts and bioactive compounds have been reported to exhibit anticancer and antitumor efficacy and could be a potential lead to the discovery

of a new anticancer drug (Iweala, 2015; Bezerra et al., 2005). It contains piperine, an alkaloid with interesting pharmacological properties which has been reported to have antibacterial, antifungal and anticancer properties, and are also capable of enhancing the effectiveness of antimicrobial and chemotherapeutic drugs (Nageswari et al., 2018; Bezerra et al., 2005; 2006; 2008).

The aim of this study is to evaluate the medicinal uses of *P. guineense* and to screen the extracts and commercial compounds against some human pathogenic fungal and bacterial strains in search of antimicrobial lead compounds. *P. guineense* is of great importance in traditional medicine, it therefore became necessarily to broaden the knowledge on the antibacterial and antifungal activity of the extracts so as to ascertain their acclaimed uses in the treatment of bacterial and fungal diseases in traditional medicine. There is need to have also a readily available HPLC and TLC methods for qualitative and quantitative determination of these bioactive compounds for possible drug candidates.

2. REVIEW OF THE LITERATURE

2.1 Botanical description

P. guineense is a perennial climbing herb that is found growing mostly in tropical regions such as Africa and other tropical zones of the world (Oyemitan et al., 2015). The plant belong to the genus *Piper* of the Piperaceae family. It is often cultivated for its fruits, leaves and roots, by local farmers who earn a living by selling it to traditional healers and the populace. It is a herb which could grow up to 20m tall, climbing by means of its adventitious roots (Besong et al., 2016). It has various parts which are utilized for different medicinal purposes. It consists of black berry fruits, seeds, leaves, rhizomes and flower buds (Besong et al., 2016). The flowers which produces the fruits are greenish yellow, the fruits when riped are reddish brown and occur in clusters and then produces the seed. The fruit is small and round in shape (Fig. 1A) and has a hot pungent taste when chewed. The fruits are sold in markets as flavouring agents (Freiesleben et al., 2015). The leaves are green and oval in shape (Fig. 1B) and are used to prepare soup and decoctions for the treatment of various ailments. It is called Uziza (Igbo), Odusa (Ibibio/ Efik) or Iyere (Yoruba) in Nigeria, West Africa. It has other common names such as Guinea pepper, Benin pepper, False cubeb and Ashanti pepper (Besong et al., 2016). *Piper* has numerous species which have been reported to have varying chemical constituents based on their diverse nature (Besong et al., 2016, Oyemitan et al., 2015). Species found in the same geographical region could have variations in their chemical constituents. It is a vegetable plant that is used to enhance the taste of food.

Fig. 1. *Piper guineense* fruits (A) and leaves (B)

2.2 Traditional medicine in West Africa

West Africa is a region that is richly endowed with medicinal plants which are used for the treatment of infectious diseases (Oguntibeju, 2018). The population which are mostly poor income earners uses medicinal plants such as *Piper guineense* and *Xylopia aethiopica* for the treatment of various diseases. The region owing to its numerous medicinal plants is a good source of a diversity of bioactive compounds which could be potential lead compounds for drug discovery (Olorunnisola, et al., 2013). According to World Health organization (WHO), greater number of people in the developing countries like in West Africa rely on medicinal plants for the treatment of infectious diseases. The population often patronize traditional medical practitioners (TMP) and herb sellers for their primary health care needs. These TMPs collects medicinal plants which are easily accessible from the wild or from their farms to prepare decoctions and various herbal formulations for the treatment of diseases. Medicinal plants growing in tropical rainforest zone are rich sources of bioactive compounds and as such the ethnobotanical information of such plants are needed for an effective use as an antimicrobial lead compounds. In West Africa, the importance of traditional medicine cannot be overemphasized because of lack of modern facilities in the rural areas (Ode et al., 2011).

2.3 Traditional and medicinal uses of *Piper guineense*

Piper guineense is useful in traditional medicine and its nutritive and medicinal potentials have been outlined in various pharmaceutical studies (Obodozie et al., 2010; Uhegbu et al., 2015; Ekundayo et al., 1988; Ene-Obong et al., 2018). The fruits, seeds and leaves are often prepared in alcohol or as decoctions together with other herbal formulations, and used in the treatment of various diseases (Besong et al., 2016; Freiesleben et al., 2015). Ethno-pharmacologically, the roots, leaves, fruits and seeds of *P. guineense* are relevant herbal products in African traditional medicine, most importantly in West Africa where it is administered to nursing mothers to stimulate breast milk production and to aid the contraction of the uterus after child birth (Okigbo and Igwe, 2007). The fruits and seed extracts from this plant have been reported to be an important ingredient in the preparation of Niprisan herbal formulation used to treat sickle-cell anaemia (Freiesleben et al., 2015; Obodozie et al., 2010). Decoctions from the fruits and seeds are used in the treatment of venereal diseases, rheumatism, gastrointestinal diseases and respiratory diseases (Udoh, 1999). *P.*

guineense extracts are used in the treatment of mental disorder and fever, and have been found to possess sedative and muscle relaxant properties (Oyemitan et al., 2015). Extracts from *P. guineense* are used as aphrodisiac and it has been reported that Yaji soup, which is often eaten as an aphrodisiac in most West African countries, is prepared from the fruits of *P. guineense* (Ibrahim et al., 2010; Asase et al., 2012). Infusions and decoctions from the fruits of *P. guineense* are administered orally to treat bronchitis, cough, and intestinal diseases (Nwozo et al., 2017). Several studies have shown that extracts of *P. guineense* could lower lipid peroxidation thereby preventing inflammation and oxidative damage and are also used in the treatment of dysentery (Ogunniran, 2009; Nwozo et al., 2017). The leaves, fruits and whole plant parts of *P. guineense* are used to prepare herbal formulation for the treatment of asthma and its related symptoms (Gbekley et al., 2017). A previous research conducted on the ethnomedicinal uses of African medicinal plants has reported that the leave extracts *P. guineense* are used in the treatment of sexually transmitted diseases (Ajibesin et al., 2011). The fruits and leaves of *P. guineense* are ground and soaked in alcohol with other herbs to prepare concoctions used for the treatment of epilepsy, convulsion and malaria (Abila et al., 1993; Umoh et al., 2013). Previous research has shown that *P. guineense* extracts are useful in the treatment of fertility disorder (Mbongue et al., 2005) and have been used to stimulate sexual behavior in an adult male rat (Kamtchouing et al., 2002). It is also used as a food preservative and as fragrance in perfume and cosmetic industries (Nwozo et al., 2017).

2.4 Antifungal activities of *P. guineense*

The effect *of P. guineense* extracts on various fungal pathogens has been reported (Dada et al., 2013; Dzoyem et al., 2014; Abiala et al., 2015). *P. guineense* is effective against pathogenic fungal strains such as *Fusarium oxysporum, Fusarium solani, Macrophomina phaseolina, Fusarium verticillioides* and *Botryodiplodia theobromae* which are responsible for rots in watermelon fruits and other vegetable fruits (Abiala et al., 2015). *P. guineense* extracts are often used as botanical fungicides by local farmers because of high cost associated with synthetic fungicides. Evaluation of the antifungal potentials of *P. guineense* extracts along with other Cameroonian spices, revealed that it is effective against *Candida albicans, Candida parapsilosis, Candida tropicalis, Candida krusei, Candida lusitaniae, Cryptococcus neoformans, Candida guilliermondii* and *Candida glabrata* (Dzoyem et al., 2014). However the cold water maceration and hot water decoctions of the plant extracts do not have activity against *Candida albicans* (Okigbo and Igwe, 2007). The activity of *P. guineense* on *Candida albicans* demonstrates that its extracts could be effectively utilized in

treatment of infections caused by opportunists *Candida strains* in antifungal therapy. The antifungal efficacy of the fruit and leaf extracts of *P. guineense* on various *Candida* strains (*Candida albicans, Candida tropicalis, Candida parapsilosis, and Candida glabrata)* supports the use of the plant in the treatment of toilet infections and sexually transmitted diseases. *P. guineense* extracts were used as a natural insecticide to control maize weevil, *Sitophilus zeamais* (Asawalam, 2006).

Furthermore, Dada et al., 2013, reported that ethanolic and hexane extracts of *P. guineense* inhibited the growth of *Aspergillus flavus* and *Aspergillus niger.* The result shows that *P. guineense* has interesting antifungal properties and can be used as a good therapeutic agent in the discovery of a new antifungal drug. Research has shown that fractions, natural compounds and extracts from *P. guineense* can be explored as antifungal agents in the prevention of skin infection (Ngane et al., 2003). The efficacy of these fractions and extracts on panel of organisms such as *Microsporum gypseum, Trichophyton mentagrophytes, Trichophyton rubrum, Aspergillus flavus, Scopulariopsis brevicaulis,* and *Cryptococcus neoformans* shows that they can be effectively used to combat diseases and infections caused by these filamentous fungi.

2.5 Antibacterial activities of *P. guineense*

Bacterial infections often result to death if not well treated, and *P. guineense* has been widely reported to exhibit antibacterial properties (Okeke et al., 2001; Konning et al., 2004; Anyanwu and Nwosu, 2014). Extracts and fractions from various parts of this plant have antibacterial activity against Gram-positive and Gram-negative bacterial strains (Tekwu et al., 2012; Dada et al., 2013). It has been observed that the extraction solvent and method of extraction play a role in the inhibitory activity of the extracts on the bacteria, and previous research have shown that hexane, methanol and ethanol extracts are more effective than the water fractions (Dada et al., 2013, Konning et al., 2004). However, essential oils from the fruits of *P. guineense* did not have activity against *Escherichia coli*, *Salmonella typhi, Klebsiella sp*., and *Pseudomonas aeruginosa* (Olonisakin et al., 2006). *P. guineense* is effective against *Mycobacterium tuberculosis* which is a threat to human life (Tekwu et al., 2012). The ethanol extracts of *P. guineense* has been reported to be effective against *Bacillus subtilis*, *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhi*, *Klebsiella pneumoniae*, and *Proteus vulgaris* (Okeke et al., 2001). The antibacterial evaluation of the plant extracts on *Streptococcus faecalis* did not show remarkable activity (Okigbo and Igwe, 2007). The antibacterial activities of *P. guineense* on *Acinetobacter* spp., *Bacillus cereus*, *Escherichia coli, Salmonella* spp., *Shigella dysenteriae, Staphylococcus aureus* by Dada et al.,

2013, revealed that bioactive compounds from the plant are good antibacterial agent and could be a lead to the discovery of a new antibacterial drug. Previous research has shown that ethanol extracts of *P. guineense* has remarkable activity against *E. coli* (Anyanwu and Nwosu, 2014), and these researchers observed that the extracts inhibited the growth of *B. subtilis*, *E. coli*, and *S. aureus* with the least activity observed in *P. aeruginosa.*

2.6 Amides and alkaloids in *P. guineense*

Amides and alkaloids from *P. guineense* and other *Piper* species are called piperamides and these piperamide compounds have gained recognition in recent years for their strong efficacy as antibacterial, antifungal, anti-inflammatory, and antitumor agents in drug discovery (Adesina et al., 2003; Nageswari et al., 2018; Bezerra et al., 2005; Scott et al, 2005). Piperine, a potent alkaloid which has been reported to have antimicrobial activity is contained in substantive amount in *P. guineense* (Adesina et al., 2003, Scott et al., 2005). Other piperamide alkaloids found in *P. guineense* include piperylin, 4, 5-dihyropiperlonguminin, piperlonguminin , and 4, 5-dihydropiperine (Scott et al., 2005). The chemical structure of the piperamide compounds found in *P. guineense* are found in publication (**II**).

3. AIMS OF THE STUDY

The general aim was to study and have more knowledge on the efficacy of extracts and bioactive compounds of *P. guineense,* a medicinal plant and a spice that is highly valued in Africa because of its numerous ethno-medicinal uses. To optimize the HPLC and TLC methods for the isolation of the bioactive compounds and to evaluate the antimicrobial activity of its extracts and pure compounds as potent antimicrobial agent.

Specific aims of the thesis were:

1. To optimize a thin-layer chromatography (TLC) and a high performance liquid chromatographic (HPLC) method for the chemical profiling, qualitative and quantitative analysis of *P. guineense* extracts setting emphasis on achieving the best possible overall separation of the main components of the extracts (for example piperine) while keeping the analysis time and solvent consumption to a minimum (**I**).

2. To investigate the antibacterial activity of various extracts and fractions from *P. guineense* using pathogenic organisms such as Gram positive (*Sarcina* sp., *Staphylococcus aureus, Bacillis cereus,* and *Proteus mirabilis*) and Gram negative bacterial (*Enterobacter aerogenes, Escherichia coli, Pseudomonas aeruginosa,* and *Salmonella enterica*) (**II**).

3. To conduct an ethnobotanical survey and to investigate the antifungal activity of various extracts and fractions from *P. guineense* using various fungal strains (*Candida albicans, Candida tropicalis, Candida parapsilosis, Candida glabrata* and *Cryptococcus neoformans)* (**III**).

4. To investigate the *in vitro* pharmacological interaction between piperine and piperlongumine when used in combination at various ratios with some conventional antimicrobials (tetracycline, rifampicin and itraconazole) against *Pseudomonas aeruginosa, Staphylococcus aureus* and *Candida albicans* (**IV**).

4. MATERIALS AND METHODS

4.1 Plant material

The fruits and leaves of *P. guineense* used in this study were collected from a rural village in Imo State, South Eastern Nigeria in 2012 and 2013 by Eunice Ego Mgbeahuruike and Mrs Amandikwa chinyere of the Federal University of Technology, Owerri, Imo state, Nigeria. Voucher specimens were prepared and the plant materials were authenticated at the Department of Crop Science of the Federal University of Technology, Owerri, Nigeria by Professor Abraham Ngwuta. Voucher specimens were deposited at 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.

4.1.1 Extraction

The plant materials were powdered with an electric grinder to obtain finely ground samples. For the TLC and HPLC optimization experiments in publication (**I**), 1.0 g each of dried powdered fruits and leaves were extracted twice by sonication with 25 mL of methanol for 10 min. The extracts were filtered with Whatman filter paper (Whatman GE Healthcare, Chicago, IL, USA) into a flask of known weight. For the antibacterial and antifungal experiments in publications (**II and III**), 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. For each extract, the filtrate was evaporated using a Rotavapor (Heidolph VV2000) combined with a water bath at +40°C. In all, the extracts were lyophilized for two days to dry completely. Macerations and hot water decoctions were also prepared from the plant samples since these preparations are used in traditional medicine. Macerations were prepared by weighing 10 g of the fruits and leaf plant materials into Erlenmeyer flasks. 100 mL of water was added and extraction was performed for 24 hours using a magnetic stirrer. The mixture was centrifuged at 2000 rpm for 15 min (Eppendorf AG centrifuge 5810R, Germany). For the decoctions, 10 g of the plant material was boiled with 100 mL of water and allowed to cool. The mixture was centrifuged for 15 min at 2000 rpm (Eppendorf AG centrifuge 5810R, Germany). Both the macerations and decoctions were carefully filtered using filter paper (Schleicher & Schuell, Ø=150 mm, Germany), and freeze dried for two days in a lyophilizer. Prior to the agar diffusion test, the freeze dried extracts were reconstituted and re-dissolved in their corresponding solvents or in MeOH to a final concentration of 50 mg/mL for the antibacterial and

antifungal screening according to the method of Anyanwu and Nwosu, (2014) and Salih et al., (2017).

4.1.2 Chemicals, instruments and pure compounds

The solvents used for the study were HPLC grades of acetonitrile purchased from Merck KGaA (Darmstadt, Germany), *n*-hexane (Merck), chloroform (Merck), ethanol (Sigma-Aldrich Corp. (Merck), toluene (Merck), ethyl acetate (Merck), acetone (Merck), cyclohexane (Sigma-Aldrich Corp. (Merck), and methanol (Merck) St. Louis, MO, USA. Ultrapure water was obtained, using a Milli-Q water system (Merck Millipore, Billerica, MA, USA). Analytical grade piperine and piperlongumine standard (≥ 97.0% purity) were purchased from TCI Europe N.V. (Zwijndrecht, Belgium). TLC aluminium sheets coated with 20 x 20 cm silica gel 60 F_{254} (Merck, Germany) and 10 cm × 10 cm glass silica gel 60 F_{254} HPTLC plates (Merck, Germany). Tetracycline hydrochloride (Sigma-Aldrich, St. Louis MO, USA), rifampicin (Sigma-Aldrich, St. Louis MO, USA), were used as standard antibiotics for antibacterial investigation, while amphotericin B and itraconazole (Sigma-Aldrich, St. Louis MO, USA) were used as positive controls for the fungal strains. Sterile Petri dishes (Ø =14 cm, VWR Finland) were used for the screening. Other materials used includes; sterile serological pipet (Falcon, Becton Labware Europe), Isosensitest agar (OXOID, Thermo Fisher Scientific), Saboraud agar (OXOID, Thermo Fisher Scientific), nutient agar (Difco, VWR Finland), sodium chloride (NaCl), sterile glass tube, sterile inoculation loops, sterile cork borers, Mueller Hinton agar (Becton Dickson, New Jersey, USA), 96 well plates (Thermo Fisher Scientific, Denmark). The instruments used were as follows: Multiskan microplate spectrometer (Thermo Fisher Scientific), rotary evaporator (Heidolph instruments, Schwabach, Germany), Varioskan plate reader (Thermo Fisher Scientific), lyophilizer (Heto LyoPro 3000, Denmark), and florescent microscope (Tokyo, Japan).

4.2 Antimicrobial screening

4.2.1 Bacterial and fungal strains

The bacterial and fungal strains were obtained from the Division of Pharmaceutical Biosciences, Faculty of Pharmacy, University of Helsinki, Finland. Most of the bacterial and fungal strains used for the screening were model pathogenic organisms that are of clinical importance. The characteristics of the bacterial and fungal strains used in the study are shown in Table 1. In all, the

growth inhibitory activity of the extracts were investigated using three Gram-positive bacterial strains (*Sarcina* sp., *Staphylococcus aureus* ATCC 25923, and *Bacillis cereus* ATCC 10987), five Gram- negative bacterial (*Enterobacter aerogenes* ATCC13048, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella enterica* ATCC 43845 and *Proteus mirabilis* 43071), and five fungal strains (*Candida albicans* ATCC 10231, *Candida glabrata* ATCC 2001, *Candida tropicalis* ATCC 750, *Candida parapsilosis* ATCC 7330, and *Cryptococcus neoformans* ATCC 10226).

Table 1 Characteristics of the bacterial and the fungal strains used in the study

Pathogen	Diseases	Symptoms
Sarcina sp.	Gastric perforation, emphysematous gastritis and gastric ulcer	Vomiting, nausea, bloating-like syndrome in animals
Staphylococcus aureus	Pneumonia, scalded skin syndrome, blood stream infection,	Fever, difficulty in breathing, abdominal pain, vomiting, nausea
Bacillus cereus	Gastrointestinal diseases and diarrheal syndromes	Diarrhea, vomiting, nausea
Enterobacter aerogenes	Hospital-acquired infections such as urinary tract infections, lower respiratory tract infections, skin and soft tissue infections.	Skin rashes and fever
Escherichia coli	infectious diarrhoea, urinary tract infections, meningitis	fever, diarrhea, abdominal pain, dysentery
Pseudomonas aeruginosa	Life-threatening nosocomial infections, blood stream infections	Fever, body pain, diarrhea
Salmonella enterica	Intestinal tract infections and stomach flu	Stomach pain, bloody stool, fever, Headache, diarrhea
Proteus mirabilis	Pneumonia	Chest pain, cough, fever
Fungal species		
Candida albicans	Candidiasis, oral thrush	Fever and painful urination, discharge, white spots in the mouth, body pain
Candida glabrata	Mucosal candidiasis and invasive yeast infections	Thick discharge with a burning sensation, itching

Candida tropicalis	Oral thrush	White spots in the mouth, general body pain
Candida parapsilosis	Urinary tract infection, joint infection and meningitis.	Fever, abdominal pain, vomiting
Cryptococcus neoformans	Cryptococcal meningitis	Headache, neck pain, fever and nausea

4.2.2 Agar disk diffusion method

Agar disk diffusion method as described in publications (**II** and **III)** were used for the antibacterial and antifungal screening. A total of twelve extracts from the fruits and leaf extracts of the plant were tested against the bacterial and fungal strains. Each of the extracts were prepared to a final concentration of 50 mg/mL (stock solution). Amphotericin-B, rifampicin, tetracycline and itraconazole were used as positive controls. The antibiotics were dissolved in methanol to a final concentration of 10 mg/mL used for the test. Twenty-five mL of sterile base agar (Antibiotic agar No. 2, Difco, VWR Finland) was applied as a bottom layer into the sterile Petri dishes using a sterile, serological pipet (Falcon, Becton Labware Europe) and allowed to solidify, thereafter twenty-five mL of Saboraud agar (OXOID, Thermo Fisher Scientific) was applied as the top layer for the fungal strains and Isosensit agar for the bacterial strains. The Petri dishes were all allowed to solidify, and then stored in +4°C. The screening started with inoculation of the fungal/bacterial strains onto solid agar slants which were incubated for 24 h (bacterial strains) or 48 h (fungal strains) at +37°C. The viable microbial cultures from the agar slants were used to prepare an inoculum for the test. Bacterial/fungi from the agar slants were transferred into 2 mL of 0.9% (w/v) sodium chloride (NaCl) solution in a sterile glass tube using a sterile inoculation loop. 1 mL of the suspension was transferred into another sterile glass tube, and the absorbance was measured at 625 nm (UV–Visible Spectrophotometer, Pharmacia LKB-Biochrom 4060). The other 1 mL of the suspension (sterile part) was diluted with the 0.9% NaCl solution so that the absorbance at 625 nm becomes 0.1 (this suspension contains approximately 1.5×10^8 CFU /mL). 200 µL of this diluted fungal/bacterial suspension was spread evenly on each Petri dish and left to dry for some seconds with the lid open. A sterile cork borer (11mm diameter) was used to make six holes equidistantly from each other on the agar surface of the Petri dishes. 200 µL of the 50 mg/mL plant extracts and 200 µL of the 10 mg/mL antibiotics were carefully pipetted into the holes respectively. Methanol, ethanol, hexane and chloroform, 200µL of each, were used as solvent controls respectively. The Petri dishes were incubated for 24 h (bacterial strains) or 48 h (fungal strains) at +37 °C. The

diameters of the zones of inhibition were measured with a caliper under a Petri dish magnifier and expressed as the mean of the diameters of three replicates ± SEM.
The Activity index (AI) of the various extracts were measured in relation to the standard antibiotics according to Fyhrquist et al., (2014).

4.2.3 Microdilution turbidimetric broth method

The microdilution turbidimetric broth method was used for MIC, MBC and MFC estimation as described in publications (**II**, **III** and **IV**). This was estimated based on the guidelines of Clinical and Laboratory Standards Institute (CLSI) (Cockerill et al., 2012). From the result obtained from the agar disk diffusion assay, minimum inhibitory concentration (MIC) was estimated for some selected extracts based on their good antibacterial/antifungal activity. MIC is considered to be the lowest concentration of an extract or compound resulting in the inhibition of at least 90% of the growth of a fungal strain. Only extracts which expressed marked antibacterial/antifungal activity in the agar disk diffusion assay were tested for MIC. For the MIC evaluation, two-fold serial dilutions of the extracts from 9.75-2500µg/mL were prepared in sterile Mueller Hinton broth (for bacterial strains) and Saboraud broth (for fungal strains). Commercial pure compounds, piperine (1mg/mL concentration in methanol) and piperlongumine (1mg/mL concentration in methanol) were also two-fold serially diluted in Mueller Hinton/Saboraud broth. Itraconazole and amphotericin-B or rifampicin and tetracycline were each two-fold serially diluted in Saboraud broth or Mueller Hinton broth from 0.48-125 µg/mL respectively. The absorbance of 1 mL of the 48 h bacterial/fungal cultures were measured for turbidity at 625 nm using a UV–Visible Spectrophotometer type 1510 (Thermo Fisher Scientific Oy). The absorbance was adjusted to 0.1 at 625 nm (approximately 1.0×10^8 CFU/mL). 100µL of this suspension A_{625}= 0.1 was further diluted 100-fold to get a working suspension or inoculum containing 1.0×10^6 CFU/mL. 100µL of this inoculum, and 100 µl of the plant extracts, pure compounds, antibiotics, and solvent controls, were pipetted into the 96 well microtiter plates. Therefore, each well contained 5×10^5 CFU/mL. The solvent controls contained a maximum of 5 % (v/v) of each solvent to be tested for toxicity. The growth control (GC wells) contained only the bacterial/fungal suspension, and the test wells (T wells) contained plant extracts or pure compounds + bacterial/fungal suspension. Moreover, sample controls wells were prepared for each plant extract/compound to be tested, and these wells contained plant extract/pure compound and the broth only. The microwell plates were incubated for 48 hours in an incubator coupled to a shaker at +37°C. The tests were done in triplicate and the % growth was expressed as the mean of these triplicates ± standard error of mean (SEM). The minimum bactericidal

concentration (MBC) or minimum fungicidal concentration (MFC) was evaluated by pipetting 100µL from those wells of the microtiter plate, which contained 2 and 4 times higher concentrations than their MIC values on Petri dishes (∅ = 9 cm) containing Isosensit/Saboraud agar, and incubating the dishes for 24 h or 48 h at +37°C. The MBC or MFC was taken as the lowest concentration where no visible growth on the Petri dish was observed after the incubation.

4.3 Methods of analytical chemistry

4.3.1 Thin-layer chromatographic conditions

100 mg of the samples were dissolved in 1 mL of methanol (stock solution), and a 5 mg/mL concentration was prepared from the stock solution for the TLC analysis in publication (**I**). The extract was applied on the plate using CAMAG linomat IV TLC spotter (CAMAG AG, Muttenz, Switzerland).TLC aluminium sheets coated with 20 x 20 cm silica gel 60 F_{254} (Merck), cut to 4 cm × 10 cm, were used for testing the various mobile-phase compositions. A CAMAG REPROSTAR 3 was used to view the plates at 254 nm. Aliquots of 5 µL of the sample solutions were applied on the plate as 6 mm bands, using the Linomat IV application device (CAMAG). The application speed was 6 sec/µL, plate width 40 mm, band 6 mm, space 6 mm, starting position 10 mm and development over a path of 8 cm. Solvents of different solvent strength (S_T) were combined in various proportions and tested under the same experimental conditions with 10 mL of the mobile phase. The solvents tested include toluene, ethyl acetate, *n*-hexane, acetone, cyclohexane and methanol.

HPTLC precoated silica gel 60 F_{254} plates were used for evaluation of the effects of the developing chamber on the separation. A CAMAG twin-trough vertical chamber and CAMAG horizontal chamber were used. HPTLC was performed on 10 cm × 10 cm glass silica gel 60 F_{254} HPTLC precoated plates (Merck). The plates were developed in an unsaturated 10 cm × 10 cm twin-trough chamber to a distance of 8 cm. The plates were documented under ultraviolet (UV) radiation at 254 nm. The optimum mobile phase of toluene-ethyl acetate 6:4 v/v was used. Three different volumes of 3, 4 and 5µL of the 5 mg/mL concentration of the fruit and leaf extracts were used. The procedure included a plate width of 100 mm, band 8 mm, space 5 mm, starting position 15 mm, 6 sec/µL and development over a path of 8 cm while 10 mL of the solvent were used for the twin-trough chamber and 2.5 mL for the horizontal chambers.

4.3.2 HPLC-UV/DAD method

For publication (**I**), the samples were analyzed with an HPLC Waters Tm 717 autosampler, equipped with a photodiode array detector set at 240 nm (Waters Corp., Milford, MA, USA). Two initial experiments were conducted at different gradient times of 30 and 60 min, with a total run time of 25 min each. The HPLC conditions were described in the original publication (**I**). The simulation with DryLab software was done with DryLab 2010 version 3.1 (LC Resources Inc., Alamo, CA, USA; in Europe: Molnar, Berlin, Germany). The retention time and peak area from the chromatographic data were input into the DryLab software data entry. Peak-matching functions and UV were used to match the peak identities. Gradient runs were predicted with the DryLab software simulation, and a resolution map was used to predict the optimum separation conditions.

For publication (**II**), the samples were analyzed with an HPLC Waters 600 E pump and a controller equipped with a 991 photodiode array detector. Samples were injected using an autosampler controlled by Agilent Chemstation software (Water Corp., Milford, USA). The HPLC conditions were described in details in publication (**II**). The UVλ absorption maxima spectra of the major components in the *P. guineense* extracts were recorded between 200 and 400 using Agilent Chemstation software and the compounds in the extracts were identified by comparing the retention times and UV spectra with that of commercial reference standard (piperine) and also with previous literature (Adesina et al., 2003; Scott et al., 2005).

4.3.3 UHPLC/ Q-TOF MS method

The masses of piperamide alkaloids were identified using UHPLC-DAD (Model 1200 Agilent Technologies)-JETSTREAM/QTOFMS (Model 6340 Agilent Technologies) equipped with a 2.1× 60 mm, 1.7 µm C18 column (Agilent technologies) equipped with a 2.1× 60 mm, 1.7 µm C18 column (Agilent technologies) according to Taulavuori et al., 2013. The gradient range was from 0 – 50% of solvents A (aqueous 1.5% tetrahydrofuran + 0.25% acetic acid) and solvent B (100 % methanol) and flow rate 0.4 mL/min. Mass spectrometry in positive and negative ion mode, depending on the compounds were used to get the (M+Na or H^-) ions.

4.3.4 Method validation

For method validation the parameters were determined, using a piperine standard solution diluted to appropriate concentrations. Parameters such as linearity, limit of detection (LOD), limit of quantification (LOQ), precision (interday and intraday) and extraction recovery were evaluated to ascertain the sensitivity and accuracy of the method. A stock solution of 1 mg/mL concentration of piperine was prepared in methanol to get six working standard solutions ranging from 2 to 10 µg/mL

(six points) for the calibration curve. For the LOD and LOQ, six working standard solutions were prepared, and a more serial dilution of the standard solution of piperine was carried out in decreasing concentrations. The precision was determined by injecting six replicates of the standard solution on the same day and on 3 consecutive days. The validation methods are described in detail in publication (**I**)

4.3.5 Fractional inhibitory concentration (FIC) index and Isobologram (IV)

The fractional inhibitory concentration (FIC) index was used to estimate the synergy (Kang et al., 2011; Abreu et al., 2017).This method supplies a graphic demonstration with linearly arranged x and y axes which is plotted using FIC (A) and FIC (B). For the FIC index calculation, the MIC values of five combination ratios each of piperamide/antimicrobial were used to calculate FIC (A) and FIC (B) using the equation below:

The FIC values were calculated as follows.

$$FIC(A) = \frac{\text{MIC value of combined piperamide and antimicrobial}}{\text{MIC value of antimicrobial alone}}$$

$$FIC(B) = \frac{\text{MIC value of combined piperamide and antimicrobial}}{\text{MIC value of piperamide alone}}$$

$$FIC\ index = FIC(A) + FIC(B)$$

A FIC index value of ≤0.5 was interpreted as synergy. The FIC index was calculated on the basis of the above-mentioned equation where FIC index = X + Y and interactions defined as; FICI ≤ 0.5, synergy; > 0.5–≤ 1.0, additive; >1.0–≤2.0, indifference; and >2.0, antagonism (Kang et al., 2011)

4.4 **Ethnobotanical survey**

The 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 (publication **III**). 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. 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. In the survey, a house to house strategy was used with the permission of the village heads and traditional rulers. The questionnaire used in the ethnobotanical survey is shown in figure 3. 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. Detailed and validated questionnaires were administered to twenty traditional healers. The questionnaires were written in English, but the local language (Igbo) was used during the interviews and conversations.

Figure 2. Map of Nigeria (http://www.maparchive.org) showing the study area Imo state shaded in a black square arrow.

Figure 3. Questionnaire for the ethnobotanical survey on the traditional use of *Piper guineense* for the treatment of fungal infections in Imo state, South Eastern Nigeria.

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 ☐

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

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

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

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

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

5. Results and discussion

5.1 HPLC and TLC methods development of *Piper guineense* (I)

The HPLC experiment conducted with dryLab simulation program gave a good and favorable resolution of the main components of the extracts. The aim which was to have the best possible overall resolution while keeping the analysis time and solvent consumption to a minimum was achieved in a minimum number of runs. With DryLab software, it was possible to develop an optimum condition for the analysis of *P. guineense* extracts. The optimized experiment which began with two initial runs of a 30 min and 60 min gradient was found to be a 19 min binary gradient with the proportion of organic solvent increasing from 39% to 80.4%, flow rate of 1 mL/min and injection volume of 20µL. This method was achieved by inputting the data (dwell volume, gradient conditions, column length, column diameter and flow rate) from the two initial experimental runs into DryLab. The HPLC conditions for these two initial gradient runs are described in details in publication (**I**) Equal volumes of the sample were injected during the two initial experimental runs in order to maintain constant peak areas. Using the experimental data obtained from these initial runs, an optimum gradient run was predicted. This was possible by the use of resolution map, peak-tracking and peak matching functions which were used to make adjustments so as to predict a new chromatogram with emphasis on analysis time, solvent consumption and optimum resolution of the peaks. The simulated separation conditions were experimentally confirmed and the simulated separation was very similar to the actual experimental separation. The method achieved in the experiment was the best separation condition, with the shortest run time and good peak shape for the analysis of *Piper guineense* extracts. Based on previous literature and by the use of UV spectra of reference standards and their retention times, the compounds were identified in the extracts. The UV spectra of the major components in the *P. guineense* extracts were taken, based on the chromatogram of the optimized 19 min run. From the result, it was observed that the overall resolution and the resolution of the critical peak pairs with the optimized method was better than or equal to one previously published method (Scott et al., 2005), albeit at the cost of a slightly longer analysis time. The HPLC experimental method used in these analyses provided satisfactory overall resolution for most of the main piperamides of *P. guineense* and the method was found to be simple, sensitive and accurate. The developed HPLC method was applied to determine the percentage content of piperine in *P. guineense* which was found to be 0.43 % w/w, linearity (0.997), interday precision (% relative standard deviation (RSD),

1.6), intraday precision (% RSD, 2.7 – 5.9), recovery (98.4%), limit of detection (0.001 μg /mL) and limit of quantification (0.003 μg /mL).

In the TLC experiment, the mobile phase composition which was systematically tested using various proportions of solvents differing in ST and PS values under the same experimental conditions according to the method of (Nyiredy, 2002), gave a good separation of the main components of the extracts. The group, ST and PS values of individually selected solvents were described in details in publication (**I**). The TLC experiment was aimed at achieving the best possible overall separation of the main components of the extracts and the mobile-phase composition giving these favorable resolution of the bands was found to be toluene: ethyl acetate (PS 6-4 corresponding to 60:40 % v/v). The selection of the solvent combinations tested during the method development were based on previous literature on the TLC analyses of extracts of various *Piper* species. Most of the solvents have been previously used in the TLC analysis of various Piper species but not *Piper guineense*. From the review of literature, bicomponent solvent mixtures containing ethyl acetate as the other solvent appeared to be the most widely used, also it was observed that acetone had been applied for the task. The effects of the developing chamber which was tested using three types of unsaturated chamber conditions: horizontal chamber in sandwich configuration, horizontal chamber in non-sandwich configuration and twin-trough vertical chamber showed that the developing chamber conditions does not affect the TLC separation efficacy in the analysis of *P. guineense* extracts. The mobile phase giving the best resolution of the bands which was found to be toluene-ethyl acetate (P_S 6:4 v/v), was applied for this task. Different volumes of 3, 4 and 5μl of the fruit and leaf extracts were applied on HPTLC precoated silica gel 60 F_{254} plates after which there was no observed effect in the separation efficacy of *P. guineense* extracts. The result shows that the choice of chamber type should be made based on any other criteria during the TLC analysis of *P. guineense* extracts.

5.1.1 Method application and validation (I)

In publication (**I**), the developed method was applied for the rapid estimation of piperine from *P. guineense*. Piperine was found to constitute 0.43% w/w of the seed of *P. guineense*. Piperine is important because of its diverse biological and therapeutic potentials in recent pharmacological studies (Chonpathompikunlert et al., 2010). In the method validation, calibration curve was linear over a range of concentrations and the linearity was favorable and fell within the concentration

range of 2–12 µg/mL (Fig. 4). The R^2 with respect to peak area was 0.997 while the slope and intercept were 75 267 and 354.84, respectively.

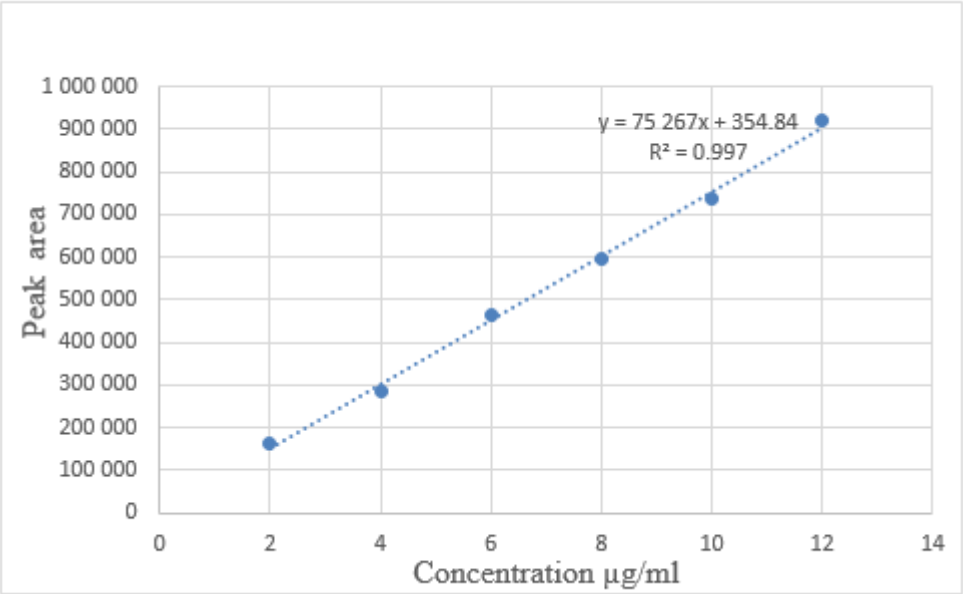

Fig. 4. Calibration curve of piperine showing the coefficient of determination (R^2)

The results obtained from the LOD (0.001 µg/mL) and LOQ (0.003 µg/mL) revealed that the method has favorable sensitivity. In the experiment, LOD was evaluated and calculated to be the lowest concentration of piperine that could be detected. From the calibration curve, these parameters were calculated as the relative standard deviation (RSD) of the response and slope (S). The interday and intraday precisions evaluated during the experiment were within the range of 1.6 – 5.9%, thus, this range was satisfactory and acceptable and revealed that the method was reproducible. The method can be used to analyze extracts and fractions produced from *P. guineense*, and also can be used as a quality-control tool by pharmaceutical industries to explore piperine and other active compounds in *P. guineense* for drug discovery and for other therapeutic purposes.

5.2 Ethnobotanical survey and field study (III)

P. guineense is predominantly used in the treatment of fungal infections by traditional healers in Imo state, South Eastern Nigeria and for other various ethno medicinal purposes (Besong et al.,

2016). The result obtained from the ethnobotanical survey shows that the leaves and fruits are the mostly used plant parts for the treatments of fungal infections in Imo state. It was observed that the oral intake of the extracts in locally produced bamboo alcohol (Kai-kai) is the most common method of administration. The traditional healers explained 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 infectious diseases. The 20 traditional healers (14 male and 6 female) were between the ages of 40 to 70 years. There were more male than female traditional healers and most of them have long been practicing traditional medicine. The findings from our study 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). Most of the traditional healers have practiced traditional medicine for over 25 years as their only source of income. They explained that they have inherited the practice from their parents. It was observed that few of the participants have university education and have recorded the information on the preparation and administration of *P. guineense* extracts on their various exercise books. The healers always obtain the plant materials for their traditional healing practice from their farms or from the markets.

The traditional healers sometimes sell *P. guineense* fruits and leaves which they have harvested from their farms in local markets as spices and medicine to the general population for the purpose of making profit. This explains that the *P. guineense* extracts are readily available and that the traditional healers do not have problems in getting the plant materials used for the herbal treatments since they are sold in the local markets. The symptoms given by all the healers on the methods of diagnosis of the fungal infections is similar, so also the method of preparations (hot infusion, decoction in combination with *Xylopia aethiopica* and then the plant material is soaked in mild alcohol). The figure below shows one of the traditional healers in his herbal clinic where he displayed various herbal preparations from *P. guineense* and He is referred to as “Doctor” in his community.

Fig.5. *P. guineense* herbal formulations displayed by a herb seller (left) in Egbu community, Owerri North Local Government Area, Imo state, South-Eastern Nigeria.

Sometimes the healers send their patients to the government hospitals if the symptoms persist as a result of their failed treatment or lack of proper understanding of the type of fungal infection to treat. It was observed that oral intake is the most common method of administration and this correlates with the findings of Maroyi, (2013) that herbal preparations are mostly administered orally by traditional healers. According to the traditional healers, the decoctions made from the fruits and leaves, prepared in mild alcohol is mostly effective when administered orally for the treatment of fungal diseases expressed by thrush on the tongue or candida vaginosis. The dosage is usually measured with a small glass tumbler which can be estimated to be about 100 mL, and it is often taken 3 to 4 times in a day. Leaves and fruits are the most frequently used plant parts for the treatments. This correlates with the findings of Rahmatullah et al., (2012), who have reported 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 demographic characteristics of the respondents are shown in figure 6. The ethnobotanical result indicates 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.

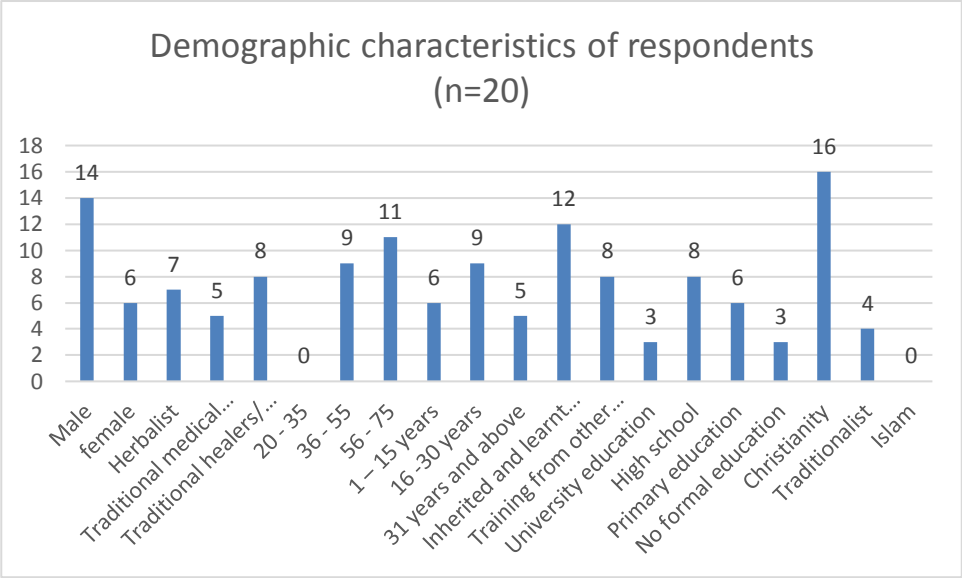

Fig 6. The demographic characteristics of the respondent shows the number of traditional healers with regards to gender, occupation, age range, years of practice (experience), sources of knowledge of traditional medicine, level of education and religion. The data figure shows the number of respondents.

5.3 Antimicrobial activity

5.3.1 Antibacterial activity

P. guineense is an African medicinal plant used by traditional healers for various medicinal purposes and more often, as herbal remedies for the treatment of symptoms related to bacterial infections, such as diarrhea, cough and rashes, and thus these uses are now justified by this study. *Piper guineense* extracts revealed potent antimicrobial efficacy against various Gram-positive and Gram-negative bacteria (**II**). Among all the bacterial strains tested in this study, *Enterobacter aerogenes* which is responsible for most hospital-acquired infections (Jha et al., 2016) was screened for the first time in this study and a large zone of inhibition (49.7 mm) was observed with the methanol extract of the fruit of the plant with MIC value of 39 µg/mL and MBC value of 78 µg/mL respectively (publication **II**). This bacterium causes urinary tract infections and lower respiratory tract infections. The other extracts of the fruit of *P. guineense* showed promising inhibitory activity against *Enterobacter aerogenes* also. The antibacterial efficacy of the extracts correlated with previous research results that plant-derived compounds and plant extracts could be potential sources for new antibacterial drugs against multi-drug-resistant (MDR) pathogens (Subramani et al., 2017;

Irshad et al., 2017). The results of the antibacterial activity of the extracts and commercial compounds are shown in Table 2.

Sarcina sp. was also tested against extracts of *P. guineense* for the first time in this study and the results revealed that the extracts were active against the bacterium. *Sarcina* sp. was the most sensitive bacterium to extracts of *P. guineens.* The hexane fruit and leaf extracts showed marked inhibition zones against *Sarcina* sp. with an MIC as low as 19 µg/mL and was thus as effective as piperine which gave an identical MIC value, this study therefore demonstrates that *Piper guineense* could be explored as a lead to a new antibacterial drug for the treatment of human infections. *Sarcina* sp. is an anaerobic Gram-positive bacterium belonging to the family *Clostridiaceae. Sarcina* has mainly been characterized as the causative agent of abomasal bloat and death of livestock (Lam-Himlin et al., 2011). There is still a debate whether *Sarcina* is pathogenic to humans, although a number of cases of human disease, including gastric perforation, emphysematous gastritis and gastric ulcer have been associated with *Sarcina* (Lam-Himlin et al., 2011; De Meij et al., 2017). The hexane extracts of both *P. guineense* fruits and leaves contain antibacterial piperamide alkaloids, which could be used as therapeutic agents in the treatment of *Sarcina* infections and other stomach related problems, including gastric ulcers, diarrhoea and foodborne diseases.

Table 2**.** Antibacterial effects of extracts of *P. guineense*, piperine and piperlongumine against Gram-negative and Gram-positive bacteria. The results were obtained with the agar diffusion method.

Extracts/ antibiotics	*E. aerogenes*	*Sarcina* sp.	*S. aureus*	*E. coli*	*B. cereus*	*P. aeruginosa*	*S. enterica*	*P. mirabilis*
Methanol fruit extract	49.7	29.8	15.7	20.7	23.7	16.3	13.3	13.7
Chloroform fruit extract	11.3	31.6	17.3	20.3	15.7	17.7	18.0	11.3
Hexane fruit extract	34.7	37.6	17.3	NA	19.7	NA	12.7	11.7
Decoction fruit	NA	10.6	NA	NA	NA	NA	NA	NA
Maceration fruit	NA	17.6	NA	NA	NA	NA	NA	NA
Ethanol fruit extract	30.2	28.3	22.6	19.8	24.5	NT	21.7	16.3
Methanol leaf extract	29.3	26.1	14.7	11.3	29.3	15.3	12.0	11.3

Chloroform leaf extract	11.3	25.6	16.7	20.3	15.3	21.3	11.7	10.7
Hexane leaf extract	17.8	33.6	15.0	NA	24.7	NA	NA	11.7
Decoction leaf	NA	10.6	NA	NA	NA	NA	NA	NA
Maceration leaf	NA	14.3	NA	NA	NA	NA	NA	NA
Ethanol leaf extract	25.0	25.1	17.5	16.3	28.2	NT	16.2	13.8
Piperine	18.7	27.6	18.3	22.3	12.7	15.7	14.7	16.0
Piperlongumine	12.7	23.6	15.3	20.0	15.7	13.3	11.3	NA
Tetracycline	38.0	39.3	48.3	40.3	50.7	45.3	39.3	34.3
Rifampicin	45.3	41.6	49.3	49.7	54.3	55.7	55.7	40.7

Diameter of the zones of inhibition in mm. NA, not active; NT, not tested.

For *Pseudomonas aeruginosa,* the methanol and chloroform extracts of *P guineense* were the most effective extracts against the bacterium with inhibition zones ranging from 15.3mm – 21.3mm (publication **II**). *Pseudomonas aeruginosa* is a Gram-negative bacterium which most of the times causes life-threatening nosocomial infections, and many combination therapy resistant strains have been reported (Lister et al., 2009; Rasamiravaka et al., 2015). The chloroform extracts gave the largest inhibition zone of 21.3 mm and the result shows that the chloroform extracts could contain non-polar compounds that may be explored as antibacterial agents for multidrug-resistant pathogens. The hexane, and water extracts were not active against *Pseudomonas aeruginosa.* The present study shows that *P. guineense* could be a source of new antibacterial agents that will be relevant for the treatment of nosocomial infections associated with *Pseudomonas aeruginosa*.

The cold water maceration and hot water decoctions of the extracts of *P. guineense* did not show any inhibitory activity in most of the bacterial strains tested, this justifies the claim by traditional healers that, the plant extracts are more active as a herbal remedy when prepared in mild alcohol than in water decoctions and further correlates with the findings of Dada et al., 2013, which reported that ethanol and hexane extracts of *P. guineense* are more effective than its water extracts. The reason for water extracts lacking antibacterial activity could be attributed to the fact that the bioactive compounds in publication (**II**) present in the other extracts, which are mostly piperamide alkaloids, are not soluble in water.

Piperine and piperlongumine commercial compounds were active against most of the bacteria investigated in our study, with piperlongumine showing the lowest MIC value of 9.7 µg/mL against *Sarcina* sp. Piperine was found to be present in high concentrations in *P. guineense* leaves and piperlongumine is known in many species of *Piper*, although we did not find it in the leaves of *P.*

guineense. Piperine and piperlongumine showed significant activity against *E. coli* with MIC values of 19 and 39 µg/mL, respectively (**II**)**.** From QTOF MS analysis conducted on the various extracts, it could be seen that the extracts were rich in alkaloids, and thus, the inhibitory activities of these extracts could be literally attributed to the presence of numerous piperamides present in *P. guineense* in publication (**II**). Piperine showed significant activity against *P. mirabilis* with an inhibition zone of 16.0 mm and a MIC value of 78 µg/mL and MBC value of 156 µg/mL (publication (**II**). Thus, piperine must be responsible for a part of the good antibacterial activity of the ethanol extracts of the seeds and leaves, since piperine was observed to be present in both extracts according to our preliminary HPLC-DAD data. Thus, piperlongumine was not active against *P. mirabilis.* Piperine and piperlongumine, were effective against *E.coli* with inhibition zones of 22.3 mm and 20.0 mm respectively and MIC values of 19 and 39 µg/mL, respectively (**II**). The extracts from *P. guineense* were susceptible to *B. cereus* with inhibition zones ranging from 15.3mm – 29.3mm (**II**). Based on the result obtained with this bacterium, the leaf extracts were more active than the seed extracts. It could be possible that the leaf contains more antibacterial compounds that could inhibit the growth of this particular bacterium. The hexane and chloroform extracts were all active against the bacterium and the result demonstrates that the more polar methanol and ethanol extracts as well as a hexane extract of the leaf contain some notable bioactive compounds that may be responsible for the significant activity against *B. cereus* when compared with the fruit extracts (**II**). This extract contains a multitude of piperamide alkaloids and their isomers, of which many could be responsible for the good activity of the hexane leaf extract.

P. guineense extracts were active against *S. aureus* with inhibition zones ranging from 14.7 – 22.67mm and the largest inhibition zone was observed with an ethanol extract of the fruits
The results of this study on the antibacterial activity of the extracts of *P. guineense* against *Staphylococcus aureus* justify the use of this plant in the treatment of common foodborne and other infections caused by this bacterium. *P. mirabilis* was found to be resistant against most of the extracts of *P. guineense* in the primary screening, using agar diffusion (**II**). From the result of the study ethanol was found to be a good solvent for extracting compounds active against *P. mirabilis.*
For *E. coli,* Low MIC of 156 µg/mL was recorded in this study against *E. coli* for an ethanol extract of the leaves of *P. guineense*. The methanol seed and leaf extracts of *P. guineense* gave large inhibition zones of 20.7 mm and 20.3 mm respectively and these results suggest that standardized *P. guineense* extracts could be used for possible alternative treatment against pathogenic multidrug-resistant *E. coli* strains.

In this study, the extracts were active against *Salmonella enterica.* The ethanol fruit extract of *P. guineense* showed the largest zone of inhibition of 21.7 mm for *S. enterica* and the ethanol leaf extract showed a clear inhibition zone of 16.2 mm and a MIC of 78 µg/mL (**II**). The more polar ethanol extracts contain antibacterial compounds that effectively inhibited the growth of this bacterium. The chloroform, hexane and methanol extracts were moderately active also with inhibition zones ranging from 18.0 – 11.7 mm.

5.3.2 Antifungal activity of *Piper guineense* (III)

The antifungal screening conducted in this study was done based on our ethnobotanical results on the uses of *P. guineense* in West-African traditional medicine for the treatment of fungal diseases (publication (**III**). In the investigation, pathogenic fungal strains which are known to be responsible for part of the diseases treated by the traditional healers were chosen for the screening. Among all the fungal strains selected, *C. albicans* is known to be the most significant human pathogenic species of yeast that can cause serious fungal diseases in humans (Brown et al., 2014).
The results from the antifungal activity of the methanol, chloroform and *n*-hexane fractions of the extracts of *P. guineense* on various candida strains (*Candida albicans, Candida tropicalis, Candida parapsilosis, and Candida glabrata)* supports the use of the plant in the treatment of toilet infections, sexually transmitted diseases and other microbial infections. This study recorded marked MIC values, as low as 39 µg/mL were recorded against some of the fungal strains. The marked antifungal activity recorded with the various extracts could be attributed to the presence of piperamide alkaloids which were found to be present in the extracts in publication (**II**). The result showed that the alkaloids present in the extracts of *P. guineense* is responsible for its antifungal efficacy and these result correlated well with previous report that piperamides piperyline, 4,5-dihydropiperylin and tetrahydropiperylin found in hexane fraction of *P. arboreum* showed promising antifungal activities with tetrahydropiperyline having the lowest MIC of 15.6 µg/ml against *C. krusei, C. parapsilosis* and *C. neoformans* (Regasini et al., 2009).The results of the antifungal activity of the various extracts of *P. guineense* and the commercial compounds are shown in Table 3. The result of the antifungal evaluation shows that the cold water maceration and hot water decoctions of the fruit and leaf extracts of *P. guineense* does not have activity against the fungal species tested in this study, thus *P. guineense* water extracts might be better to use in combination with other medicinal plants as herbal remedy for treatment of fungal infections in traditional medicine as seen in publication (**III**).

Table 3. Antifungal effects of extracts of *P. guineense*, piperine and piperlongumine against four pathogenic strains of yeast and *Cryptococcus neoformans*. The results were obtained with the agar diffusion method.

Plant extracts and antibiotics	*Candida glabrata*	*Candida albicans*	*Candida parapsilosis*	*Candida tropicalis*	*Cryptococcus neoformans*
Methanol fruit extract	24.3	20.3	18.0	21.3	19.7
Chloroform fruit extract	23.3	17.7	14.7	16.2	18.2
Hexane fruit extract	203	14.3	15.3	11.0	14.3
Decoction fruit	NA	NA	NA	NA	NA
Maceration fruit	NA	NA	NA	NA	NA
Ethanol fruit extract	NT	21.8	NT	19.8	22.2
Methanol leaf extract	20.7	19.7	28.7	17.3	21.3
Chloroform leaf extract	11.7	18.0	13.7	15.5	17.3
Hexane leaf extract	13.7	21.7	17.8	12.0	11.7
Decoction leaf	NA	NA	NA	NA	NA
Maceration leaf	NA	NA	NA	NA	NA
Ethanol leaf extract	NT	19.8	NT	18.2	23.8
Piperine	17.3	18.3	23.3	16.7	11.0
Piperlongumine	14.7	19.3	16.3	18.0	11.8
Itraconazole	16.3	25.3	29.7	26.2	29.7
Amphotericin B	34.3	35.7	43.7	29.3	38.0

The diameter of the zones of inhibition in mm. NA, not active; NT, not tested.

From the study, it was observed that the leaf extracts showed a different spectrum of activity compared to the fruits. For example, the hexane leaf extract showed an inhibition zone of 21.7 mm against *C. albicans*, whereas the hexane fruit extract only gave an IZ of 14.3 mm against this fungus (**III**). The result could be supported by our ethnobotanical survey results, where the traditional healers explained that the leaves of *P. guineense* are sometimes more active in the treatment of some of the fungal infections and are therefore used more frequently than the fruit for some specific symptoms of fungal infection and the results are in accordance with Rahmatullah et al., (2012), who argued that the leaf extracts of medicinal plants are used more often in traditional medicine because they are usually more effective than extracts made from other plant parts. The extracts were active

against *C. albicans* which is an opportunistic pathogenic fungus that is capable of causing serious systemic infections in humans most especially individuals with compromised immune defences, such as HIV/AIDS patients (Brown et al., 2014) (**III**). From the ethnobotanical survey, *P. guineense* extracts when soaked in mild alcohol can be used for the treatment of vaginal candidiasis which is often caused by *C. albicans* (**III**). Piperlongumine was found to be very active against *C. albicans* with a MIC value of 39 µg/mL, while piperine gave a MIC of 78 µg/mL. The piperamide alkaloids were also active against all the other tested fungal strains (**III**). The result obtained from our study demonstrates that piperlongumine and piperine could be scaffolds for new natural plant derived antifungal agents to combat multi-drug resistance in *Candida* strains. Marked growth inhibitory activity was recorded against *C. glabrata* which is another significant human pathogenic species of yeast. Previous research has shown that *C. glabrata* is a pathogenic fungal strain which is capable of causing systemic infections which is often characterized with high mortality rate (Pfaller et al., 2004; 2007). The largest inhibition zones were recorded with the methanol and chloroform extracts against *C. glabrata* (IZ ranging from 24.3 mm to 20.7 mm). From the result, a low MIC value of 0.48 µg/mL was recorded with amphotericin B against *C. glabrata*, and this 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).

The efficacy of *P. guineense* extracts and its piperamide alkaloids on the yeast tested in this study demonstrates that its extracts and alkaloids could be effectively utilized in combinations with conventional antifungals for the treatment of fungal infections (**III**). The result further demonstrates that the extracts and the piperamide compounds could be possible antifungal agents for the discovery of a new antifungal drug for the treatment of systemic infections associated with the tested fungal strains.

5.4 UHPLC/QTOF-MS results

From the present study**,** piperamides were found to be predominantly the major bioactive compounds present in *P. guineense* extracts. The extracts contained piperamide compounds such as piperine, dihydropiperine, piperylin, piperlonguminine, dihydropiperlonguminine, wisanine, and dihydrowisanine**.** The results from the HPLC-DAD, UHPLC/Q-TOF MS and antimicrobial activity indicates that these piperamides compounds identified in the extracts were responsible for the antimicrobial efficacy of *P. guineense* and the result is in agreement with other previous authors (Juliani et al., 2013; Besong et al., 2016). Altogether 18 compounds were identified in the extracts (**II**). Among these compounds, the previously known piperamide alkaloids piperine,

dihydropiperine, piperylin, dihydropiperylin or piperlonguminine, dihydro-piperlonguminine, wisanine, dihydrowisanine and various derivatives of piperine were identified and their mass spectrometric data were compared to previous literature on piperamide alkaloids in *Piper* species (Kotte et al., 2014; Rao et al., 2011; Adesina et al., 2003; Liu et al., 2015).

5.5 Synergistic effects of piperine/piperlongumine and antimicrobials

Piperine and piperlongumine were screened singly and in combination with conventional antimicrobials to evaluate their synergetic, additive or antagonistic interactions against *Staphylococcus aureus*, *Pseudomonas aeruginosa,* and *Candida albicans* (**IV**). The aim was to investigate the synergistic effects of piperine and piperlongumine when used singly and in combination with conventional antimicrobials against *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Candida albicans. Staphylococcus aureus, Pseudomonas aeruginosa,* and *Candida albicans* are human pathogens that are capable of causing systemic infection in humans. It has been reported that 90 to 95% of *Staphylococcus aureus* strains are resistant to most conventional antibiotics (Hemaiswarya et al., 2008; Kang et al., 2011). The Fractional inhibitory concentration (FIC) index was used to determine the synergistic interaction between the commercial piperamides and the antimicrobials. This method has been reported to be one of the most accurate methods to determine synergistic interactions when the two inhibitors are studied in various combinations (Tallarida, 2006; van Vuuren and Viljoen, 2011; van Vuuren et al., 2009). Synergistic effect is said to be observed when the FIC index value of the compound of interest is less or equal to 0.5 (Kang et al., 2011). For piperlongumine, there was synergistic effect at the ratio of 5:5 of rifampicin and piperlongumine against *Staphylococcus aureus* (**IV**). This result demonstrates that the pharmacological interaction between a bioactive compound and a conventional antimicrobial could be affected by the ratio and concentration at which the two are combined. However antagonistic interactions were observed between piperlongumine and tetracycline in all the ratio of combinations tested against *S. aureus*. There is synergistic effect observed between piperine and rifampicin and there is also synergistic effect between piperine and tetracycline against *S. aureus*. Rifampicin is an antibiotic that is constantly used in the treatment of systemic bacterial infections in antimicrobial therapy and this antibiotic is characterized with numerous adverse effects when used consecutively for 10-14 days (Dhingra et al., 2004; Nageswari et al., 2018).

6. Conclusion

From this study, DryLab simulation program was successfully used to develop a rapid HPLC method and an improved choice of mobile-phase for the analysis of *P. guineense* extracts.
Also from this study, it was observed that *P. guineense* extracts used for various medicinal purposes and as herbal remedies, could be a potential lead to the discovery of a new antimicrobial drug. This study demonstrated that extracts of *P. guineense*, enriched in alkaloids, have potent antibacterial and antifungal activity against a panel of Gram-positive and Gram-negative bacteria, as well as fungal strains, including significant human pathogens. Through the results obtained from this study it has been established that alkaloids are the main bioactive constituents present in *P. guineense* extracts. The water extracts were found to be devoid of these alkaloids, and thus inactive against most of the studied bacterial and fungal strains tested in the study. The results revealed that *P. guineense* alcohol, chloroform and hexane extracts are potent sources of piperamide compounds which could help to combat bacterial and fungal infections, and might be used as an alternative to or in combinations with synthetic antibiotics to address the problem of increasing microbial resistance to synthetic antibiotics. Further research should be conducted to test the antibacterial and antifungal mechanisms of action of piperamide compounds. It is also important to investigate the piperamide compounds for their anti-biofilm activities, including quorum sensing. The inhibitory activity observed with the various fractions and extracts against the tested bacterial and fungal strains warrants further exploration of the bioactive compounds from *P. guineense* as molecular scaffolds for new therapeutic agents in modern antimicrobial therapy. Further research could also be focused on evaluating *P. guineense* extracts in combination with *Xylopia aethiopica* and other medicinal plants for their antibacterial and antifungal effects. For these investigations, herbal formulations identical to those used to treat infectious diseases by traditional healers in African traditional medicine could be used.

Moreso, the antibacterial and 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 bacterial strains, *C. albicans* and non- albicans *Candida* strains supports the use of *P. guineense* in the treatment of bacterial and fungal infections in traditional medicine and demonstrates that these piperamide compounds can be utilized as therapeutic agents or scaffolds in the production of new antibacterial and antifungal drugs to treat infectious diseases. From this study, it could be deduced that the extracts and piperamide compounds exert promising antibacterial and antifungal properties against pathogenic bacterial strains, *Candida albicans* and other non-albicans *Candida* strains. Moreover, the extracts showed marked growth inhibitory profile against *Cryptococcus neoformans*

which is known to cause life-threatening meningitis in immunocompromised individuals. Based on this current knowledge on alkaloids of *P. guineense* as antibacterial and antifungal agents, we recommend that additional research should be done to evaluate the *in vivo* antibacterial and antifungal properties of the extracts and piperamide compounds from *P. guineense* with some animal models. Additional research is also needed to ascertain the antibacterial and antifungal mechanism of action of the piperamide alkaloids.

The marked inhibitory activity recorded with piperine and piperlongumine against the bacterial strains, *C. albicans*, and non-albicans *Candida* strains prompted us to study the piperamides in combination with currently known conventional antibiotics, which the results of the study revealed that piperine and piperlongumine could possibly enhance the effect of the currently available antibacterial and antifungal drugs for the treatment of bacterial and fungal infections.

7. References

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