University of Fort Hare

Together in Excellence

PHARMACOLOGICAL EVALUATION OF *PHRAGMANTHERA CAPITATA* (SPRENGEL) BALLE, LORANTHACEAE: A PARASITIC MISTLETOE GROWING ON RUBBER TREES

FRANKLIN UANGBAOJE OHIKHENA

DEPARTMENT OF BOTANY

FACULTY OF SCIENCE AND AGRICULTURE

UNIVERSITY OF FORT HARE

ALICE 5700, SOUTH AFRICA

2017

PHARMACOLOGICAL EVALUATION OF *PHRAGMANTHERA CAPITATA* (SPRENGEL) BALLE, LORANTHACEAE: A PARASITIC MISTLETOE GROWING ON RUBBER TREES

FRANKLIN UANGBAOJE OHIKHENA

A THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN ETHNOBOTANY

DEPARTMENT OF BOTANY
FACULTY OF SCIENCE AND AGRICULTURE
UNIVERSITY OF FORT HARE, ALICE,
SOUTH AFRICA

SUPERVISOR: PROF ANTHONY JIDE AFOLAYAN
CO-SUPERVISOR: DR OLUBUNMI ABOSEDE WINTOLA

MAY 2017

DECLARATION

I, Franklin Uangbaoje Ohikhena, declare that this thesis, submitted to the University of Fort Hare for the degree of Doctor of Philosophy in Ethnobotany in the Faculty of Science and Agriculture, is my own work; and that this work has not been submitted to any other institution for the award of any academic degree.

I declare that I followed the rules and conventions concerning referencing and citation in scientific writing.

I also declare that all sources of materials used for this thesis have been duly acknowledged and accurately referenced.

Again, I declare that I am fully aware of the University of Fort Hare policy on plagiarism and I have taken every precaution to comply with the regulations of the University.

Name: Franklin Uangbaoje Ohikhena **Signature:** ....

Institution: University of Fort Hare **Date:** May, 2017

We confirm that the work reported here was carried out by the above-named candidate under our supervision.

Prof Anthony Jide Afolayan

Signature: …………………………. Date: …………………………

Dr Olubunmi Abosede Wintola

Signature: .... Date: ....

DEDICATION

This thesis is dedicated to God Almighty for sustaining me through this study. I also dedicate this work to my dearest wife: Hope Orobosa Franklin-Ohikhena, my little princess: Ofure Abigail Franklin-Ohikhena and to my parents and siblings for their support, care and sacrifices which saw me through this study.

ACKNOWLEDGEMENTS

I am grateful to my Supervisor, Prof Anthony J Afolayan for his advice, guidance and constructive criticisms that brought the best out of this work. To my Co-Supervisor, Dr Olubunmi A Wintola, I wish to express my heartfelt appreciation to you for guiding me through my assays and always prompt to read and correct my write up in due course. You were more than just a co-supervisor; you were a colleague, a friend and a big sister to me. God bless you ma.

I wish to appreciate all the Medicinal Plants and Economic Development (MPED) Research Centre past and present group members. Special thanks to Dr Wilfred Mbeng, Dr Josephine Sharaibi, Dr Callistus Bvenura, Dr Gloria Otunola, Dr Emmanuel Ajayi, Dr Samuel Odeyemi, Dr Ggenga Adeogun, Vuyokasi Mgobozi, Zimasa Dubeni, Dr Leye Akinleye, Dr Jonas Sagbo, Dr Linda Sowunmi, Yanga Mhlomi, Siyasanga Mnciva, Zomsa Yako, Dr Ogochukwu, Oladayo Idris, Mrs Sherifat and Taiwo Abifarin. To my past and present office mates; Dr Cromwell Kibiti, Dr Mojisola Asowata, Funmi Adegbaju, Muhali Jimoh, Tomi Lois Olatunji, Barnabas Oyeyinka and the chairman himself, Jeremiah Oshiomhome Unuofin. How can I forget Maureen Mangoale, Bosede Elizabeth Famewo and Mama P (Funke Paulina Adijat Ogundola) AKA Tea mama. I appreciate you all.

I wish to say thank you to Dr Uyi Omoruyi of the Chemistry Department, UFH, for his assistance in my admission. Thank you sir.

I acknowledge the financial support of the Govan Mbeki Research Development Centre, University of Fort Hare, South Africa. My appreciation also goes to the Management, staff and my colleagues at the Rubber Research Institute of Nigeria (RRIN), Iyanomo. Dr and Dr (Mrs) Crosdel Emuedo (my second parents), Edwina Olohirere Uzunuigbe, Dido Chukwuka, Joy Omorogbe, Onyinmwen, Pst Desmond, not forgetting Kemi now in Canada. To my RRIN brothers, Emmanuel Osadolor and Stanley Omorogbe, words fail me to appreciate you but know that I hold you guys in high esteem.

I thank the Pastorate and members of the Redeemed Christian Church of God (RCCG), Alice, most especially the Men and Organizing members for making me feel comfortable in God's house. I wish to especially appreciate Mike Ojemaye for his love, support and encouragement through this journey.

To my parents, Mr Roland and Mrs Rosaline Ohikhena and my siblings; Engr Presley, Clarisbel Enabulu, Ebibu, GodsPower, GodsGift, Bayo and Princess, I appreciate you all. To my parents-in-law and all my siblings-in-law, I duly acknowledge you all.

I emotionally wish to acknowledge my beloved wife, my priceless jewel and queen, Hope Orobosa Franklin-Ohikhena whom during the course of this study I never set eyes on hitherto because she was bearing and weaning our baby, Ofure Abigail Osagbemwonrue Franklin-Ohikhena whom also, I have not seen even at submitting this thesis. I thank you, both for your patience, love, resilience, prayers and belief which propelled me through this course. I love you. God bless you guys richly for me.

If your name was not mentioned here, it does not mean you are less important or I have forgotten you and even if I did, God shall never forget you and know that you are a part of my success story and I do really appreciate you.

ABSTRACT

Rubber tree (*Hevea brasiliensis* Muell.) is the world's major source of natural rubber and a major non-oil, agricultural export commodity in the producing countries in Africa. However, it is highly susceptible to specific mistletoes of the Loranthaceae family. *Phragmanthera capitata* (Sprengel) Balle is the major mistletoe parasitizing the rubber tree plantations in Africa and hence, alongside with other mistletoes, has attracted indiscriminate destruction from plantation owners. Indiscriminate destruction of plants could be as a result of ignorance of the importance or benefit(s) of the plants and this could lead to their extinction if not checked. Hence, there is the need for scientific documentation of plants and their uses because, from the pharmacological perception, the destruction of a plant could result in the permanent loss of a potential drug. Therefore, this study was aimed at documenting and evaluating the pharmacological potentials of *P. capitata* collected from rubber tree.

Fresh leaves were gently rinsed with water and dried. Dried leaves were pulverised and kept at 4°C when not in use. Pulverised leaves were extracted with acetone, methanol, ethanol and water and concentrated to give the respective solvent crude extracts used in most of the assays. From the extraction, Methanol had the highest yield of 21.5%, water had 10.8% and acetone was 6.87% while ethanol had the lowest yield of 4.3%. Standard spectrophotometry assays on the extracts were used to quantify some major phytochemicals of pharmacological interest and also to determine the antioxidant potentials of the sample. The result revealed high amounts of phenols (175.53 ± 0.32 mg GAE/g to 218.62 ± 3.38 mg GAE/g), flavonoids (197.06 ± 3.80 mg QE/mg to 679.82 ± 6.26 mg QE/g) and proanthocyanidins (128.83 ± 1.69 mg CE/g to 645.68 ± 6.35 mg CE/g) with the acetone extract having the highest amount of the phytochemicals while the aqueous extract had the least. The results of the antioxidant assays revealed that the sample had high radical scavenging potentials with comparable inhibitory concentrations to standard antioxidant drugs.

The proximate and mineral analysis on the pulverised sample showed that it is rich in carbohydrate with a composition of 57.73 ± 0.33 g/100 g. Protein, fat and fibre compositions were 12.50 ± 0.50 g/100 g, 3.34 ± 0.18 g/100 g and 11.66 ± 0.54 g/100g. The total energy composition was 310.97 ± 2.30 Kcal/100 g. The mineral composition revealed high potassium level of 1047.83 ± 34 mg/100g. Calcium (6.22.58 ± 0.01 mg/100g), magnesium (361.15 ± 0.01 mg/100 g) and phosphorous (115.40 ± 0.01 mg/100 g) were also present in appreciable amounts. The toxicity of the four extracts was assayed using brine shrimp. The LC_{50} in all the solvent extracts was greater than 1 mg/mL which according to Meyer's index and other indexes signify "no toxicity" for crude plant extracts.

Antimicrobial activity of the extracts was done using the minimal inhibitory concentration (MIC) assay against 10 bacteria (*Enterococcus faecalis*, *Staphylococcus aureus, Bacillus subtilis*, *Bacillus cereus, Streptococcus pyogenes*, *Vibrio cholera, Klebsiella pneumonia*, *Pseudomonas aeruginosa*, *Salmonella typhi* and *Escherichia coli*) and 4 fungi (*Trichophyton mucoides*, *Trichophyton tonsurans*, *Candida albicans* and *Aspergillus niger*) of human pathogenic strains. The MIC of the antibacterial assay ranged from 1.25 mg/mL to 5 mg/mL for the organic extracts while no activity was observed in the aqueous extract at the highest concentration tested. However, the aqueous extract had a great inhibitory activity on all four of the tested fungi with a MIC of ≤ 0.3125 mg/mL to 1.25 mg/mL while only two fungi (*Trichophyton Tonsurans* and *Aspergillus niger*) were susceptible to the organic extracts. The MIC of the TB causative pathogen, *Mycobacterium tuberculosis* (H37Rv) strain ranged from 25 µg/mL (acetone and aqueous extracts) to 50 µg/mL in the ethanol extract while it was > 50 µg/mL (highest concentration tested) in the methanol extract.

This study revealed that the sample has the potential in phytotherapy against oxidative stresses (inflammations) caused by free radicals. It could also be a useful herb in combating diseases caused by pathogenic organisms. The high anti-*Mycobacterium tuberculosis* activity observed

in this study suggests it could be a plant with high promise in the fight against tuberculosis. The toxicity evaluation signifies it is safe for further plant-based pharmaceutical explorations. This study also validated some reported ethnopharmacological uses of this plant and call for its protection from indiscriminate destruction in rubber plantations and other crop gardens/plantations in the world.

TABLE OF CONTENTS	Pages

DECLARATION .... **ii**
DEDICATION .... **iii**
ACKNOWLEDGEMENTS .... **iii**
ABSTRACT .... **v**
TABLE OF CONTENTS .... **viii**
INTRODUCTION AND LITERATURE REVIEW **1**
Background 3
Herbal Medicine 3
Herbal Medicine in Africa 5
Mistletoe 6
Phragmanthera capitata (Sprengel) Balle 12
Justification for this research work 16
Overall aim of the study 18
Specific objectives of this study 18
Structure of the thesis 18
References 20
QUANTITATIVE PHYTOCHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITIES OF *PHRAGMANTHERA CAPITATA* EXTRACTED WITH DIFFERENT SOLVENTS **27**
Background of the study 29
Methodology 30
Results 36
Discussion 46
Conclusion 49
References 50
PROXIMATE COMPOSITION AND MINERAL ANALYSIS OF *PHRAGMANTHERA CAPITATA* **52**
Abstract 54
Introduction 55
Materials and Methods 55
Results and Discussion 57
Conclusion 60
References 60
GC-MS PROFILING OF THE ESSENTIAL OILS OF *PHRAGMANTHERA CAPITATA* **63**
Background of the study 65
Methodology 66
Result and Discussion 67

References 79
TOXICITY ASSESSMENT OF DIFFERENT SOLVENT EXTRACTS OF *PHRAGMANTHERA CAPITATA* ON BRINE SHRIMP (*ARTEMIA SALINA*) 83
Abstract 85
Introduction 86
Materials and methods 86
Location and collection of sample 86
Result.. 87
Discussion 92
Conclusion.. 93
References 94
EVALUATION OF THE ANTI-BACTERIAL AND ANTI-FUNGAL PROPERTIES OF *PHRAGMANTHERA CAPITATA* USING AGAR DILUTION AND A 96-WELL BROTH MICRODILUTION TECHNIQUES 95
Background of the study 97
Materials and Methods 98
Result.. 102
Discussion 109
Conclusion.. 112
References 113
EVALUATION OF THE ANTI-*MYCOBACTERIUM TUBERCULOSIS* POTENTIALS OF *PHRAGMANTHERA CAPITATA* 115
Background of the study 117
Methodology 118
Result and Discussion 119
Conclusion.. 122
References 122
***IN- VITRO* ANTI-DIABETIC EVALUATION OF *PHRAGMANTHERA CAPITATA* 126**
Background of the study 128
Methodology 129
Statistical analysis 131
Result.. 132
Discussion 134
Conclusion.. 136
References 136
GENERAL DISCUSSION AND CONCLUSION.. 139
Discussion 140
Conclusion.. 142
References 143

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

CONTENTS	Pages

Background 3
Herbal Medicine 3
African Herbal Medicine 5
Mistletoes 6
Folkloric and Ethnopharmacological uses of mistletoes 7
Ecology of mistletoe 10
The African mistletoe — Loranthaceae 11
Phytochemistry of some African Loranthaceae 11
Phragmanthera capitata (Sprengel) Balle. 12
Ethnomedicinal uses of *Phragmanthera capitata* 14
Rubber tree (*Hevea brasiliensis* Muell) and *Phragmanthera capitata* 15
Justification for this research work 16
Overall aim of the study 18
Specific objectives of this study 18
Structure of the thesis 18
References 20

Background

Historically, plants have played a pivotal role as natural sources of novel compounds with potentials for the development of safe, effective and affordable drugs (Iwalokun *et al.,* 2011). The genesis of the use of medicinal plants by man was instinctive as it is in the case with animals (Stojanoski, 1999). The widespread use of herbal remedies and their preparations in healthcare have been traced to the occurrence of natural products with medicinal properties (Parekh, 2007). In every culture and in every age, there are different theories of diseases and different systems employed in medicine, but botanical remedies are universal (Parekh, 2007). Until the advent of synthetic drugs, plants had been the source of treatment and prophylaxis (Kelly, 2009). Nonetheless, the decreasing efficacy and the increasing contraindications of synthetic drugs have made the usage of natural drugs a cynosure again (Petrovska, 2012). However, the rise in the global disease burden in recent times has resulted in a simultaneous increase in the lack of adequate affordable healthcare mostly in impoverished regions of the world. The use of plants as medicine has been the major hope for affordable healthcare in these regions and in the fight against the alarming rise in the global disease burden. The evaluation and proper documentation of potential plant species with medicinal values cannot be overemphasised; hence, this study was born on this premise.

Herbal Medicine

During the last decade, the use of herbal medicine has expanded globally and has gained popularity. It has not only been used for the primary healthcare of the poor in developing countries but has also been used in countries where conventional medicine is predominant in the national healthcare systems (WHO, 2000). About 70% of the world's population depends on medicinal plants for their primary healthcare. Among the 250,000 estimated plants species in the world, an equivalent of about 35 to 75% species have been used worldwide and about 14 to 28% has been used as medicaments (Mamedov, 2012; Padulosi *et al.,* 2002; Akerele, 1992; Farnsworth and Soejarto, 1991). Recently, more than 50 major therapeutic drugs in the global

markets originated from tropical plants (Mamedov, 2012; Padula *et al.,* 1999). Chemical and biological diversity observed in plants is a potential reservoir to a limitless renewable source of new drugs development (Mamedov, 2012).

The use of herbal medicine or phytotherapy was derived from the accumulation of botanical wisdom by indigenous people (Mamedov, 2012; Shankar and Liao, 2004; Sumner, 2000). In fact, drugs derived from plants that are currently prescribed in the developed countries were discovered by using the ethnobotanical approach of studying the uses of traditional herbs (Balick and Cox, 1997; Farnsworth, 1992).

According to the World Health Organization (2003), herbal medicine has gained a documented popularity in all parts of the developing world and also gaining rapid acceptance in the developed countries. Over 50% of the population in Europe, North America and other developed nations of the world have used herbal therapy at least for once. About 60% of the Hong Kong population has used the services of a herbalist. Herbal preparations have accounted for 30 to 50% of the total medicinal consumption in China. 75% of the people living with HIV/AIDS in San Francisco, London and South Africa have used herbal therapy. In Canada and Germany, 70% and 90% of the respective populations have used herbal medicine at some point in their lives. From 1995 to 2000, the number of medical doctors who had gone for special training in the use of natural remedy medicine almost doubled to 10,800 in Germany (WHO, 2003). In France and Australia, 49% and 46% of the respective population use herbal Medicine (Abdullahi, 2011). Reports from the United States have revealed that about 158 million adult population use complementary medicine. About 17 billion USD was spent on herbal remedies in 2000 in the United States and 230 million USD spent annually on alternative medicine in the United Kingdom. The global herbal medicine market currently stands at over 60 billion USD annually and the trend is steadily increasing (WHO, 2003).

Herbal Medicine in Africa

Africa is considered as the cradle of mankind and blessed with a vast biological and cultural diversity. Africa is believed to be the oldest and most assorted of all the healing systems (Mahomoodally, 2013; Gurib-Fakim, 2006). Prior to the colonial era and the advent of orthodox medicine by the Europeans, millions of Africans have relied solely on herbal medicine as the only available healthcare system in both rural and urban communities (Abdullahi, 2011; Romero-Daza, 2002). The difficulty or inability in accessing modern healthcare which may be due to cost or inadequate health service providers and the lack of effective Western medical treatment for some ailments like malaria and HIV/AIDS which although are worldwide diseases, but with Africa having the worst hit, might have triggered the continued interest in herbal therapy in African healthcare system (Mahomoodally, 2013).

Africa is endowed with an enormous diversity of about 40,000 to 45,000 plants species of which about 5,000 have been exploited in the African Traditional Medicine system (Mahomoodally, 2013). Research has shown that African medicinal plants accumulate important secondary metabolites to be able to withstand the sun's ultraviolent rays typical of the tropics and also to ward off different pathogenic attacks. Hence, these plants show more ability to amass chemoprotective biocompounds to survive in this harsh environment than species from the northern hemisphere. Abegaz *et al.* (2004) study on different species of *Dorstenia* showed that only species from the tropical rainforest in Central Africa had more biological activities than related ones from outside the tropics (Mahomoodally, 2013; Manach *et al.,* 2004; Farnsworth *et al.,* 1985).

Although there had been simultaneous and contradictory attitudes or feelings to herbal medicine in Africa (Abdullahi, 2011; Bello, 2006; Feierman, 2002), herbalism is still practised even in modern-day Africa after aeons of its existence without much-reported cases of adverse effects (Okigbo and Mmeka, 2006). According to the WHO (2003) report, up to 80% of the African

population relies on herbal medicine for primary healthcare. In Ghana, Mali, Zambia and Nigeria, the first choice of treatment for about 60% of children with high fever as a result of malaria is the use of herbal remedies at home. According to Carpentier *et al.* (1995), there is an increasing demand for herbal therapy in Africa for the management of rheumatic and neurological complaints. About 27 million black South Africans use herbal medicines for a variety of ailments (Abdullahi, 2011). Convulsion also known as “degedege” in Tanzania has been extensively managed and treated with plant products and over 70% of Ghanaians rely on phytotherapy (Abdullahi, 2011; Makundi *et al.,* 2006; Roberts, 2001). In Nigeria, some patients suffering from hypertension had integrated herbs into their conventional treatments (Amira and Okubadejo, 2007).

Aside from the inadequate technical specifications and quality control standards which have fraught the use of herbal medicinal system in Africa, there is also a pressing concern for the rapid loss of natural habitats of some medicinal plant species as a result of anthropogenic activities and the fast erosion of traditional knowledge (Gurib-Fakim and Mahomoodally, 2013; Mahomoodally, 2013). The highest rate of deforestation is observed in the African continent and in spite of her endowed biodiversity, the continent has only a few drugs commercialised globally to her credit (Chintamunnee and Mahomoodally, 2012; Atawodi, 2005). Hence, there is the need for scientific validation, documentation and sustainable exploitation of African medicinal plants. One of the various group of plants which has been exploited for their medicinal values for centuries in Europe but had suffered a dearth of medicinal exploration in Africa is the Mistletoe.

Mistletoe

The term “mistletoe” is not strictly a taxonomic term. It donates a group of polyphyletic shrubby parasitic flowering plants in the order, Santalales. They attach to the aerial stem or branches of shrubs and trees (Mathiasen *et al.,* 2008; Nickrent, 2002). Molecular phylogenetic

studies have revealed that mistletoes evolved from their root parasitic ancestors on five different occasions and have also independently evolved in five separate families viz: Viscaceae, Amphorogynaceae, Santalaceae, Loranthaceae and Misodendraceae. Presently, mistletoes are represented by 88 genera and about 1600 species (Table 1). Loranthaceae (over 900 species) and Viscaceae (about 550 species) are the most abundant and diverse mistletoe families (Nickrent, 2011; Nickrent *et al.,* 2010).

Table 1: Mistletoes: number of genera and species

S/N	Families	Number of Genera	Number of species
1	Misodendraceae	1	10
2	Loranthaceae	73	990
3	Santalaceae[a]	3	11
4	Amphorogynaceae[b]	4	37
5	Viscaceae	7	550
	Total	**88**	**1598**

[a]Traditionally classified as Eremolepidaceae.
[b]Traditionally considered as part of Santalaceae (Nickrent, 2011).

Folkloric and Ethnopharmacological uses of mistletoes

Mistletoes from the earliest times has been one of the magical, mysterious, sacred and revered plants in the European folklore. It was believed to bestow life and fertility and acts as protection against poison and as an aphrodisiac (http://www.theholidayspot.com/christmas /history/mistletoe.htm). The custom of using mistletoe to decorate homes at Christmas and other festive periods is a survival of the European beliefs and traditions. Branches are cut and hung from the ceilings to ward off evil spirits. In some other countries, they are placed over houses and stable doors to prevent the entrance of witches. There was also a belief that mistletoe could be used as a fire extinguisher (Olawale, 2012).

Almost all cultures and continents at some point in their history have used mistletoes in folk medicine. The use of mistletoes for holiday affection is only a minor example of the various

ways in which they have been used throughout history and in different regions. In the last century, awareness of the medical potential of mistletoe has resurged as its efficacy in treating cancers has been investigated (Evans, 2005). It is seen in folklore medicine as a panacea and has been used in various forms to treat cancers, infertility, menopausal symptoms, epilepsy, smallpox, ulcer, nervous tension, asthma, headache, hypertension and dermatitis etc (Ameer *et al.,* 2015; Zainuddin and Sul'ain, 2015; Dibong *et al.,* 2009).

According to Adesina *et al.* (2013), the ethnomedicinal applications of mistletoes had for a long time been in the hands of very few herbal practitioners. They claimed it is used to counter sorcery and has magical powers to treat mental disorders, urogenital problems, sterility and rheumatism related pains. Presently in Europe, mistletoe is mostly used for the treatment and management of cancer, physical and mental conditions. It is either used alone or in combination with other drugs or medication for cancer therapy. Some HIV/AIDS Non-Governmental Organisations have reported that mistletoe therapy restores immune systems (National Cancer Institute, 2016; Ogunmefun *et al.,* 2015a and b). In Germany, mistletoe extracts are used for traditional oncology therapy. It has also found application for the treatment of skin diseases and prostate cancer in the Palestine traditional medicine system (Ogunmefun *et al.,* 2015b). Ken'ichi *et al.* (2006) reported that species of Loranthaceae made into tea were believed to heal bone fractures and body pains. In Southwestern Ethiopia, leaves of *Tapinanthus globiferous* is mixed with cold water and taken orally for the treatment of "Tanachaa" (tumor) (Yineger and Yewhalaw, 2007); in Cameroon, the fresh leaves are macerated with an equal amount of the root bark of *Boswella odorata* in 5L of local beer and a glassful taken two times a day for about 2 weeks for the treatment of syphilis (Noumi and Eloumou, 2011). The fresh twigs of *Tapinanthus globiferus* also called "Hadhal" in Saudi Arabia has been used in all kinds of livestock to treat their fever and also to remove their placentals after parturition (Adesina *et al.,* 2013; Sher and Alyemeni, 2011).

In South Africa, *Viscum capensis* is sold as a "Cape Mistletoe Tea" for the treatment of asthma, bronchitis and irregular menstruation (Afrinatural Holdings, 2012) and the Woodrose formed from the association of the mistletoe in the genera, *Erianthemum* and *Pedistylis* (Loranthaceae) are detached, polished and sold as curios (Mathiasen *et al.,* 2008). In Nigeria, *Loranthus micranthus* has been widely used as ethnomedicine for the treatment of hypertension, diabetes, schizophrenia and as an immune system booster (Osadebe and Omeje, 2009). The National Health Product Services (NAHEPS) Limited in southern Nigeria has produced a tea from mistletoe which is sold as "NAHEPS Tea"; it is believed that this tea promotes good health by enhancing the body natural immune system (Adesina *et al.,* 2013). Some of these ethnomedicinal uses have already been supported and acclaimed by several investigations (Osadebe and Omeje, 2009; Griggs, 1991; Nwude and Ibrahim, 1980).

Different products of mistletoe with the trade names; Helixor, Iscador, Eurixor, Isorel, Iscucin, Plenosol and abnoba VISCUM have been sold across the European market for the treatment of infertility, epilepsy, hypertension and arthritis (National Cancer Institute, 2016; Adesina *et al.,* 2013; Kienle and Kiene, 2007).

Studies have demonstrated that composition and biological activities of mistletoe are dependent on harvesting period, host tree species (National Cancer Institute, 2016; Adesina *et al.,* 2013; Scheer *et al.,* 1992) and geographical locations. Leaves of a particular mistletoe are usually prepared alone as medicaments but at some instances, leaves of a particular mistletoe differentiated by their host are prepared together as a mixture. This is because the bioactivity of mistletoe is chiefly dependent on the host species as certain host species confer some activities on the mistletoe for the cure of a particular ailment than others (Ogunmefun *et al.,* 2015a; Adesina *et al.,* 2013; Burkill, 1985).

Ecology of mistletoe

Mistletoe is often considered a pest that kills trees and devalues natural habitats, but recently, it has been recognised as an ecological keystone species; an organism with a disproportionately pervasive influence over its community (Wikipedia, 2015; Watson, 2001). A broad array of animals, insects and even fungi depends on mistletoe for food; consuming the leaves, young shoots, flowers and fruits. This interaction helps in transferring pollen between host plants and also helps in dispersing the sticky seeds of the mistletoe (Wikipedia, 2015)

A study of mistletoe in junipers concluded that more juniper berries sprout in stands where mistletoe is present, as the mistletoe attracts berry-eating birds that also feed on juniper berries (Milius, 2002). The dense evergreen "witches' brooms" formed by the dwarf mistletoes (*Arceuthobium* species) of western North America is an excellent habitat for roosting and nesting of the northern spotted owl and the marbled murrelet (Wikipedia, 2015). In Colorado, a pine forest with mistletoe had a greater diversity of bird species and a high population of elk and deer than pine forests without mistletoe stands (Bennetts *et al.,* 1996). Such interactions lead to dramatic influences on diversity, as areas with greater mistletoe densities support higher diversities of animals. March and Watson (2007) reported the ability of mistletoe to replenish the host and the surrounding organisms with nutrients through litterfall. The report is based on the fact that mistletoe litter contains a high proportion of many elements most especially phosphorus and potassium. Thus, rather than being a pest, mistletoe can have a positive effect on biodiversity, providing high-quality food and habitat for a broad range of animals in forests and woodlands worldwide. Also, their vast network of interactions with other organisms can be utilised as sensitive indicators of community structure integrity and ecosystem health (Wikipedia, 2015; Mathiasen *et al.,* 2008).

The African mistletoe — Loranthaceae

Loranthaceae and Viscaceae are the major families of the mistletoe. Both families were initially classified as subfamilies of Loranthaceae but are now considered to have originated separately. Loranthaceae is monophyletic and by far the widest distributed family in the Order, Santalales. They are widely distributed from the tropics to the temperate regions (Nickrent, 2011; Mathiasen *et al.,* 2008; Polhill and Wiens, 1999) excluding the extremely cold zones.

Most African mistletoe belongs to the Loranthaceae family. Seven genera of the Loranthaceae: *Helixanthera, Berhautia, Englerina, Globimetula, Agelanthus, Tapinanthus* and *Phragmanthera* are recognised in Western Africa (Adesina *et al.,* 2013; Burkill, 1985). These genera are hemiparasitic on a wide variety of economic, cultivated and non-cultivated shrub or trees which include; *Vitellaria paradoxa, Azadirachta indica, Theobroma cacao,* (Adesina *et al.,* 2013), *Kola acuminata*, *Baphia nitida*, *Persia americana*, *Irvingia gabonensis*, *Citrus simensis*, *Pentacletrama crophylla*, *Treculiarafricana*, *Ficus exasperata* (Osadebe *et al.,* 2012; Ali et al*.,* 2005), *Accacia karroo, Ziziphus mucronata*, *Ehretia rigida* (Okubamicheal *et al.,* 2001; Dean *et al.,* 1994) and *Hevea brasiliensis* etc (Begho *et al.,* 2007).

Phytochemistry of some African Loranthaceae

Extensive phytochemical evaluations of *Loranthus micranthus* extracts demonstrated the presence of various phytoconstituents and compounds. Crude leaf methanolic extract of *L. micranthus* harvested from *P. americana* was found to possess terpenoids, steroids, oils, proteins, resins, flavonoids, tannins, saponins, alkaloids, reducing sugar, acidic compounds, glycosides and carbohydrates (Osadebe *et al.,* 2010). Lohézic-Le *et al.* (2002), study on *L. ferrugineus* as cited by Ameer *et al.* (2015), revealed the presence of flavonoids and a high amount of condensed tannins. The study also reported the isolation of three natural flavonol compounds from the ethyl acetate fraction in addition to the isolation of a flavonol glycoside, 4”-O-acetylquercitrin.

Iwalokun *et al.* (2011) investigated the phytochemicals of n-butanol, chloroform, ethyl acetate, and water fractions of the methanolic extract of *L. micranthus* leaves of Kolanut tree (*K. acuminata*). Moderate and high levels of steroids and terpenoids were detected in the n-butanol fraction, while a moderate level of phenols and tannins were reported in the chloroform fraction. Reducing sugars and tannins were detected in all the fractions they studied. Flavonoids and saponins were only present in the ethyl acetate and water fractions respectively. They reported that terpenoids were present in low to moderate levels in the chloroform and water fractions, while they were not detected in the ethyl acetate fraction. Phytochemical studies of *L. micranthus* leaves harvested from six different host trees: *P. americana, K. acuminate, B. nitida, P. macrophylla, A. indica* and *I. gabonensis,* revealed that alkaloids are more in the extracts of *K. acuminata, P. americana,* and *I. gabonensis*.

The effect of seasonal variation on the phytoconstituents of the African mistletoe, *L. micranthus* was evident in a study on the petroleum ether leaf extract of *L. micranthus* parasitic on *P. americana* harvested in January, April, July, and November. The results showed the presence of alkaloids only in the April and July extracts (Osadebe *et al.,* 2007). In Africa, pharmacological investigation of mistletoe is very scanty as most of the attention is channelled on crop protection: how to reduce the devastating effect of mistletoe on the host (Dibong *et al.,* 2009) and its possible eradication from existence if possible.

***Phragmanthera capitata* (Sprengel) Balle**

The genus, *Phragmanthera,* comprises 33 species (http://www.theplantlist.org/browse/A/ Loranthaceae/Phragmanthera/) which include *Phragmanthera capitata* (Sprengel) Balle. It belongs to the Kingdom: Plantae (plants); Subkingdom: Tracheobionta (vascular plants); Superdivision: Spermatophyta (seed plants); Division: Magnoliophyta (flowering plants); Class: Magnoliopsida (Dicotyledons); Subclass: Rosidae; Order: Santalales; Family: Loranthaceae; Genus: *Phragmanthera* Tiegh.; Species: *Phragmanthera capitata*.

Phragmanthera capitata is a woody aerial hemiparasitic shrub of about 2 m long. The leaves are entire, short-stalked and opposite with pack of yellow corolla with reddish-pink tips that look like a matchstick. The berries are green when young and red when ripe (Figure 1). It is found in secondary jungles and bush savannas in the tropical regions of Africa. These regions include; South Tropical Africa of Angola, through the West Tropical Regions of Benin, Cote D'Ivoire, Ghana, Guinea, Liberia, Sierra Leone, Togo, Nigeria and the West-Central Tropical Regions of Burundi, Cameroon, Central African Republic, DR Congo, Equatorial Guinea, Gabon, Rwanda (Ogunmefun *et al.,* 2013; Polhill and Wiens, 1999, 1998). It is parasitic on a number of hosts which include *Theobroma cacao, Cola nida and acuminate, Irvingia gabonensis, Citrus maxima, Manikarazapota, Persia americana, Psidium guajava*, *Coffea* spp and *Heavea brasiliensi*s etc (Ogunmefun *et al.,* 2015a and b, 2013; Adesina *et al.,* 2013; Dibong *et al.,* 2010, 2009; EngoneObiang *et al.,* 2009).

Figure 1: *Phragmanthera capitata* (Sprengel) Balle showing the leaves, inflorescence (flowers) and seeds. Pic by Franklin Ohikhena

Ethnomedicinal uses of *Phragmanthera capitata*

A survey on the uses of *P. capitata* by Dibong *et al.* (2009) in Cameroon reported that the leaves prepared as an infusion or marc are used for the management and treatment of over 22 diseases in the Logbessou region of North Douala. These ailments include; nerve attacks, convulsion, chronic muscular pains, diabetes, respiratory disorders, rheumatism, epilepsy, dizziness, uterine haemorrhage, hypertension, hypotension, back pains, kidney pains, menopause, headache, heart palpitation, general purifications, irregular menstruation and nose bleeding. Another ethnobotanical survey of *P. capitata* conducted in Nigeria also reported its usage for the treatment and management of insomnia, infertility, hypertension and diabetes etc. These surveys information were gathered from traditional and herbal practitioners in the countries sampled (Ogunmefun *et al.,* 2015b, 2013). Some *in-vitro* and *in-vivo* works have be conducted to validate some of its ethnopharmacology uses. Ogunmefum *et al.* (2015a) reported the antimicrobial activity of *P. capitata* parasitic on *Theobroma cacao* and *Cola nitida.* Though antimicrobial activities were shown, they were very weak as the study reported minimal inhibitory concentration (MIC) values of 100 mg/mL to 200 mg/mL. The work also showed that host plants play significant roles in the phytoconstituents and/or activities of *P. capitata.* The leaves of *P. capitata* have been reported to reduce the levels of plasma inorganic phosphatase, haemoglobin, muscle glycogen and blood sugar (Fasanu and Oyedapo, 2008). Takem *et al.* (2014) reported the anti-secretory, gastroprotective and anti-ulcer activities of aqueous extract of *P. capitata* harvested from Avocado in albino rats. As with most mistletoe, distinction in biological activities of a specific species is made between host plants and not always necessary between the mistletoe. Hence, the host species and even locations are very important in the investigation of the ethnopharmacological activities of *P. capitata*.

Rubber tree (*Hevea brasiliensis* Muell) and *Phragmanthera capitata*

Rubber tree (*Hevea brasiliensis* Muell. Arg.) (Figure 2) that is grown primarily for its latex (milky colloidal aqueous dispersion of rubber particles), is the world's major source of natural rubber. In Gabon, it was first introduced during the World War II to supply rubber for the Allied countries during the war. Its production stopped immediately after the war and was reintroduced in the 1980s to help diversify the Gabon economy which was over-reliant on crude oil (EngoneObiang *et al.,* 2009). The plant was introduced into Nigeria in 1895 and is now widely cultivated on more than 200,000 ha of land in the rain forest belt in the South of the country, with smallholders accounting for over 70% of the planted area (Shaib *et al.,* 1997). In Nigeria, natural rubber is a major non-oil, agricultural export commodity (Esekhade *et al.,* 2003). However, the rubber tree now cultivated in most tropical nations of the world is susceptible to various specific parasitic plants belonging to the Loranthaceae family. For example, in China by *Taxillus chinensis* and *Dendrophthoe pentandra* (Zhiwei *et al.,* 1995) and in most African plantations like Cameroon (Dibong *et al.,* 2010), Gabon (EngoneObiang *et al.,* 2009) and Nigeria (Akinlabi *et al.,* 2005; Gill and Oyinbe, 1990) by *Phragmanthera capitata* (EngoneObiang *et al.,* 2009).

Figure 2: (A) Rubber plantation showing healthy and (B) infected with *P. capitata* stands.
Pic by Franklin Ohikhena

Begho *et al.* (2007) reported that the species of mistletoe observed on rubber trees in Nigeria were the yellow-flowered *Phragmanthera capitata* (present in all plots examined) and *Agelanthus brunneus* (which was scarcely seen). They also reported *Phragmanthera* as the only phanerogamic parasite on rubber trees in Rubber Research Institute of Nigeria plantations. In the reports of EngoneObiang *et al.* (2009), data collected in the area of Mitzic, Gabon, in the industrial and smallholding plantations of rubber trees, showed Loranthaceae were very common and that among them, *Phragmanthera capitata* was by far the most widespread (95%).

Studies carried out by various researchers on rubber plantations infected with mistletoe in Rubber Research Institute of Nigeria, Iyanomo, revealed that there is no significant effect on the quality of natural rubber latex from such mistletoe-infected trees. The report also went further to recommend that lattices from mistletoe-infected trees should not be commercially inferior to latex from uninfected trees (Akinlabi *et al.,* 2005). In other studies, some plantations with severe mistletoe infestation gave higher latex yield by volume of latex (cm^3) and dry weight (g) of cup lump (Ogbebor *et al.,* 2007). The findings of EngoneObiang and Salle (2006), suggested that there is no need for the eradication of *P. capitata* parasitizing rubber trees as there was no statistical difference in the latex yield and that the parasite most often times parasitizes the larger trees which can accommodate it.

Justification for this research work

Generally, mistletoe is seen as notorious, noxious and a devastating parasite in gardens/plantations which poses serious loss to economically valuable tree crops like cocoa, kola nut, coffee, rubber tree and some medicinal plants whether growing in the wild forests, gardens or orchards etc (Adesina *et al.,* 2013). They tend to reduce the value of the cultivated plants in amounts more than the thresholds the host can accommodate by drawing water and nutrients from them. Hence, the first action that comes to mind is to eradicate them as soon as they appear.

In Nigeria and other rubber growing nations of Africa and the world in general with the mistletoe scourge, expensive, laborious and time-consuming programmes are been used and sort after for its eradication without any consideration to the other benefits it may possibly confer. The known method of removing mistletoe from host trees is by pruning (cutting off completely) the infected part(s) (Dibong *et al.,* 2010) thereby losing important physiological host plant parts in the process. This practice could lead to low biomass accumulation by the host and create an avenue for the introduction of other pests and diseases. If this trend of destroying mistletoe continues, there is the chance of losing the function(s) they play in our ecosystem. This could also lead to their extinction as already evident in the report of the International Union for the Conservation of Nature (IUCN) that some species of both Loranthaceae and Viscaceae are on the RED LIST of Threatened Species and calling for conservation (IUCN, 2015).

This study is born from the premise that although *P. capitata* is a hemiparasite of rubber plantation and other economic crops, it could also serve other purposes: it is believed that when the host leaves falls during the drier seasons, mistletoe could act as a photosynthetic organ providing some nutrition to the host (Mathiasen *et al.,* 2008). Also, when rubber tree is treated with ethephon and other chemicals to stimulate latex production, the presence of *P. capitata* could steadily improve the rubber tree metabolism to increase latex yield (EngoneObiang and Salle, 2006). It also can play an ecologically important association with rubber trees; aiding pollination when birds that feed on the berries help in transferring pollens from one flower to another or within flowers. It also has importance in cultural and traditional medicine. Hence, if these values can be channelled with well-documented proof of their phytotherapeutic importance, it can be used sustainably with proper harvesting from rubber trees. This will aid in maintaining a synergistic relationship with the host plants while providing its ecological, cultural and medicinal functions to man and the environment. In no doubt, this will serve in maintaining a threshold which the rubber tree can accommodate without much pronounced

negative effect(s). This study, therefore, tends to scientifically investigate the various medicinal values of *P. capitata* harvested from rubber trees as no attention has been given to this aspect other than its negative effects on rubber plantations.

Overall aim of the study

The aim of this study was to evaluate, validate and document the pharmacological importance of *Phragmanthera capitata* that is parasitic on rubber trees and how these values could be used as an alternate source of medicine.

Specific objectives of this study

1. To quantitatively evaluate the phytochemical constituents and to screen for the free radical scavenging/antioxidant potentials of the mistletoe.
2. To evaluate the nutritive and mineral value(s) of the mistletoe.
3. To extract and identify the compounds in the essential oils of the mistletoe using GCMS.
4. To investigate the possible toxicity of the mistletoe using Brine Shrimp.
5. To evaluate the antibacterial and antifungal potentials of the mistletoe.
6. To screen for the anti-*Mycobacterium tuberculosis* potential of the mistletoe in the fight against tuberculosis.
7. To investigate the anti-diabetic properties of the mistletoe

Structure of the thesis

This thesis is composed of discrete chapters that have been published, accepted or under review or prepared for manuscript in different peer-reviewed accredited journals.

Chapter 1 is the introduction and literature review which also outlines the aim and objectives of the study. It highlights the uses of plants as herbal medicine, a review of mistletoe, its importance and uses in Africa. The specific mistletoe of concern, *Phragmanthera capitata* and its host, *Hevea brasiliensis* (Rubber tree), were discussed and how the study was born. Chapter

2 is an evaluation of the quantitative phytochemical and antioxidant activities of the mistletoe. The ability of an extract to scavenge free radicals is a determinant of the phytochemicals and the quantity present. Evaluation of the phytochemicals and antioxidant potentials of *P. capitata* gives an indication of its ethnomedicinal values. The effects of different solvents were screened to find out which solvent is best for the extraction of the phytochemicals and their corresponding antioxidant potentials. Chapter 3 is an evaluation of the proximate, mineral and anti-nutrient of *P. capitata.* Highly nutritious diets could boost the immune system in fighting and warding off sicknesses and diseases. Hence, this chapter evaluated these parameters in relation to medicinal significance. Chapter 4 attempts to look at the chemical profiles of the essential oil from the plant. Chapter 5 is a preliminary toxicity assessment of *P. capitata*. According to the WHO specification for medicinal plants, it must be potent against the target disease and should not be toxic. Toxicity may be due in part the amount consumed and the presence of toxic bio-compounds in the plants. Toxicity information may give an insight into the required ingestion dosage while taking note of the effective therapeutic concentration(s). There had been reports of toxicity associated with mistletoe most especially the berries. Hence, there is a need for this species to be screened for its possible toxicity. Chapter 6 is an evaluation of the potential antifungal and antibacterial activities of the plant against some human pathogenic organisms. Mistletoe is known as a panacea and therapeutic against all kinds of human pathogenic diseases. Chapter 7 is the evaluation of the plant extracts as a possible anti-mycobacterial agent. Chapter 8 is an *in-vitro* anti-diabetic evaluation of the sample with emphasis on the inhibition of both α- amylase and α- glucosidase inhibition as possible mechanisms of action. This is with an attempt to validate one of the ethnopharmacological uses of mistletoe. Chapter 9 is the General discussion and conclusion of the study.

References

Abdullahi, A.A., 2011. Trends and Challenges of Traditional Medicine in Africa. African Journal of Traditional, Complementary and Alternative Medicine 8(5), 115–123. DOI: 10.4314/ajtcam.v8i5S.5.

Abegaz, B.M., Ngadjui, B.T., Folefo, G.N., Fotso, S., Ambassa, P., Bezabih, M., Dongo, E., Rise, F., Petersen, D., 2004. Prenylated flavonoids, monoterpenoid furanocoumarins and other constituents from the twigs of *Dorstenia elliptica* (Moraceae). Phytochemistry 65(2), 221–226. DOI:10.1016/j.phytochem.2003.10.028

Adesina, S.K., Illoh, H.C., Johnny, I.I., Jacobs, I.E., 2013. African mistletoes (Loranthaceae); ethnopharmacology, chemistry and medicinal values: an update. African Journal of Traditional, Complementary and Alternative Medicine. 10(4), 161-70. PMCID: PMC3794408

Afrinatural Holdings, 2012. Herbal teas. http://www.afrinatural.com/zizamele-range/herbal-teas. Copyright© 2012

Akerele, O., 1992. Importance of medicinal plants: WHO's programme. In: Natural Resources and Human Health: plants of medicinal and nutritional value. Elsevier, Amsterdam, Netherlands, 63-77.

Akinlabi, A.K., Aigbodion, A.I, Akpaja, E.O., Bakare, O., Akinlabi, J.O., 2005. Characterisation of natural rubber latex from mistletoe-infested *Hevea* trees of NIG 804 clones. Journal of Rubber Research 8, 153159.

Ali, F.H., Intesar, T.N., Khylood, W.A., Saad, A.H., 2005. Hematopoietic toxicity of *Loranthus europaeus* chloroform extract: *in vitro* study. International Journal of Comprehensive Pharmacy 17, 345–52.

Ameer, O.Z., Salman, I.M., Quek, K.J., Asmawi, M.Z., 2015. *Loranthus f errugineus*: a Mistletoe from Traditional Uses to Laboratory Bench. Journal of Pharmacopuncture 18(1), 7–18. DOI: 10.3831/KPI.2015.18.001.

Amira, O.C., Okubadejo, N.U., 2007. 'Frequency of Complementary and Alternative Medicine Utilization in Hypertensive Patients Attending an Urban Tertiary Care Centre in Nigeria.' BMC Complementary and Alternative Medicine 7(30), 1–5.

Atawodi, S.E., 2005. Antioxidant potential of African medicinal plants. African Journal of Biotechnology 4(2), 128–133.

Balick, M.J Cox, P.A., 1997. Plants, People, and Culture: The Science of Ethnobotany, Scientific American Library.

Begho, E.R., Omokhafe, K.O., Omo-Ikerodah, E.E., Akpaja, E.O., 2007. Some Observations on the Fruit Set and Incidence of Mistletoes on Rubber Trees in Nigeria. American-Eurasian Journal of Sustainable Agriculture 1(1), 13-18.

Bello, R.A., 2006. Integrating the Traditional and Modern Healthcare System in Nigeria: A Policy Option for Better Access to Healthcare Delivery In: Saliu H, Jimoh A, Arosanyin T (Eds) The National Question and Some Selected Topical Issues on Nigeria. Ibadan: Vantage Publishers.

Benetts, R.E., White, G.C., Hawksworth, F.G., Severs, S.E., 1996. The influence of dwarf mistletoe on birth communities in Colorado ponderosa pine forests. Ecological Applications 899-909.

Burkill, H.M., 1985. The useful Plants of West Tropical Africa. Vol 3: families J-L. Royal Botanical Gardens, Kew. 3: 548–560.

Burkill, H.M., 1995. The useful plants of West Tropical Africa. 2nd Edition. Volume 3, Families J–L. Royal Botanic Gardens, Kew, Richmond, United Kingdom. 857 pp.

Carpentier, L., Prazuck, T., Vincent-Ballereau, F., Ouedraogo, L.T., Lafaix, C., 1995. Choice of Traditional or Modern Treatment in West Burkina Faso. World Health Forum 16,198–210.

Chintamunnee, V., Mahomoodally, M.F., 2012. Herbal medicine commonly used against infectious diseases in the tropical island of Mauritius. Journal Herbal Medicine 2, 113–125.

Dean, W.R.J., Midgley, J.J., Stock, W.D., 1994. The distribution of mistletoes in South Africa: patterns of species ricnhness and host choice. Journal of Biogeography 21, 503-510.

Dibong, S.D., EngoneObiang, N.L., Ndongo, D., Priso, R.J., Taffouo, V., Fankem, H., Salle, G., Missoup, A.D., Boussim, I.J., Amougou, A., 2009. An assessment on the uses of Loranthaceae in ethnopharmacology in Cameroon: A case study made in Logbessou, North of Douala. Journal of Medicinal Plants Research 3(8), 592-595.

Dibong, S.D., Mony, R., Ndiang, Z., Ondoua, J.M., Boussim, I.J., Bilong, B., Amougou, A., 2010. The struggle against *Phragmanthera capitata* (Sprengel) S. Balle (Loranthaceae) parasite of agroecosystems’ fruit trees in Cameroon. Journal of Agricultural Biotechnology and Sustainable Development 2(5), 76-81.

EngoneObiang, N.L., Sallé, G., 2006. Is there any point to eradicate *Phragmanthera capitata* parasitizing African rubber trees? Comptesrendus-Biologies 329(3), 185-195. DOI:10.1016/j.crvi.2006.01.007.

EngoneObiang, N.L., Sallé, G., Eschbach, J.M., Salomon, M., 2009. Susceptibility of rubber trees to Loranthaceae in Gabon. Journal of Plant Interaction 4 (3), 233-240. DOI: 10.1080/17429140902962613.

Esekhade, T., Orimoloye, J.R., Ugwa, I.K., Idoko, S.O., 2003. Potentials of multiple cropping systems in young rubber plantations. Journal of Sustainable Agriculture 22(4), 79-94

Evans, J., 2005. Mistletoe: Good for More Than Free Kisses. Herbal Gram. 68:50-59 American Botanical Council.

Farnsworth, N.R., Akerele, O., Bingel, A.S., Soejarto, D.D., Guo, Z., 1985. Medicinal plants in therapy. Bulletin of the World Health Organization 63(6), 965–981.

Farnsworth, N.R., Soejarto, D.D., 1991. Global importance of medicinal plants. In: Conservation of Medicinal Plants. Cambridge University Press, UK, 25-52.

Fasanu, P.O., Oyedapo, O.O., 2008. *Phragmanthin*-peptide from fresh leaves of African mistletoe (*Phragmanthera incana*): purification and metabolic activities. In: Govil J.N., Singh V.K., Bhardwaj R, (Eds). Recent Progress in Medicinal Plants 19: Phytopharmacology and Therapeutic Values 28, 39–47.

Feierman, S., 2002 'Traditional Medicine in Africa: Colonial Transformations' New York Academy of Medicine. Reported by Carter, G.M. The Foundation for the Integrative AIDS Research.

Fransworth, N.R., 1992. Preclinical assessment of medicinal plants. Natural Resources and Human Health. Elsevier Science Publishers BV, 87-91.

Gill, L.S., Onyibe, H.I., 1990. Mistletoes on rubber trees in Nigeria. Haustorium 23, 1-2

Griggs, P., 1991. Mistletoe, myth, magic and medicine, The Biochemist 13, 3–4.

Gurib-Fakim, A., 2006. Medicinal plants: traditions of yesterday and drugs of tomorrow. Molecular Aspects of Medicine 27(1), 1–93.

Gurib-Fakim, A., Mahomoodally, M.F., 2013. African flora as potential sources of medicinal plants: towards the chemotherapy of major parasitic and other infectious diseases- a review. Jordan Journal of Biological Sciences (6), 77–84.

International Union for the Conservation of Nature. 2015. Red List of Threatened Species. Version 2015.1. <www.iucnredlist.org>. Downloaded 04 June 2015.

Iwalokun, B.A., Hodonu, S., Nwoke, S., Ojo, O., Agomo, P.U., 2011. Evaluation of the possible mechanisms of antihyper-tensive activity of *Loranthus micranthus*: an African mistletoe. Biochemistry Research International. DOI: 10.1155/2011/159439.

Kelly, K., 2009. History of medicine. New York: Facts on file, pp 29–50

Ken'ichi, M., Kazuhiro, N., Norimasa, N., Dawa, D., Laximi, T., Atsushi, W., Fumi, M., Toshiro, B., Gaku, M., 2006. Report of investigation for Wild Edible Plants and their

Traditional Knowledge in Bhutan. Journal of the Faculty of Agriculture, Shinshu University 42(1–2), 37–47

Kienle, G.S., Kiene, H., 2007. Complementary cancer therapy; a systematic review of prospective clinical trials on anthroposophic Mistletoe extracts. European Journal of Medical Research 12, 103–119. PMID: 17507307.

Kienle, G.S., Glockmann, A., Schink, M., Kiene, H., 2009. *Viscum album* L. extracts in breast and gynaecological cancers: a systematic review of clinical and preclinical research. Journal of Experimental and Clinical Cancer Research 28(1), 79. Doi: 10.1186/1756-9966-28-79.

Lohézic-Le-Dévéhat, F., Tomasi, S., Fontanel, D., Boustie, J., 2002. Flavonols from *Scurrula ferruginea* Danser (Loranthaceae). Zeitschrift für Naturforschung C – A Journal of Biosciences 57, 1092-5.

Mahomoodally, M.F., 2013. Traditional medicines in Africa: An appraisal of ten potent African medicinal plants. Evidence-Based Complementary and. Alternative Medicine Doi: 10.1155/2013/617459.

Makundi, E.A., Malebo, H.M., Mhame, P., Kitua, A.Y., Warsame, M., 2006. Role of Traditional Healers in the Management of Severe Malaria among Children below Five Years of Age: the Case of Kilosa and Handeni Districts, Tanzania. Malaria Journal 5(58), 1–9.

Mamedov, N., 2012. Medicinal Plants Studies: History, Challenges and Prospective. Medicinal and Aromatic Plants 1, 8. Doi: 10.4172/2167-0412.1000e133.

Manach, C., Scalbert, A., Morand, C., Rémésy, C., Jiménez, L., 2004. Polyphenols: food sources and bioavailability. The American Journal of Clinical Nutrition 79(5), 727–747.

March, W.A., Watson, D.M., 2007. Parasites boost productivity: Effects of mistletoe on litter fall dynamics in a temperate Australian forest. Oecologia 154, 339-347.

Mathiasen, R.L., Nickrent, D.L., Shaw, D.C., Watson, D.M., 2008. Mistletoes: Pathology, Systematics, Ecology, and Management. Plant Disease 92(7), 988-1006. DOI: 10.1094/PDIS-92-7-0988.

Milius, S., 2002. Mistletoe, of All Things, Helps Juniper Trees. Science News. 161.1:6

National Cancer Institute, 2016. Mistletoe Extracts (PDQ®)–Health Professional Version. Updated October 19th, 2016. Webpage accessed 06th January, 2017. https://www.cancer.gov/about-cancer/treatment/cam/hp/mistletoe.

Nickrent, D.L., 2002. Mistletoe phylogenetics: Current relationships gained from analysis of DNA sequences. Pp. 48-57 in: Proceedings of the Western International Forest Disease Work Conference, August 14-18, 2000. Waikoloa, Hawai'i. 253 pp.

Nickrent, D.L., 2011. Santalales (Including Mistletoes). In: Encyclopedia of Life Sciences (ELS). John Wiley & Sons, Ltd: Chichester. DOI: 10.1002/9780470015902.a0003714.pub2.

Nickrent, D.L., Malécot, V., Vidal-Russell, R., Der, J.P., 2010. A revised classification of Santalales. Taxon 59(2), 538–558.

Noumi, E., Eloumou, M.E.R., 2011. Syphilis ailment; Prevalence and herbal remedies in Ebolowa subdivision (South region, Cameroon). International Journal of Pharmaceutical and Biomedical Sciences 2(1), 20–28.

Nwude, N., Ibrahim, M.A., 1980. Plants used in traditional veterinary medical practice in Nigeria. Journal of Veterinary Pharmacology and Therapeutics 3(4), 261–273.

Ogbebor, N.O., Omorusi, V.I., Evueh, G.A., 2007. Evaluation of nine *Hevea brasiliensis* clones for mistletoe infestation and the effect on latex yield. Natural Rubber Materials 20(1-2), 87-89.

Ogunmefun, O.T., Ekundayo, E.A., Ogunnusi, T.A., Olowoyeye, A.H., Fasola, T.R., Saba, A.B., 2015a. Antimicrobial Activities of *Phragmanthera incana* (Schum.) Balle, a Mistletoe Species Harvested from Two Host Plants against Selected Pathogenic Microbes. Annual Review and Research in Biology 8(3), 1-10. DOI: 10.9734/ARRB/2015/14947.

Ogunmefun, O.T., Fasola, T.R., Saba, A.B., Oridupa, O.A., 2013. The Ethnobotanical, Phytochemical and Mineral Analyses of *Phragmanthera Incana* (Klotzsch), a Species of Mistletoe Growing on Three Plant Hosts in South-Western Nigeria. International Journal of Biomedical Science 9(1), 33–40. PMCID: PMC3644413

Ogunmefun, O.T., Olatunji, B.P., Adarabioyo, M.I., 2015b. Ethnomedicinal survey on the uses of mistletoe in South-western Nigeria. European Journal of Medicinal Plants 8 (4), 224-230. DOI: 10.9734/EJMP/2015/11048.

Okigbo, R.N., Mmeka, E.C., 2006. An Appraisal of Phytomedicine in Africa. KMITL Science and Technology Journal 6(2), 83–94.

Okubamichael, D.Y., Griffiths, M.E., Ward, D., 2001. Host specificity, nutrient and water dynamics of the mistletoe *Viscum rotundifolium* and its potential host species in the Kalahari of South Africa. Journal of Arid Environments 75, 898-902.

Olawale, A.K., 2012. Healing power of mistletoe for diabetes, high blood pressure. Vanguard online community. Posted April 11th, 2012. http:// community.vanguardngr.com/forum/ topics/healing-power-of-mistletoe-fordiabetes-high-blood-pressure

Osadebe, P.O., Abba, C.C., Agbo, M.O., 2012. Anti-motility effects of extracts and fractions of Eastern Nigeria mistletoe (*Loranthus micranthus* Linn). Asian Pacific Journal of Tropical Medicine 5, 412–20.

Osadebe, P.O., Omeje, E.O., 2009. Comparative acute toxicities and immunomodulatory potentials of five Eastern Nigeria mistletoes, Journal of Ethnopharmacology 126 (2), 287–293.

Osadebe, P.O., Omeje, E.O., Nworu, S.C., 2010. Antidiabetic principles of *Loranthus micranthus* Linn. parasitic on *Persea americana.* Asian Pacific Journal of Tropical Medicine 3(8), 619–623.

Osadebe, P.O., Dieke, C.A., Okoye, F.B.C., 2007. A study of the seasonal variation in the antimicrobial constituents of the leaves of *Loranthus micranthus* sourced from *Persea americana*. Planta Medica 73, 205–210.

Padula, De., Bunyapraphatsara, L.S., Lemmens, R.H.M.J., 1999. Plant Resources of South East Asia. PROSEA, Bogor, Indonesia 21, 1.

Padulosi, S., Leaman, D., Quek, P., 2002. Challenges and opportunities in enhancing the conservation and use of medicinal and aromatic plants. Journal of Herbs, Spices and Medicinal Plants 9, 243-267.

Parekh, J.T., 2007. Potency of some medicinal flora: Phytochemical and Pharmacological Evaluation. PhD Thesis, Saurashtra University. http://etheses.saurashtrauniversity.edu/id/eprint/581.

Petrovska, B.B., 2012. Historical review of medicinal plants' usage. Pharmacognosy Reviews 6(11), 1–5. Doi: 10.4103/0973-7847.95849.

Polhill R and Wiens D. 1998. Mistletoes of Africa. The Royal Botanic Garden, Kew, UK. 370.

Polhill RM and Wiens D. 1999. Flora of Tropical East Africa-Loranthaceae. Vol. 179. CRC Press.

Roberts, H., 2001. ACCRA: A Way Forward for Mental Healthcare in Ghana? Lancet. 357(9271), 1859. DOI: 10.1016/S0140-6736(00)05020-0.

Romero-Daza, N., 2002. Traditional Medicine in Africa. Annals of the American Academy of Political and Social Science. 583, 173–176.

Scheer, R., Scheffler, A., Errenst, M., 1992. Two harvesting times, summer and winter: are they essential for preparing pharmaceuticals from mistletoe (*Viscum album*)? Planta Medica 58(7), 594–599.

Shaib, B., Aliyu, A., Bakshi, J.S., 1997. Nigeria National Agriculture Research Strategy Plan 1996-2010. Federal Ministry of Agriculture and Natural Resources, Abuja, Nigeria. Intec Printers Limited, Ibadan

Shankar, K., Liao, L.P., 2004. Traditional systems of medicine. Physical Medicine and Rehabilitation Clinics of North America 15, 725-747.

Sher, H., Alyemeni, M.N., 2011. Pharmaceutically important plants used in traditional system of Arab medicine for the treatment of livestock ailments in the kingdom of Saudi Arabia. African Journal of Biotechnology 10(45), 9153–9159.

Stojanoski, N., 1999. Development of health culture in Veles and its region from the past to the end of the 20th Century. Veles: Society of science and art 1999, 13–34.

Sumner, J., 2000. The natural history of medicinal plants. Timber Press, Portland, USA.

Takem, L.P., Udi, P.M., Poh, C.F., 2014. Anti-secretory, gastro protective and anti-ulcer activities of aqueous extract of *Phragmanthera capitata* S. Balle in rats. International Journal of Pharmaceutical Sciences and Research 5(8), 3560-65. Doi: 10.13040/IJPSR.0975-8232.5 (8).3560-65.

Watson, D.M., 2001. Mistletoe: a keystone resource in forests and woodlands worldwide. Annual Review of Ecology, Evolution, and Systematics 32, 219–249.

WHO, 2000. General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine. WHO/EDM/TRM/2000.1. Geneva

WHO, 2003. Traditional medicine. Revised May, 2003. http://www.who.int/mediacentre /factsheets/2003/fs134/en/

WHO, 2013. Traditional Medicine Strategy, 2014-2023. http://www.who.int/medicines/ publications/traditional/trm_strategy14_23/en

Wikipedia, 2015. Mistletoe. http://en.wikipedia.org/wiki/Mistletoe. Last edited 10 May, 2015.

Yineger, H., Yewhalaw, D., 2007. Traditional medicinal plant knowledge and use by local healers in Sekoru District, Jimma Zone, Southwestern Ethiopia. Journal of Ethnobiology and Ethnomedicine 3, 24. Doi: 10.1186/1746-4269-3-24.

Zainuddin, N.A.S.N., Sul'ain, M.D., 2015. Phytochemical Analysis, Toxicity and Cytotoxicity Evaluation of *Dendropthoe Pentandra* Leaves Extracts. International Journal of Applied Biology and Pharmaceutical 6(1), 108-116.

Zhiwei, F., Xingguo, D., Yufang, Z., Yide, S., 1995. Chemical control of Chinese *Taxillus* on rubber trees. The Planter 71, 459-468.

CHAPTER TWO

QUANTITATIVE PHYTOCHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITIES OF *PHRAGMANTHERA CAPITATA* EXTRACTED WITH DIFFERENT SOLVENTS

This chapter has been accepted for publication in the Pharmacognosy Research journal

CHAPTER TWO

QUANTITATIVE PHYTOCHEMICAL CONSTITUENTS AND ANTIOXIDANT ACTIVITIES OF *PHRAGMANTHERA CAPITATA* EXTRACTED WITH DIFFERENT SOLVENTS

CONTENTS **Pages**

Background of the study 29
Methodology 30
Location and collection of sample 30
Extraction procedure 30
Reagents and chemicals used 31
In-vitro quantitative phytochemical evaluation 31
Phenolic acid determination 31
Flavonoid determination 32
Proanthocyanidin (Condensed Tannin) 32
In- Vitro anti-oxidant analyses 33
DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay 33
ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid) radical scavenging activity 34
Ferric reducing power of the extracts 34
Nitric oxide scavenging activity 35
Total Antioxidant Capacity (TAC) (Phosphomolybdenum assay) 35
Statistical analysis 36
Results 36
Phytochemical evaluation 36
Phenol content 36
Flavonoid content 36
Proanthocyanidin (condensed tannin) content 37
In-vitro antioxidant compositions 37
DPPH free radical scavenging assay 37
ABTS radical scavenging assay 40
Ferric reducing antioxidant power assay (FRAP) 41
Nitric oxide scavenging assay 42
Total antioxidant capacity (TAC) 43
Discussion 46
Conclusion 49
References 50

Background of the study

Phytochemicals are naturally occurring and biologically active plant compounds that have potential disease inhibiting capabilities. These phytochemicals are believed to combat diseases due to their antioxidant effect (Afolabi *et al.,* 2007). As a way of responding to environmental stress, many plants produce antioxidant compounds such as the polyphenolics. These antioxidants are phytochemicals which absorb and neutralise free radicals, quenching singlet and triplet oxygen or inducing expression of peroxides and other toxic metabolites. Hence, antioxidants have protective ability from damages caused by free radical-induced stress as a result of oxidation (Iloki-Assanga *et al.,* 2015).

Plants are the major source of active chemical constituents against diseases. Most of the world's population still relies on alternative medicines for the treatment of a large number of serious diseases (Hussain *et al.,* 2014). Medicinal plants represent a lot of untapped reservoirs of drugs, and the structural diversity of their components makes a valuable source of novel compounds. Hence, there is a growing interest by natural product scientists in the utilisation of phytochemicals and are intensifying efforts towards the evaluation of these valuable medicinal plants.

One of the different families of medicinal plants that have long been exploited in various herbal folklores is the Loranthaceae. It is one of the five families in the group of plants called "mistletoe". Species of the Loranthaceae have been extensively used in alternative medicine for the treatment and management of several ailments/diseases (Ameer *et al.,* 2015). Chemical and pharmacological studies of this family have identified several compounds such as; flavonoids, alkaloids, lectins, polypeptides, argenine, glycosides, gallic acid and Loranthin (newly identified flavocoumarin) (Ameer *et al.,* 2015).

Phragmanthera capitata (Sprengel) Balle which is a mistletoe in the Loranthaceae family has been used in herbal medicine in Africa. It is the major Loranthaceae species used by the

traditional healers in the Logbessou region in Cameroon. It has been used for the management and treatment of several ailments as described in the previous chapter (Dibong *et al.,* 2009).

In Africa, much attention in mistletoe research is in crop protection and little attention has been given to its potential role in ethnopharmacology. However, there is the need for scientific screening of the African mistletoe for its potential in therapeutics. Different solvents have been used in extracting and isolating bio-constituents in plants and the group of compounds extracted or isolated is highly solvent dependent (Iloki-Assanga *et al.,* 2015). Prior to this study, no report on the quantitative phytochemical evaluation of *P. capitata* parasitic on rubber trees has been investigated. Hence, the aim of this study was to quantitatively examine the phytochemical constituents and their corresponding antioxidant activities of *Phragmanthera capitata* extracted with different solvents.

Methodology

Location and collection of the sample

The leaves of *Phragmanthera capitata* were collected from mature rubber plantations in the Rubber Research Institute of Nigeria. The site is located on latitude 6° 00'- 6°15' N; longitude 5°30'- 5°45' E and at about 27 m above sea level. The sample was authenticated by Dr Emmanuel I. Aigbokhan of the Plant Biology and Biotechnology Department, University of Benin, Nigeria. A voucher specimen (UBH10284) was deposited at the UNIBEN herbarium for future reference.

Extraction procedure

The leaves were removed from the twigs, gently rinsed to remove dust and dirt, air-dried at room temperature (mean morning and night temperature of 24°C and mean noon temperature of 27°C) in a well-aerated atmosphere and prevented from direct sunlight to avoid denaturation of vital phytoconstituents. Dried leaves were pulverised. 300 g each of the ground sample was soaked in separate conical flasks with 1.2 L of the organic solvents which include acetone,

methanol, ethanol and water and shaken in an orbital shaker (Orbital Incubator Shaker, Gallenkamp) for 24 hours. The crude extracts were filtered using a Buchner funnel and Whatman No. 1 filter paper. The acetone, methanol and ethanol extracts were further concentrated to dryness to remove the solvents under reduced pressure using a rotary evaporator (Strike 202 Steroglass, Italy) while the aqueous filtrate obtained was concentrated using a freeze dryer (Vir Tis benchtop K, Vir Tis Co., Gardiner, NY).

Reagents and chemicals used

Solvents and chemicals used include; Folin-Ciocalteu, anhydrous sodium carbonate (Na_2CO_3), Aluminium trichloride ($AlCl_3$), Sodium nitrite ($NaNO_2$), Sodium chloride, 2,2diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), vanillin, aluminum chloride ($AlCl_3$), potassium acetate (CH_3CO_2K), ferric chloride ($FeCl_2$), BHT, ascorbic acid, rutin, n-butanol, diethyl ether, ammonia solution, acetone, methanol, ethanol, hydrochloric acid, sodium hydroxide, phosphate buffer, potassium ferricyanide [$K_3Fe(CN)_6$], ammonium molybdate, sodium phosphate, trichloroacetic acid (TCA), glacial acetic acid (CH_3COOH), sodium nitroprusside ($Na_2[Fe(CN)_5NO]_2H_2O$). These chemicals were purchased from Merck and Sigma-Aldrich, Gauteng, South Africa. All the chemicals used in this study were of analytical grade.

***In-vitro* quantitative phytochemical evaluation**

Phenolic acid determination

Phenol determination was estimated spectrophotometrically using the Folin-Ciocalteu's method as described by Samatha et al. (2012) with some modifications. 0.5 mL of the plant extracts (1 mg/mL), standard gallic acid (0.02mg/ mL to 0.1 mg/mL) and the solvent of dissolution (control) was pipetted in different test tubes. To this, 2.5 mL of 10% (v/v) Folin-Ciocalteu's reagent was added and the mixture was vortexed. The reaction was allowed to stand at room temperature for about 5 mins. After 5 mins, 2 mL of 7.5% (w/v) anhydrous sodium carbonate

was added to the solution, vortexed and incubated at 40°C for 30 mins. The control solution was used as a blank. After incubation, the absorbance was measured at 765 nm using a UV- 3000 PC spectrophotometer. The experiment was done in triplicate. The phenol content was extrapolated from the gallic acid standard/calibration graph equation; $y = 8.07x + 0.1$, R^2 0.9981, and was expressed as mg gallic acid equivalent (GAE)/g from the equation CV/m; where "C" is the concentration as derived from the calibration curve equation in mg/mL, "V" is the volume of the extract used in the assay in mL and "m" is the mass of the extract used in the assay in "g".

Flavonoid determination

The Aluminium chloride colorimetric assay was used to determine the flavonoid content according to the method described by Kamtekar et al. (2014) with little modification. This method is based on the quantification of the yellow-orange colour produced by the interaction of flavonoid with $AlCl_3$. Briefly, 0.5 mL (1 mg/mL) aliquots of the solvent fractions, different concentrations (0.2 to 1 mg/mL) of quercetin standard and the solvent of dissolution were placed in different test tubes. 2 mL of distilled water was added to the test tubes after which, 0.15 mL of 5% sodium nitrite was also added to the mixture. The mixture was allowed to stand for 6 mins. After 6 mins, 0.15 mL of 10% $AlCl_3$ was added to the solution, allowed to stand for 5 mins followed by the addition of 1mL of 1 M sodium hydroxide. The solution was made up to 5 mL with distilled water and measured using a spectrometer at 420 nm. The control solution was used as a blank. The experiment was done in triplicate. The flavonoid content was calculated using the calibration curve equation, $y = 1.064x$, $R^2 = 0.9976$ and expressed as mg of quercetin equivalent (QE)/g using the formula CV/m in the same manner as described in the phenolics assay above.

Proanthocyanidin (Condensed Tannin) determination

Total proanthocyanidin was determined based on the procedure of Oyedemi *et al.,* (2010). To 0.5 mL of 1 mg/mL of the extract solution, different concentrations (0.02 mg/mL to 1 mg/mL)

of the standard catechin and the solvent of dissolution (control), was added 3 mL of vanillin-methanol (4% w/v) and 1.5 mL of hydrochloric acid and vortexed. The mixture was allowed to stand for 15 min at room temperature. The control was used as a blank. The absorbance was measured at 500 nm using a UV- 3000 PC spectrophotometer. The experiment was done in triplicate. Proanthocyanidin content was evaluated using the calibration curve equation: y = 0.9038 x + 0.0449, R^2 0.9951 and was expressed as mg catechin equivalent (CE)/g using the formula, CV/m as earlier mentioned in phenol determination.

In- Vitro **anti-oxidant analyses**

The antioxidant activities of *P. capitata* were determined using DPPH, ABTS, reducing power, nitric oxide and phosphomolybdate (Total antioxidant capacity) assays.

DPPH (2, 2-diphenyl-1-picrylhydrazyl) radical scavenging activity assay

The method of Kibiti and Afolayan (2015) was used for the determination of DPPH free radical scavenging activity. Briefly, a solution of 0.135 mM DPPH radical in methanol was prepared. A 1 mL of this solution was mixed with 1 mL (5 µg/mL to 80 µg/mL) each of the plant fractions/standard drugs (BHT, vitamin C). The reaction mixture was then vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at 517 nm. The actual decrease in absorbance was measured against that of the control. The scavenging ability of the plant extract was then calculated using the equation:

DPPH Scavenging activity (%) = [(Abs control – Abs sample)/ (Abs control)] ×100;

Where; Abs control is the absorbance of DPPH + methanol; Abs sample is the absorbance of DPPH radical + sample/or standard.

ABTS (2, 2'-azino-bis (3-ethylbenzothiazoline)-6-sulfonic acid) radical scavenging activity

The method described by Wintola and Afolayan (2011) was adopted for the determination of ABTS activity of the plant extract. The working solution was prepared by mixing two stock solutions of 7 mM ABTS and 2.45 mM potassium persulfate in equal amounts (1:1) and allowed to react for 12 to 18 h at room temperature in the dark to form a green-coloured ABTS radical ($ABTS^+$). The resulting solution was further diluted by mixing 1 mL of the $ABTS^+$ solution with about 50 mL of methanol to obtain an absorbance of 0.700 ± 0.006 at 734 nm (the $ABTS^+$ + methanol solution is adjusted by either adding the $ABTS^+$ if the absorbance were lower or adding methanol if the absorbance were higher than the stipulated unit). After obtaining the desired absorbance, 1 mL of the resultant solution is then mixed with 1 mL of the plant extract/or standard drugs of different concentrations (5 µg/mL to 80 µg/mL). After 7 min, the reduction in absorbance was measured at 734 nm using a spectrophotometer. The percentage inhibition of $ABTS^+$ by the extract was calculated from the following equation:

$$\% \text{ inhibition} = [(\text{Abs control} - \text{Abs sample})] / (\text{Abs control})] \times 100.$$

Ferric reducing power of the extracts

The reducing power was evaluated according to the method described by Aiyegoro and Okoh (2010). The mixture containing 2.5 mL of 0.2 M phosphate buffer (pH 6.6) and 2.5 mL 1% potassium hexacyanoferrate [$K_3Fe(CN)_6$] (w/v) was added to 1.0 mL of the extracts and standards (25 µg/mL to 400 µg/mL). The resulting mixture was incubated for 20 min at 50°C, followed by the addition of 2.5 mL of 10% Trichloroacetic acid (TCA) (w/v) to terminate the reaction. The mixture was centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was removed and mixed with 2.5 ml of distilled water and 0.5 ml of 0.1% freshly prepared ferric chloride, $FeCl_3$, (w/v) was added. The mixture was allowed to stand for 10 min and the absorbance was measured at 700 nm. A mixture with distilled water instead of the sample served as the control. Increased absorbance of the reaction mixture indicated higher reducing

power of the plant fractions. The percentage inhibition of the sample and the standard drug was calculated using the formula:

% inhibition = [(absorbance of sample – absorbance of control)/ (absorbance of sample)] × 100

Nitric oxide scavenging activity

A volume of 2 mL of 10 mM sodium nitroprusside prepared in 0.5 mM phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant fractions, Vit C and BHT individually at 25 µg/mL to 400 µg/mL. The mixture was incubated at 25°C for 150 min. 0.5 mL of the incubated solution was mixed with 0.5 mL of Griess reagent [1 mL sulfanilic acid reagent (0.33% prepared in 20% glacial acetic acid) with 1 mL of naphthalene diamine dichloride (0.1% w/v) at room temperature for 5 min]. The mixture was incubated at room temperature for 30 min, followed by the measurement of the absorbance at 540 nm. A solution containing water instead of the extract/standard was used as a control (Wintola and Afolayan, 2011). The amount of nitric oxide radicals inhibited by the extract was calculated using the following equation:

NO radical scavenging activity (%) = [(Abs control – Abs sample)]/(Abs control)] × 100, where Abs control is the absorbance of NO radicals + methanol and Abs sample is the absorbance of NO radical + extract or standard.

Total Antioxidant Capacity (TAC) (Phosphomolybdenum assay)

The total antioxidant capacity of the plant fractions was determined by phosphomolybdenum method according to the protocol described by Olugbami *et al.,* (2015). 0.3 mL of the different plant fractions and standard drugs (25 µg/mL to 400 µg/mL) were taken in test tubes and dissolved in 3 mL of reagent solution (0.6 M sulphuric acid, 4 mM ammonium molybdate and 28 mM sodium phosphate). Then the test tubes were covered and incubated in a water bath at 95°C for 95 min. The mixture was allowed to cool to room temperature and the absorbance was measured at 695 nm. A mixture containing distilled water instead of the samples served as

control. Ascorbic acid and gallic acid were used as standard drugs. A higher absorbance indicates higher total antioxidant potential. The percentage inhibition was calculated thus:

[(absorbance of sample – absorbance of control)/ (absorbance of sample)] $\times$ 100

All the experiments were carried out in triplicates.

Statistical analysis

All data were expressed as mean ± standard deviation (SD) of three replications. Statistical analysis was performed by ANOVA. Where the data showed significance ($p < 0.05$), a mean separation was done using the Fischer's LSD with the aid of GENSTAT 8 statistical package.

Results

The resultant percentage yield after extraction with the various solvents were: 21.5% (64.5 g) for the methanol extract, 10.8% (32.5 g) for the aqueous extract, 6.87% (20.6 g) for the acetone extract and 4.3% (12.9 g) for the ethanol extract.

Phytochemical evaluation

Phenol content

The results of the phenolic content were expressed in mg of gallic acid equivalent per gram (mg GAE/g). The acetone extract had the highest phenol content of 218.62 ± 3.38 mg GAE/g while the aqueous extract had the lowest amount (175.53 ± 0.32 mg GAE/g). The methanolic and ethanolic extracts were 203.78 ±1.95 mg GAE/g and 214.39 ± 4.09 mg GAE/g respectively. There was no significant difference between the acetone and ethanol extract ($p > 0.05$) (Figure 1).

Flavonoid content

The flavonoid content of the crude extracts was determined with reference to the standard quercetin and expressed as its equivalent (mg QE/g). The result of the evaluation also showed the acetone fraction to be significantly higher (679.82 ± 6.26 mg QE/g) than the other solvent

fractions ($p < 0.05$). Ethanol, methanolic and aqueous fractions had values of 548.87 ± 4.10 mg QE/g, 407.90 ± 0.94 mg QE/g and 197.06 ± 3.80 mg QE/mg respectively. All the solvent fractions were significantly different from each other ($p < 0.05$) (Figure 1).

Proanthocyanidin (condensed tannin) content

The result of the proanthocyanidin was expressed as mg catechin equivalent per gram (mg CE/g). Acetone extract had a significantly higher amount (645.68 ± 6.35 mg CE/g) than the other solvent extracts ($p < 0.05$). The methanol, ethanol and aqueous extracts were 194.00 ± 6.20 mg CE/g, 402.15 ± 7.55 mg CE/g and 128.83 ± 1.69 mg CE/g respectively. All the solvent fractions were significantly different from one another ($p < 0.05$) (Figure 1).

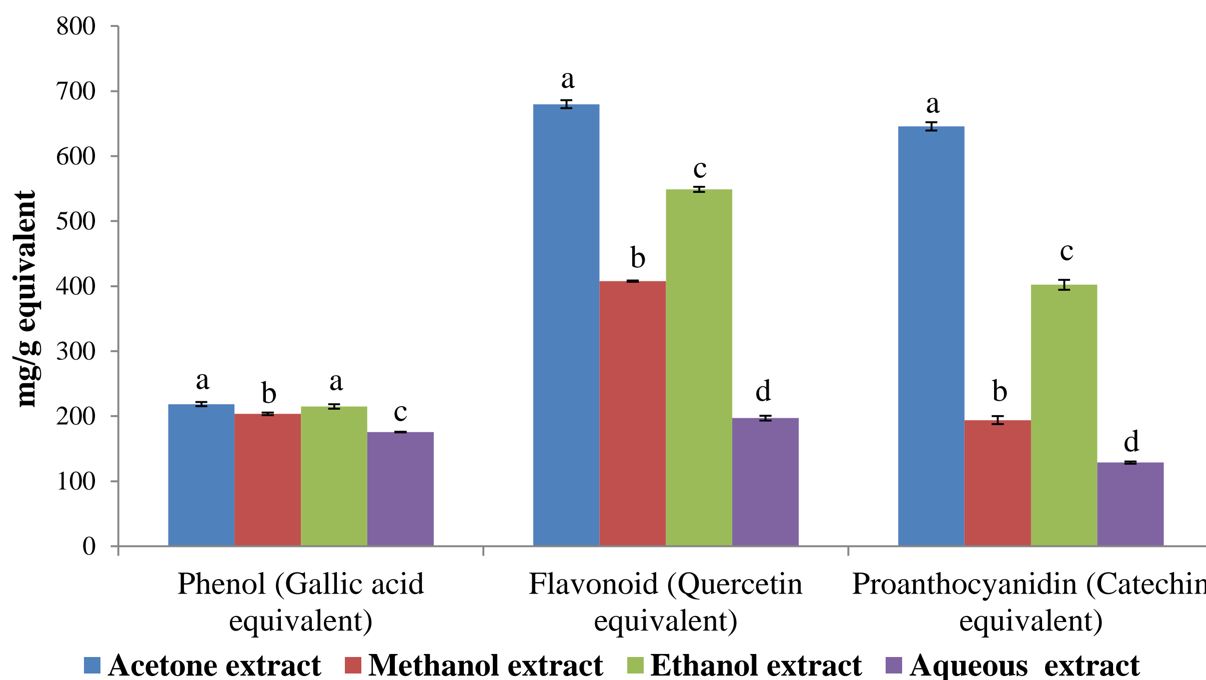


Figure 1: Phytochemical content of the different solvent fractions of *P. capitata* in standard equivalents. Values are mean ± SD of three replications. Set of bars with different letters are significantly different ($p < 0.05$).

In-vitro antioxidant compositions

DPPH free radical scavenging assay

The DPPH radical scavenging activity of the different solvent extracts in comparison to known antioxidants (Vit C and BHT) and their respective concentrations that scavenged 50% (IC_{50}) of

the radicals are presented in Figure 2 and Table 1 respectively. The scavenging activity of all the solvent fractions and standard drug increased with increase in concentration. At 40 µg/mL, almost all the solvent fractions had over 50% inhibitory activity (64.54 ± 2.09% to 82.73 ± 0.30%) on the DPPH radical except for the aqueous fraction with an activity of 27.59 ± 1.21% (Figure 2). The IC_{50} (the ability of the fractions to scavenge or inhibit 50% of the radicals) ranged from 24.5 µg/mL in the acetone fraction to 67.2 µg/mL in the aqueous extract. The decreasing scavenging activity of the extracts and the standard drugs based on the IC_{50} was in the order; Vit C > Acetone > Ethanol > Methanol > BHT > Aqueous (Table 1).

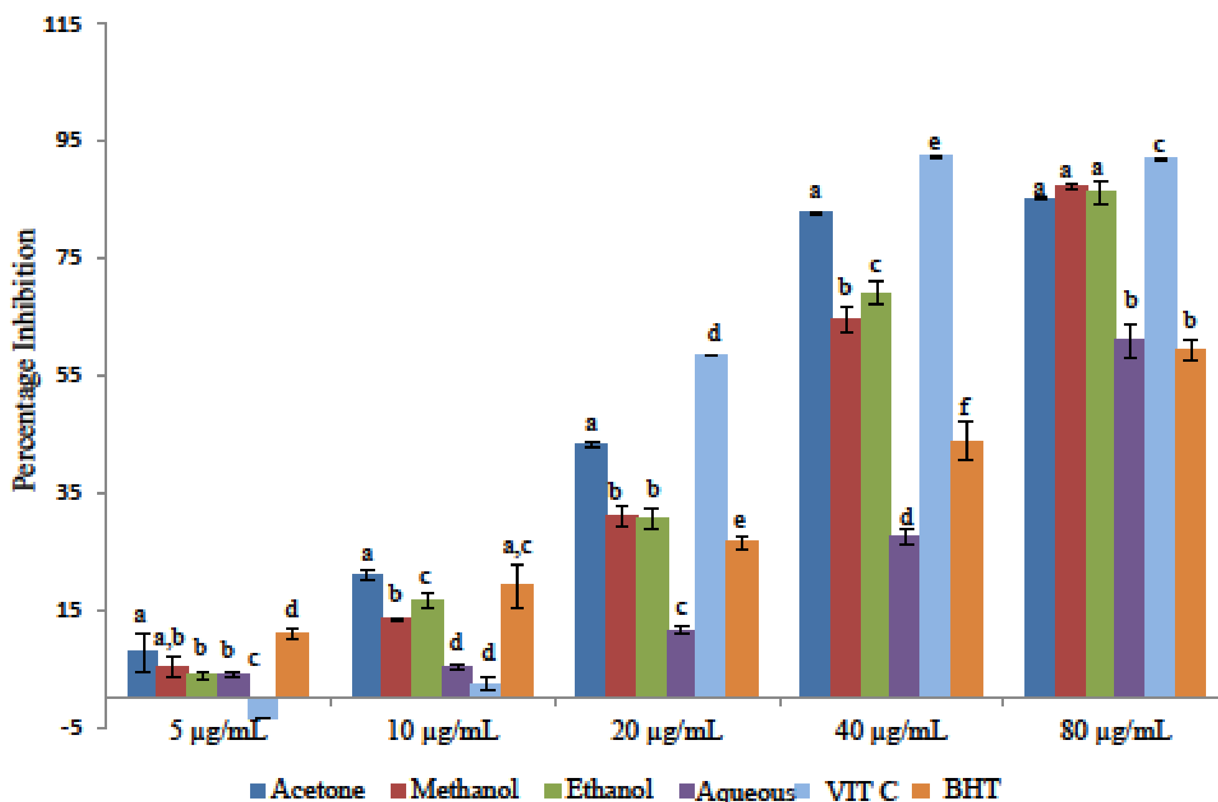


Figure 2: DPPH radical scavenging activity of the different solvent fractions of *P. capitata*. Values are mean ± SD of three replications. Set of bars with different letters are significantly different ($p < 0.05$).

Table 1: IC_{50} values of the solvent fractions of *P. capitata* leaves and standard drugs

	DPPH		**ABTS**		**FRAP**		**Nitric Oxide**		**Phosphomolybdenum**	
	IC_{50} µg/mL	**R^2**	**IC_{50} µg/mL**	**R^2**	**IC_{50} µg/mL**	**R^2**	**IC_{50} µg/mL**	**R^2**	**IC_{50} µg/mL**	**R^2**
Acetone	24.5	0.9928	1.9*	0.8972	318	0.9677	23*	0.9796	85	0.998
Methanol	27.4	0.9585	5.7	0.9189	302	0.9748	1*	0.9773	124	0.974
Ethanol	26.4	0.9594	1.9*	0.7373	308	0.9423	41.9	0.981	87	0.979
Aqueous	67.2	0.9948	6.8	0.9551	374	0.9887	34	0.9727	144	0.994
Vit C	18.2	0.928	22	0.959	89	0.9906	10*	0.9999	18*	0.959
BHT	56	0.9661	4.6*	0.9966	NA	0.8942	27	0.9924	-	-
Gallic acid	**-**	-	-	-	-	-	-	-	2*	0.938

IC_{50} is the concentration (µg/mL) required to scavenge/inhibit 50% of the radical, **R^2**: coefficient of determination; values obtained from regression lines with 95% confidence level, **NA**: not active, asterisk (*****): values are lower than the least concentration evaluated and dash (**-**)**:** Values not determined.

ABTS radical scavenging assay

There was also a concentration-dependent response in this assay. All the solvent extracts showed great ABTS radical scavenging activity at very low concentrations. At the lowest concentration (5 µg/mL), the acetone and ethanol extracts had inhibitory activity on the $ABTS^+$ of over 50% (63.18 ± 8.19% and 58.61 ± 1.80% respectively). At 10 µg/mL, all the solvent fractions and BHT had exceeded 60% scavenging activity. At 40 µg/mL, there was complete inhibition (100%) of all the solvent extracts and standard drugs (Figure 3). The IC_{50} values ranged from < 5 µg/mL in the acetone and ethanol fractions (1.9 µg/mL) and BHT (4.6 µg/mL), to 22 µg/mL in Vit C (Table 1). The order of decreasing scavenging activity of the extracts and the standard drugs based on the IC_{50} is in the order; Acetone ≥ Ethanol > BHT > Methanol > Aqueous > Vit C.

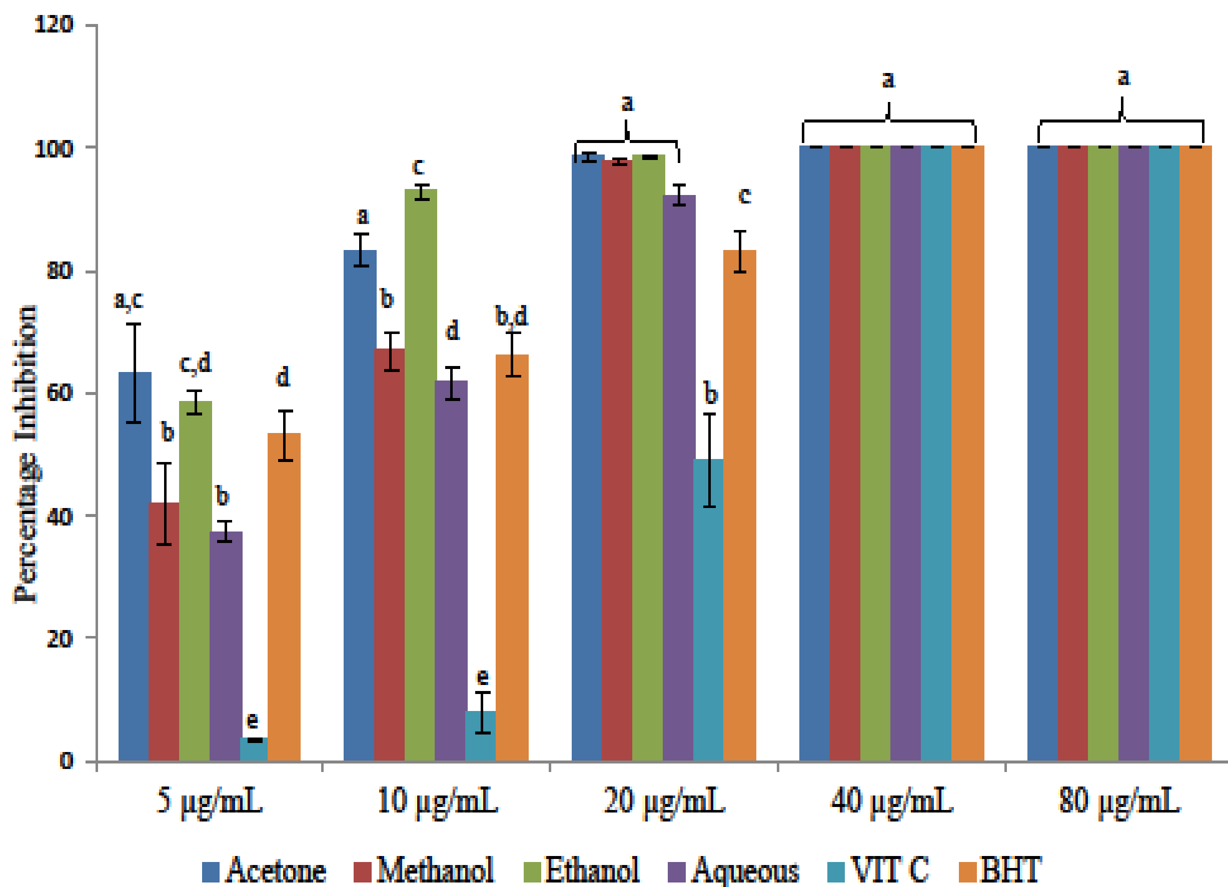


Figure 3: ABTS radical scavenging activity of the different solvent fractions of *P. capitata*. Values are mean ± SD of three replications. Set of bars with different letters are significantly different ($p < 0.05$).

Ferric reducing antioxidant power assay (FRAP)

The reducing power of the solvent extracts on ferric to ferrous gradually increased with increase in concentration. It was at the highest test concentration (400 µg/mL) that all the extracts had 50% and above reducing power why vit C had 50% reducing power at 100 µg/mL and BHT was not active in this assay (Figure 4). The IC_{50} obtained for the solvent fractions and standard ranged from 89 µg/mL in Vit C to >> 400 µg/mL in BHT (Table 1). The increase in the reducing power activity of the solvent extracts and standard drugs as obtained from the IC_{50} is in the order; Vit C > Methanol > Ethanol > Acetone > Aqueous > BHT.

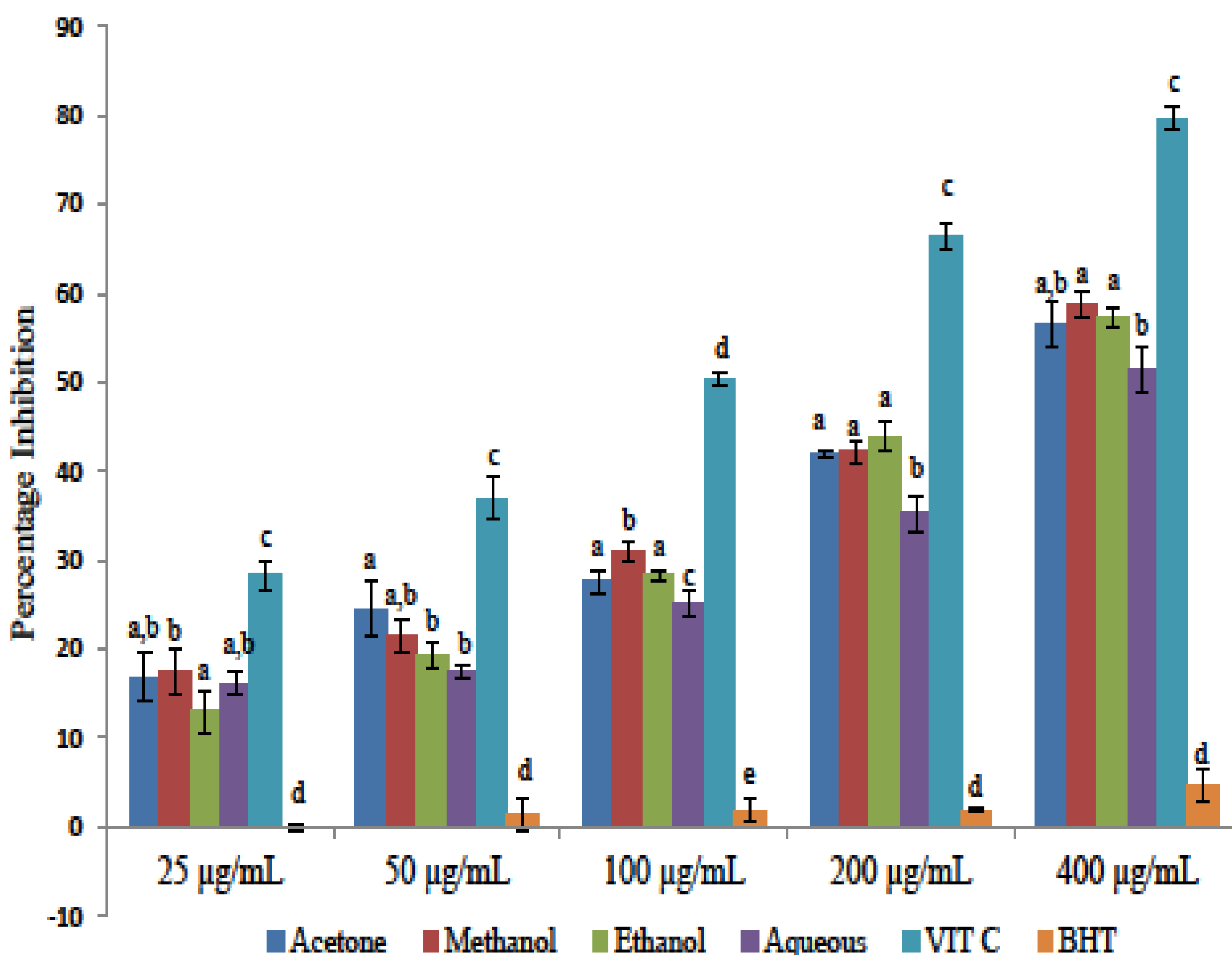


Figure 4: Ferric reducing activity of the different solvent fractions of *P. capitata*. Values are mean ± SD of three replications. Set of bars with different letters are significantly different ($p < 0.05$).

Nitric oxide scavenging assay

The result of the nitric oxide scavenging activity of the different solvents showed that the acetone fraction had a scavenging activity which ranged from 46.41 ± 4.58% at the lowest concentration (25 µg/mL) to 73.15 ± 2.79% at 200 µg/mL. The methanol extract scavenging activity ranged from 64.99 ± 1.72% at the lowest concentration (25 µg/mL) to 75.26 ± 0.56% at 200 µg/mL. The ethanol scavenging activity ranged from 49.07 ± 0.43% at the lowest (25 µg/mL) concentration to 68.73 ± 3.95% at the highest concentration (400 µg/mL). Aqueous extract had an activity which ranged from 49.07 ± 2.56% at 25 µg/mL to 68.02 ± 4.32% at 400 µg/mL. The standard drugs, however, had their highest activities at 50 µg/mL (67.91 ± 2.97%) for Vit C and 100 µg/mL (60.292 ± 5.27%) for BHT (Figure 5). The scavenging activity as recorded from the IC_{50} values is in the order; Methanol > Vit C > Acetone > BHT > Aqueous > Ethanol (Table 1).

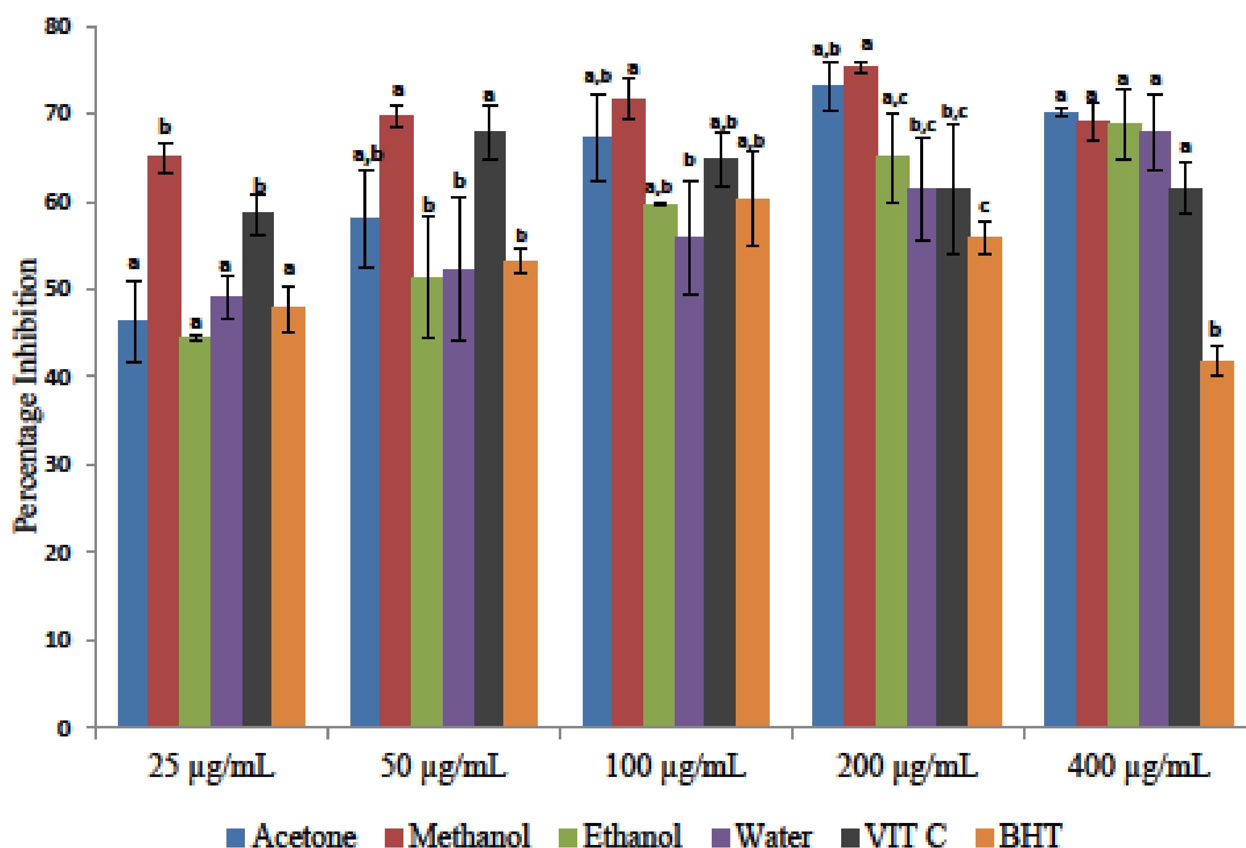


Figure 5: Nitric oxide scavenging activity of the different solvent fractions of *P. capitata*. Values are mean ± SD of three replications. Set of bars with different letters are significantly different ($p < 0.05$).

Total antioxidant capacity (TAC)

The total antioxidant capacity of *P. capitata* was measured by phosphomolybdenum method. The antioxidant capacity of all the solvent fractions increased with an increase in concentration. The acetone fraction ranged from 26.48 ± 2.12% at 25 µg/mL to 78.64 ± 0.77% at 400 µg/mL; methenaol extract fraction had a TAC from 19.66 ± 5.82% at 0.025 mg/mL to 75.49 ± 0.79% at 0.4 mg/mL; ethanol extract TAC was from 30.07 ± 2.47% at 25 µg/mL to 78.46 ± 0.12% at 400 µg/mL and the aqueous extract TAC ranged from 12.27 ± 5.05% at 25 µg/mL to 70.30 ± 0.60% at 400 µg/mL (Figure 6). The IC_{50} of the solvent fractions and the standard drugs in the order of decreasing TAC are; Gallic acid > Vit C > Acetone > Ethanol > Methanol > Aqueous (Table 1).

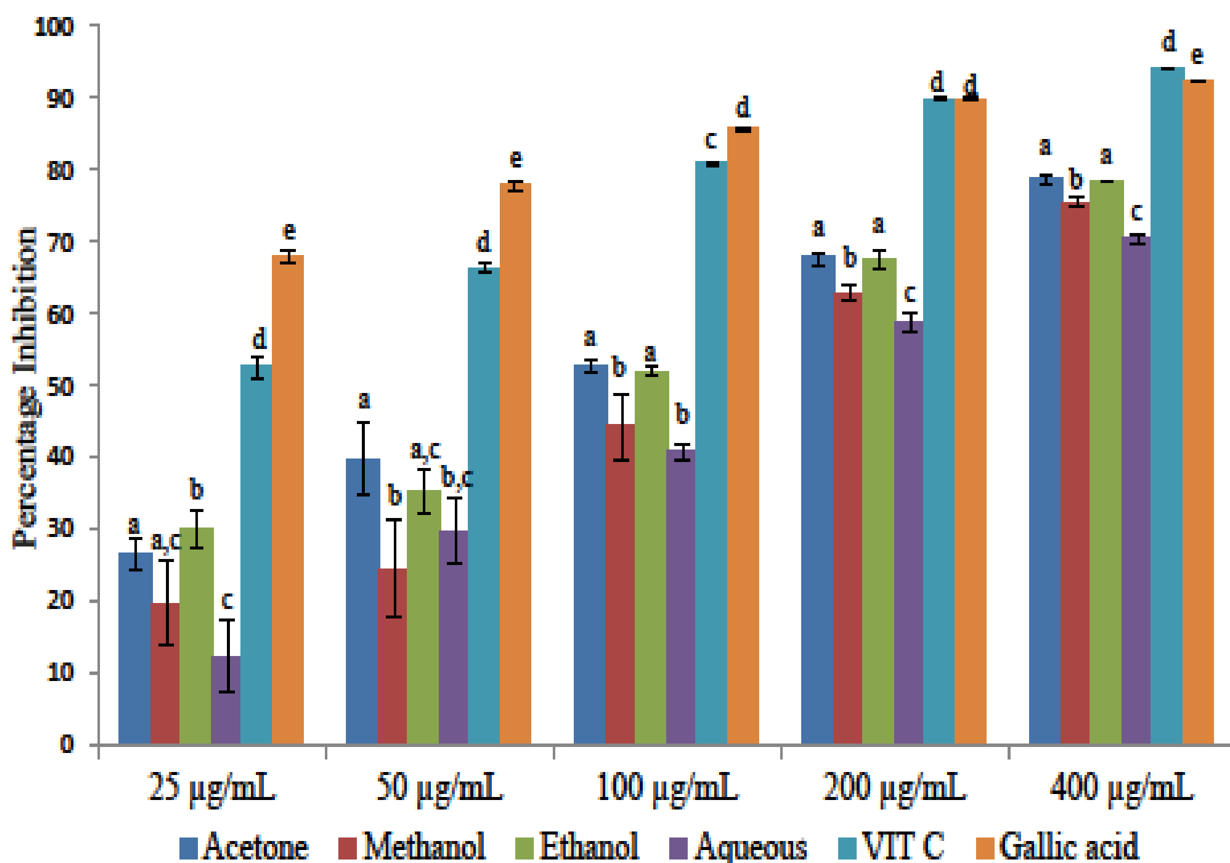


Figure 6: Total Antioxidant capacity (TAC) of the different solvent fractions of *P. capitata*. Values are mean ± SD of three replications. Set of bars with different letters are significantly different ($p < 0.05$).

The relationship between the phytochemical contents and the IC_{50} of the antioxidant activities is as shown in Figure 7(A-E). The phenol contents had a high linear correlation of $R^2 = 0.9228$ and 0.8658 based on DPPH and TAC assays respectively. Flavonoid also had a high linear correlation with TAC ($R^2 = 0.9228$) but with DPPH, it showed a high power correlation of $R^2 = 0.9098$. Proanthocyanidin content also had a high exponential correlation of $R^2 = 0.9405$ with TAC assay.

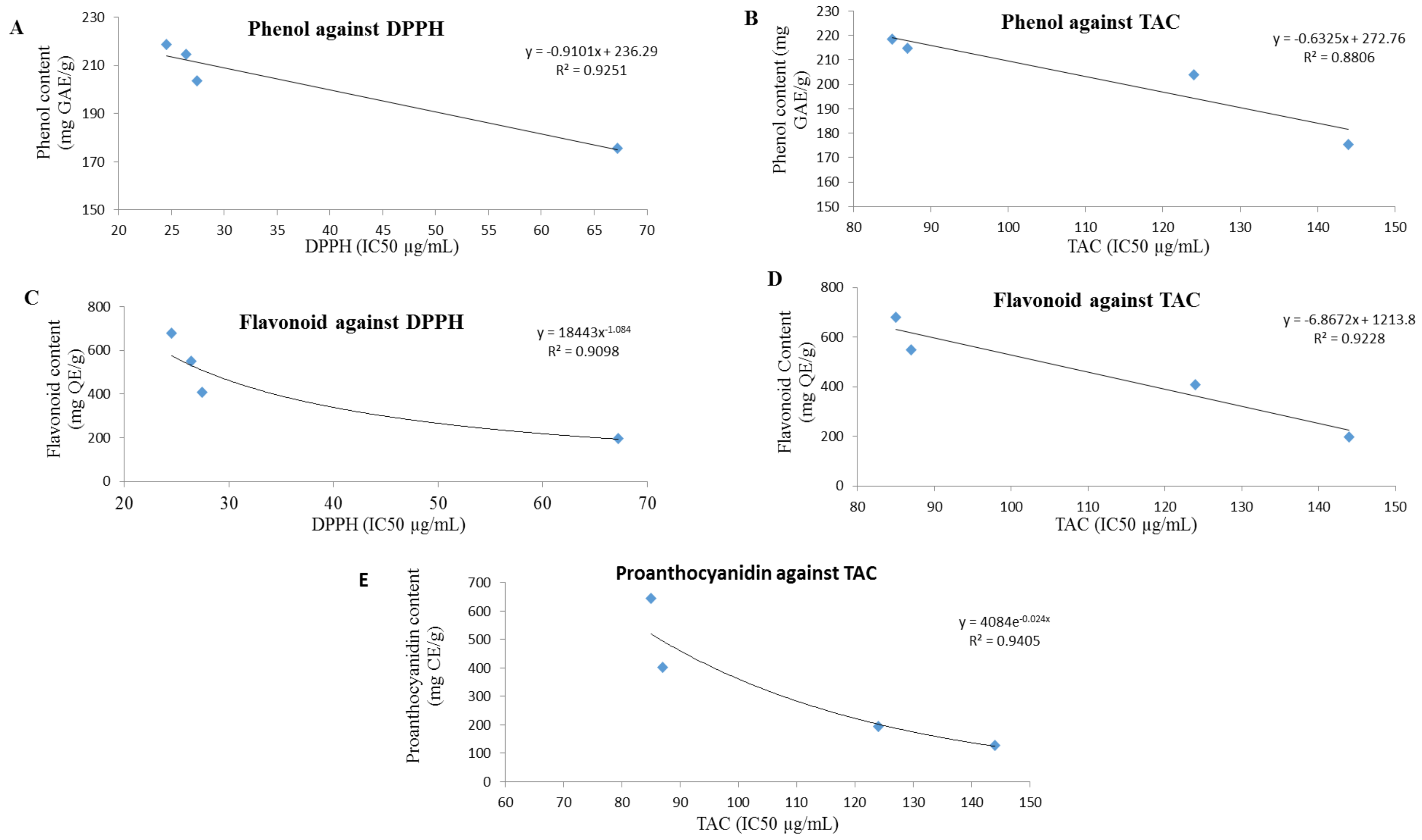


Figure 7: Correlation of the polyphenolic compounds against the IC_{50} of the antioxidant.

Discussion

Phenols have been recorded to exhibit great antioxidant activities and this has been attributed to the presence of the hydrogen group which acts as a hydrogen donor. Consequently, as a result of their redox property, they function as a reducing agent and act as radical scavengers (Wintola and Afolayan, 2011). The values of the phenol content in this study were evaluated based on the amount of Gallic Acid Equivalent (GAE) per gram in the sample. The values of the phenolic content obtained confirmed that the solvents had different abilities in extracting the phenols from the sample. The acetone fraction, though not significantly different from the ethanol fraction ($p < 0.05$), extracted higher levels of the phenols in *P. capitata* at the same amount of evaluation. It should be noted that phenolic compounds are often associated with diverse biomolecules (polysaccharides, proteins, terpenes, chlorophyll, inorganic compounds) and therefore, a solvent suitable for the extraction of a particular class/group of compounds must be used based on the structural features and related level of solubility of a target molecule (Ghasemzadadeh *et al.,* 2011).

Like phenols, flavonoids are secondary metabolites with polyphenolic structure. They are also water soluble and have been reported to exhibit great antioxidant activities than vitamins C, E and carotenoids (Ghasemzadadeh and Ghasemzadadeh, 2011). The flavonoid was estimated using Quercetin as the reference. It was observed that the effect of the different solvents on the flavonoid content was similar to that of the phenol. The acetone fraction was significantly higher than the rest of the solvent extracts compared ($p < 0.05$) (Figure 1); hence, it forms the best solvent for the extraction of flavonoid from this *P. capitata*. However, water (aqueous) which is the cheapest solvent and readily available, had the lowest quantity of flavonoid in relation to quercetin equivalent.

Condensed tannin also called proanthocyanidin, are a group of polyphenolic bioflavonoids (Wintola and Afolayan, 2011). They are ubiquitous and present as the second most abundant

natural phenolic after lignin. Proanthocyanidins are of great interest in nutrition and medicine because of their potent antioxidant capacity and possible protective effects on human health (Liwei *et al.,* 2004)**.** The result of this study showed that *P. capitata* is very rich in proanthocyanidin content in reference to catechin. Once again, the acetone extracts had a higher significant proanthocyanidin content than the other solvent extracts ($p < 0.05$) and hence a solvent of choice for the extraction of proanthocyanidins.

Different *in-vitro* methods were also employed to examine the effects of the acetone, methanol, ethanol and aqueous on the antioxidant activities of the sample. DPPH radical scavenging model is widely employed to evaluate antioxidant activities of natural compounds and plant extracts. The degree of discolouration from purple to light yellow indicates the scavenging potential of the extract which is a result of the hydrogen donating ability. This assay is very sensitive and can detect active ingredients at very low concentrations (Quy Diem *et al.,* 2014). The result of this experimentation revealed that all the solvent fractions had high DPPH scavenging activities even at very low concentrations (5 µg/mL to 80 µg/mL). IC_{50} which is the concentration of the extract required to scavenge 50% of the radical is an indicator which reflects the activity of an extract. The lower the IC_{50} value, the higher the scavenging activity of the extract. In this study, all the solvent fractions exhibited strong DPPH radical scavenging abilities. The IC_{50} values for the acetone (24.5 µg/mL), methanol (27.4 µg/mL) and ethanol fractions (26.4 µg/mL), were very close to the standard Vit C (18.2 µg/mL) and lower than BHT (56 µg/mL). This result is in conformity with the results of the phytochemical analysis with a positive correlation of the polyphenolics (phenol, flavonoid and proanthocyanidin) with DPPH and TAC. Solvent fractions with higher polyphenolics contents had lower IC_{50} values hence exhibited higher scavenging activity (Figure 7).

The ability of the solvent fractions to scavenge the ABTS radical was also evaluated. ABTS reacts with persulphate to form a bluish-green colour. When the ABTS radical so formed reacts

with an H-donor, it decolourises. All the solvent fractions showed great scavenging activity on the ABTS radical at very low concentrations. The IC_{50} values of all the solvent fractions, which ranged from < 5 µg/mL to 6.8 µg/mL, were far lesser than the value for Vit C (22 µg/mL) (Table 1). In this study, it was observed that the acetone and the ethanol extract had an IC_{50} lesser than the least concentration which indicates their greater effectiveness in scavenging ABTS radicals than the methanol and aqueous fractions.

The ferric reducing power of the solvent fractions was determined by the direct electron donation in the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}). The presence of the different solvent fractions caused a reduction of the Fe^{3+} complex to Fe^{2+} and was monitored at 700 nm. The antioxidant activity on FRAP was much lower than DPPH and ABTS as much higher concentrations were required to achieve the desired IC_{50} values for all the solvent extracts and standards used. The trend on the effects on FRAP was not directly proportional to the results of the polyphenolic contents of the plant as observed in DPPH and ABTS. The methanol fraction in this assay, had the strongest FRAP (IC_{50} = 302 µg/mL) followed by the ethanol (IC_{50} = 308 µg/mL), acetone (IC_{50} = 318 µg/mL) and aqueous (IC_{50} = 374 µg/mL) in that order (Table 1).

During the nitric oxide assay, sodium nitroprusside decomposes in aqueous solution at physiological pH producing NO, making it an ideal assay to mimic the human body system in scavenging the free radical (Pacher *et al.,* 2007). During this assay, nitrite is formed when NO generated from sodium nitroprusside reacts with oxygen. So, it can be deduced that the plant fractions inhibit nitrite formation by directly competing with oxygen and other nitrogen oxides such as NO (Boora *et al.,* 2014). The IC_{50} values like in the FRAP, does not have a direct proportion to the polyphenolic compounds. The methanol fraction gave the best NO scavenging ability with an IC_{50} value lesser than the least concentration evaluated (< 25 µg/mL) (Table 1).

The total antioxidant capacity (TAC) of the solvent fractions was determined based on the reduction of molybdenum (VI) to molybdenum (V) and the subsequent formation of a green

phosphate/molybdenum (V) complex at acidic pH. All the solvent fractions had a steady increase in TAC as the concentrations increased. At 100 µg/mL, the acetone and ethanol fractions had already reduced > 50% molybdenum (VI) to molybdenum (V). Based on the IC_{50}, the standard drugs (Vit C and gallic acid) were more potent than the solvent fractions with values far lesser than the lowest concentration used for this assay. However, the effect of the different solvent showed acetone to be more potent with an IC_{50} value of 84 µg/mL followed by the ethanol fraction with a value of 87 µg/mL. Methanol and aqueous fractions showed lesser TAC values with IC50 values of 124 µg/mL and 144 µg/mL respectively. There was a positive correlation of the polyphenolic content with the IC50 values of TAC.

Conclusion

This study revealed the effect of different solvents on the extraction, phytochemical content and antioxidant activities of *P. capitata* harvested from rubber tree. From the result of this findings, if the work were just to extract components for preparatory work only without bioassay, methanol would be the solvent of choice as it gave better yield than the rest solvents. If the intention were for activity screening, acetone will be preferred as it had the highest polyphenols and antioxidant activities.

Acetone has the ability to dissolve both hydrophilic and lipophilic compounds and is miscible with water, less volatile and a very useful extractant in bioassays. It is easy to handle at different stages of bioassays and preferred to methanol, ethanol and water even when more hydrophilic components are investigated. This is because acetone extracts highly polar components and a very useful extractant for dried plant materials (Eloff, 1998).

The results of this experimentation showed that *P. capitata* has high amounts of polyphenolic compounds and in turn, exhibited great antioxidant activities in all the solvent fractions tested. This plant may have great importance in combating oxidative stresses and hence, expanding its application in health delivery is recommended.

References

Afolabi, C., Akinmoladun, E., Ibukun, O., Emmanuel, A., Obuotor, E.M., 2007. Phytochemical constituent and antioxidant activity of extract from the leaves of *Ocimum gratissimum.* Scientific Research and Essays 2 (5), 163-166.

Aiyegoro, O.A., Okoh, A.I., 2010. Preliminary phytochemical screening and in vitro antioxidant activities of the aqueous extract of *Helichrysum longifolium.* BMC Complementary and Alternative Medicine 10, 21. DOI: 10.1186/1472-6882-10-21.

Ameer, O.Z., Salman, I.M., Quek, K.J., Asmawi, M.Z., 2015. *Loranthus ferrugineus:* a Mistletoe from traditional uses tolaboratory bench. Journal of Pharmacopuncture. DOI: 10.3831/KPI.2015.18.001.

Boora, F., Chirisa, E., Mukanganyama, S., 2014. Evaluation of nitrite radical scavenging properties of selected Zimbabwean plant extracts and their phytoconstituents. Journal of Food Processing. DOI: 10.1155/2014/918018.

Dibong, S.D., Engone, O.N.L., Ndongo, D., Priso, R.J., Taffouo, V., Fankem, H., Salle, G., Missoup, A.L., Boussim, I.J., Amougou, A., 2009. An assessment on the uses of *Loranthaceae* in ethnopharmacology in Cameroon: A case study made in Logbessou, North of Douala. Journal of Medicinal Plants Research 3(8), 592-595.

Eloff, J.N., 1998. Which extractant should be used for the screening and isolation of antimicrobial components from plants? Journal of Ethnopharmacology 60(1), 1–8. DOI:10.1016/S0378-8741(97)00123-2.

Ghasemzadadeh, A., Ghasemzadadeh, N., 2011. Flavonoids and Phenolic acids: Role and biochemical activity in plants and human. Journal of Medicinal Plants Research 5(31), 6697-6703. DOI: 10.5897/JMPR11.1404.

Ghasemzadadeh, A., Jaafar, H., Rahmat, A., 2011. Effects of solvent types on phenolics and flavonoids content and antioxidant activities in two varieties of young ginger (*Zingiber officinale* Roscoe) extracts. Journal of Medicinal Plants Research 5(7), 1147-1154.

Hussain, J., Ali, L., Khan, A.L., Rehman, N.U., Kim, J-S., Al-Harrasi, A., Jabeen, F., 2014. Isolation and Bioactivities of the Flavonoids Morin and Morin-3-*O*-ß-D-glucopyranoside from *Acridocarpus orientalis*—A Wild Arabian Medicinal Plant. Molecules. DOI: 10.3390/molecules191117763.

Iloki-Assanga, S.B., Lewis-Lujan, L.M., Lara-Espinoza, C.L., Gill-Salido, A.A., Fernandez-Angulo, D., Rubio-Pino, J.L., Haines, D.D., 2015. Solvent effects on the phytochemical constituent profiles and antioxidant activities, using four different extraction formulations

for analysis of *Bucida buceras* L. and *Phoradendron californicum.* BMC Researh Notes 8, 396. DOI 10.1186/s13104-015-1388-1.

Kamtekar, S., Keer, V., Patil, V., 2014. Estimation of phenolic content, flavonoid content, antioxidant and alpha amylase inhibitory activity of marketed polyherbal formulation. Journal of Applied Pharmaceutical Science 4(9), 61-65. DOI: 10.7324/JAPS.2014.40911.

Kibiti, C.M., Afolayan, A.J., 2015. Preliminary Phytochemical Screening and Biological Activities of Bulbine abyssinica Used in the Folk Medicine in the Eastern Cape Province, South Africa. Evidence-Based Complementary and Alternative Medicine. DOI: 10.1155/2015/617607

Liwei, G., Kelm, M.A., Hammerstone, J.F., Beecher, G., Holden, J., Haytowitz, D., Gebhardt, S., Prior, R.L., 2004. Concentrations of Proanthocyanidins in Common Foods and Estimations of Normal Consumption. Journal of Nutrition 134, 613-617.

Olugbami, J.O., Gbadegesin, M.A., Odunola, O.A., 2015. *In-vitro* free radical scavenging and antioxidant properties of ethanol extract of *Terminalia glaucescens*. Pharmacognosy Research *7*(1), 49–56. DOI:10.4103/0974-8490.147200.

Oyedemi, S.O., Bradley, G., Afolayan, A.J., 2010. *In vitro* and *In vivo* antioxidant activities of aqueous extract of *Strychonos henningsii* Gilg. African Journal of Pharmacy and Pharmacology 4,70-8.

Pacher, P., Beckman, J.S., Liaudet, L., 2007. Nitric oxide and peroxynitrite in health and disease. Physiological Reviews 87(1), 315-424.

Quy Diem, Do., Angkawijaya, A.E., Tran-Nguyen, P.L., Huynh, L.H., Soetaredjo, F.E., Ismadji, S., Yi-Hsu, J., 2014. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of *Limnophila aromatic.* Journal of Food and Drug Analysis 22, 296-302. DOI: 10.1016/j.jfda.2013.11.001.

Samatha, T., Shyamsundarachary, R., Srinivis, P., Swamy, N.R., 2012. Quantification of total phenolic and flavonoid contents in extracts of Oroxylum indicum L. Kurz. Asian Journal of Pharmaceutical and Clinical Research 5(4), 177-179.

Wintola, O.A., Afolayan, A.J., 2011. Phytochemical constituents and antioxidant activities of the whole leaf extract of *Aloe ferox* Mill. Pharmacognosy Magazine 7, 325-333.

CHAPTER THREE

PROXIMATE COMPOSITION AND MINERAL ANALYSIS OF *PHRAGMANTHERA CAPITATA*

This chapter has been published in the Research Journal of Botany

CHAPTER THREE

PROXIMATE COMPOSITION AND MINERAL ANALYSIS OF *PHRAGMANTHERA CAPITATA*

CONTENTS	Pages

Abstract 54
Introduction 55
Materials and Methods 55
  Nutritive composition analyses 55
  Energy content 56
  Anti-Nutritive composition 56
    Determination of oxalate content 56
    Determination of phytic acid 56
    Determination of Saponins 56
    Determination of Alkaloids 56
  Macro and micro-minerals analysis 56
    Digestion and mineral analysis 56
  Statistical analysis of data 57
Results and Discussion 57
  Proximate composition 57
  Mineral composition of *P capitata* 58
  Anti-nutritional composition of *P. capitata* 59
Conclusion 60
References 60

OPEN ACCESS Research Journal of Botany

ISSN 1816-4919
DOI: 10.3923/rjb.2017.23.31

Research Article
Proximate Composition and Mineral Analysis of *Phragmanthera capitata* (Sprengel) Balle, a Mistletoe Growing on Rubber Tree

Franklin Uangbaoje Ohikhena, Olubunmi Abosede Wintola and Anthony Jide Afolayan

Medicinal Plants and Economic Development (MPED) Research Center, Department of Botany, University of Fort Hare, Alice, Eastern Cape, South Africa

Abstract
Background: Leaves of *Phragmanthera capitata* (Sprengel) Balle growing on rubber trees were evaluated in order to determine its nutritional, anti-nutritional and mineral compositions. **Materials and Methods:** Proximate and anti-nutrient analyses were performed using standard analytical methods while mineral contents were determined using Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). **Result:** The result of the proximate analysis showed that carbohydrate was more with a composition of 57.73±0.33 g/100 g. Protein, fat and fibre compositions were 12.50±0.50, 3.34±0.18 and 11.66±0.54 g/100 g. The total energy composition was 310.97±2.30 kcal/100 g. The mineral composition revealed that the leaves of the plant were very rich in basic minerals with high potassium level of 1047.83±34 mg/100 g. Calcium (6.22.58±0.01 mg/100 g), magnesium (361.15±0.01 mg/100 g) and phosphorous (115.40±0.01 mg/100 g) were also present in appreciable amount. The anti-nutrients evaluated had appreciable amounts in phytate (0.15±0.23%), oxalate (2.99±0.61%), saponin (3.46±0.01%) and alkaloid (4.20±0.11%). **Conclusion:** The study revealed that *Phragmanthera capitata* could serve as a source of essential nutrients which can go a long way in ameliorating most nutritional challenges and may contribute remarkably to the amount of nutrients in human.

Key words: Proximate, anti-nutrients, mineral, mistletoe, *Phragmanthera capitata*

Received: September 17, 2016 Accepted: November 15, 2016 Published: December 15, 2016

Citatio n: Franklin Uangbaoje Ohikhena, Olubunmi Abosede Wintola and Anthony Jide Afolayan, 2017. Proximate composition and mineral analysis of *Phragmanthera capitata* (Sprengel) Balle, a mistletoe growing on rubber tree. Res. J. Bot., 12: 23-31.

Corresponding Author: Anthony Jide Afolayan, Medicinal Plants and Economic Development (MPED) Research Center, Department of Botany, University of Fort Hare, Alice, Eastern Cape, South Africa

Copyright: © 2017 Franklin Uangbaoje Ohikhena *et al.* This is an open access article distributed under the terms of the creative commons attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Phragmanthera capitata (Sprengel) Balle is a mistletoe in the Loranthaceae family. It is a woody parasitic shrub with pendent branches of about 2 m long and has a yellow corolla with a pink-red tip often associated with ants' nests. It grows on trees in secondary jungles, plantations and bush Savannah areas, from Sierra Leone to Western Cameroons, Fernando Po and extending across the Congo basin to Zaïre, Nigeria, Gabon, Ivory Coast and Angola[1]. As with most mistletoe, *P. capitata* is an obligate hemi-parasitic plant which attaches to and penetrates the stems and branches of its host tree or shrub by a structure called the haustorium, through which it absorbs water and mineral nutrients, even though it can produce its own food through photosynthesis[2,3]. They are found parasitizing many economic important tree crops which include shear butter, neem, cocoa, avocado, kola and rubber to mention but a few[4].

Mistletoes have been widely used in various cultures in almost every continent to treat various ailments or as a diuretic agent[5]. It is commonly consumed in parts of West Africa for the treatment of hypertension, ulcers, epilepsy, diabetes, weakness of vision and for promoting muscular relaxation before delivery[6]. Some rural farmers use the leaves as feed for their goats and other livestock that have newly given birth to young ones[7,8]. In the ecosystem, mistletoe attracts avian frugivores and other broad array of animals depend on it for food, consuming the leaves and young shoots therefore, providing high quality food for a wide range of animals in forests and woodlands worldwide[9].

It is worthwhile to note that consumption of numerous types of edible plants as sources of food could be beneficial to nutritionally marginal population especially in developing countries where poverty and climate is causing havoc to the rural populace. In many countries, the supply of minerals is inadequate to meet the mineral requirements of farm animals and the growing human population. Minerals cannot be synthesized by animals/humans and must be provided from plants or mineral-rich water[10] and hence, research is looking inward to the use of non-conventional leafy plants as possible sources of cheap nutritional supplement[11].

The use of mistletoe in pharmaceuticals as drugs and other therapeutic agents for the treatment of divers' kinds of ailments are well known. Their phyto-composition and biological activities which are chiefly dependent on host species[4] are well reported and documented, hence it is widely known as "cure all"[12]. Despite its vast medicinal usage, little or no report is known about its nutritional values hence the aim of this study was to determine the nutritional and mineral compositions of *P. capitata* that is growing on rubber trees and also to providing scientific data based on our findings in relation to its dietary/nutritional and medicinal application(s).

MATERIALS AND METHODS

Leaves of the mistletoe, *P. capitata* (Fig. 1, 2) were collected from mature rubber plantations in Rubber Research Institute of Nigeria, Iyanomo, located on latitude 6°00'-6°15' N, longitude 5°30'-5°45' E and on altitude 27 m a.s.l. in Benin city, which lies on the wet lowland rainforest of Edo State, Nigeria. Leaves were removed from the twigs, properly rinsed and air-dried at room temperature (mean morning and night temperature of 24°C and mean noon temperature of 27°C) in a well aerated atmosphere and prevented from direct sunlight to avoid denaturation of vital phyto-constituents. Dried leaves were pulverized using an electric motor blender and kept in an air-tight glassware container and stored at 4°C until when needed. The pulverized sample was used for all the analyses and all the analyses were carried out in triplicate.

Nutritive composition analysis: The moisture content was determined by the drying method and ash content by

Fig. 1: *Phragmanthera capitata* (Sprengel) Balle growing on rubber tree (*Hevea brasiliensis* L.)

Fig. 2: Leaves of *Phragmanthera capitata* (Sprengel) Balle

incinerating in a muffle furnace at 550°C as described by AgriLASA[13]. Dietary fibre was determined by acid/base digestion as described by Aina *et al.*[14]. Crude fat was extracted with ether, the nitrogen content of the plant was determined using the method described by Bvenura and Afolayan[15] by means of the Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES). The value of nitrogen obtained was multiplied by 6.25 to give the percentage of crude protein[16].

The carbohydrate content was determined by weight difference between 100 and the sum of the percentage of protein, fat, ash, moisture and dietary fibre.

Energy content: The kilocalorie (kcal/100 g) value estimation was done by summing the multiplied values for crude protein, crude lipid (excluding crude fibre) and carbohydrate respectively at Atwater factors (4, 9 and 4 kcal) as thus:

$$\text{Energy value (kcal/100 g)} = (\text{Crude protein}\times 4)+(\text{Crude fat}\times 9)+(\text{Total carbohydrate}\times 4)$$

Anti-nutritive composition

Determination of oxalate content: The modified titration method of Day and Underwood[17] was used to determine the oxalate content of the plant. One gram of the pulverized sample was weighed into a conical flask. Seventy five milliliters of 3 M H_2SO_4 was added and stirred with a magnetic stirrer for an hour. This was filtered and 25 mL aliquot of the filtrate was collected and warm to 80-90°C and kept above 70°C at all times. The hot aliquot was titrated against 0.05 M of $KMnO_4$ until an extremely faint pale pink colour persisted for 15-30 sec. The oxalate content was calculated by taking 1 mL of 0.05 M of $KMnO_4$ as equivalent to 2.2 mg oxalate.

Determination of phytic acid: Phytic acid was determined as described by Damilola *et al.*[18]. Two grams of the sample was weighed into a 250 mL conical flask. Hundred milliliters of 2% HCl was used to soak the sample for 3 h and then filtered through What man No. 1 filter paper. Twenty five milliliters aliquot of the filtrate was placed in a separate 250 mL conical flask and 5 mL of 0.3% ammonium thiocyanate solution as indicator. About 53.5 mL of distilled water was added to give the desired acidity. This was then titrated with standard iron III chloride solution which contains 0.00195 g iron mL^{-1} until a brownish yellow colour persisted for 5 min phytic acid was calculated thus:

$$\text{Phytic acid (\%)} = \text{Titre value}\times 0.00195\times 1.19\times 100$$

Determination of saponins: Saponin content was determined as described by Obadoni and Ochuko[19]. Briefly, 20 g of the pulverized plant sample was added to 200 mL of 20% ethanol and kept on a shaker for 30 min and was then heated in a water bath at 55°C for 4 h. The resulting mixture was filtered and the residue re-extracted with another 200 mL of 20% aqueous ethanol. The filtrate mixture was combined and reduced to 40 mL in a water bath at 90°C. The concentrate was transferred into a separatory funnel and 20 mL of diethyl ether was added, shook vigorously. The ether layer which was the upper layer was discarded and the aqueous (bottom) layer retained in a beaker. The retained layer was re-introduced into the saparatory funnel and 60 mL of n-butanol was added and shook vigorously. The butanol extract which is the upper layer was retained while the bottom layer was discarded. The butanol layer was washed twice with 10 mL of 5% aqueous sodium chloride. The remaining solution was collected and heated in a water bath and evaporated to dryness to constant weight at 40°C in an oven. The saponin content was calculated using the equation:

$$\text{Saponin content (\%)} = \frac{\text{Weight of residue}}{\text{Weight of original sample}}\times 100$$

Determination of alkaloids: The alkaloid content was determined according to the method of Omoruyig *et al.*[20]. Briefly, 5 g of plant extract was mixed with 200 mL of 10% acetic acid in ethanol. The mixture was covered and allowed to stand for 4 h. This was filtered and the filtrate was concentrated on a water bath to one-fourth of its original volume. Concentrated ammonium hydroxide was added in drops to the extract until precipitation (cloudy fume) was completed. The whole solution was allowed to settle, the collected precipitates washed with dilute ammonium hydroxide and then filtered. The residue collected was dried and weighed. The alkaloid content was determined using this equation:

$$\text{Alkaloid (\%)} = \frac{\text{Weight of precipitate}}{\text{Weight of original sample}}\times 100$$

Macro and micro-minerals analysis

Digestion and mineral analysis: The method described by Bvenura and Afolayan[15] was used for the digestion of plant material. Briefly, a digestion mixture comprising of selenium powder, sulphuric acid and salicylic acid were prepared. About 0.3 g of the ground plant material was placed in dry, clean

digestion tubes. A volume of 2.5 mL of the digestion mixture was added to each tube and allowed to react at room temperature for 2 h. The tubes were heated in a block digester at 110°C for 60 min. The tubes were allowed to cool and three successive portions of 1 mL hydrogen peroxide added at 10 sec intervals due to the volatility of the reaction. The tubes were returned to the block digester at a temperature of 330°C and were removed from the block digester when the digest turned clear in colour. The tubes were allowed to cool to room temperature, contents transferred into 50 mL volumetric flasks and then deionized water was added to attain volumes of 50 mL. Standards were prepared for all the individual elements to be analyzed.

The macro-minerals (Calcium, magnesium, potassium, sodium and phosphorus) and micro-minerals (Iron, zinc, aluminum, manganese and copper) were determined using the Inductively Coupled Plasma-Optical Emission Spectrometer (ICP-OES, Varian 710–ES series, SMM Instruments, Cape Town, South Africa)[15]. All analyses were carried out in triplicates.

Statistical analysis of data: All experiments were done in triplicates and the results expressed as Mean±SD using the Microsoft excel 2010 spreadsheet.

RESULTS AND DISCUSSION

Proximate composition: Results of the proximate composition of *P. capitata* leaves were shown in Table 1. Parameters determined include; moisture content, total ash, fat, dietary fibre, crude protein and carbohydrate. There was relatively lower moisture content (7.36±0.70 g/100 g) in the sample which is comparable to values obtained in five cowpea verities (6.80±0.22 to 9.10±0.41 g/100 g) as reported by Owolabi *et al.*[21]. This low moisture level indicates a longer shelf life because moisture content determines the suitability of food products before consumption because it affects the physical and chemical aspects of food which relates to the freshness and stability for storage[22].

The ash content (7.41±0.07 g/100 g) evaluated from this sample was relatively higher than all five varieties of cowpea (3.38±0.93 to 4.46±0.41 g/100 g), Indian spinach (5.02 g/100 g), *Telfaira occidentale* (8.54 g/100 g) and all *Cissus populnea* stem (6.5±0.7-8.8±0.5 g/100 g) collected from three different locations as reported by Owolabi *et al.*[21], Asaolu *et al.*[10] and Adebowale *et al.*[23], respectively. The ash content of the sample (7.41±0.07 g/100 g) was relatively high indicating its richness in mineral nutrients. Ash content is a measure of

Table 1: Proximate composition of *Phragmanthera capitata* (Sprengel) Balle

Parameters	Composition (g/100 g dry weight)
Moisture content	7.36±0.07
Total ash	7.41±0.07
Crude fat	3.34±0.18
Crude fibre	11.66±0.54
Crude protein	12.50±0.50
Carbohydrates	57.73±0.33
Energy value (kcal/100 g)	310.97±2.30

Values are expressed as Mean±SD, n = 3

the total amount of minerals present in the plant. These minerals act as inorganic co-factors in metabolic processes which means that without these inorganic co-factors, there could be impaired metabolism[24].

The dietary fibre content of the sample (11.66±0.54 g/100 g) is higher than Indian spinach (6.05 g/100 g), bush-buck (4.02 g/100 g), scent leaf (7.04 g/100 g), *Amaranthus hybidus* (8.05 g/100 g) and *Telfaria occidentale* (11.05 g/100 g) but slightly lower than *Vernonia amygdalina* (Bitter leaf) and *Hibiscus sabdariffa* with values of 12.08 and 12.04% respectively as reported by Asaolu *et al.*[10]. The dietary fibre recorded in the sample is lower than the Recommended Daily Intake (RDI) for men (31.00-38 g day^{-1}), females (25-26 g day^{-1}), pregnant women (28 g day^{-1}) and lactating mothers (29 g day^{-1})[25]. Nutritionally, relatively high dietary fibre evaluated in the sample is beneficial because it provides the buck necessary for proper peristaltic action and aids the absorption of trace elements in the gut and reduces cholesterol absorption[23,26].

3The protein content in the sample was 12.50±0.50 g/100 g and it was the second highest proximate composition of the sample. Though below the Recommended Dietary Allowance (RDA) for adult males and females (56 g day^{-1})[27], it is enough to provide 19.64% day^{-1} if 100 g (dry weight) of the sample were consumed. The protein content of the sample was quiet lower than reported results for different leafy vegetables consumed in Africa as reported by Asaolu *et al.*[10] and also lower than the five cowpea varieties (19.84±0.18-26.61±0.48 g/100 g) as reported by Owolabi *et al.*[21] but was higher than *Peperomia pellucida* (10.63±0.07 g/100 g) as reported by Ooi *et al.*[28]. Nutritionally, protein is the major component of all cells in the body and functions in growth, movement and body defense. It also serves the purpose of enzymatic catalyst and mediates metabolic and energy regulation[25,29].

The result showed that the fat (lipid) content of the sample was 3.34±0.18 g/100 g and it was the lowest nutritional composition evaluated in this study indicating the low level of fat in the sample which is far below the recommended percentage calorie requirement for fat per day which ranges from 20-35% calories (kcal/100 g). An active

male requiring 3,000 cal per day[27] would need to consume 600-1050 cal or 67-117 g of fat day^{-1}. This sample could be a good choice for people requiring low fatty food source.

Carbohydrate had the highest nutritional composition of 57.73±0.33 g/100 g. This value is comparable to the five varieties of cowpea (56.24±0.51 to 63.30±0.33 g/100 g) recorded by Owolabi *et al.*[21] and higher than values obtained for *Peperomia pellucida* (46.58±2.74 g/100 g) and stem flour of *Cissus populnea* (43.7±2.5 to 48.1±3.5 g/100 g) as reported by Ooi *et al.*[28] and Adebowale *et al.*[23], respectively. The carbohydrate content of the sample (57.73±0.33 g/100 g) is lower than the DRI for carbohydrate (130 g day^{-1}) but can supplement 44.41% of the daily requirement for carbohydrate if 100 g were consumed (Dietary relative intakes)[25]. Nutritionally, this sample can provide readily accessible fuel, serve as source of energy for the body, for physical performance, breathing, maintaining body temperature and for contraction and relaxation of the heart muscles[29], it can also help in breaking down fatty acids and prevent ketosis, maintaining digestive health and gives food good flavourings.

The total energy derived from this plant as calculated was 310.97±2.30 kcal/100 g (Table 1) which is below the recommended daily energy intake of 1,000 kcal for sedentary children of ages 2-3 years and 1,000-1400 kcal day^{-1} for active children under the same age bracket, sedentary female adults (19-30 years) would need about 2,000 kcal day^{-1} and active female adults of the same ages would require 2,400 kcal day^{-1} to meet their daily intake. For sedentary men (19-30 years) and active men (14-18 years), their energy requirement per day is 2,800-3,200 kcal, respectively[25]. The higher the energy value of a food sample, the lesser the amount required to meet the required energy intake. This plant on the whole is a good energy source as all the required energy needed in human is supplemented from various food products.

Mineral composition of *P. capitata*: Table 2 shows the result of the mineral composition of the sample. The results showed that the sample is a good source of both macro and micro minerals. The minerals evaluated are in the other; K>Ca>Mg>P>Fe>Mn>Na>Zn>Cu.

The amount of K (1047.83±0.01 mg/100 g) in the sample is relatively lower than the Recommended Dietary Allowances (RDA, 4700 mg day^{-1}) for K and also lower than the values reported in *Peperomia pellucida* (6977±4.24 mg/100 g)[28] and *Cissus populnea* stem (2679±13 mg/100 g)[23] but higher than values recorded in *Cicer arietinum* (870 mg/100 g)[30], cowpea (15.67±1.17 to 18.69±0.29 mg/100 g)[21], Indian spinach (16.85 mg/100 g), bitter leaf (73.25 mg/100 g), scent leaf (86.24 mg/100 g) and *Telfaria occidentalis* (130.24 mg/100 g)[10]. The Na content (19.23±3.71 mg/100 g) in the sample is far lower than the RDA (1500 g day^{-1}) and lower than almost all the traditional vegetables consumed in South Africa as reported by Odhav *et al.*[31]. The K and Na both function for proper fluid balance, nerve transmission and muscle contraction[32].

Table 2: Mineral composition of *Phragmanthera capitata* (Spreng.) Balle

Mineral elements	Composition (mg/100 g)
Calcium (Ca)	622.58±0.01
Magnesium (Mg)	361.15±0.01
Potassium (K)	1047.83±0.03
Phosphorous (P)	115.40±0.01
Sodium (Na)	19.23±3.71
Zinc (Zn)	2.49±0.14
Copper (Cu)	1.81±0.07
Manganese (Mn)	27.57±0.60
Iron (Fe)	105.15±8.11

Values are expressed as Mean±SD, n = 3

Calcium is the second highest mineral element in the sample with a value of 622.58±0.01 mg/100 g and plays a major role in building and maintaining strong bones and teeth, it also serves vital roles in nerve transmission, constriction and dilation of blood vessels, muscle contraction[27], normal functioning of blood coagulation, milk clotting[33], regulation of cell permeability, blood pressure regulation and immune system health[32]. Deficiency of calcium causes rickets, osteoporosis, back pain, indigestion, irritability, premenstrual tension and cramping of the uterus[34]. The daily Recommended Dietary Allowance (RDA) for Ca is 1000 mg day^{-1} for males and females aged 19-30 years which is a bite higher than the 622.58±0.01 mg/100 g obtained in the sample but it can conveniently supply 62.258% of the daily allowance hence it is a good supplement for people with Ca deficiency.

The presence of Mg in the sample per 100 g was 361.15±0.01 mg which is sufficiently enough to supply the RDA of Mg per day for most of life stage groups which requires[25] 80-420 mg day^{-1}, hence the sample is a good source of Mg. Magnesium is important in the formation and function of bones, muscles and prevents high blood pressure and depression. It is also needed for making proteins, muscle contraction, nerve transmission and immune system health and plays important role in enzyme activity and prevents heart diseases[33]. The Mg is vital in strengthening cell membrane structure and modulates glucose transport across cell membranes[35]. Studies have shown that Mg supplementation improves insulin sensitivity in diabetic patients and it can improve insulin sensitivity in obese individuals who are at risk of type 2 diabetes mellitus[36].

The composition of phosphorous in the sample was 115.40±0.01 mg/100 g which is below the RDA of 700 mg day^{-1} for adults and also lower than chickpea seeds (226 mg/100 g) as reported by Alajaji and El-Adawy[30] and most vegetables consumed in South Africa as reported by Odhav *et al.*[31], phosphorous is important for healthy bones and teeth and it is found in every cell and maintains normal cell growth and repairs; it maintains blood sugar level, acid-base balance and normal heart beat level[32,33].

The body also needs micro (trace) minerals in very small amounts. Iron is considered to be a trace mineral, although the amount needed is somewhat more than for other micro minerals. The micro minerals assayed for are Zn, Cu, Mn and Fe. Iren (Fe) is the highest micro mineral produced by the sample with a composition of 105.15±8.11 mg/100 g which is far above the RDA of 8 mg day^{-1} for adult male and up to 28 mg day^{-1} for pregnant women. So, about 29.44 g of the sample is sufficiently enough to supply the RDA of Fe for pregnant women. The Fe is a major constituent of hemoglobin and a carrier of oxygen in the blood. The Fe is also important in tendon and ligament formation, certain chemicals in the brain are controlled by the presence and absence of iron and also needed for energy metabolism[32,37]. The Fe deficiency causes anaemia, weakness, depression, poor resistance to infection and in women may cause infertility[32] and hence this sample can be a good source for people with fertility challenges and other Fe deficiency crisis.

Zinc (Zn) content in the sample was 2.49±0.14 mg/100 g which is lower than the RDA of 8 mg for adult males, 18 mg for adult females and 27 mg for pregnant women[25]. The Zn is in part of many enzymes, needed for making protein and genetic material has a function in taste perception, wound healing, normal fetal development, production of sperm, normal growth and sexual maturation, immune system health[32]. This sample therefore can be a good Zn supplement for people with infertility challenges.

Copper is part of the enzymes such as cytochrome oxidase, lysyl oxidase and ceruloplasm, needed for iron metabolism in the blood[33]. The Cu deficiency can cause cardiac abnormalities in human and animals, anemia and neutropenia[38]. The amount of Cu in the sample (1.81±0.07 mg/100 g) is above the RDA of 0.9 mg day^{-1} for adult males and females, 1 mg day^{-1} for pregnant women and 1.3 mg day^{-1} for lactating mothers hence it's a good source of Cu and highly recommended for people with Cu deficiency.

Manganese acts as a cofactor and constituents of several enzymes involved in metabolic processes necessary for the skeletal development, reproductive function and growth. It is a cofactor of oxidative phosphorylation enzymes whose activity increases insulin secretion to improve glucose tolerance under diabetic condition[39]. This element is also involved in urea formation, metabolism of amino acids, cholesterol and carbohydrates[40]. The composition of Mn in the sample was 27.57 mg/100 g which is above the RDA of 2.3 mg for adult males, 1.8 mg for adult females, 2 mg for pregnant women and 2.6 mg for lactating mothers[25]. So, in providing the daily Mn requirement from the sample, adult males would need 8.34 g day^{-1}, adult females 6.53 g day^{-1}, pregnant women 7.25g day^{-1} and lactating mothers would need 9.43 g.

Table 3: Anti-nutrient composition of *Phragmanthera capitata* (Sprengel) Balle

Parameters	Values (%)
Phytic acid	0.15±0.23
Oxalate	2.99±0.61
Saponins	3.46±0.01
Alkaloids	4.20±0.11

Values are expressed as Mean±SD, n = 3

Anti-nutritional composition of *P. capitata*: The summary of the anti-nutritional composition of the sample is as shown in Table 3. Four anti-nutrients were studied, alkaloid was the most abundant with a percentage value of 4.20±0.11% which was closely followed by saponin (3.46±0.11%) and oxalate (2.99±0.61%). Phytic acid was the least with a percentage value of 0.15±0.23%.

The anti-nutrient activities of alkaloid are observed at high level intake/dosage to exert toxic and adverse effects to humans, especially in physiological and neurological activities. However, dosage differentiates between toxicity and pharmacological effects of alkaloids[41]. The percentage of alkaloid (4.2%) in the sample is higher than *Clerodendrum volubile* (0.79%)[11], *Cissus populnea* (0.23%)[23], red and white cocoyam leaves (1.44±0.08 and 1.50±0.05%, respectively)[18].

Oxalate composition of 2.99±0.61% in the sample was higher than 0.583±0.04 and 0.828±0.07% (converted values) of red and white cocoyam leaves respectively as reported by Damilola *et al.*[18]. The oxalate content of foods is of interest because consumption of high oxalate diet may result in hyperoxaluria thereby increasing the risk of kidney stones. Iron oxalate crystals cause significant oxidative damage and diminish iron stores needed for red blood cell formation while kidney stones are caused by calcium oxalate. Oxalate on the long run, can also act as a chelator and can chelate toxic metals such as mercury and lead[42] and can act as an antioxidant.

Phytic acid (phytate) though very low (0.15±0.23%) was also recorded in the sample (Table 2). This value is very small

to cause any adverse anti-nutrient effect to phosphorus absorption in diet. Phytate chelates metal ions such as calcium, magnesium, zinc, copper, iron and molybdenum to form insoluble complexes that are not readily absorbed from the gastrointestinal tract[42,43]. The greatest effect of phytic acid on human nutrition is its reduction of zinc bioavailability[43]. Its antioxidant ability can be used in the food industry as a unique and versatile food preservative as it can increase nutritive value, prolong shelf life and prevent discoloration when added to fruits, vegetables, cheese, noodle, soy sauces, juices, bread, alcoholic beverages, meat fishmeal pastes and canned sea foods. Phytate can also act as an anti-cancer agent against colon, soft tissue, metastatic lung cancer, breast and prostate cancer[44]. It has been reported to reduce blood glucose and possess health benefits to diabetic patients and can also prevent kidney stone formation[45], hence this plant has the potential to serve as a remedy for people with the scare of high oxalate consumption which can lead to kidney stone formation.

The saponin content of the species was 3.46±0.01 5%. Saponin is a heterogeneous group of naturally occurring foam-producing triterpene or steroidal glycosides that occur in a wide range of plants[46]. Some biological effects of saponin in animals include erythrocyte haemolysis, reduction of blot (ruminant), inhibition of smooth muscles activity, enzyme inhibition and reduction in nutrient absorption, alteration of cell wall permeability and therefore produce some toxic effects when ingested, it also binds with the cell of the small intestine thereby affecting the absorption of nutrients across the intestinal wall[42,43]. Aside its anti-nutrient effects, saponin have been reported to have various biological benefits such as, anti-inflammatory, anti-diabetic, anti-HIV, anti-atherosclerotic and serve as protective functions like gastro-protective, hepatoprotective and hypolipidemic. Reports have also shown saponins to be effective in maintaining liver function, lowering blood cholesterol, preventing peptic ulcer, osteoporosis as well as platelet agglutination[42].

CONCLUSION

The study revealed that *Phragmanthera capitata*, a mistletoe growing on rubber tree has the potential of contributing useful amount of nutrients to human and animal diets. The anti-nutrients present were abite higher than most found in some conventional vegetables but preparation techniques like soaking, boiling and cooking reduce anti-nutrient thereby making their effect negligible. This plant can serve as a supplement to many mineral deficiencies and also as a source of medicine to a number of ailments, hence, mistletoe is known as a remedy for all kinds of diseases and sicknesses. Instead of been considered a pest, it should be seen as a plant with great potential in the food/nutritional and pharmaceutical industries. Further studies on its toxicity are ongoing to ascertain if any, its possible adverse effects.

SIGNIFICANT STATEMENTS

This study provides insights into the nutritional composition of *Phragmanthera capitata*, a mistletoe widely exploited in Africa. This study reveals important mineral compositions even higher than those found in some conventional vegetables. It is rich in carbohydrate which serves as a major source of energy and substantial amount of fibre, protein and fat. The findings of this study revealed that this species could be used to boast the immune system due to its rich mineral and nutrient compositions and hence could be the reason to its therapeutic application in folkloric medicine. This study also gives a baseline data on its nutritional composition as most research is on crop protection because of its parasitic mode of association.

ACKNOWLEDGMENT

The authors wish to acknowledge the financial support of Govan Mbeki Research Development Centre, University of Fort Hare, South Africa and Rubber Research Institute of Nigeria, Iyanomo.

REFERENCES

1. Takem, L.P., G.A. Eshiet, O.G. Ogom and U.U. Mbang, 2014. Exploratory and anxiety potentials of aqueous extract of *Phragmanthera capitata*. J. Phytopharmacol., 3: 400-404.
2. Hassan, A.F., I.T. Numan, K.W. Al-Sammarrae and S.A. Hussain, 2005. Hematopoietic toxicity of *Loranthus europaeus* chloroform extract: *In vitro* study. Int. J. Comprehen. Pharm., 17: 345-352.
3. Agbo, M.O., C.S. Nworu, F.B.C. Okoye and P.O. Osadebe, 2014. Isolation and structure elucidaton of polyphenols from *Loranthus micranthus* Linn. parasitic on Hevea brasiliensis with antiinflammatory property. EXCLI J., 13: 859-868.
4. Adesina, S.K., H.C. Illoh, I.I. Johnny and I.E. Jacobs, 2013. African mistletoes (Loranthaceae); ethnopharmacology, chemistry and medicinal values: An update. Afr. J. Tradit. Complement. Altern. Med., 10: 161-170.
5. Jadhav, N., C.R. Patil, K.B. Chaudhari, J.P. Wagh, S.J. Surana and R.B. Jadhav, 2010. Diuretic and natriuretic activity of two mistletoe species in rats. Pharmacogn. Res., 2: 50-57.

6. Bassey, M.E., 2012. Phytochemical investigations of *Tapinanthus globiferus* (Loranthaceae) from two hosts and the taxonomic implications. Int. J. Chem. Environ. Pharm. Res., 3: 174-177.
7. Ishiwu, C.N., J.E. Obiegbuna and N.M. Aniagolu, 2013. Evaluation of chemical properties of mistletoe leaves from three different trees (Avocado, African oil bean and Kola). Nig. Food J., 31: 1-7.
8. Sher, H. and M.N. Alyemeni, 2011. Pharmaceutically important plants used in traditional system of Arab medicine for the treatment of livestock ailments in the kingdom of Saudi Arabia. Afr. J. Biotechnol., 10: 9153-9159.
9. Mathiasen, R.L., D.L. Nickrent, D.C. Shaw and D.M. Watson, 2008. Mistletoes: Pathology, systematics, ecology and management. Plant Dis., 92: 988-1006.
10. Asaolu, S.S., O.S. Adefemi, I.G. Oyakilome, K.E. Ajibulu and M.F. Asaolu, 2012. Proximate and mineral composition of Nigerian leafy vegetables. J. Food Res., 3: 214-218.
11. Erukainure, O.L., O.V. Oke, A.J. Ajiboye and O.Y. Okafor, 2011. Nutritional qualities and phytochemical constituents of *Clerodendrum volubile*, a tropical non-conventional vegetable. Int. Food Res. J., 18: 1393-1399.
12. Adodo, A., 2004. Nature Power: A Christian Approach to Herbal Medicine. 3rd Edn., Generation Press, Surulere, Lagos, ISBN-13: 9789780601874, Pages: 209.
13. AgriLASA., 2007. Method No. 6.1.1 for Feeds and Plants. In: Agrilasa Handbook of Feeds and Plant Analysis, Vol. 1, 2nd Edn., Palic, P., A.S. Claasens, J. Collier, A. Loock and D. Hattingh (Eds.)., Agri Laboratory Association of South Africa, Pretoria.
14. Aina, V.O., B. Sambo, A. Zakari, H.M.S. Haruna, K. Umar, R.M. Akinboboye and A. Mohammed, 2012. Determination of nutritional and anti-nutritional content of *Vitis vinifera* (Grapes) grown in Bomo (Area C) Zaira, Nigeria. Adv. J. Food Sci. Technol., 4: 445-448.
15. Bvenura, C. and A.J. Afolayan, 2012. Heavy metal contamination of vegetables cultivated in home gardens in the Eastern Cape. South Afr. J. Sci., 108: 1-6.
16. Magomya, A.M., D. Kubmarawa, J.A. Ndahi and G.G. Yebpella, 2014. Determination of plant proteins via the Kjeldahl method and amino acid analysis: A comparative study. Int. J. Sci. Technol. Res., 3: 68-72.
17. Day, Jr. R.A. and A.L. Underwood, 1986. Quantitative Analysis. 5th Edn., Prentice-Hall Publication, London, UK., Pages: 701.
18. Damilola, O.L., O.B. Joseph, A. Olufemi and I.A. Amoo, 2013. Chemical composition of red and white cocoyam (*Colocosiaesculenta*) leaves. Int. J. Sci. Res., 11: 121-125.
19. Obadoni, B.O. and P.O. Ochuko, 2002. Phytochemical studies and comparative efficacy of the crude extracts of some haemostatic plants in Edo and Delta states of Nigeria. Global J. Pure Applied Sci., 8: 203-208.
20. Omoruyig, B.E., G. Bradley and A.J. Afolayan, 2012. Antioxidant and phytochemical properties of *Carpobrotus edulis* (L.) bolus leaf used for the management of common infections in HIV/AIDS patients in Eastern Cape Province. BMC. Complement Altern. Med., Vol. 12. 10.1186/1472-6882-12-215.
21. Owolabi, A.O., U.S. Ndidi, B.D. James and F.A. Amune, 2012. Proximate, antinutrient and mineral composition of five varieties (improved and local) of Cowpea, *Vigna unguiculata*, commonly consumed in samaru community, Zaria-Nigeria. Asian J. Food Sci. Technol., 4: 70-72.
22. Igwe, O.U. and D.E. Okwu, 2013. Investigation of the chemical composition of *Brachystegia eurycoma* harms plant parts used in herbal medicine. Int. Res. J. Pharm. Applied Sci., 3: 51-55.
23. Adebowale, K.O., L.M. Nwokocha and W.B. Agbaje, 2013. Composition of *Cissus populnea* stem. J. Food Compos. Anal., 30: 41-46.
24. Jonathan, A.A. and A.S. Funmilola, 2014. Nutritional and anti-nutritional composition of *Bridelia ferruginea* Benth (Euphorbiaceae) stem bark sample. Int. J. Scient. Res. Knowl., 2: 92-104.
25. Institute of Medicine, 2006. Dietary Reference Intakes: The Essential Guide to Nutrient Requirements. National Academies Press, Washington DC, USA., ISBN: 978-0-309-15742-1, Page: 1344.
26. Dhingra, D., M. Michael, H. Rajput and R.T. Patil, 2012. Dietary fibre in foods: A review. J. Food Sci. Technol., 49: 255-266.
27. USDA. and DHHS., 2010. Dietary Guidelines for Americans. 7th Edn., U.S. Government Printing Office, Washington, DC., USA., Pages: 112.
28. Ooi, D.J., S. Iqbal and M. Ismail, 2012. Proximate composition, nutritional attributes and mineral composition of *Peperomia pellucida* L. (Ketumpangan Air) grown in Malaysia. Molecules, 17: 11139-11145.
29. Hussain, J., A.L. Khan, Najeeb ur Rehman, Zainullah, F. Khan, S.T. Hussain and Z.K. Shinwari, 2009. Proximate and nutrient investigations of selected medicinal plants species of Pakistan. Pak. J. Nutr., 8: 620-624.
30. Alajaji, S.A. and T.A. El-Adawy, 2006. Nutritional composition of chickpea (*Cicer arietinum* L.) as affected by microwave cooking and other traditional cooking methods. J. Food Compos. Anal., 19: 806-812.
31. Odhav, B., S. Beekrum, U.S. Akula and H. Baijnath, 2007. Preliminary assessment of nutritional value of traditional leafy vegetables in KwaZulu-Natal, South Africa. J. Food Compos. Anal., 20: 430-435.
32. WebMD, 2014. Vitamins and supplements minerals: Their functions and sources guide. WebMD Medical Reference, Healthwise.

33. Shivraj, H.N. and C.N.N. Khobragade, 2009. Determination of nutritive value and mineral elements of some important medicinal plants from Western Part of India. J. Med. Plants, 8: 79-88.
34. Hasling, C., K. Sondergaard, P. Charles and L. Mosekilde, 1992. Calcium metabolism in postmenopausal osteoporotic women is determined by dietary calcium and coffee intake. J. Nutr., 122: 1119-1126.
35. Jahnen-Dechent, W. and M. Ketteler, 2012. Magnesium basics. Clin. Kidney J., 5: 3-14.
36. Volpe, S.L., 2013. Magnesium in disease prevention and overall health. Adv Nutr., 4: 378S-383S.
37. Gaeta, A. and R.C. Hider, 2005. The crucial role of metal ions in neurodegeneration: The basis for a promising therapeutic strategy. Br. J. Pharmacol., 146: 1041-1059.
38. Smith, J.C., 1987. Copper nutritive and cardiovascular integrity. Proceedings of the University of Missouri's 21st Annual Conference on Trace Substances in Environmental Health, May 24-28, 1987, St. Louis, Mo., USA., pp: 499-513.
39. Lee, S.H., H.A. Jouihan, R.C. Cooksey, D. Jones, H.J. Kim, D.R. Winge and D.A. McClain, 2013. Manganese supplementation protects against diet-induced diabetes in wild type mice by enhancing insulin secretion. Endocrinology, 154: 1029-1038.
40. Zablocka-Slowinska, K. and H. Grajeta, 2012. The role of manganese in etiopathogenesis and prevention of selected diseases. Postepy. Hig. Med. Dosw., 66: 549-553.
41. Makkar, H.P.S., S. Siddhuraju and K. Becker, 2007. Methods in Molecular Biology: Plant Secondary Metabolites. Humana Press, Totowa, New Jersey, USA.
42. Egbuna, C. and J.C. Ifemeje, 2015. Biological functions and anti-nutritional effects of phytochemicals in living system. IOSR J. Pharm. Biol. Sci., 10: 10-19.
43. Akande, K.E., U.D. Doma, H.O. Agu and H.M. Adamu, 2010. Major antinutrients found in plant protein sources: Their effect on nutrition. Pak. J. Nutr., 9: 827-832.
44. Liu, Z., F. Cheng and G. Zhang, 2005. Grain phytic acid content in japonica rice as affected by cultivar and environment and its relation to protein content. Food Chem., 89: 49-52.
45. Dost, K. and O. Tokul, 2006. Determination of phytic acid in wheat and wheat products by reverse phase high performance liquid chromatography. Anal. Chim. Acta, 558: 22-27.
46. Jenkins, K.J. and A.S. Atwal, 1994. Effects of dietary saponins on fecal bile acids and neutral sterols and availability of vitamins A and E in the chick. J. Nutr. Biochem., 5: 134-137.

CHAPTER FOUR

GC-MS PROFILING OF THE ESSENTIAL OILS OF *PHRAGMANTHERA CAPITATA*

CHAPTER FOUR

GC-MS PROFILING OF THE ESSENTIAL OILS OF *PHRAGMANTHERA CAPITATA*

CONTENTS **Pages**

Background of the study 65
Methodology 66
  Plant collection and identification 66
  Essential oil extraction... 66
    Hydro-distillation Technique 66
    Solvent-free microwave extraction (SFME) 66
  Gas chromatography-Mass spectrometry (GC-MS) analysis 67
Result and Discussion 67
References 79

Background of the study

Essential oils also called volatile oils, ethereal oils, aetherolea or oil from the named plant, are a complex mixture of concentrated hydrophobic liquid containing volatile aromatic compounds. It is called "essential" because it contains the essence of the plant's fragrance (Gautam *et al.,* 2014; Celiktas *et al.,* 2007).

Essential oils have been known to mankind for hundreds of years and even millennia. Long before the utilisation of essential oils as fragrance, its importance as a remedy had been identified. It gained recognition in the 9th century and became widespread in the 16th and 17th centuries when their uses as flavour, aroma and in medicine were known (Lawal and Ogunwande, 2013; Baser and Buchbauer, 2010).

Interest in essential oils has sky-rocked in recent times because of their perceived biological activities: Antioxidative, antimicrobial and antiparasitic properties (Nwiloh, *et al.,* 2014), and has now become a part of everyday's life. They are employed in different biological, physical and chemical industries: as food and cigarette flavourings, feed additives, compounding of cosmetics and perfumes and as pharmaceuticals. Most recently, they have been employed as biocides and insect repellents and currently, it is widely used in aromatherapy (massaging and bathing) (Lawal and Ogunwande, 2013; Lee *et al.,* 2012; Baser and Buchbauer, 2010).

Many novel compounds of biological and pharmacological importance identified, characterised and isolated from the essential oils of different plant species from Africa origin have been extensively studied. Essential oils have been extracted from different parts of the plant (leaves, stem, flowers and roots) and the oils so extracted from different parts of the same plant may have completely different compositions. Different methods: distillation, expression, solvent extraction, absolute oil extraction, resin tapping, carbon dioxide extraction, cold pressing and solvent free microwave extraction, and solvents have been explored in the extraction of essential oils in plants (Wikipedia, 2016; Hamid *et al.,* 2011).

Despite the wide ethnopharmacological usage and report on the chemical compositions of the crude extracts of *P. capitata* as described in previous chapters, there was no report of the essential oil extraction and compositions prior to this study. Hence, this study was aimed at evaluating the essential oil compositions of the dried leaves of *P. capitata* using hydrodistillation with two extraction media (water and normal saline) and solvent-free microwave extraction (SFME) techniques.

Methodology

Plant collection and identification

Plant collection and identification were carried out as previously described in chapter two

Essential oil extraction

Hydro-distillation Technique

200 g each of the dried leaves were immersed in 2.5 L of distilled water and normal saline and hydro-distilled using the Clevenger apparatus fitted with a condenser and connected to a heat-resistant 5-L round bottom flask. Heat at 50°C was supplied to the mantle and the essential oils were collected after 3 h of continuous heat.

Solvent-free microwave extraction (SFME)

This was done with a Milestone DryDIST (2004) apparatus. The multimode reactor has a twin magnetron (2×800 W, 2450 MHz) with a maximum delivered power of 500W in 5W increments. A rotating microwave diffuser ensures homogeneous microwave distribution throughout the plasma coated PTFE cavity. The temperature was monitored by an external infrared sensor. Constant conditions of temperature and water were guaranteed by the reflux of condensed water, achieved by a circulating cooling system at 5°C. 200 g of the leaves were placed into the reactor without the addition of water or solvent. The extraction of the essential oil was complete in 40 min. The oils collected in both extraction methods were analysed using GC–MS (Ajayi *et al.,* 2016).

Gas chromatography-Mass spectrometry (GC-MS) analysis

GC-MS analyses were performed using an Agilent 7890B GC system coupled with an Agilent 5977A mass selective detector (MSD) (Chemetrix, Pty, Ltd, Agilent Technologies, DE, Germany) and a Zebron-5MS (cross-linked 5% phenylmethyl polysiloxane) column (ZB-5MS 30m x 0.25 mm x 0.25 μm). GC grade helium was used as carrier gas at a flow rate of 2 ml/min and splitless 1 ml injections were used. The injector, source and oven temperatures were set at 280°C, 120 and 70°C, respectively. The ramp settings were; 15°C/min to 120°C, then 10°C/min to 180°C, then 20°C/ min to 270°C and held for 3 min. The identification of the chemical constituents of the essential oil was determined by their GC retention time, and comparison of mass spectra obtained with those stored in the NIST11 library.

Result and Discussion

This study represents the first report of the chemical composition of the essential oils (EO) extracted from the dried leaves of *Phragmanthera capitata* parasitic on rubber tree. In this study, a solvent free microwave (no solvent) and Clevenger apparatus (distilled water and normal saline as solvents) extractors were used in the extraction of the essential oils. This is with an attempt to ascertain if extraction methods and/or solvents have an effect on the overall oil compositions.

The total number of compounds identified in all three essential oils in this study is shown in Table 1. The compounds that are common in all the essential oils are represented in Table 2 while Table 3 is the percentage compositions of the compounds common only to the oil extracted from the solvent-free microwave extractor. In Table 4, the compounds common only to the essential oils extracted by hydrodistillation (distilled water and normal saline) are shown. The percentage compositions of all the essential oils are in respect to a particular oil and so, in Tables 1, 2 and 4, the percentage area (% Area) may not be used to ascertain in terms of exact comparison/correlation, the amount or quantity of the compounds, but it speaks best to the amount of a particular oil composition. Fifty-six compounds (38 from the solvent-free microwave, 28 each from the hydrodistillation) were identified in all the oils as analysed by the GC-MS. Terpenes/terpenoids formed almost 50% of the total classified oils and the

monoterpenes/monoterpenoids constituted over 90% of the total EO (Zuzarte and Salgueiro, 2015). The mass spectra of all the compounds are represented in the GC-MS chromatograms in Figure 1.

Table 1: Characterization and composition of the essential oils extracted from *P. capitata* using Solvent-free microwave extractor (SFME) and hydrodistillation

SN	RT	Compound	SFME	Hydrodistillation		Class of compound	Molecular Weight	Formula
				DH_2O	NS			
			(% Area)					
1.	3.169	Heptane, 3,5-dimethyl-	1.44	2.54	2.12	Alkanes	128	C_9H_{20}
2.	3.297	2-Hexenal, (E)-	-	-	2.43	Aldehydes	98	$C_6H_{10}O$
3.	3.359	Heptane, 2,4-dimethyl-	4.47	9.36	6.70	Alkanes	128	C_9H_{20}
4.	3.408	Ethylbenzene	4.92	8.68	7.76	Benzene (monoterpernoid)	106	C_8H_{10}
5.	3.457	Xylene	11.15	19.36	17.22	Benzene	318	$C_{24}H_{30}$
6.	3.938	2-Nonanone, 9-[(tetrahydro-2H-pyra n-2-yl)oxy]-	2.35	-	-	NA	242	$C_{14}H_{26}O_3$
7.	4.160	Propanedinitrile, dicyclohexyl-	5.54	-	-	NA	230	$C_{15}H_{22} N_2$
8.	4.169	Benzaldehyde	-	1.59	2.94	Aldehyde	106	C_7H_6O
9.	4.222	Phenol	0.30	-	-	Phenols/benzenoids	94	C_6H_5OH
10.	4.371	Furan, 2-pentyl	-	1.02	0.92	Heteroaromatic	138	$C_9H_{14}O$
11.	4.357	2-Pentene, 3,4-dimethyl-, (E)-	0.80	-	-		98	C_7H_{14}
12.	4.530	2,4-Heptadienal, (E, E)-			0.75	Aldehydes	110	$C_7H_{10}O$
13.	4.687	Benzene, 1-methyl-3-(1-methylethyl)- β-Cymene	0.14	1.56	1.06	Monoterpene	134	$C_{10}H_{14}$
14.	4.726	D-Limonene	-	0.81	0.62	Monoterpene	136	$C_{10}H_{16}$
15.	4.844	Benzene acetaldehyde	-	-	1.98	Benzanoid	146	$C_{10}H_{10}O$
16.	5.068	Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-yl carbonate	0.32	1.25	1.74	NA	242	$C_{13}H_{22}O_4$
17.	5.206	2-methoxy Phenol (Guaiacol)	1.23	-	-	Methoxyphenols	124	$C_7H_8O_2$
18.	5.270	Nonanal	-	2.03	-	Monoterpanoid	142	$C_9H_{18}O$
19.	5.737	Para-ethylphenol	0.32	-	-	Monoterpanoid	122	$C_8H_{10}O$
20.	5.984	4'-Methylacetophenone	0.27	-	-	Monoterpanoid	134	$C_9H_{10}O$
21.	6.047	Naphthalene	3.10	17.36	12.61	Benzenoids	128	$C_{10}H_8$

22.	6.115	Safranal	-	1.32	1.50	Monoterpanoid	150	$C_{10}H_{14}O$
23.	6.122	o-Tolualdehyde	0.46	-	-	Monoterpanoid	120	C_8H_8O
24.	6.207	2-Allyl-4-methylphenol (2-Allyl-p-cresol)	-	0.85	0.99	Monoterpenoid	148	$C_{10}H_{12}O$
25.	6.415	Cumaldehyde	0.48	1.36	1.20	Monoterpenoid	148	$C_{10}H_{12}O$
26.	6.488	Acetaldehyde, (3,3-dimethylcyclohexylidene)-, (E)-	0.30	-	-	Monoterpenoid	152	$C_{10}H_{16}O$
27.	6.490	2-Cyclohexen-1-one, 5-methyl-2-(1-methylethyl)-	-	-	1.50	Monoterpenoid	152	$C_{10}H_{16}O$
28.	6.659	p-Ethylguaiacol	0.41	-	-	Monoterpanoid	152	$C_9H_{12}O_2$
29.	6.724	Tridecane	-	2.14	3.28	Alkanes (sesquiterpene)	184	$C_{13}H_{28}$
30.	6.762	Thiosemicarbazide, 4-(1-adamantylcarbonyl)-	-	-	1.09	NA	253	$C_{12}H_{19}N_3OS$
31.	6.863	Benzocycloheptatriene	-	0.61	0.55	Naphthalene	142	$C_{11}H_{10}$
32.	6.793	Indole	0.55	-	-	Indole	117	C_8H_7N
33.	6.913	2-Methoxy-4-vinylphenol	0.62	-	-	Methoxyphenol	150	$C_9H_{10}O_2$
34.	7.149	Phenol, 2,6-dimethoxy- (Syringol)	2.90	-	-	Methoxyphenol (Ether)	154	$C_8H_{10}O_3$
35.	7.265	1, 1, 5-Trimethyl-1, 2-dihydronaphthalene	0.91	-	-	Naphthalenes (Sesquiterpenoid)	172	$C_{13}H_{16}$
36.	7.405	2-Propenoic acid, 3-phenyl-, methyl ester Methyl cinnamate	0.57	-	-	Monoterpenoids	162	$C_{10}H_{10}O_2$
37.	7.523	2',3',4' Trimethoxyacetophenone	0.64	-	-	NA	210	$C_{11}H_{14}O_4$
38.	7.626	Naphthalene, 2,6-dimethyl-	0.23	1.19	-	Naphthalene	156	$C_{12}H_{12}$
39.	7.754	Naphthalene, 2,7-dimethyl-	-	2.95	-	Naphthalene	156	$C_{12}H_{12}$
40.	7.835	Isoeugenol	1.21	-		Monoterpenoids	164	$C_{10}H_{12}O_2$
41.	8.059	Pentadecane	1.03	2.68	2.08	Sesquiterpenoid	212	$C_{15}H_{32}$
42.	8.095	β-ionone	1.05	2.85	4.03	Sesquiterpenoid	192	$C_{13}H_{20}O$
43.	9.248	Pentadecane	2.03	-	-	Alkanes (sesquiterpenoid)	212	$C_{15}H_{32}$
44.	9.288	(4-Acetylphenyl) phenylmethane	-	1.25	-	Sesquiterpenoid	210	$C_{15}H_{14}O$
45.	9.353	Tetradecanal	3.58	3.80	5.91	Alkanes	212	$C_{14}H_{28}O$

						(sesquiterpenoid)		
46.	9.422	Acorenone 1		2.47	4.24	Sesquiterpenoid	220	$C_{15}H_{24}O$
47.	9.423	Methyl eudesmate	4.99	-	-	Ester	226	$C_{11}H_{14}O_5$
48.	9.789	Octadecane	0.21	-	-	Acyclic alkane	254	$C_{18}H_{38}$
49.	9.816	2-Iodoadamantane	0.50	1.05	1.30		262	$C_{10}H_{15}I$
50.	10.017	cis-Pinane	22.82	-	-	Monoterpenoids	138	$C_{10}H_{18}$
51.	10.052	(±)-Phytone	3.08	1.08	1.64	Sesquiterpenoid	268	$C_{18}H_{36}O$
52.	10.232	Diheptyl Phthalate	-	0.51	0.82	Ester	362	$C_{22}H_{34}O_4$
53.	10.349	α-Linolenic acid	6.73	7.69	11.00	Polyunsaturated fatty acids (Fatty Acyls)	278	$C_{18}H_{30}O_2$
54.	10.447	Methyl hexadecanoic acid	3.08	-	-	Esters	270	$C_{17}H_{34}O_2$
55.	10.468	Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)-	-	0.64	-	NA	281	$C_{13}H_{10}F_3N_3O$
56.	11.387	Phytol	5.31	-	-	Diterpenoid	296	$C_{20}H_{40}O$

RT: Retention time, DH_2O: Distilled water, NS: Normal Saline, NA: Not Available in library.

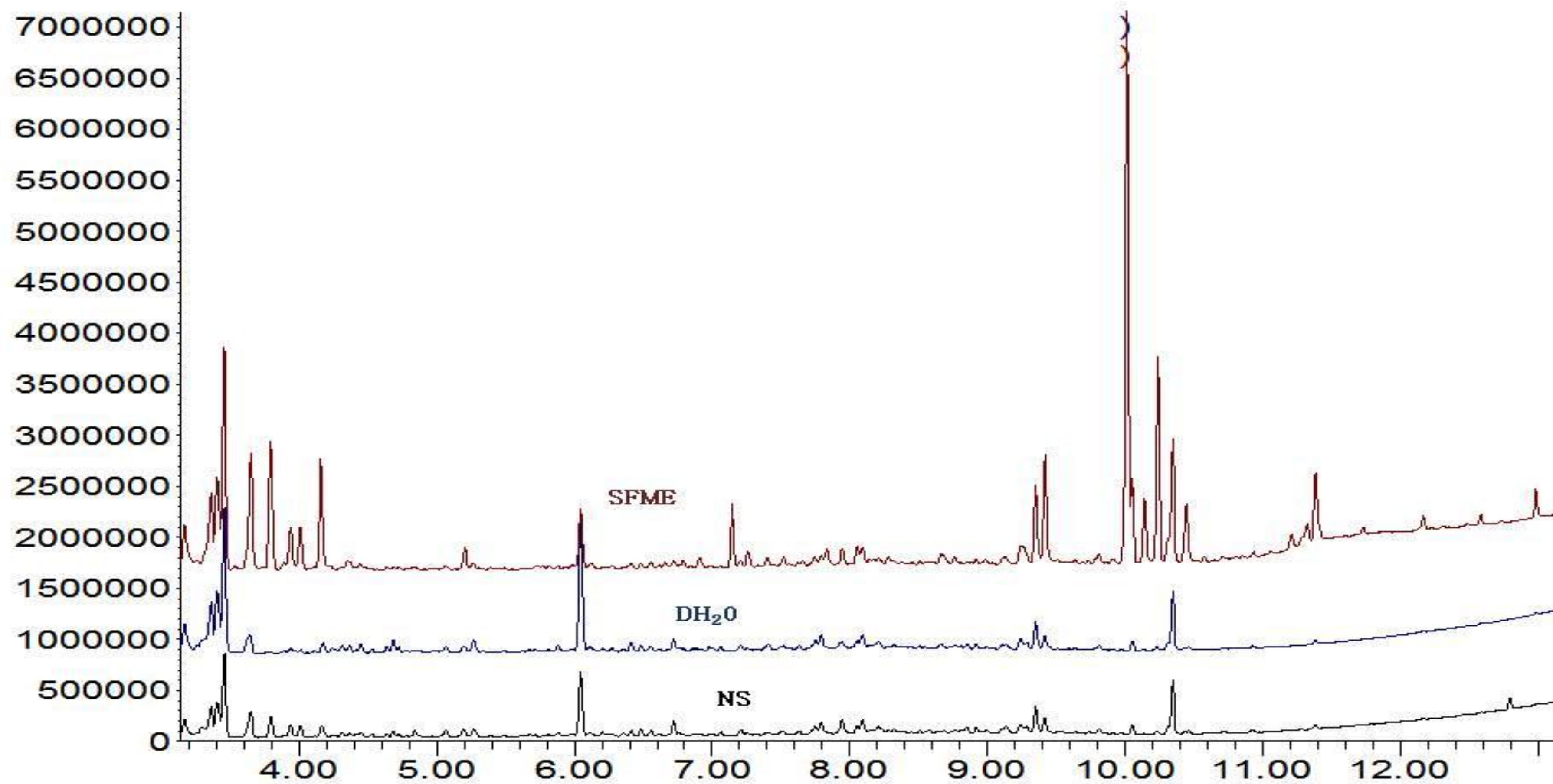


Figure 1: GC-MS total-ion chromatograms overlay of the essential oils of *P. capitata* extracted with the Solvent-Free Microwave Extractor (SFME) and hydrodistillation techniques using Distilled Water (DH_2O) and Normal Saline (NS) as solvents.

Fourteen compounds as shown in Table 2 were present in all three essential oils of the plant: Compounds extracted with the solvent-free microwave formed 41.97% while compounds extracted with the hydrodistillation formed 80.61% (distilled water) and 76.38% (normal saline).

Table 2: Compounds common to all the essential oils *of P. capitata*

SN	Compound	SFME	Hydrodistillation	
			DH_2O	NS
		(% Area)		
1.	Heptane, 3,5-dimethyl-	1.44	2.54	2.12
2.	Heptane, 2,4-dimethyl-	4.47	9.36	6.70
3.	Ethylbenzene	4.92	8.68	7.76
4.	Xylene	11.15	19.36	17.22
5.	Benzene, 1-methyl-3-(1-methylethyl)- β-Cymene	0.14	1.56	1.06
6.	Ethyl 2-(5-methyl-5-vinyltetrahydrofuran-2-yl)propan-2-yl carbonate	0.32	1.25	1.74
7.	Naphthalene	3.10	17.36	12.61
8.	Cumaldehyde	0.48	1.36	1.20
9.	Pentadecane	1.03	2.68	2.08
10.	β-ionone	1.05	2.85	4.03
11.	Tetradecanal	3.58	3.80	5.91
12.	2-Iodoadamantane	0.50	1.05	1.30
13.	2-Pentadecanone, 6,10,14-trimethyl (±)-Phytone	3.08	1.08	1.64
14.	α-Linolenic acid	6.73	7.69	11.00
	Total (%)	**41.97**	**80.61**	**76.38**

SFME: Solvent–Free Microwave Extraction, DH_2O: Distilled water, NS: Normal Saline

Among the compounds present in all the essential oils, Xylene was the most abundant with compositions of 11.15%, 19.36% and 17.22% for solvent-free, distilled water and normal saline respectively (Table 2 and Figure 2). It is an aromatic hydrocarbon mostly used in industry and medical laboratories as a solvent. It is found naturally in petroleum, coal, and wood tar. It is a colourless, flammable liquid, often used as a solvent in printing, paint, leather and rubber industries (Kandyala *et al.,* 2010). The high content of xylene and some other

compounds: Heptane, 3, 5-dimethyl-, Heptane, 2, 4-dimethyl- and Ethylbenzene, could have been influenced by the host species, *Hevea brasiliensis;* the world's natural source of rubber and also may be responsible for fire outbreak most especially in the dryer seasons in the tropics (Mathiasen *et al.,* 2008).

Alpha (α) linolenic acid is the second highest abundant compound common to all the EOs with compositions of 6.73%, 7.69% and 11.00% respectively for solvent-free, distilled water and normal saline (Table 2). It is a polyunsaturated omega-3-fatty acid found in fish and plant sources (vegetable oils, seeds and nuts). It is an essential fatty acid needed by humans but cannot be synthesised in the body and as such, must be acquired through diet. It is used for the treatment of cardiovascular diseases (coronary heart disease, ischemic heart disease, myocardial infarction, cardiac arrest, heart failure and stroke), reduces cholesterol levels and acts as an anti-inflammatory agent (Rajaram, 2014; Pan, et al 2012). It is also believed to have a reducing effect on hip bone fracture (Orchard *et al.,* 2013; Farina *et al.,* 2011). It plays a role in the reduction of type-2-diabetes with the suggestion that it improves insulin sensitivity (Wu *et al.,* 2012; Muramatsu *et al.,* 2010; Bloedon *et al.,* 2008). One of its notable mechanisms of action in combating cardiovascular diseases is by reduction of blood pressure and improving endothelial function (Rajaram *et al.,* 2014).

The compounds found only in the essential oil extracted with the solvent-free microwave (SFM) extractor are depicted in Table 3. Twenty-three compounds making 57.80% of the total oil from SFME was exclusive to it. Cis-Pinane (22.82%) (Figure 2) was the most abundant compound in all the three essential oils composition (Table 1). Its major use is as an ingredient of fragrance.

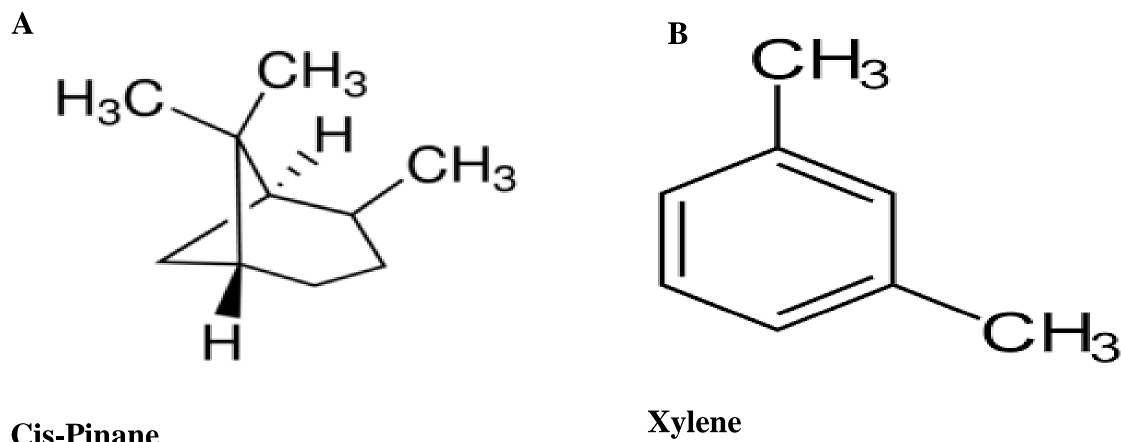
Table 3: Compounds exclusive to the essential oils extracted with the Solvent-Free Microwave Extractor

SN	Compound	% Area
1.	2-Nonanone, 9-[(tetrahydro-2H-pyra n-2-yl)oxy]-	2.35
2.	Propanedinitrile, dicyclohexyl-	5.54
3.	Phenol	0.30
4.	2-Pentene, 3,4-dimethyl-, (E)-	0.80
5.	2-methoxy Phenol (Guaiacol)	1.23
6.	Para-ethylphenol	0.32
7.	4'-Methylacetophenone	0.27
8.	o-Tolualdehyde	0.46
9.	Acetaldehyde, (3,3-dimethylcyclohexylidene)-, (E)-	0.30
10.	p-Ethylguaiacol	0.41
11.	Indole	0.55
12.	2-Methoxy-4-vinylphenol	0.62
13.	Phenol, 2,6-dimethoxy- (Syringol)	2.90
14.	1, 1, 5-Trimethyl-1, 2-dihydronaphthalene	0.91
15.	2-Propenoic acid, 3-phenyl-, methyl ester Methyl cinnamate	0.57
16.	2',3',4' Trimethoxyacetophenone	0.64
17.	Isoeugenol	1.21
18.	Pentadecane	2.03
19.	Benzoic acid, 3,4,5-trimethoxy-, methyl ester	4.99
20.	Octadecane	0.21
21.	cis-Pinane	22.82
22.	Methyl hexadecanoic acid	3.08
23.	Phytol	5.31
	Total (%)	**57.80**

Syringol and guaiacol with the percentage compositions of 1.23% and 2.90% respectively are closely related and derived from the pyrolysis of lignin. They are the components of wood and charcoal smoke. They form the main aroma of smoked and grilled foods. Syringol is responsible for the smoky aroma while guaiacol gives the taste (ACS, 2015). While syringol is

mainly a flavourant, guaiacol on the other hand, aside being a flavourant, is used medically as an expectorant, local anaesthetic, antiseptic (ACS, 2011) and for the treatment of pulmonary tuberculosis (Nammack and Tiber, 1937; Coghill, 1896). The presence of guaiacol could contribute to the anti-*Mycobacterium tuberculosis* activity observed in the crude extracts of *P. capitata* in chapter seven of this study. Isoeugenol is a monoterpenoid which is used as flavourant and storage agent in cosmetics and food products. It also possesses some medicinal properties as a prooxidant and antioxidant agent (Atsumi *et al.,* 2005). It acts as an anti-inflammatory agent by modulating ionomycin-induced cytokine release (Galbiati *et al.,* 2012). Different studies have shown its antioxidant activities by inhibiting the lipopolysaccharide-dependent production of nitric oxide and also have an anti-microbial activity (Sharifi-Rad *et al.,* 2015; Li *et al.,* 2006).

Another compound of interest with considerable percentage composition (3.08%) in the essential oil extracted from the solvent-free microwave is the Methyl hexadecanoic acid. It acts as an antioxidant, nematicide, hypocholesterolemic, pesticide, hemolytic-5-alpha reductase inhibitor (Rajeswari *et al.,* 2012). One compound which has elicited interest and extensively used in medicinal application is phytol. Its composition in the SFME was 5.31%. It is a diterpenoid with an inhibitory effect on *Staphylococcus aureus* (Inoue *et al.,* 2005); a causative agent of some skin infections, urinary tract infection, pneumonia, osteomyelitis and mastitis, etc. Phytol is an oxidant which reduces hydroxyl radical and nitric oxide production in the body (Santos *et al.,* 2013). It also has an anti-rheumatic/arthritis, anti-diuretic effects and it is known to be a good anti-cancer agent (Rajeswari *et al.,* 2012), it reduces cytokine production and oxidative stress (Silva *et al.,* 2013). Other compounds common only to the SFME in substantial amounts are; 2-Nonanone, 9-[(tetrahydro-2H-pyra n-2-yl) oxy] - (2.35%), Propanedinitrile, dicyclohexyl- (5.54%), Pentadecane (2.03%) and Benzoic acid, 3, 4, 5-trimethoxy-, methyl ester (4.99%).



Cis-Pinane **Xylene**

Figure 2: A**:** The compound with the highest composition (present only in the solvent-free microwave extraction) and B: compound with the second highest composition (present in all the essential oils of *P. capitata*)

The percentage composition of compounds common only to the essential oils (Distilled water and Normal saline) extracted using hydrodistillation are shown in Table 4. Cumulatively, 18 compounds were identified in the oils while nine compounds were found common in the oils.

Table 4: Compounds found only in the hydrodistillation extraction using distilled water (DH2O) and Normal saline (NS) as solvents

SN	Compound	DH_2O	NS
		% Area	
1.	2-Hexenal, (E)-		2.43
2.	Benzaldehyde	1.59	2.94
3.	Furan, 2-pentyl	1.02	0.92
4.	2,4-Heptadienal, (E, E)-		0.75
5.	D-Limonene	0.81	0.62
6.	Benzene acetaldehyde		1.98
7.	Nonanal	2.03	
8.	Safranal	1.32	1.50
9.	2-Allyl-4-methylphenol (2-Allyl-p-cresol)	0.85	0.99
10.	2-Cyclohexen-1-one, 5-methyl-2-(1 methylethyl)-		1.50
11.	Tridecane	2.14	3.28
12.	Thiosemicarbazide, 4-(1-adamantylcarbonyl)-		1.09
13.	Benzocycloheptatriene	0.61	0.55
14.	Naphthalene, 2,7-dimethyl-	2.95	
15.	(4-Acetylphenyl) phenylmethane	1.25	
16.	Acorenone 1	2.47	4.24

17.	Diheptyl Phthalate	0.51	0.82
18.	Pyridine-3-carboxamide, oxime, N-(2-trifluoromethylphenyl)-	0.64	
	Total (%)	**18.20**	**23.62**
	Number of compounds	**13**	**14**

Benzaldehyde with the percentage composition of 1.59% and 2.94% for distilled and normal saline respectively is the simplest representative of the aromatic aldehydes. It is a colourless liquid with a characteristic almond odour. It is used as a flavourant in food and scented products like cosmetics. It is a precursor to other organic compounds used in pharmaceuticals and plastic industries. (Anderson, 2006). It is also used in beekeeping as a bee repellent during honey harvest. (Evans and Butler, 2010). The percentage compositions of D-Limonene in the oils were 0.81% and 0.62% respectively for distilled and normal saline. It is a natural monoterpene which had been extensively studied for its chemotherapeutic and minimal toxicity activities. Its wide application has been in cancer therapy. It has shown great anti-cancer activity in *in-vitro, in-vivo* and clinical trials. It has also shown promise in gallstone dissolution and Heartburn relief (Sun, 2007; Vigushin *et al.,* 1998; Elegbede *et al.,* 1984). The composition of Acorenone 1 was 2.47% and 4.24% in the distilled water and normal saline solvents respectively. This compound has not been extensively studied and hence, little is known about its medicinal application but the report of Bader *et al.* (2003) mentioned its activity against *Bacillus subtilis* and cytotoxic effect on monkey kidney cell-line.

Safranal with the composition of 1.32% and 1.50% in the distilled and normal saline solvents, is famous for its saffron odour and flavour. It has been used as an antimicrobial agent. It has a protective effect against indomethin-induced gastric ulcers and effective against specific cancer cell-lines. Its effects on the Central Nervous System have been widely investigated. It has shown anticonvulsive, antidepressant, antianxiety and hypnotic effects. It also has shown anti-ischemia and antihypertensive effect (Rezaee and Hosseinzadeh, 2013). Other compounds

common to both distilled and normal saline respectively are Benzaldehyde 1.59% and 2.94%, Furan, 2-pentyl 1.02% and 0.92%, Tridecane 2.14% and 3.28%, Benzocycloheptatriene 0.61% and 0.55% and Diheptyl Phthalate 0.51% and 0.82%.

Conclusion

This study being the first account of the essential oils extracted from *Phragmanthera capitata,* was able to reveal some compounds with reported biological activities and industrial usage. In recent days, mistletoe study is geared towards cancer research in Europe and extensively used as an antihypertensive agent in traditional medicine in Africa. The presence of some of the compounds in the essential oils of this plant could contribute to its wide reported ethnopharmacological applications; most especially in cancer and hypertension management.

This study also revealed that different extraction methods and solvents could influence the composition of essential oils of *P. capitata.*

References

ACS (American Chemical Society), 2011. Molecule of the week, Guaiacol. Available at: https://www.acs.org/content/acs/en/molecule-of-the-week/archive/g/molecule-of-the-week-guaiacol.html. June 20, 2011.

ACS (American Chemical Society), 2015. Molecule of the week, Syringol. Available at: https://www.acs.org/content/acs/en/molecule-of-the-week/archive/s/syringol.html. August 31, 2015.

Ajayi., E.O., Sadimenko, A.P. Afolayan, A.J., 2016. GC–MS evaluation of *Cymbopogon citratus* (DC) Stapf oil obtained using modified hydrodistillation and microwave extraction methods. Food Chemistry 209, 262–266.

Andersen, A., 2006. Final report on the safety assessment of benzaldehyde. International Journal of Toxicology 25 Suppl 1, 11–27. Doi: 10.1080/10915810600716612. PMID 16835129.

Atsumi, T., Fujisawa, S. and Tonosaki, K.A., 2005. Comparative study of the antioxidant / prooxidant activities of eugenol and isoeugenol with various concentrations and oxidation conditions. Toxicology in Vitro 19(8), 1025–1033. Doi.org/10.1016/j.tiv.2005.04.012.

Bader, A., Caponi, C., Cioni, P.L., Flamini, G., Morelli, I., 2003. Acorenone in the essential oil of flowering aerial parts of *Seseli tortuosum* L. Flavour and Fragrance Journal 18, 57–58. Doi: 10.1002/ffj.1154.

Baser, K.H.C., Buchbauer, G., 2010. Handbook of Essential Oils: Science, Technology and Applications. CRC Press, Boca Raton, London, New York.

Bloedon, L.T., Balikai, S., Chittams, J., Cunnane, S.C., Berlin, J.A., Rader, D.J., Szapary, P.O., 2008. Flaxseed and cardiovascular risk factors: results from a double blind, randomized, controlled clinical trial. Journal of the American College of Nutrition 27, 65–74. PMID: 18460483.

Celiktas, O.Y., Kocabas, E., Bedir, E., Sukan, F.V., Ozek, T., Baser, H.C., 2007. Antimicrobial activities of methanol extracts and essential oils of *Rosmarinus officinalis*, depending on location and seasonal variations, Food Chemistry 100 (2), 553–559.

Coghill, S.J.G., 1896. The Hypodermic Use of Guaiacol in Acute Pulmonary Tuberculosis. British Medical Journal 1(1836), 586-590.

Elegbede, J.A., Elson, C.E., Qureshi, A., Tanner, M.A., Gould, M.N., 1984. Inhibition of DMBA-induced mammary cancer by the monoterpene d-limonene. Carcinogenesis 5 (5), 661-664. Doi.:10.1093/carcin/5.5.661.

Evans, E., Butler, C., 2010. Why Do Bees Buzz? Fascinating Answers to Questions about Bees. Rutgers University Press. pp. 177–178.

Farina, E.K., Kiel, D.P., Roubenoff, R., Schaefer, E.J., Cuppies, L.A., Tucker, K.L., 2011. Dietary intakes of arachidonic acid and alpha-linolenic acid are associated with reduced risk of hip fracture in older adults. Journal of Nutrition 141, 1146–53.

Galbiati, V., Carne, A., Mitjans, M., Galli, C.L., Marinovich, M., Corsini, E., 2012. Isoeugenol destabilizes IL-8 mRNA expression in THP-1 cells through induction of the negative regulator of mRNA stability tristetraprolin. Archives of Toxicology 86(2), 239-48. Doi: 10.1007/s00204-011-0758-2.

Gautam, N., Mantha, A.K., Mittal, S., 2014. Essential Oils and Their Constituents as Anticancer Agents: A Mechanistic View. BioMed Research International. Article ID 154106, 23 pages Doi:10.1155/2014/154106.

Hamid, A.A., Aiyelaagbe, O.O., Usman, L.A., 2011. Essential oils: its medicinal and pharmacological uses. International Journal of Current Research 3 (2), 086-098.

Inoue, Y., Hada, T., Shiraishi, A., Hirose, K., Hamashima, H., Kobayashi, S., 2005. Biphasic effects of geranylgeraniol, teprenone, and phytol on the growth of *Staphylococcus aureus*. Antimicrobial Agents and Chemotherapy 49(5), 1770–1774. Doi: 10.1128/AAC.49.5.1770-1774.2005.

Kandyala, R., Raghavendra, S.P.C., Rajasekharan, S.T., 2010. Xylene: An overview of its health hazards and preventive measures. Journal of Oral and Maxillofacial Pathology 14(1), 1–5. Doi: 10.4103/0973-029X.64299.

Lawal, O.A., Ogunwande, I.A., 2013. Essential Oils from the Medicinal Plants of Africa. In book: Medicinal Plant Research in Africa. Pp 203-224. DOI: 10.1016/B978-0-12-405927-6.00005-9.

Lee, M.S., Choi, J., Posadzki, P., Ernst, E., 2012. Aromatherapy for healthcare: An overview of systematic reviews. Maturitas. 71 (3), 257–260. PMID 22285469.

Li, W., Tsubouchi, R., Qiao, S., Haneda, M., Murakami, K., Yoshino, M., 2006. Inhibitory action of eugenol compounds on the production of nitric oxide in RAW264.7 macrophages. Biomedical Research 27(2), 69-74. PMID: 16707845.

Mathiasen, R.L., Nickrent, D.L., Shaw, D.C. and Watson, D.M. (2008). Mistletoes: Pathology, Systematics, Ecology, and Management. Plant. Dis. 92(7):988-1006. DOI: 10.1094/PDIS-92-7-0988.

Muramatsu, T., Yatsuya, H., Toyoshima, H., Sasaki, S., Li, Y., Otsuka, R., Wada, K., Hotta, Y., Mitsuhashi, H., Matsushita, K., Murohara, T., Tamakoshi, K., 2010. Higher dietary intake of alpha-linolenic acid is associated with lower insulin resistance in middle-aged Japanese. Preventive Medicine 50, 272–6. Doi: 10.1016/j.ypmed.2010.02.014

Nammack, C.H., Tiber, A.M., 1937. The treatment of lung abscess by means of guaiacol intravenously: an analysis of twenty cases. Journal of the American Medical Association 109(5), 330-336. DOI: 10.1001/jama.1937.02780310008004

Nwiloh, B.I., Monago, C.C., Uwakwe, A.A., 2014. Chemical composition of essential oil from the fiddleheads of *Pteridium aquilinum* L. Kuhn found in Ogoni. Journal of Medicinal Plants Research 8(1), 77-80. Doi: 10.5897/JMPR2013.5093.

Orchard, T.S., Ing, S.W., Lu, B., Belury, M.A., Johnson, K., Wactawski-Wende, J., Jackson, R.D., 2013. The association of red blood cell n-3 and n-6 fatty acids with bone mineral density and hip fracture risk in the women's health initiative. Journal of Bone and Mineral Research 28, 505–15.

Pan, A., Chen, M., Chowdhury, R., Wu, J.H., Sun, Q., Campos, H., Mozaffarian, D., Hu, F.B., 2012. A-Linolenic acid and risk of cardiovascular disease: a systematic review and meta-analysis. The American Journal of Clinical Nutrition 96, 1262–73

Rajaram, S., 2014. Health benefits of plant-derived α-linolenic acid. The American Journal of Clinical Nutrition 100 (1), 443S-8S. Doi: 10.3945/ajcn.113.071514.

Rajeswari, G., Murugan, M., Mohan, V.R., 2012. GC-MS analysis of bioactive components of *Hugonia mystax* L. (Linaceae). Research Journal of Pharmaceutical, Biological and Chemical Sciences 3 (4), 301.

Rezaee, R., Hosseinzadeh, H., 2013. Safranal: From an Aromatic Natural Product to a Rewarding Pharmacological Agent. Iranian Journal of Basic Medical Sciences 6(1), 12–26. PMCID: PMC3637901.

Santos, C.C., Salvadori, M.S., Mota, V.G., Costa, L.M., de Almeida, A.A., de Oliveira, G.A., Costa, J.P., de Sousa, D.P., de Freitas, R.M., de Almeida, R.N., 2013. Antinociceptive and Antioxidant Activities of Phytol In Vivo and In Vitro Models. Neuroscience - Journal Article ID 949452, 9 pages. Doi:10.1155/2013/949452.

Sharifi-Rad, J., Hoseini-Alfatemi, S.M., Sharifi-Rad, M., Sharifi-Rad, M., riti, M., Sharifi-Rad, M., Sharifi-Rad, R., Raeisi, S., 2015. Phytochemical Compositions and Biological Activities of Essential Oil from *Xanthium strumarium* L. Molecules. 20, 7034-7047. Doi: 10.3390/molecules20047034.

Silva, R.O., Sousa, F.B., Damasceno, S.R.B., Carvalho, N.S., Silva, V.G., Oliveira, F.R., Sousa, D.P., Aragão, K.S., Barbosa, A.L., Freitas, R.M., Medeiros, J.V., 2013. Phytol, a diterpene alcohol, inhibits the inflammatory response by reducing cytokine production and oxidative stress. Fundamental and Clinical Pharmacology. Doi: 10.1111/fcp.12049.

Sun, J., 2007. D-Limonene: Safety and Clinical Applications. Alternative medicine review 12(3), 259-264. PMID: 18072821.

Vigushin, D.M., Poon, G.K., Boddy, A., English, J., Halbert, G.W., Pagonis, C., Jarman, M., Coombes, C.R., 1998. Cancer Research Campaign Phase I/II Clinical Trials Committee. Phase I and pharmacokinetic study of d-limonene in patients with advanced cancer. Cancer Chemotherapy and Pharmacology 42(2), 111–117. Doi: 10.1007/s002800050793.

Wu, J.H., Micha, R., Imamura, F., Pan, A., Biggs, M.L., Aiaz, O., Diousse, L., Hu, F.B., Mozaffarian, D., 2012. Omega-3 fatty acids and incident type 2 diabetes: a systematic review and meta-analysis. British Journal of Nutrition 107, S214–27. Doi: 10.1017/S0007114512001602.

Zuzarte, M., Salgueiro, L.M., 2015. Essential Oils Chemistry. In: Bioactive Essential Oils and Cancer. D. P. de Sousa (Ed.). Springer International Publishing, 15, 292p. Doi: 10.1007/978-3-319-19144-7_2.

CHAPTER FIVE

TOXICITY ASSESSMENT OF DIFFERENT SOLVENT EXTRACTS OF *PHRAGMANTHERA CAPITATA* ON BRINE SHRIMP (*ARTEMIA SALINA*)

This chapter has been published in the International Journal of Pharmacology

CHAPTER FIVE

TOXICITY ASSESSMENT OF DIFFERENT SOLVENT EXTRACTS OF *PHRAGMANTHERA CAPITATA* ON BRINE SHRIMP (*ARTEMIA SALINA*)

CONTENTS **Pages**

Abstract 85
Introduction 86
Materials and methods 86
Location and collection of sample 86
  Extraction procedure 86
  Preparation of the assay 87
  Artemia salina hatching assay 87
  Artemia salina lethality assay 87
  Data analysis 87
Result 87
  Brine shrimp hatchability assay 87
  Brine shrimp lethality assay (BSLA) 90
Discussion 92
Conclusion 93
References 94

OPEN ACCESS International Journal of Pharmacology

ISSN 1811-7775
DOI: 10.3923/ijp.2016.701.710

Research Article

Toxicity Assessment of Different Solvent Extracts of the Medicinal Plant, *Phragmanthera capitata* (Sprengel) Balle on Brine Shrimp (*Artemia salina*)

Franklin Uangbaoje Ohikhena, Olubunmi Abosede Wintola and Anthony Jide Afolayan

Medicinal Plants and Economic Development Research Center, Department of Botany, University of Fort Hare, Alice, Eastern Cape, South Africa

Abstract

Background: There has been a surge in the use of medicinal plants in the past decades; hence, the screening of potential cytotoxic compounds is of utmost importance to guarantee their safe use in alternative medicine. Different solvent extracts of the leaves of *Phragmanthera capitata* (Sprengel) Balle, a parasitic mistletoe growing on rubber tree were evaluated for possible toxicity. **Methodology:** Brine shrimp (*Artemia salina*) hatchability (based on the Minimum Inhibitory Concentration (MIC_{50}) of the extracts to inhibit 50% hatching of the cysts) and lethality (based on the Lethal Concentration (LC_{50}) of the extracts to kill 50% of the hatched cysts (nauplii)) assays were employed to evaluate the possible toxicity of the species. **Results:** Lowest hatching percentage was recorded in the ethanolic extract (34.40%) with an MIC_{50} value of 0.14 mg mL^{-1} and the highest hatching success was observed in the aqueous extract (59.33%) with an MIC_{50} value of 0.59 mg mL^{-1}. All the extracts hatching success were significantly higher than the positive control (Amoxicillin) ($p<0.05$). The LC_{50} for the lethality assay in all the solvent extracts was greater than 1 mg mL^{-1}. **Conclusion:** Based on Meyer's toxicity index, $LC_{50}>1$ mg mL^{-1} were considered non-toxic hence, all the solvent extracts tested showed that they were not toxic and can be further explored for the development of plant-based pharmaceuticals drugs. Further *in vivo* and cell lines cytotoxicity test is recommended to substantiate these findings.

Key words: *Phragmanthera capitata*, toxicity, brine shrimp, hatchability, lethality, *Artemia salina*, nauplii, cyst, extracts

Received: May 21, 2016 Accepted: July 19, 2016 Published: September 15, 2016

Citation: Franklin Uangbaoje Ohikhena, Olubunmi Abosede Wintola and Anthony Jide Afolayan, 2016. Toxicity assessment of different solvent extracts of the medicinal plant, *Phragmanthera capitata* (sprengel) balle on brine shrimp (*Artemia salina*). Int. J. Pharmacol., 12: 701-710.

Corresponding Author: Anthony Jide Afolayan, Medicinal Plants and Economic Development Research Center, Department of Botany, University of Fort Hare, Alice, Eastern Cape, South Africa

Copyright: © 2016 Franklin Uangbaoje Ohikhena *et al*. This is an open access article distributed under the terms of the creative commons attribution License, which permits unrestricted use, distribution and reproduction in any medium, provided the original author and source are credited.

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

There has been a surge in public interest in herbal therapies both in developing and developed countries with herbal remedies being available not only in drug stores, but now also in food stores and supermarkets. It is estimated that upto four billion people (representing 80% of the world's population) living in the developing world rely on herbal medicinal products as a primary source of healthcare. Medicinal plants have provided a source of inspiration for novel therapeutic drugs, as plant derived medicines have immensely contributed to the health and well being of humans[1]. The medicinal value of plants is chiefly due to the phytochemicals like alkaloids, essential oils, tannins, resins and many others that are present in them which produce a physiological action on the human body[2].

In spite of the positive perception of patients on the use of herbal medicines, their alleged satisfaction with therapeutic outcomes as well as their disappointment with conventional allopathic or orthodox medicines in terms of effectiveness and/or safety, the problem of safety of herbal remedies continues to remain a major issue of concern. The general perception that herbal remedies or drugs are very safe and devoid of adverse effects is not only untrue, but also misleading. Herbs have been reported to produce a wide range of undesirable or adverse reactions some of which could cause serious injuries, abortion of pregnancy, dizziness, vomiting, diarrhoea, abdominal pain, fast heart beat, ulcer, loss of appetite, life-threatening conditions and even death[1].

Much of the information on medicinal herbs made available to consumers is not backed by credible scientific data. For this reason, study is carried out to determine the toxicity of medicinal plants[3] and in recommendation from the World Health Organization (WHO)[4], in order to reduce adverse effects from the consumption of herbal medicines, there is need for a thorough scientific validation on the toxicity of these plants.

There had been a number of toxicity tests in which the responses have been measured in invertebrates. These tests have the advantages of being cost effective, reproducible, easy to experiment and environmentally relevant[5]. A general bioassay that appears capable of detecting a broad spectrum of bioactivity present in plant crude extracts is the brine shrimp (*Artemia salina*) lethality assay. Brine shrimp is used as an indicator for the detection of anti-tumor, fungi toxins, plant extract toxicity, heavy metals, cyanobacteria toxins, pesticidal compounds and cytotoxicity of dental materials[2,6]. This assay has also been noted as a useful tool for the isolation of bioactive compounds from plants extracts[7]. Most study is based on Brine Shrimp Lethality Assay (BSLA) on the hatched cysts (nauplii) for toxicity analysis of natural products but a few other researchers have used the assay on the inhibition of hatching of the cysts[5,6,8].

Phragmanthera capitata (Spreng) Balle is an obligate hemi-parasitic plant which attaches to and penetrates the stems and branches of its host tree or shrub by a structure called the haustorium. It is a mistletoe in the Loranthaceae family. It is a woody parasitic shrub with pendent branches of about 2 m long. It has a yellow corolla with a pink-red tip and often associated with ant's nests. It grows on trees in secondary jungles, plantations and bush savannah areas; from Sierra Leone to Western Cameroons, Fernando Po and extending across the Congo basin to Zaïre, Nigeria, Gabon, Ivory Coast and Angola[9,10]. As with most mistletoe, it is a medicinal plant utilized in the treatment of a wide range of ailments across Africa and the world at large. The leaves, twig (stem) and/or combination of both parts have been used in different preparations and doses in folklore medicine to treat diverse ailments ranging from insomnia, diabetes, hypertension, infertility, gastrointestinal disorders, anxiety, bacteria/fungi infections, arthritis, epilepsy, cancer, etc.[10,11]. However, based on literatures, there had been no report on the possible toxicity of *P. capitata* despite its numerous folkloric applications.

From the foregoing therefore, the objectives of this study was to evaluate the potential toxicity of the acetone, methanol, ethanol and aqueous extracts of *P. capitata* using the brine shrimp hatchability and lethality assays (BSH and BSLA) in order to ascertain/validate its numerous ethno-pharmacological safe use and provide data base for the preliminary toxicity of *P. capitata* growing on rubber tree.

MATERIALS AND METHODS

Location and collection of sample: Leaves of mistletoe were collected from mature rubber plantations in Rubber Research Institute of Nigeria, Iyanomo, located on latitude 6°00'-6°15' N; longitude 5°30'-5°45'E and on altitude 27 ma.s.l., in Benin City, which lies on the wet lowland rainforest of Edo State, Nigeria.

Extraction procedure: Leaves were removed from the twigs, gently rinsed to remove dust and dirt, air-dried at room temperature (mean morning and night temperature of 24°C and mean noon temperature of 27°C) in a well aerated atmosphere and prevented from direct sunlight to avoid denaturation of vital phyto-constituents. Dried leaves were pulverized. The ground sample was put into separate conical

flasks containing acetone, methanol, ethanol and water, shaken in an orbital shaker (Orbital Incubator Shaker, Gallenkamp) for 24 h. The crude extracts were filtered using a Buchner funnel and Whatman No. 1 filter paper. The acetone, methanol and ethanol extracts were further concentrated to dryness to remove the solvents under reduced pressure using a rotary evaporator (Strike 202 Steroglass, Italy) while the aqueous filtrate obtained was concentrated using a freeze dryer (Vir Tis benchtop K, Vir Tis Co., Gardiner, NY). The acetone, methanol and ethanol extracts were reconstituted in their parent solvents to yield a 100 mg mL^{-1} stock solution while the aqueous extract was directly prepared in filtered sea water.

Preparation of the assay: The method described by Kibiti and Afolayan[12] was employed with little modifications. Five petri dishes containing 30 mL of the extracts were prepared in filtered sea water by first dissolving them in an infinitesimal amount of the parent solvents to yield a two-fold dilution series of concentrations (1, 0.5, 0.25, 0.125 and 0.0625 mg mL^{-1}). A positive control was also prepared by dissolving amoxicillin in sea water in the same concentrations as the plant extracts. Sea water only served as the negative control. The set up was allowed to stand for 30 min to allow the solvents to evaporate.

***Artemia salina* hatching assay:** This assay was evaluated as described by Kibiti and Afolayan[12]. A density of ten *A. salina* cysts was stocked in each of the petri dishes containing 30 mL of the prepared two-fold concentrations (1-0.0625 mg mL^{-1}) of the plant fractions and positive control. The petri dishes were partly covered, incubated at 30°C and under constant illumination for 72 h. The number of free nauplii in each petri dish was counted after every 12 h till end of 72 h. The percentage of hatchability was assessed by comparing the number of hatched nauplii with the total number of cysts stocked.

***Artemia salina* lethality assay:** *Artemia salina* cysts were hatched in sea water and 10 nauplii were pipetted into each petri dish containing the two-fold concentrations of the extracts and control as in the hatchability above. The petri dishes were then examined and the number of living nauplii (that exhibited movement during several seconds of observation) was counted after every 12 h and the set up was allowed to stand for 72 h under constant illumination. The percentage of mortality was calculated as:

$$\text{Mortality (\%)} = \frac{\text{Total nauplii-Alive nauplii}}{\text{Total nauplii}} \times 100$$

Data analysis: The percentage hatchability success and mortality data obtained from the 5 different concentrations of each fraction and control experiments were used to construct the dose-response curves. These were used to determine their corresponding MIC_{50} and LC_{50} values. The minimum inhibitory concentration 50 (MIC_{50}) was determined as the concentration of the plant extract/control drug that inhibited hatching of 50% of the cysts. The LC_{50} was taken as the concentration required for producing 50% mortality of the nauplii. The MIC_{50} and LC_{50} values were determined from the best-fit line obtained by regression analysis of the percentage hatchability and lethality versus the concentration. The statistical analysis was done on GENSTAT 8. A two-way analysis of variance (ANOVA) followed by Fischer's least significant different (for means separation) was used to test the effect of concentration and time of exposure of the plant extracts on the hatchability success of the cysts and mortality of the larvae, respectively.

RESULTS

Brine shrimp hatchability assay: The hatching success of *A. salina* incubated with different plant extracts and control is as shown in Fig. 1 with the aqueous extract having a significantly higher hatching success (59.33%) than the rest of the solvent extracts including both the positive control (amoxicillin) (23.47%) and sea water (46.67%) ($p<0.05$). The hatching success of the cysts in the acetone (37.87%) and methanol (35.47%) extracts showed non-significant difference from each other but the acetonic extract was significantly higher than the ethanolic extract (34.40%) which had non-significant difference from the methanol extract ($p>0.05$).

The effect of different solvent concentrations on the hatching success of the cyst was also evaluated and the result is depicted in Fig. 2a and b. Figure 2a shows the activities of the different plant extracts/positive control at varying concentrations to the hatching success of the cysts. The percentage hatching success of cysts incubated with the acetone extract showed significant differences at varying concentrations. The lowest concentration (0.0625 mg mL^{-1}) had the highest hatching percentage (67.33%) and it was not significantly different from the cysts incubated at 0.25 mg mL^{-1} with a hatching success of 59.33%. There was zero percent (0%) hatchability at the highest

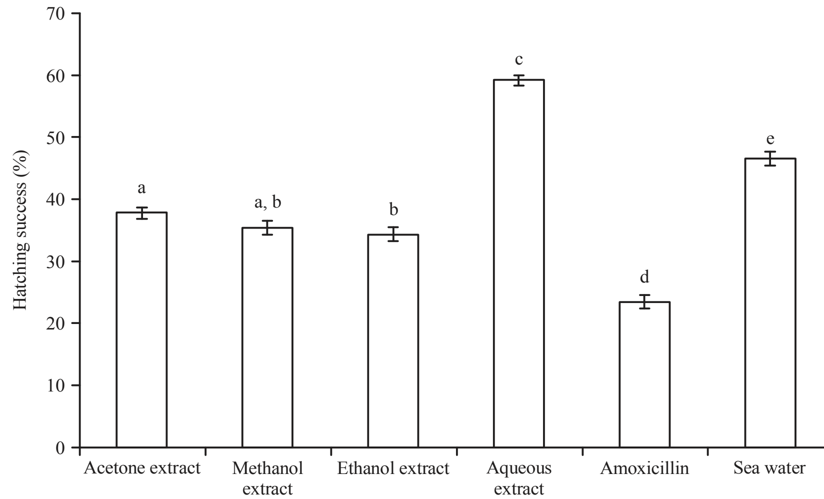


Fig. 1: Percentage hatching success of *Artemia salina* cysts incubated in different solvent extracts and controls. The values are means of five concentrations for each plant extract/control±SD of three replicates. Bars with different letters are significantly different ($p<0.05$)

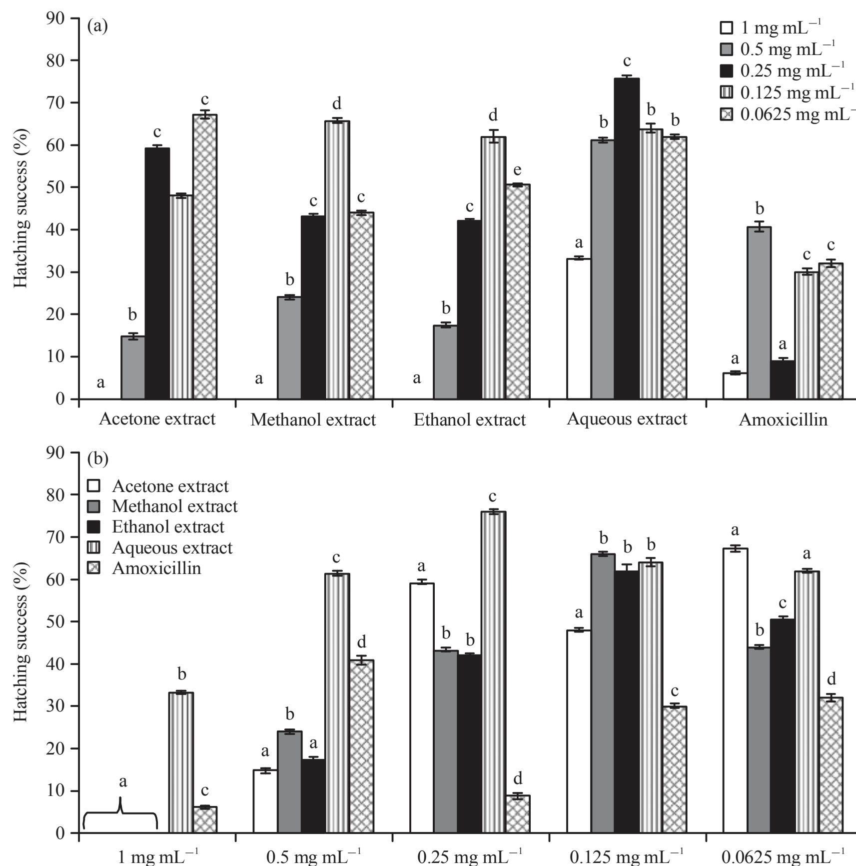


Fig. 2(a-b): Percentage hatching success of *Artemia salina* cysts incubated in different concentrations of the plant extracts and control. The values are means of the replicates (at different hours) for the concentrations for each plant extract/control±SD of three replicates. Set of bars with different letters is significantly different ($p<0.05$), (a) Set of bars represents the effect of the solvent extracts/positive control at varying concentrations on the hatching success of the cysts and (b) Set of bars represents the effect of the solvent extracts/positive control to a particular concentration on the hatching success of the cysts

concentration of 1 mg mL^{-1}. The methanol, ethanol and aqueous extracts had significant higher hatching percentage of the cysts at 0.125 mg mL^{-1} (66, 62 and 64%, respectively). There was also 0% hatchability at the highest concentration of 1 mg mL^{-1} in the methanol and ethanolic extracts. While, there was non-significant difference at 0.25 and 0.0625 mg mL^{-1} in the methanolic extract but the same was not true in the ethanolic extract that showed significant difference at all concentrations tested ($p<0.05$). The aqueous extract had non-significant difference at 0.5, 0.125 and 0.0625 mg mL^{-1}. There was a dose-dependent response in the positive control with an anomalous significantly higher hatchability at 0.5 mg mL^{-1}.

Figure 2b is an expression of the percentage hatchability of the solvent extracts in response to particular concentrations. At 1 mg mL^{-1}, acetone, methanol and ethanol extracts had 0% hatchability while the aqueous extract had a significant higher percentage hatchability of 33.33%, all the extracts were significantly different from the positive control. The aqueous extract also had significant higher hatching success (61.33 and 76%, respectively) at 0.5 and 0.25 mg mL^{-1}. There was non-significant hatchability success between acetone and ethanol extracts at 0.5 mg mL^{-1} while at 0.25 mg mL^{-1}, methanol and ethanol was not different significantly from each other. At 0.125 mg mL^{-1}, methanol, ethanol and aqueous extracts showed non-significant difference and at 0.0625 mg mL^{-1}, acetone and aqueous extracts were not significantly different from each other ($p<0.05$).

The effect of exposure time on the hatching success on *A. salina* is shown in Fig. 3a and b. Figure 3a showed the response of the cysts in each solvent extract to varying time of exposure. The same trend was observed in all the extracts and controls tested. There was a lower significant hatching success at 24 h in all the extracts and controls. There were no

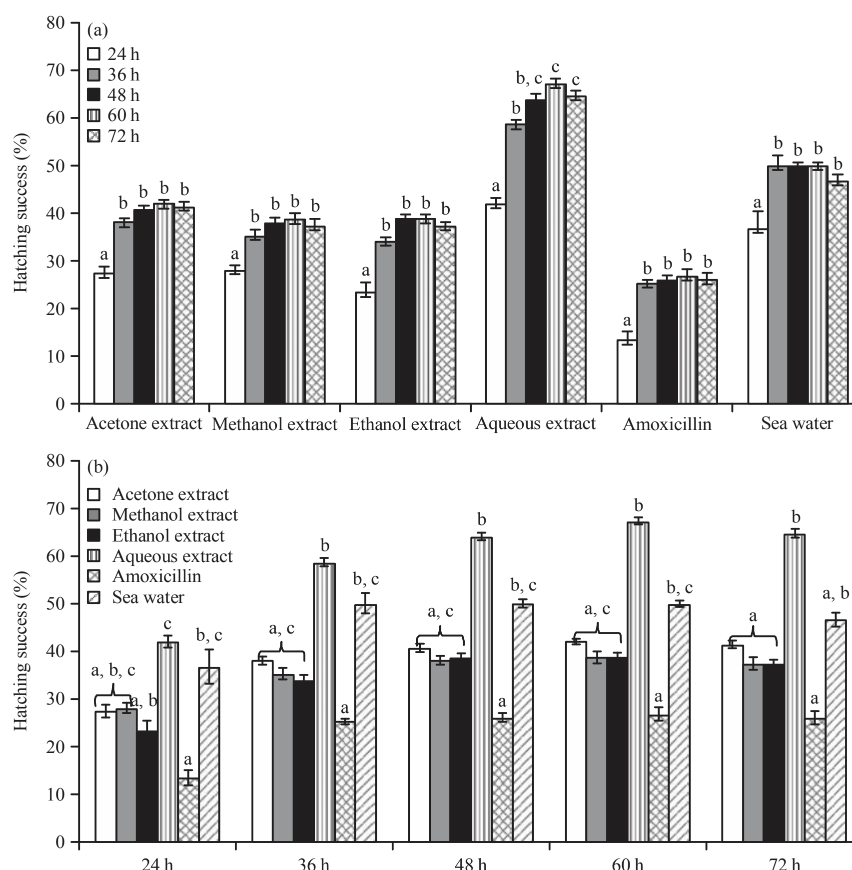


Fig. 3(a-b): Percentage hatching success of *Artemia salina* cysts incubated at different durations in the plant extracts/controls. The values are means of replicates (of all the concentrations) for each plant extract/control±SD of three replicates. Set of bars with different letters are significantly different ($p<0.05$), (a) Set of bars represents the effect of the solvent extracts/positive control at varying time of exposure on the hatching success of the cysts and (b) Set of bars represents the effect of the solvent extracts/positive control to a particular time of exposure on the hatching success of the cysts

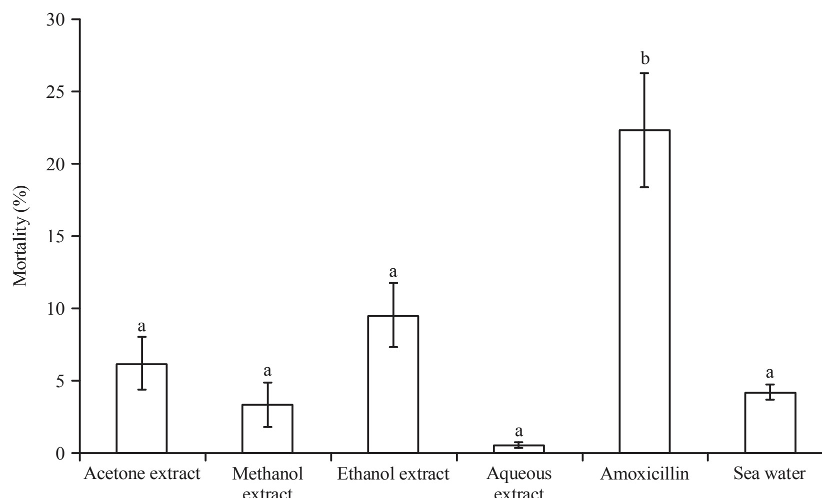


Fig. 4: Percentage mortality of *Artemia salina* nauplii incubated in different plant extracts and controls. Means are values of five concentrations for each plant fraction/control±SD of three replicates. Bars with different letters are significantly different (p<0.05)

Table 1: Hatchability and Lethality of *Artemia salina* in different plant extracts as shown by their MIC_{50} and LC_{50} values, respectively

	Hatchability assay		Lethality assay	
	MIC_{50} (mg mL^{-1})	R^2	LC_{50} (mg mL^{-1})	R^2
Acetone extract	0.15	0.8314	>1	0.9398
Methanol extract	0.21	0.9985	>1	0.9398
Ethanol extract	0.14	0.8995	>1	0.9778
Aqueous extract	0.59	0.8325	>1	0.8068
Amoxicillin	<0.0625	0.7971	0.89	0.9604

MIC_{50} and LC_{50} are defined as the concentration (mg mL^{-1}) of the plant extracts and positive control (Amoxicillin) sufficient to obtain 50% of hatching inhibition of the cysts and nauplii mortality of *Artemia salina*, respectively. The R^2 is the coefficient of determination from the regression equation

significant differences in hatching success from 36-72 h in most of the extracts (p>0.05) and controls except in the aqueous extract where there was a lower significant difference at 36 h from both 60 and 72 h (p<0.05).

In Fig. 3b, the response of the cyst in different solvent extract to a particular time exposure was shown. The aqueous extract had a higher hatching success at all levels of exposure but was not significantly different from sea water at all levels of exposure (p>0.05). The acetone, methanol and ethanol extracts showed non-significant difference from one another at all levels of exposure (p>0.05). Though the test drug had the lowest hatching success in all the levels of exposure time, the same was not significantly different from the acetone, methanol and ethanol extracts (p>0.05). The test drug was significantly lower than the aqueous extract and sea water at all the levels of exposure to time (p<0.05) except at 72 h where it was not significantly different from the sea water hatching success (p>0.05).

The inhibitory effects of the different solvent extracts and positive control on the hatchability success were expressed as MIC_{50} (Table 1) which represents the potential of the extracts to inhibit hatching of the cysts by 50% (that is 50% hatching success). The positive control exhibited more potent inhibitory activity (76.53%) with a MIC_{50} value of <0.0625 mg mL^{-1} while the extracts had inhibitory effect in the other: Ethanol>acetone>methanol>aqueous.

Brine shrimp lethality assay (BSLA): The percentage lethality/mortality of *A. salina* larvae (nauplii) incubated in different solvent extracts of *P. capitata* and controls are shown in Fig. 4. There was a significantly higher mortality percentage (22.33%) of the nauplii incubated with the test drug than the extracts and sea water (p<0.05). Although, there was non-significant difference (p>0.05) between the extracts and the sea water, the aqueous extract had the least mortality of 0.50% while the ethanolic extract had the highest mortality of 9.50%.

The effect of varying concentrations of the plant fractions on the mortality of larvae is shown in Fig. 5a and b. The degree of mortality of nauplii was in a concentration dependent fashion. The highest mortality was observed in all the extracts at 1 mg mL^{-1} while the control had a maximum mortality (100%) at 1 mg mL^{-1}. There was 0% mortality of the nauplii at concentrations of 0.0625-0.5 mg mL^{-1} in the methanolic and aqueous extracts. There was also 0% mortality at concentrations of 0.125 and 0.0625 mg mL^{-1} in the acetone extract while the ethanolic extract had 0% only at 0.0625 mg mL^{-1} (Fig. 5a).

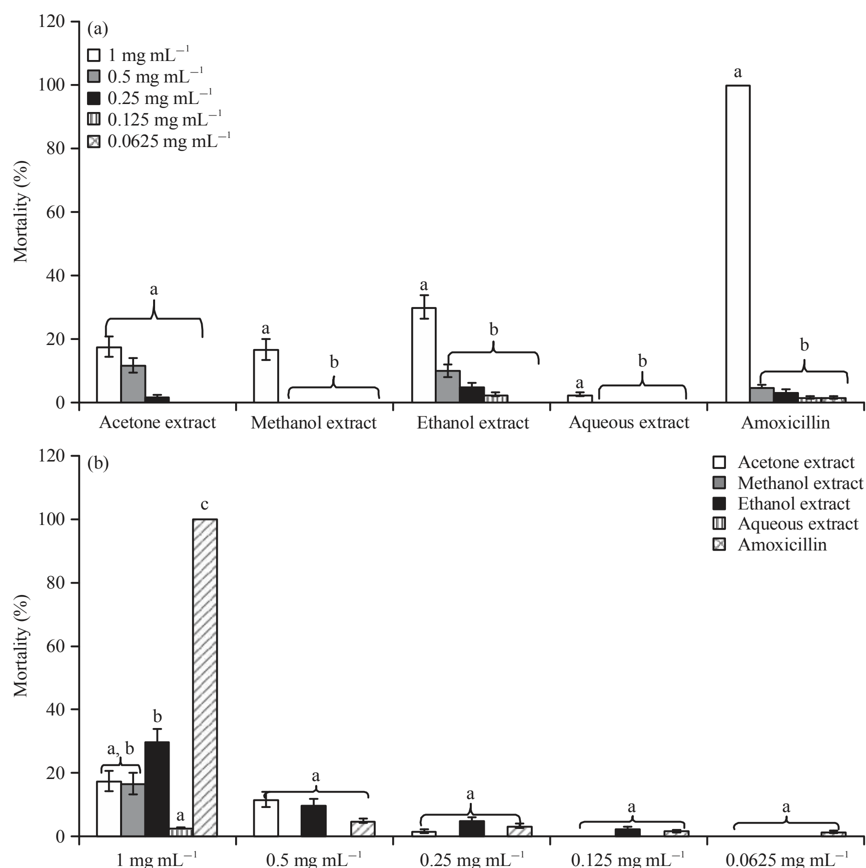


Fig. 5(a-b): Percentage mortality of *Artemia salina* cysts incubated in different concentrations of the plant extracts and control. The values are means of the replicates (at different hours) for the concentrations for each plant extract/control±SD of three replicates. Set of bars with different letters are significantly different ($p<0.05$), (a) Set of bars represents the effect of the solvent extracts/positive control at varying concentrations on the mortality of the nauplii and (b) Set of bars represents the effect of the solvent extracts/positive control to a particular concentration on the mortality of the nauplii

Figure 5b illustrates the relationship of the solvent extracts and control in respect of a particular concentration. At 1 mg mL^{-1}, the positive control had a higher significant mortality (100%) from the extracts ($p<0.05$). Though the ethanolic extract had the highest mortality of the nauplii (30%) but it was not significantly different from the acetone (6.17%) and methanolic (3.33%) extracts ($p>0.05$) but significantly higher than the aqueous extract (2.5%) ($p<0.05$). At 0.5-0.0625 mg mL^{-1}, all the plant extracts and control had no significant difference from one another ($p>0.05$).

The percentage mortality due to exposure time is captured in Fig. 6a and b. The result showed that the percentage mortality was time dependent as the longer the nauplii were exposed to the plant extracts, the higher the mortality (Fig. 6a). Exposure of the nauplii from 12-48 h in the acetone and methanol extracts showed no mortality but further exposure gave a non-significant mortality at 60 h and significant mortality at 72 h (Fig. 6a). Significant mortality in the ethanol extract was only recorded after 72 h while the aqueous extract had non-significant mortality although, ($p<0.05$). The test drugs showed a higher significant mortality of the nauplii at 12-60 h ($p<0.05$). The extracts gave non-significant different mortality from one another and sea water at 12-60 h ($p<0.05$) (Fig. 6b).

The expected dose/concentration expected to kill 50% nauplii was calculated and presented in Table 1. The test drug had the highest lethal dose of 0.89 mg mL^{-1} while, all the plant extracts had lethal doses >1 mg mL^{-1}.

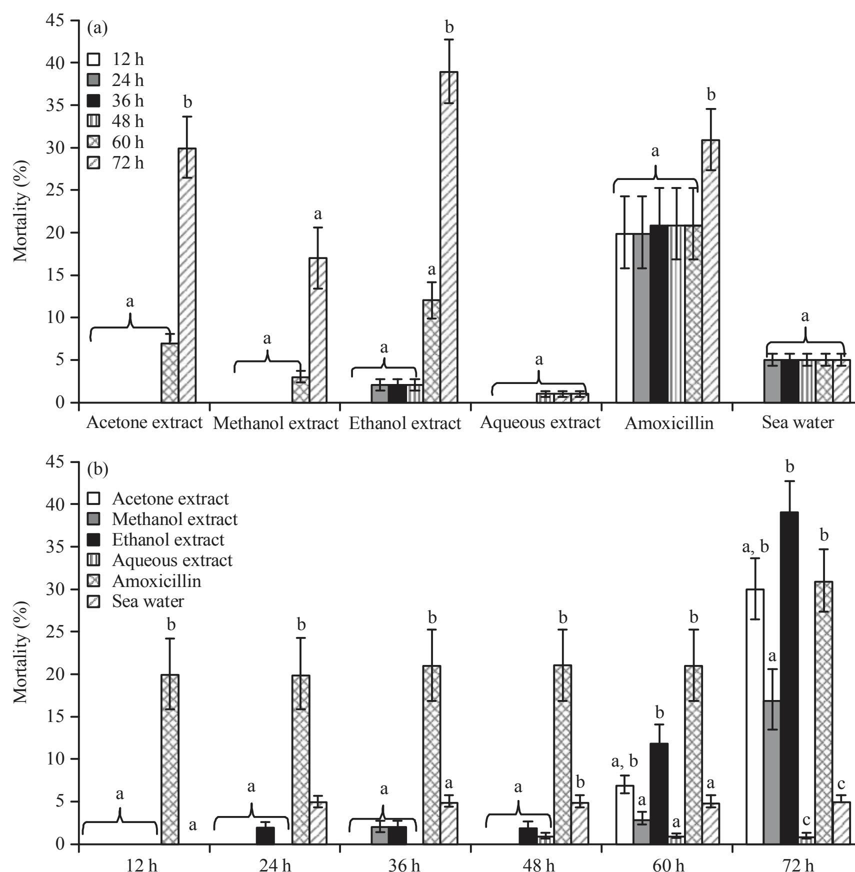


Fig. 6(a-b): Percentage mortality of *Artemia salina* cysts incubated in different time durations in the plant extracts/controls. The values are means of replicates (of all the concentrations) for each plant extract/control±SD. Set of bars with different letters are significantly different (p<0.05), (a) Set of bars represents the effect of the solvent extracts/positive control at varying time of exposure on the mortality of the nauplii and (b) Set of bars represents the effect of the solvent extracts/positive control to a particular time of exposure on the mortality of the nauplii

DISCUSSION

For the past 30 years, *A. salina* (brine shrimp) nauplii have been used as the first line of preliminary evaluation of the general toxicity of herbal remedies[6]. In this study, the toxicity of *P. capitata* was evaluated using both the inhibition of hatching of the cysts and mortality of the hatched cysts (nauplii) in different concentrations of plant extracts and controls. The hatching success of *A. salina* cysts incubated with aqueous extracts had the highest hatching success which means it had the least inhibitory hatching activity (40.67%) and presumably less toxic than the rest solvent extracts (Fig. 1). This could explain why most traditional herbal medicines are prepared using water as a solvent because it is not or less toxic[8].

There was an increase in hatching success of the cysts incubated in the plant extracts as the concentration decreases, but a maximum hatching success was achieved at 0.25 mg mL^{-1} and a further decrease in concentration resulted in decrease in hatching success (Fig. 2a, b). *Artemia salina* has a resistant cyst stage which is tolerant of a wide range of salinity from saturated saline to almost fresh water[5,13] and until the dormancy is broken, hatching will not occur hence, at 0.25 mg mL^{-1}, the plant extracts exhibited an optimum breaking of the dormancy of the cysts and further increase or decrease in the concentration exhibited an inhibitory action on the cysts. From the results, it is observed that the extracts at 1 mg mL^{-1} exhibited a toxic/inhibitory effect therefore preventing the cysts from hatching.

The hatching success of the cysts in response to exposure time as shown in Fig. 3a suggests that after 36 h of incubation in the various plant extracts, no further significant hatching success was observed (p<0.05) suggesting that 36-48 h is the best hatching time for brine shrimp which is also in agreement with the reports of Meyer *et al.*[14]. There was a fairly low hatching success of the plant extracts on the cysts except for

the aqueous extract which had a hatching success of over 50% at 36 h and above. The low hatchability success observed with this plant species could be attributed to the presence of chemical metabolites which probably may cause the eggs to further encyst in response to the chemical toxins.

According to Otang *et al.*[5] the resistance of the brine shrimp cysts to unfavourable environmental conditions makes the hatchability assay less desirable hence the lethality assay is a more appropriate test for the preliminary screening of herbal toxicity because it has the advantage of circumventing the toxin tolerance of the cyst stage as the nauplii that are very sensitive to toxins are used[13].

In accordance to Meyer *et al.*[14] and Bastos *et al.*[15] with respect to brine shrimp lethality test, the criterion of toxicity for plant remedies is as follows; the plant extract showing LC_{50} values greater than 1000 µg mL^{-1} (1 mg mL^{-1}) are considered non-toxic, LC_{50} values equal/greater than 500 µg mL^{-1} (0.5 mg mL^{-1}) but not greater than 1000 µg mL^{-1} are considered to have weak toxicity while those having LC_{50} values less than 500 µg mL^{-1} are considered toxic. The BSLA result of all the solvent extracts of *P. capitata* leaf showed that the extracts were not toxic with LC_{50}>1 mg mL^{-1} (Table 1) hence, these extracts may be considered safe for consumption as a herbal medicine. On the other hand, this non-toxic result could be discouraging as an alternative for the treatment and management of cancer/tumor, as brine shrimp lethality test is usually an indicator for the preliminary screening of bioactivity including for anticancer[16]. This result is in agreement with the results obtained from Indonesian mistletoe, *Dendrophthoe pentandra*, growing on different host plants and *Macrosolen cochinchinensis* growing on *Artocarpus heterophyllus*[16] which had LC_{50} values >1000 µg mL^{-1}.

There was a proportionate relationship of the concentration to the degree of lethality of the nauplii (Fig. 5a, b). The degree of mortality increased with increase in concentration as the maximum mortality of 17.5, 16.7, 30 and 2.5% occurred at the highest concentration of 1 mg mL^{-1} incubations of acetone, ethanol, ethanol and aqueous extracts, respectively while the test drug had a 100% mortality of the nauplii. Conversely, least mortality of 0% was observed in all the solvent extracts at the least concentration of 0.0625 mg mL^{-1} (Fig. 5a, b). The percentage mortality of the nauplii in all the extracts at concentration range from 0.0625-0.5 mg mL^{-1} was non-significant ($p>0.05$) except at 1 mg mL^{-1} where there was significance between the aqueous extract (2.5%) and the ethanolic extract (30%) (Fig. 5b). The administration of different concentration range was to set a baseline between safe and lethal limits in order to prevent the effect of acute overdose in future *in vivo* trials as simple zoological invertebrates are used for convenient toxicological screening system of medicinal plants[5].

The effect of the plant extracts on the nauplii over duration of time was done to ascertain the maximum sensitivity of nauplii on the toxic metabolites/chemical compounds present in the different solvent extracts. Mortality of the nauplii was first observed at 60 h of exposure to the extracts and an exponential significant lethality at 72 h ($p<0.05$) in the extracts except for the aqueous extract. Lewis[17] sited by Carballo *et al.*[6] reported that maximum sensitivity of the nauplii to test compounds is reached at the second and third instar stages and this is after 48 h of incubation, this is in agreement with this present findings that mortality was hardly noticed before 48 h in the plant extracts. According to Otang *et al.*[5], toxic effects of plant toxins can be delayed and thus suggesting that long exposure time is advisable for the evaluation of toxicological risks of plant extracts with brine shrimp lethality assay. The delayed mortality effect observed at 72 h suggests that the different plant extracts may have some nutritive values that may have acted as food and also the presence of less toxic compounds to brine shrimp.

CONCLUSION

This study showed that the different solvent extracts of *P. capitata* growing on rubber tree are not toxic (LC_{50}>1 mg mL^{-1}) in the brine shrimp lethality assay hence, it may be considered safe for use in traditional/alternative medicine. However, further *in vivo*, *in vitro* and cancer cell lines toxicity tests are required to further substantiate these claims and also to ascertain if it has anticancer potentials because there had been a report on *Macrosolen cochinchinensis* (a mistletoe) which had no toxicity on brine shrimp but showed cytotoxicity in cell lines. The indication being that the cytotoxic compounds in the extracts might be selectively toxic on cancer cells and hopefully less toxic to normal cells[16].

ACKNOWLEDGMENTS

Authors wish to acknowledge the financial support of Govan Mbeki Research and Development Centre, University of Fort Hare, Eastern Cape, South Africa and Rubber Research Institute of Nigeria.

REFERENCES

1. Ekor, M., 2014. The growing use of herbal medicines: Issues relating to adverse reactions and challenges in monitoring safety. Front Pharmacol., Vol. 4. 10.3389/fphar.2013.00177.
2. Olowa, F.L. and M.O. Nuneza, 2013. Brine shrimp lethality assay of the ethanolic extracts of three selected species of medicinal plants from Iligan City, Philippines. Int. Res. J. Biol. Sci., 2: 74-77.
3. Sahgal, G., S. Ramanathan, S. Sasidharan, M.N. Mordi, S. Ismail and S.M. Mansor, 2010. Brine shrimp lethality and acute oral toxicity studies on *Swietenia mahagoni* (Linn.) Jacq. seed methanolic extract. Pharmacogn. Res., 2: 215-220.
4. WHO., 2014. WHO guidelines on safety monitoring of herbal medicines in pharmacovigilance systems. World Health Organization, Geneva, Switzerland.
5. Otang, M.W., S.D. Grierson and N.R. Ndip, 2013. Assessment of potential toxicity of three South African medicinal plants using the brine shrimp (*Artemia salina*) assay. Afr. J. Pharm. Pharmacol., 7: 1272-1279.
6. Carballo, J.L., Z.L. Hernandez-Inda, P. Perez and M.D. Garcia-Gravalos, 2002. A comparison between two brine shrimp assays to detect *in vitro* cytotoxicity in marine natural products. BMC Biotechnol., Vol. 2. 10.1186/1472-6750-2-17.
7. Sam, T.W., 1993. Toxicity Testing using Brine Shrimp *Artemia salina*. In: Bioactive Natural Products Detection, Isolation and Structural Determination, Colegate, S.M. and R.J. Molyneux (Eds.). CRC Press, Boca Raton, FL., ISBN: 9780849343728, pp: 442-456.
8. Wintola, O.A., S. Arowosegbe and A.J. Afolayan, 2015. Toxicological investigations of *Aloe ferox* Mill. extracts using brine shrimp (*Artemia salina* L.) assay. Pak. J. Pharm. Sci., 28: 635-640.
9. Takem, L.P., G.A. Eshiet, O.G. Ogom and U.U. Mbang, 2014. Exploratory and anxiety potentials of aqueous extract of *Phragmanthera capitata*. J. Phytopharmacol., 3: 400-404.
10. Takem, L.P., B.A.S. Lawal, P.M. Udia and F.V. Udoh, 2015. Assessment of glycaemic property of *Phragmanthera capitata*. Int. J. Pharm. Pharmaceut. Res., 4: 1-8.
11. Ogunmefun, O.T., T.R. Fasola, A.B. Saba and O.A. Oridupa, 2013. The ethnobotanical, phytochemical and mineral analyses of *Phragmanthera incana* (Klotzsch), a species of mistletoe growing on three plant hosts in South-Western Nigeria. Int. J. Biomed. Sci., 9: 33-40.
12. Kibiti, C.M. and A.J. Afolayan, 2016. Antifungal activity and brine shrimp toxicity assessment of *Bulbine abyssinica* used in the folk medicine in the Eastern Cape Province, South Africa. Bangladesh J. Pharmacol., 11: 469-477.
13. Caldwell, G.S., M.G. Bentley and P.J.W. Olive, 2003. The use of a brine shrimp (*Artemia salina*) bioassay to assess the toxicity of diatom extracts and short chain aldehydes. Toxicon, 42: 301-306.
14. Meyer, B.N., N.R. Ferrigni, J.E. Putnam, L.B. Jacobsen, D.E. Nichols and J.L. McLaughlin, 1982. Brine shrimp: A convenient general bioassay for active plant constituents. Planta Medica, 45: 31-34.
15. Bastos, M.L.A., M.R.F. Lima, L.M. Conserva, V.S. Andrade, E.M.M. Rocha and R.P.L. Lemos, 2009. Studies on the antimicrobial activity and brine shrimp toxicity of *Zeyheria tuberculosa* (Vell.) Bur. (Bignoniaceae) extracts and their main constituents. Ann. Clin. Microbiol. Antimicrob., Vol. 8. 10.1186/1476-0711-8-16.
16. Artanti, N., T. Firmansyah and A. Darmawan, 2012. Bioactivities evaluation of Indonesian mistletoes (*Dendrophthoe pentandra* (L.) Miq.) leaves extracts. J. Applied Pharmaceut. Sci., 2: 24-27.
17. Lewis, G.E., 1995. Testing the toxicity of extracts of Southern African plants using brine shrimp (*Artemia salina*). S. Afr. J. Sci., 91: 382-384.

CHAPTER SIX

EVALUATION OF THE ANTI-BACTERIAL AND ANTI-FUNGAL PROPERTIES OF *PHRAGMANTHERA CAPITATA* USING AGAR DILUTION AND A 96-WELL BROTH MICRODILUTION TECHNIQUES

This chapter has been accepted for publication in the Journal of Botany

CHAPTER SIX

EVALUATION OF THE ANTI-BACTERIAL AND ANTI-FUNGAL PROPERTIES OF *PHRAGMANTHERA CAPITATA* USING AGAR DILUTION AND 96-WELL BROTH MICRODILUTION TECHNIQUES

CONTENTS **Pages**

Background of the study 97
Materials and Methods 98
Collection and extraction 98
Rationale for the selection of the microorganisms 98
Microbial strains 98
Preparation of bacterial inoculum 99
Preparation of fungal inoculum 99
Dilution assays 99
Preparation of extract 100
Resazurin (Alamar Blue) Preparation 100
Agar dilution assay 100
Broth microdilution assay 101
Minimum Inhibitory/Bactericidal Concentrations (MIC/MBC) 101
Result 102
Discussion 109
Antibacterial assay 110
Anti-fungal activity 112
Conclusion 112
References 113

Background of the study

The advent of antibiotics has proved to be the main tool in combating microbial infections and have greatly improved the health-related qualities of human life. Since the discovery of antibiotics and their uses in chemotherapy, there has been a notion in the health sector that this would lead to the eventual eradication of infectious diseases. However, over the past decades, over-reliance and use of antibiotics have led to the emergence and dissemination of multi-drug resistant strains of several groups of microorganisms (Bhalodia and Shukla, 2011; Khan *et al.,* 2009).

Due to the increase in resistant clinical isolates, there is a paramount need to develop new and innovative antimicrobial agents (Djeussi *et al.,* 2013). Coates *et al.,* (2002) cited by Khan *et al.* (2009), stated that even new families of antimicrobial agents may result in short life expectancy due to the rapid and widespread of the emergence of resistance to newly introduced antibiotics. Therefore, researchers are looking for new leads in the discovery of better alternatives against multi-drug resistant microbial strains. Among the potential sources of new agents, plants have long been investigated owing to their popular use as remedies for diverse infectious diseases because they contain many bioactive compounds that could be of interest in therapeutics (Djeussi *et al.,* 2013). Herbal drugs research is increasing on a daily basis not only because they serve as lead to the formulation of new preventive or curative drugs, but because they are affordable and believed to be safer than orthodox drugs (Balouiri *et al.,* 2016; Ogunmefun *et al.,* 2015; Olajuyigbe and Afolayan, 2012).

One of the groups of plants popular for its folkloric usage and for the treatments of all kinds of ailments is the mistletoe. They are also commonly called as “heal all” because of the belief that they can cure all diseases (Adodo, 2004). Amongst know families of the mistletoe, Loranthaceae is widely distributed and extensively exploited in Africa for its diverse therapeutic values. Some of the recognised therapeutic applications include; antitumor, cough,

headache, tightening of the uterus after childbirth, antiviral, anticancer, antinociception and antimicrobial etc. (Ameer *et al.,* 2015; Moghadamtousi *et al.,* 2013).

Prior to this study, there had been a dearth or no information on the antimicrobial activities of *Phragmanthera capitata* growing on rubber tree save the report of Ogunmefun *et al.* (2015) on *Phragmanhera incana* growing on Kolanut and Cocoa. This may be due to the fact that most studies on mistletoe are focused on its parasitic habit on host plants. This chapter, therefore, is aimed at investigating the anti-bacterial and anti-fungal activities of *P. capitata* on different human and animal pathogenic strains of microorganisms using the agar and 96-well broth dilution techniques.

Materials and Methods

Collection and extraction of the *Phragmanther capitata*

Collection and extraction of the *P. capitata* were done as previously described in chapter two.

Rationale for the selection of the microorganisms

The bacteria and fungi used for this work were selected based on their roles as opportunistic pathogens to humans and animals and their association with stomach disorders, diarrhoea, dysentery, wound and other infections and primarily to validate the ethnopharmacological claims of *P. capitata* as a remedy to these diseases (Ogunmefun *et al.,* 2015)

Microbial strains

Five gram-positive strains: *Enterococcus faecalis* (ATCC 29212), *Staphylococcus aureus* (OK), *Bacillus subtilis* KZN, *Bacillus cereus, Streptococcus pyogenes* and 5 gram negative strains: *Vibrio cholera, Klebsiella pneumonia* (ATCC 4352), *Pseudomonas aeruginosa* (ATCC 19582), *Salmonella typhi* (OK) and *Escherichia coli* (ATCC 8739) were used for the antibacterial activity. The fungi isolates used were *Trichophyton mucoides* ATCC 201382, *Trichophyton tonsurans* ATCC 28942, *Candida albicans* (ATCC 10231) and *Aspergillus niger* ATCC 16888.

All the organisms used were obtained from the Medicinal Plants and Economic Development (MPED) Research Centre, Botany Department, University of Fort Hare, South Africa.

Preparation of bacterial inoculum

Direct colony suspension method was used in preparing the inoculum. Three to five morphologically similar colonies from fresh Müller Hinton Agar plates were transferred with a loop into about 5 mL of normal saline in a capped test tube and vortex. The suspension formed was adjusted to give a turbidity equivalent to that of a 0.5 McFarland standard ($BaSO_4$ prepared spectrophotometrically) to give an approximate 1.5×10^8 CFU/mL. The adjusted colony was then diluted in a ratio 1:100 in Müller Hinton Broth to give a colony suspension of 1×10^6 CFU/mL. Final suspensions of 1×10^4 CFU/spot and 3-7 $\times 10^5$ CFU/mL were used for the agar and broth dilutions respectively.

Preparation of fungal inoculum

Fungal strains were freshly sub-cultured on sterile Sabouraud Dextrose Agar and incubated at 30°C for 2-5 days. The resultant cells and spores were washed into sterile Normal Saline and the turbidity adjusted to a 0.5 McFarland standard equivalent. This resulted in a 1×10^6 CFU/mL. The suspension is further diluted in a 1:10 ratio in Sabouraud Dextrose Broth to give a turbidity of 5×10^5 CFU/mL.

Dilution assays

Agar dilution and broth microdilution assays as described by Wiegand *et al.* (2008) and the European Committee for Antimicrobial Susceptibility Testing (EUCAST) (2003) which are modifications from the guidelines of the Clinical and Laboratory Standard Institute (NCLI), were used for this study.

Preparation of the plant extract

A 10 mL stock solution of 500 mg/mL was prepared by first dissolving the crude extract in 1 mL of DMSO and made up with either Müller Hinton or Sabouraud Dextrose Broth for anti-bacterial and anti-fungal respectively was prepared. Two-fold serial dilutions of the stock (250, 125, 62.5, 31.23, 15.625 and 7.8125 mg/mL) were also prepared in broth. Standard drugs (ciprofloxacin and nystatin for antibacterial and antifungal respectively) were also prepared in 2-fold serial dilutions according to guidelines of the Clinical and Laboratory Standard Institute (CLSI).

Resazurin (Alamar Blue) Preparation

Resazurin was obtained as a tablet and prepared according to the manufacturer's specification. A tablet was dissolved in 50 mL of sterilised distilled water and vortex. A ratio 1:10 final volume was used for the assay.

Agar dilution assay

Müller Hinton and Sabouraud Dextrose Agar were separately prepared according to the manufacturer's description for antibacterial and fungi screening respectively. The agar was autoclaved at 121° C for 15 min and allowed to cool to 50°C in a water bath. About 0.5 mL of the 2-fold serial dilutions were added to the molten agar (24.5 mL) in the water bath, swirled and poured into petri dishes and allowed to cool and solidify. 10 µL each from both the prepared bacterial and fungal inoculum was delivered on the solidified agar surface to give the desired final inoculum of 1×10^4 CFU/spot and 1×10^3 CFU/mL respectively. The extract concentrations for the antibacterial ranged from 5 mg/mL to 0.1563 mg/mL while for the antifungal, a range of 10 mg/mL to 0.3125 mg/mL was used. The concentration of Ciprofloxacin (antibacterial standard) ranged from 64 µg/mL to 2 µg/mL while Nystatin (antifungal standard) ranged from 16 µg/mL to 0.5 µg/mL. Bacteria plates were incubated at 37°C and readings were taken between 16-20 hrs and after 3 days of incubation; fungi plates

were incubated at 30°C and initial readings were taken after 2 to 3 days and the second readings were taken after 5 days.

Broth microdilution assay

Müller Hinton Broth for antibacterial screening was also tested using the 96-well microtitre plate with lid. The extracts and the standard drug were prepared in a concentration twice the desired final concentration as it will be diluted with an equal amount of bacteria in broth. Briefly, 200 µL of the prepared extracts and standard drug in broth was introduced into the first wells in columns 1-10 (in row A). Rows B-H in columns 1-10 had 100 µL of broth alone while rows A-H in column 11 had 200 µL of broth and 100 µL of broth was in A-H in column 12. Two-fold serial dilutions using a multi-channelled micropipette was done systematically down the columns 1-10 (from rows B-H). 100 µL was removed from the starting concentrations (Columns 1-10 in Row A) and taken to the next row with the 100 µL broth, mix well and 100 µL taken from it to the next well until the last row (H) where it was discarded. This brings the final volume in all the test wells with the extracts and the standard drugs to 100 µL except the 11th column which had 200 µL of the broth that served as sterility control. An equal volume (100 µL) of the 1×10^{6} CFU/mL bacterial inoculum was transferred into all the wells except the 11th column to give the desired final inoculum load of 5×10^{5} CFU/mL. Column 12 served as growth control (drug-free). The extracts concentrations ranged from 10 mg/mL to 0.078 mg/mL while ciprofloxacin ranged from 2 µg/mL to 0.0156 µg/mL. Microtiter plates were incubated at 37°C for 18-20 hrs. After incubation, 20 µL of Alamar blue (resazurin) was added to all the wells and incubation for few minutes to observe any colour changes.

Minimum Inhibitory/Bactericidal Concentrations (MIC/MBC)

The Minimum Inhibitory Concentrations were determined visually in the agar and broth dilutions as the lowest concentrations of the extracts at which no bacterial/fungal growth was visible (or greatly reduced in comparison to the controlled growth in the antifungal assay) or

colour changed from blue to pink in the case of the resazurin broth assay. Minimum Bactericidal Concentrations were determined by sub-culturing wells with no colour change on fresh agar plates and incubated at 37°C for 16 to 20 hrs. After the incubation, the lowest concentration that did not show any visible growth was taken as the MBC.

Result

The results of the antibacterial Minimum Inhibitory Concentration (MIC) using agar dilution and resazurin broth microdilution assays (Figure 1) are shown in Table 1. The result revealed that both gram-positive (+ve) and negative (-ve) bacteria tested were susceptible to the crude extracts of *P. capitata.* The gram-negative bacteria were more susceptible to the crude extracts in both methods assayed for but more defined in the agar dilution. The MIC values in the agar dilution ranged from 1.25 mg/mL to 2.5 mg/mL for all the gram-negative bacteria except for *K. pneumonia* (in the methanol and ethanol extracts) and *S. typhi* (in the methanol extract) which had MIC values of 5 mg/mL. The aqueous extract had the lowest activity compared to the organic solvent extracts but also exhibited better gram –ve activity in the agar dilution method with a MIC value of 5 mg/mL in *E. coli, S. typhi* and *V. cholera.* However, there was no activity recorded for the aqueous extract in the broth dilution technique. The standard drug (Ciprofloxacin) showed great antibacterial activity with a MIC value ranging from 0.0625 μg/mL to 0.25 μg/mL in the broth dilution and ≤ 2 μg/mL (lest concentration tested) in the agar dilution.

Table 1: Minimum Inhibitory Concentrations (MICs) of the different solvent extracts of *P. capitata* on selected gram-negative and gram-positive bacteria using the agar and Resazurin broth dilution assays

	Agar Dilution (16-20 hrs)					Resazurin Broth Microdilution				
	Act	Met	Eth	Aqu	Cip	Act	Met	Eth	Aqu	Cip
	mg/mL				µg/mL	mg/mL				µg/mL
Enterococcus faecalis (+ve)	2.5	2.5	5	> 5	≤ 2	2.5	2.5	5	> 10	0.25
Staphylococcus aureus (+ve)	5	5	5	> 5	≤ 2	5	5	5	> 10	0.0625
Bacillus subtilis (+ve)	5	5	5	> 5	≤ 2	2.5	2.5	2.5	> 10	0.0625
Bacillus cereus (+ve)	2.5	2.5	2.5	> 5	≤ 2	2.5	2.5	5	> 10	0.125
Streptococcus pyogenes(+ve)	5	5	5	> 5	≤ 2	5	5	5	> 10	0.0625
Vibrio cholera (-ve)	2.5	2.5	2.5	5	≤ 2	2.5	2.5	5	> 10	0.0625
Klebsiella pneumonia (-ve)	2.5	5	5	> 5	≤ 2	2.5	2.5	2.5	> 10	0.25
Pseudomonas aeruginosa (-ve)	2.5	2.5	2.5	> 5	≤ 2	2.5	2.5	2.5	> 10	0.25
Salmonella typhi (-ve)	2.5	5	1.25	5	≤ 2	5	5	5	> 10	0.0625
Escherichia coli (-ve)	2.5	2.5	1.25	5	≤ 2	2.5	1.25	2.5	> 10	0.0625

Annotations: Act (acetone extract), Met (methanol extract), Eth (ethanol extract), Aqu (aqueous extract), Cip (ciprofloxacin), “>” (value greater than the highest concentration tested) and “≤” (value lesser than or equal to the lowest concentration tested).

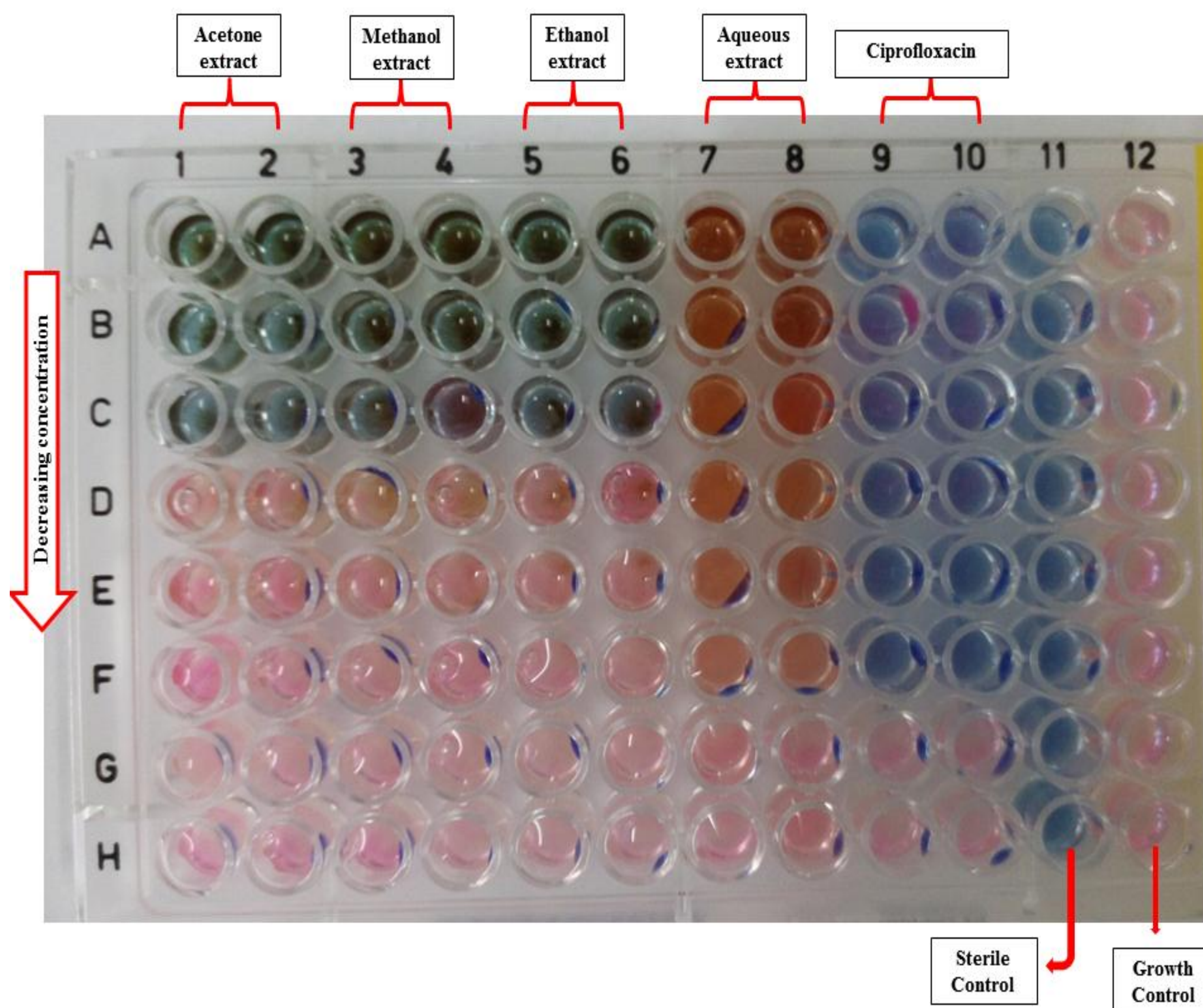


Figure 1: A schematic representation of the 96-Well Resazurin Broth Microdilution Model

Annotations: The blue colouration indicates inhibition of growth; Pink indicates organisms are active.

Table 2 is the result of the bactericidal activity of the extracts and agar dilution MIC after three days of incubation. The organic solvent extracts of *Phragmanthera capitata* showed more bactericidal activity on *Escherichia coli* with a MBC value of 2.5 mg/mL while there was no bactericidal activity on *P. aeruginosa.* The MBC for the organic extracts ranged from 2.5 mg/mL to 10 mg/mL with acetone having the best activity. The result of the agar dilution technique incubated for a prolonged time (3 days) showed a comparable result to the MBC with a range of 2.5 mg/mL to values greater than the highest concentration tested (> 5 mg/mL) for the crude extracts. Ciprofloxacin also showed a great lethal activity on almost all the organisms

at the concentrations tested except for *E. faecalis* that survived the highest dosage for this test. However, variation was observed in the organisms incubated for a prolonged period of time with ciprofloxacin in the agar. While at 4 mg/mL, *V. cholera* and *K. pneumonia* continued to grow, the same organisms were killed in the bactericidal test.

Table 2: Minimum Bactericidal Concentrations (MBCs) and MIC of the Agar dilution after 3 days of incubation of the different solvent extracts of *P. capitata* on selected gram-negative and gram-positive bacteria

	MBC					Agar Dilution (After 3 Days)				
	Act	Met	Eth	Aqu	Cip	Act	Met	Eth	Aqu	Cip
	mg/mL				µg/mL	mg/mL				µg/mL
Enterococcus faecalis (+ve)	5	5	5	> 10	> 2	5	5	5	> 5	4
Staphylococcus aureus (+ve)	5	10	10	> 10	0.0625	5	> 5	> 5	> 5	≤ 2
Bacillus subtilis (+ve)	5	10	10	> 10	0.125	5	> 5	> 5	> 5	≤ 2
Bacillus cereus (+ve)	5	5	10	> 10	> 2	5	5	> 5	> 5	≤ 2
Streptococcus pyogenes(+ve)	5	10	10	> 10	0.0625	5	> 5	> 5	> 5	≤ 2
Vibrio cholera (-ve)	5	5	5	> 10	0.0625	5	5	5	> 5	4
Klebsiella pneumonia (-ve)	5	5	5	> 10	1	5	5	5	> 5	4
Pseudomonas aeruginosa (-ve)	10	10	10	> 10	0.25	> 5	> 5	> 5	> 5	≤ 2
Salmonella typhi (-ve)	5	10	10	> 10	0.0625	5	> 5	> 5	> 5	≤ 2
Escherichia coli (-ve)	2.5	2.5	2.5	> 10	0.0625	2.5	2.5	2.5	> 5	≤ 2

Annotations: Act (acetone extract), Met (methanol extract), Eth (ethanol extract), Aqu (aqueous extract), Cip (ciprofloxacin), ">" (value greater than the highest concentration tested) and "≤" (value lesser than or equal to the lowest concentration tested)

The antifungal activities of the solvent extracts of *P. capitata* on some selected human pathogenic fungi are as shown in Table 3. The MICs were read twice; between 2-3 days and after 5 days of incubation. The MIC for *C. albicans* and *A. niger* were taken as the concentrations which showed little spot to no growth in comparison to the control (Figure 2). The result revealed that *T. mucoides* and *C. albicans* were resistant to the organic solvent extracts of the plant. *Trichophyton tonsurans* and *A. niger* were, however, susceptible to the organic solvent extracts. The MICs for acetone, methanol and ethanol extracts on *T. tonsurans* were 1.25 mg/mL, 10 mg/mL and 5 mg/mL respectively. Acetone and ethanol extracts had a MIC of 5 mg/mL while methanol extract had MIC value of 10 mg/mL on *A. niger*. The result after 5 days of incubation was similar to the first observation except in the ethanol extract where the MIC increased to 10 mg/mL in both *T. tonsurans* and *A. niger*. However, a very high susceptibility was observed in the aqueous extract in all the fungi assayed for and remained unchanged after 5 days of incubation. The MIC of the aqueous extract was 1.25 mg/mL on *T. mucoides* and ≤ 0.3125 mg/mL (lowest concentration tested) on *T. tonsurans, C. albicans* and *A. niger*. Nystatin also showed a high MIC on the tested organisms with MIC which ranged from 4 µg/mL to 8 µg/mL after 2-3 days of incubation and greater than 16 mg/mL in *C. albicans* after 5 days of incubation.

Table 3: Minimum Inhibitory Concentrations (MIC) of the different solvent extracts of *P. capitata* on selected human pathogenic fungi

	2-3days after incubation					5days after incubation				
	Act	**Met**	**Eth**	**Aqu**	**Nys**	**Act**	**Met**	**Eth**	**Aqu**	**Nys**
	mg/mL				**µg/mL**	**mg/mL**				**µg/mL**
Trichophyton mucoides	>10	>10	>10	1.25	4	>10	>10	>10	1.25	4
Trichophyton tonsurans	1.25	10	5	≤0.3125	4	1.25	10	10	≤0.3125	4
Candida albicans	>10	>10	> 10	≤0.3125	4	>10	>10	>10	≤0.3125	>16
Aspergillus niger	5	10	5	≤0.3125	8	5	10	10	≤0.3125	8

Annotations: Act (acetone extract), Met (methanol extract), Eth (ethanol extract), Aqu (aqueous extract), Nys (nystatin), “>” (value greater than the highest concentration tested) and “≤” (value lesser than or equal to the lowest concentration tested).

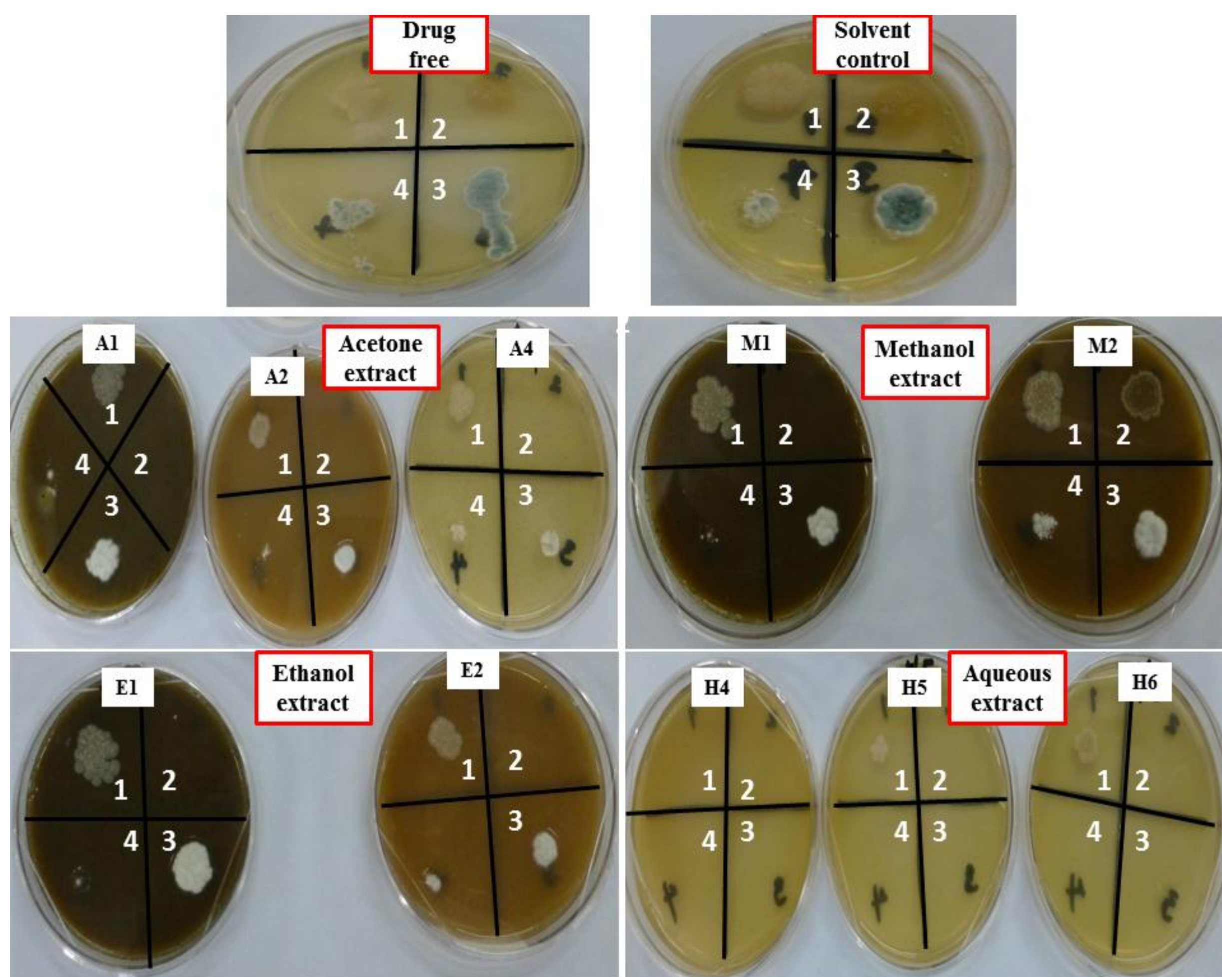


Figure 2: Representation of the anti-fungal MIC determination of *P. capitata* against the tested fungi using the Agar Dilution technique**.**

Annotations: values 1-4 are the fungal used which are; 1= *Trichophyton mucoides*; 2= *Trichophyton tonsurans;* 3= *Candida albicans* and 4= *Aspergillus niger.* A1, A2 & A4, M1& M2, E1 & E2 and H4, H5 & H6, are the different concentrations used with the highest concentration being 1 (10 mg/ml) and the least was 6 (0.3125 mg/mL). Spots on plates are fungi growth indicating resistance of the organism(s) at that concentration.

Discussion

In ethnopharmacological research, antimicrobial susceptibility tests are carried out to determine how effective potential anti-microbial agents from biological extracts could be against different pathogenic microorganisms. These tests are used to screen plant extracts for antimicrobial activities and also used to determine or ascertain the usefulness of antimicrobial agents in fighting infections by determining their MIC (Ncube *et al.,* 2007). According to the EUCAST

document (2003), *in-vitro* susceptibility tests are carried out on pathogenic microorganisms with suspicion of belonging to species that have displayed resistance to commonly used antimicrobial agents. These tests are also very useful for the surveillance of resistance, epidemiology of susceptibility and to compare new and existing antimicrobial agents. These parameters are very vital in clinical practice in classifying the tested microorganisms as clinically susceptible, intermediate or resistant to the test antimicrobial agents (Wiegand *et al.,* 2008).

Different standard methods have been used to evaluate the antimicrobial activities of plants crude extracts. However, dilution methods have been favoured over others for the determination of MIC (broth and agar dilutions) and MBC (broth dilution) (Wiegand *et al.,* 2008). Presently, a new method using the oxidation–reduction colorimetric indicator resazurin is been used for the determination of drug resistance and MICs of antimicrobial agents against Pathogenic organisms (Ncube *et al.,* 2007; Nateche *et al.,* 2006). Resazurin, which is blue in its oxidised state, turns pink when reduced by viable cells (Figure 1) and can easily be detected with the naked eyes and the MIC determined even without the aid of a spectrophotometer. This work explores the use of resazurin for the broth microdilution method for the antibacterial MIC determination

Antibacterial assay

The agar and broth dilution techniques used in this study revealed that they are both effective antibacterial techniques for the determination of MIC. However, the resazurin broth microdilution technique proved to be more sensitive as it could detect the slightest activity of the organisms where the agar dilution could not; this was evidently observed in the aqueous extract (*V. cholera, S. typhi* and *E. coli*) in the antibacterial assay (Table 1 and Figure 1).

According to early works on parasitic plants, Hawksworth (1996) reported that parasitic plants scarcely utilise their photosynthate and depends mostly on the nutrient absorbed from the host.

Hence, the bio-compounds are chiefly dependent on its host. This was evident in the antimicrobial works of Ogunmefun *et al.* (2015), Deeni and Sadiq (2002) and Efuntoye *et al.* (2010) which showed different antimicrobial activities of the same plant on different hosts. This study in comparison to the works of Ogunmefun *et al.,* (2015), showed that *Phragmanthera capitata* of rubber tree (*Hevea brasiliensis*) have greater promise for its anti-bacteria potential (MIC of 1.25 mg/mL to 5 mg/mL in the organic solvents) than the ones harvested from kolanut and cocoa (MIC of 100 mg/mL to 200 mg/mL). While *Bacillus* sp*, K. pneumonia* and *E. coli* were resistant in *P. capitata* from kolanut and cocoa (Ogunmefun *et al.,* 2015), these bacteria were highly susceptible to the same species collected from rubber trees in this work.

The bactericidal ability of *P. capitata* parasitic on rubber tree was also assayed for and the result revealed that this plant will not only inhibit these bacteria but also has the potential to kill them at appreciable concentrations (Table 2). Hence, *P. capitata* parasitic on rubber tree has potential as an antibiotic in the pharmaceutical industry and can serve as an alternative remedy to the diseases caused by these bacteria.

In evaluating the effect of the extract on the bacteria in the agar dilution method, incubation was further prolonged for 3 days and the results obtained were in comparison with the MBC. Some organisms that were initially inhibited by the extracts after 20 hrs however recovered and continued growth (Table 2). This could be as a result of the antibacterial bio-compounds in the extract(s) becoming weak and less active and therefore bacteria that were still alive recovered and continued to grow. This observed outcome could give some insights into the shelf life or duration of action of the extracts against microorganisms. This simply explains that where organisms continued growth, the extracts at those concentrations were not bactericidal to them. Alternatively, organisms that continued growth may not have been properly dissolved in the agar and hence when the activity of the extract reduced beyond their threshold, they re-grew.

This could be a major advantage of the broth over the agar dilution method as the organism is highly soluble or mixes very well with the broth as they are both liquids.

Antifungal assay

The activity of the crude extracts of *P. capitata* was also tested against pathogenic fungi using the agar dilution technique. Four pathogenic fungi were tested but only two viz; *T. tonsurans* and *A. niger* were susceptible to the organic extracts. While *A. niger* was susceptible in this work, the same was resistant in the reports of Ogunmefun *et al.,* (2015) suggesting that *P. capitata* from rubber tree may be more potent than the ones collected from kolanut and cocoa as an anti-fungal agent. The result on *C. albicans* from this work is in support of Ogunmefun *et al.,* (2015) which did not show any activity on the organic solvents used. Interestingly, while the aqueous extract seldom showed anti-bacterial activity, the same had a very high anti-fungal activity as it inhibited all four tested fungi (Table 3 and Figure 2, H4). This could suggest that water was able to extract the anti-fungal agent in the plant than the organic solvent extracts. There were no marked changes observed after prolonged incubation of the plates for 5 days and where changes occurred, the same reason as above could justify it.

Conclusion

The outcome of this work clearly revealed that *P. capitata* harvested from rubber trees has the ability to inhibit both gram-positive and negative bacteria effectively and also exhibited anti-fungi ability and with great promise for use as an anti-microbial agent in folklore medicine. While it was recorded that water was a weak anti-bacterial extractant in this study, contrarily, water would be recommended if the target were fungi. This work further supported the claimed ethnopharmacological uses of *P. capitata* against gastrointestinal infections and other opportunistic diseases of humans and animals. Also worth noting is the potential of *P. capitata* to could serve as an alternative remedy in therapeutics as most of the organisms used for this work have had some reports of resistance to conventional drugs.

References

Adodo, A. 2004. Nature Power, a Christian Approach to Herbal Medicine. Benedictine Publication Nigeria, (3rd Edn), Edo State. 7th Printing by Generation Press Ltd, Surulere, Lagos. p 103-111.

Ameer, O.Z., Salman, I.M., Quek, K.J., Asmawi, M.Z., 2015. *Loranthus ferrugineus*: a Mistletoe from traditional uses to laboratory bench. Journal of Pharmacopuncture. DOI: 10.3831/KPI.2015.18.001

Balouiri, M., Saiki, M., Ibnsouda, S.K., 2016. Methods for *in vitro* evaluating antimicrobial activity: A review. Journal of Pharmaceutical Analysis 6920, 71 – 79. DOI:10.1016/j.jpha.2015.11.005.

Bhalodia, N.R., Shukla, V.J., 2011. Antibacterial and Antifungal activities from leaf extracts of *Cassia Fistula* L. An Ethnomedicinal Plant. Journal of Advanced Pharmaceutical Technology and Research 2(2), 104–109.

Coates, A., Hu, Y., Bax, R., Page, C., 2002. The future challenges facing the development of new antimicrobial drugs. Nature Reviews Drug Discovery 1, 895-910.

Deeni, Y.Y., Sadiq, N.M., 2002. Antimicrobial properties and phytochemical constituents of the leaves of African mistletoe (*Tapinanthus dodoneifolius* (DC) Danser) (Loranthaceae): an ethnomedicinal plant of Hausaland, Northern Nigeria. Journal of Ethnopharmacology 83(3), 235-240. DOI:10.1016/S0378-8741(02)00244-1

Djeussi, D.E., Noumedem, J.A., Seukep, J.A., Fankam, A.G., Voukeng, I.K., Tankeo, S.B., Nkuete, A.H., Kuete, V., 2013. Antibacterial activities of selected edible plants extracts against multidrug-resistant Gram-negative bacteria. BMC Complementary and Alternative Medicine. 3, 164. DOI: 10.1186/1472-6882-13-164.

Efuntoye, M.O., Ayodele, A.E., Thomas, B.T., Ajay, T.O., 2010. Does host plant affect the antibacterial activity of *Tapinanthus bangwensis* (Engl. and K. Krause) Danser (Loranthaceae)? Journal of Medicinal Plants Research. 4(13), 1281-1284. DOI:10.5897/JMPR10.017.

European Committee for Antimicrobial Susceptibility Testing (EUCAST)., 2003. Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. European Society of Clinical Microbiology and Infectious Diseases, CMI 9, 1-7

Hawksworth, F.G., Wiens, D.G., 1996. Dwarf mistletoe: Biology, Pathology and Systematics. Agricultural Handbook 709, United States Department of Agriculture, Forest Service. Washington D.C. 410p.

Khan, R., Islam, B., Akram, M., Shakil, S., Ahmad, A., Ali, S.M., Siddiqui, M., Khan, A.U., 2009. Antimicrobial activity of five herbal extracts against Multi-Drug Resistant (MDR) Strains of bacteria and fungus of clinical origin. Molecules 14, 586-597. DOI: 10.3390/molecules14020586.

Moghadamtousi, S.Z., Hajrezaei, M., Kadir, H.A., Zandi, K., 2013. *Loranthus micranthus* Linn.: Biological Activities and Phytochemistry. Evidence-Based Complementary and Alternative Medicine. Article ID 273712,. DOI: 10.1155/2013/273712.

Nateche, F., Martin, A., Baraka, S., Palomino, J.C., Khaled, S., Portaels, F., 2006. Application of the resazurin microtitre assay for detection of multidrug resistance in *Mycobacterium tuberculosis* in Algiers. Journal of Medical Microbiology 55, 857–860. DOI:10.1099/jmm.0.46513-0.

Ncube, N.S., Afolayan, A.J., Okoh, A.I., 2007. Assessment techniques of antimicrobial properties of natural compounds of plant origin: current methods and future trends. African Journal of Biotechnology 7 (12), 1797-1806 17.

Ogunmefun, O.T., Ekundayo, E.A., Ogunnusi, T.A., Olowoyeye, A.H., Fasola, T.R., Saba, A.B., 2015. Antimicrobial Activities of *Phragmanthera incana* (Schum.) Balle, a mistletoe species harvested from two host plants against selected pathogenic microbes. Annual Research and Review in Biology 8(3), 1-10, DOI: 10.9734/ARRB/2015/14947.

Olajuyigbe, O.O., Afolayan, A.J., 2012. *In-vitro* pharmacological activity of the crude acetone extract of *Erythrina caffra* Thunb: antibacterial and antifungal assessment. Journal of Medicinal Plants Research 6 (9), 1713-1720. DOI: 10.5897/JMPR11.1517.

Wiegand, I., Hilpert, K., Hancock, R.E.W., 2008. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature Protocols 3 (2), 163-175.

CHAPTER SEVEN

EVALUATION OF THE ANTI-*MYCOBACTERIUM TUBERCULOSIS* POTENTIALS OF *PHRAGMANTHERA CAPITATA*

CHAPTER SEVEN

EVALUATION OF THE ANTI-*MYCOBACTERIUM TUBERCULOSIS* POTENTIALS OF *PHRAGMANTHERA CAPITATA*

CONTENTS **Pages**

Background of the study 117
Methodology 118
Collection and extraction of the sample 118
Microbial strain, culture and assay 118
Result and Discussion 119
Conclusion 122
References 122

Background of the study

Tuberculosis (TB), an infectious disease usually caused by the bacterium, *Mycobacterium tuberculosis,* is one of the leading causes of human morbidity and mortality (Nguta *et al.,* 2016; Sánchez and Kouznetsov 2010). According to the 2016 Factsheet on TB by the World Health Organization (WHO), TB is one of the top ten causes of death worldwide. The report stated that, of the 10.4 million people who took ill with TB, 1.4 million died from the disease (excluding those with HIV) in 2015. An estimated 1 million children (0 to 14 years) were infected with TB and 170,000 excluding those with HIV died of the infection in 2015 (WHO, 2016).

Tuberculosis is prevalent in every part of the world. Currently, a reported one-third of the world's population has latent TB (infected but asymptomatic) and a 10% lifetime risk of developing into an active TB. Active TB is transmitted from an infected person through the air when they cough, sneeze or spit, to about 10 to 15 other people within a year. The Asian continent accounts for 61% while Africa accounts for 26% of the world TB cases. Six countries in the order of prevalence: India, Indonesia, China, Nigeria, Pakistan and South Africa, account for 60% of the new TB cases (WHO, 2016; Gupta *et al.,* 2014).

Rifampicin, Isoniazid, Streptomycin and Ethambutol were introduced almost three decades ago in TB control programme for the treatment of TB. The indiscriminate use: inappropriate, incorrect prescription by healthcare providers, poor quality drugs and patients stopping treatment prematurely had led to the alarming rise in the development of multidrug resistance (MDR-TB), the more severely extensive-resistant (XDR-TD) and currently, totally drug-resistant (TDR-TB) TB strains (WHO, 2016; Nguta *et al.,* 2016; Proksch *et al.,* 2015; Singh *et al.,* 2013). No new anti-TB drug had been released for decades now and MDR-TB, XDR-TB and TDR-TB are on the increase, hence, there is an urgent need for new leads in the development of novel, effective, safe and affordable drugs against all forms of resistant TB

strains. Amongst different natural products used in ethnopharmacology, plant-based products have been extensively utilised because of its vast reservoir of phytochemicals explored for a wide range of infections and anti-mycobacterium compounds have been derived from them (Nguta *et al.,* 2015).

Phragmanthera capitata (Sprengel) Balle has been used in herbal medicine for the treatment and management of TB related ailments and different infections and diseases in Africa (Takem *et al.,* 2015). Arising from the foregoing, this chapter was aimed at investing the possible anti-*Mycobacterium tuberculosis* potential of *P. capitata* using the automated BACTEC MGIT 960 system.

Methodology

Collection and extraction of *Phragmanthera capitata*

Collection and extraction of *P. capitata* were carried out as previously described in chapter two

Microbial strain, culture and assay

The American Type Culture Collection of *Mycobacterium tuberculosis* strain H37R_V (ATCC 25618) was used for this assay. Suspension of virulent *M. tuberculosis* (H37Rv) strain was grown using mycobacterial growth indicator tubes (MGIT). The inoculum was prepared in Lowenstein–Jensen slants. An inoculum of less than 15 days old was prepared from the culture grown on Lowenstein-Jensen medium in normal saline and adjusted to a 1.0 McFarland standard. The suspension was then vortexed and allowed to stand for about 20 min for larger particles to settle to the bottom of the tube. After settling, the supernatant was transferred to a fresh sterile tube and adjusted to a 0.5 McFarland standard. The adjusted inoculum was further diluted in a ratio 1:5. 0.5 mL of the diluted suspension was inoculated in Middlebrook 7H12 (MGIT 960 system, Becton Dickinson, Sparks, USA) tubes containing aliquots (100 µl) of the test and control compounds. Final DMSO concentration was ≤ 1.2%. The tubes were incubated at 37°C in MGIT system. The growth units (GU) were monitored for six days. The growth was

monitored through fluorescent changes due to oxygen consumption in the medium during active growth.

For the determination of the Minimal Inhibitory Concentration, 1% mycobacterial control culture was prepared in a drug-free; with 1.2% DMSO; isoniazid (INH) and extracts in MGIT tubes and the MIC of the compound determined relative to the growth units of the control (GU). The MIC was determined as the lowest test compound concentration that equals or lower than GU of the 1% mycobacterial culture. All the extracts were tested at two-fold decreasing concentrations (50, 25, 12.5, 6.25, 3.125 and 1.5625 µg/mL).

Result and Discussion

Ethnobotanical studies on the use of *P. capitata* and other mistletoe of the Loranthaceae have reported their pharmacological application on a variety of ailments but most have been silent on their use for the treatment of tuberculosis except for the report of Ibekwe *et al.* (2014), which identified a mistletoe species (*Tapinanthus sessilifolia* Polh. & Wiens) with anti-tuberculosis activity. Yet, they are known as a panacea or "cure-all" (National Institute of Health, 2015; Ogunmefun *et al.,* 2013; Adodo, 2004). This study is the first evaluation of the anti-tuberculosis potential of *P. capitata* irrespective of the host and amongst the reported few on mistletoes generally.

There is no clear-cut reference for crude plant extract activity on tuberculosis. The sensitivity is subject to the methodology employed in the test. Different researchers have used various standards for the interpretation of anti-mycobacterial activity of herbal drugs. There is not really an explicatory reason to these set standards in most of these cases (Mohamad *et al.,* 2011). According to Borges-Argáeza *et al.* (2007) and Gautam *et al.* (2007), MIC of ≤ 100 µg/mL were considered active against *M. tuberculosis* and other *Mycobacterium* spp. Luo *et al.* (2011), set activity at MIC ≤ 125 µg/mL. Tosun *et al.* (2005) and Camacho-Corona *et al.* (2008) set a MIC value of ≤ 200 µg/mL while a value of ≤ 500 µg/mL and ≤ 1600 µg/mL were considered

as active by Newton *et al.* (2002); Ibekwe *et al.* (2014) and Mohamad *et al.* (2011) respectively. In this present study, the highest tested concentration was 50 µg/mL and comparison was done in respect to MIC values by previous works.

Different methods have been employed for the evaluation of anti-tuberculosis screening of medicinal plants. Franzblau *et al*. (2012), Sánchez and Kouznetsov (2010), Khalifa (2013) and many other researchers have reported different methodologies for the anti-tuberculosis screening of different compounds. The BACTEC 460 which uses the radioisotope was considered a gold-standard for measuring the growth of intracellular mycobacteria in macrophages. However, the use of BACTEC 460 was discontinued by the manufacturer due to safety issues involved in the handling of radioactive elements. As an alternative to the BACTEC 460 system, a BACTEC Mycobacteria Growth Indicator Tube (MGIT) 960 system was used for this assay (Jhamb *et al.,* 2014). Its performance is equivalent to the Radiometric BACTEC 460, plating and other methods (Jhamb *et al.,* 2014; Adjers-Koskela and Katila, 2003; Bemer *et al.,* 2002). This system is fully automated, safe (not radioactive), not labour intensive, higher throughput, not invasive and highly efficient in *M. tuberculosis* screening (http://www.bd.com/ds/productCenter/MT-BactecMgit960.asp).

The result of the *in-vitro* anti-*Mycobacterium tuberculosis* screening of the different fractions of *P. capitata* is as shown in Table 1. The MIC values of the solvent fractions ranged from 25 µg/mL to 50 µg/mL. The acetone and the aqueous extracts had the best activity with a MIC value of 25 µg/mL, while the ethanol extract had a MIC value of 50 µg/mL. The methanol extract did not show any activity at the highest concentration evaluated (Table 1). The result from this study is an indication that *P. capitata* harvested from rubber tree has the potential as an anti-mycobacterial agent. With comparison to the different ranges of MIC values set above by different researchers on crude plant extracts on *Mycobacterium tuberculosis,* this species activity can be termed as highly active (Mohamad *et al.,* 2011). The result also revealed that

extraction solvent has an effect on the mycobacterial bioactivity of the extracts. It can be deduced that acetone and water extracted more of the active bio-compounds against *M. tuberculosis* than ethanol and methanol. This solvent effect also supports our earlier antifungal study where the aqueous extract had a lower MIC (better antifungal properties) than the other solvents.

Table 1: MIC determination against *M. tb* H37Rv growth using BACTEC MGIT 960 system

Extract	**MIC in µg/mL**
Acetone	25
Methanol	> 50
Ethanol	50
Aqueous	25
Isoniazid	0.05

This study revealed the potential of *P. capitata* against the virulent *M. tuberculosis* strain. In comparison to other established anti-tuberculosis medicinal plants in Malaysia (Mohamad *et al.,* 2011), Turkey (Askun *et al.,* 2013; Tosun *et al.,* 2005), Cameroon (Nkenfou *et al.,* 2015), South Africa (Mativandlela *et al.,* 2008) and Nigeria (Ibekwe *et al.,* 2014), *P. capitata* has shown to be more effective than most of the enumerated species. Notably, in one of these evaluations, a mistletoe species, *Tapinanthus sessilifolia,* (host could not be ascertained) of the Loranthaceae family, used in Nigeria for the treatment of tuberculosis by herbal practitioners, a high activity against the H37Rv strain (MIC 128 µg/mL) (Ibekwe *et al.,* 2014) was recorded. This high activity may suggest the potential of the African mistletoe (Loranthaceae) for the treatment of tuberculosis. Hence, further anti-tuberculosis screening on the African Mistletoe to evaluate their potentials in the fight against tuberculosis is of the essence as Africa is highly ladened with the TB scourge (WHO, 2016)

Conclusion

This study highlights the results of *P. capitata* harvested from rubber tree plantation against the virulent strain of *Mycobacterium tuberculosis.* It provides a preliminary scientific validation of the use of this species against some tuberculosis associated symptoms like cough, respiratory dysfunctions, fever and headaches (Ogunmefun *et al.,* 2013; Dibong *et al.,* 2009). Further screening of *P. capitata* parasitic in different host plants and other African mistletoe for possible use in the development of anti-mycobacterial drugs is needed in the fight against TB.

References

Adjers-Koskela, K., Katila, M.L., 2003. Susceptibility testing with the manual mycobacteria growth indicator tube (MGIT) and the MGIT 960 system provides rapid and reliable verification of multidrug-resistant tuberculosis. Journal of Clinical Microbiology 41, 1235–1239: DOI: 10.1128/JCM.41.3.1235-1239.2003.

Adodo, A., 2004. Nature Power, a Christian Approach to Herbal Medicine. 3rd Edn. Benedictine Publication Nigeria. pp. 103–111. Edo State. 7th Printing by Generation Press Ltd, Surulere, Lagos.

Askun, T., Satil, F., Tumen, G., Yalcin, O., Modanlioglu, S., 2012. Antimycobacterial activity of some different Lamiaceae plant extracts containing flavonoids and other phenolic compounds. INTECH Open Access Publisher

Askun, T., Tekwu, E.M., Satil, F., Modanlioglu, S., Aydeniz H., 2013. Preliminary antimycobacterial study on selected Turkish plants (Lamiaceae) against *Mycobacterium tuberculosis* and search for some phenolic constituents. BMC Complementary and Alternative Medicine 13, 365. Doi: 10.1186/1472-6882-13-365.

Bemer, P., Palicova, F., Rusch-Gerdes, S., Drugeon, H.B, Pfyffer, G.E., 2002. Multicenter evaluation of fully automated BACTEC mycobacteria growth indicator tube 960 system for susceptibility testing of *Mycobacterium tuberculosis*. Journal of Clinical Microbiology 40, 150–154. DOI: 10.1128/JCM.40.1.150-154.2002

Borges-Argáeza, R., Canche-Chaya, C.I., Pena-Rodrígueza, L.M., Said-Fernándezb, S., Molina-Salinas, G.M., 2007. Antimicrobial activity of *Diospyros anisandra*. Fitoterapia 78, 370–372

Camacho-Corona, M.d.R., Ramírez-Cabrera, M.A., González-Santiago, O., GarzaGonzález, E.I.D., Palacios, P., Luna-Herrera, J., 2008. Activity against drug resistant-tuberculosis

strains of plants used in Mexican traditional medicine to treat tuberculosis and other respiratory diseases. Phytotherapy Res., 22, 82–85

Dibong, S.D., EngoneObiang, N.L., Ndongo, D., Priso, R.J., Taffouo, V., Fankem, H., Salle, G., Missoup, A.D., Boussim, I.J., Amougou, A., 2009. An assessment on the uses of Loranthaceae in ethnopharmacology in Cameroon: A case study made in Logbessou, North of Douala. Journal of Medicinal Plants Research 3(8), 592-595.

Franzblau, S.G., DeGroote M.A., Cho, S.H., Andries, K., Nuermberger, E., Orme, I.M., Mdlulie, K., Angulo-Barturen, I., Dick, T., Dartois, V., Lenaert, A.J., 2012. Comprehensive analysis of methods used for the evaluation of compounds against *Mycobacterium tuberculosis*. Tuberculosis 92(6), 453–488. DOI: 10.1016/j.tube.2012.07.003.

Gautam, R., Saklani, A., Jachak, S.M., 2007. Indian medicinal plants as a source of antimycobacterial agents. Journal of Ethnopharmacology 110, 200–234

Gupta, P., Bhatter, P., D'souza, D., Tolani, M., Daswani, P, Tetali, P., Birdi, T., 2014. Evaluating the anti *Mycobacterium tuberculosis* activity of *Alpinia galanga* (L.) Willd. axenically under reducing oxygen conditions and in intracellular assays. BMC Complementary and Alternatary Medicine 14, 84: DOI**:** 10.1186/1472-6882-14-84

Ibekwe, N.N., Nvau, J.B., Oladosu, P.O., Usman, A.M., Ibrahim, K., Boshoff, H., Dowd, C.S., Orisadipe, A.T., Aiyelaagbe, O., Adesomoju, A.A., Barry, C.E., Okogun, J., in collaboration with 73 Visited Herbalists., 2014. Some Nigerian Anti-Tuberculosis Ethnomedicines: A Preliminary Efficacy Assessment. Journal of Ethnopharmacology 155(1), 524–532. doi:10.1016/j.jep.2014.05.059.

Jhamb, S.S., Goyal, A., Singh, P.P., 2014. Determination of the activity of standard anti-tuberculosis drugs against intramacrophage *Mycobacterium tuberculosis*, *in vitro*: MGIT 960 as a viable alternative for BACTEC 460. Brazilian Journal of Infectious Diseases 18(8), 336–340. DOI: 10.1016/j.bjid.2013.12.004.

Khalifa, R.A., Nasser, M.S., Goma, A.A., Osman, N.M., Salem, H.M., 2013. Resazurin Microtiter Assay Plate method for detection of susceptibility of multidrug resistant Mycobacterium tuberculosis to second-line anti-tuberculous drugs. Egyptian Journal of Chest Disease and Tuberculosis 62(2), 241–247: DOI: 10.1016/j.ejcdt.2013.05.008

Luo, X., Pires, D., Aínsa, J.A., Gracia, B., Mulhovo, S., Duarte, A, Anes, E., Ferreira, M-J.U., 2011. Antimycobacterial evaluation and preliminary phytochemical investigation of selected medicinal plants traditionally used in Mozambique. Journal of Ethnopharmacology 137 (1), 114–120: DOI: 10.1016/j.jep.2011.04.062.

Mativandlela, S.P.N., Meyer, J.J.M., Hussein, A.A., Houghton, P.J., Hamilton, C.J., Lall, N., 2008. Activity against *Mycobacterium smegmatis* and *M. tuberculosis* by Extract of South African Medicinal Plants. Phytotherapy Research 22, 841–845. DOI: 10.1002/ptr.2378.

Mohamad, S., Zin, N.M., Wahab, H.A., Ibrahim, P., Sulaiman, S.F., Zahariluddin, A.S., Noor, S.S., 2011. Antituberculosis potential of some ethnobotanically selected Malaysian plants. Journal of Ethnopharmacology 133, 1021–1026. DOI: 10.1016/j.jep.2010.11.037

National Institutes of Health (NIH), 2015. Mistletoe Extracts (PDQ®)–Patient Version. National Cancer Institute. https://www.cancer.gov/about-cancer/treatment/cam/ patient /mistletoe-pdq

Newton, S.M., Lau, C., Gurcha, S.S., Besra, G.S., Wright, C.W., 2002. The evaluation of forty-three plant species for in vitro antimycobacterial activities; isolation of active constituents from Psoralea corylifolia and Sanguinaria canadensis. Journal of Ethnopharmacology 79, 57–67.

Nguta, J.M., Appiah-Opong, R., Nyarko, A.K., Yeboah-Manu, D., Addo. P.G.A., 2015. Current perspectives in drug discovery against tuberculosis from natural products. International Journal of Mycobacteriology 4(3), 165–183: DOI: 10.1016/j.ijmyco.2015.05.004.

Nguta, J.M., Appiah-Opong, R., Nyarko, A.K., Yeboah-Manu, D., Addo, P.G.A., Otchere, I., Kissi-Twuma, A., 2016. Antimycobacterial and cytotoxicity activity of selected medicinal plant extracts. Journal of Ethnopharmacology 182, 10–15: http://dx.doi.org/10.1016/j.jep.2016.02.010.

Nkenfou, C.N., Mawabo, I.K., Notedji, A., Nkenfou, J., Fokou, P.V., Jouda, J.B., Kuiate, J-R., 2015. In vitro antimycobacterial activity of six Cameroonian medicinal plants using microplate alamarBlue assay. International Journal of Mycobacteriology 4(4), 306–311. Doi: 10.1016/j.ijmyco.2015.08.004.

Ogunmefun, O.T., Fasola, T.R., Saba, A.B., Oridupa, O.A., 2013. The Ethnobotanical, Phytochemical and Mineral Analyses of Phragmanthera Incana (Klotzsch), a Species of Mistletoe Growing on Three Plant Hosts in South-Western Nigeria. International Journal of Biomedical Science 9(1), 33–40. PMCID: PMC3644413.

Proksch, P., Georgios, D., Rainer, K., Hendrik, K., Rudolf, H., Nicole, J., Victor, W., Wenhan, L., 2015. Callyaerins from the marine sponge *Callyspongia aerizusa*: cyclic peptides with antitubercular activity. Journal of Natural Products. DOI: 10.1021/acs.jnatprod.5b00266

Sánchez, J.G., and Kouznetsov, V.V., 2010. Antimycobacterial susceptibility testing methods for natural products research. Brazilian Journal of Microbiology 41(2), 270–277: DOI: 10.1590/S1517-83822010000200001.

Singh, R., Hussain, S., Verma, R., Sharma, P., 2013. Anti-mycobacterial screening of five Indian medicinal plants and partial purification of active extracts of *Cassia sophera* and *Urtica dioica.* Asian Pacific Journal of Tropical Disease 6(5), 366-71: DOI: 10.1016/S1995-7645(13)60040-1.

Takem, L.P., Babatunde A.S. Lawal, B.A.S., Udia, P.M., Udoh, F.V., 2015. Assessment of Glycaemic Property of *Phragmanthera capitata*. International Journal of Pharmacy and Pharmaceutical Research, Human Journal 4(4), 1-8.

Tosun, F., Akyüz Kızılay, Ç., Şener, B., Vural, M., 2005. The Evaluation of Plants from Turkey for *in-Vitro* Antimycobacterial Activity. Pharmaceutical Biology 43(1), 58–63. DOI: 10.1080/13880200590903372.

World Health Organization (WHO), 2016. Tuberculosis. Fact sheet. Revised October, 2016. http://www.who.int/mediacentre/factsheets/fs104/en/.

CHAPTER EIGHT

IN- VITRO ANTI-DIABETIC EVALUATION OF *PHRAGMANTHERA CAPITATA*

This chapter has been accepted for publication in the Journal of Herbs, Spices and Medicinal Plants

CHAPTER EIGHT

IN- VITRO ANTI-DIABETIC EVALUATION OF *PHRAGMANTHERA CAPITATA*

CONTENTS **Pages**

Background of the study 128
Methodology 129
Alpha amylase inhibition assay 129
Alpha glucosidase inhibition assay.. 131
Statistical analysis 131
Result.. 132
Discussion 134
Conclusion... 136
References 136

Background of the study

Diabetes mellitus which is a complex disease characterized by hyperglycemia (Lee and Jeon, 2013) is an abnormal metabolism of carbohydrate, protein and fat (Kazeem *et al.,* 2013). This progressive disorder of glucose metabolism eventually leads to different severe vascular changes with secondary complications that may be hard to manage and threat (Rayar and Manivannan, 2015). The inadequate synthesis of insulin and the insulin resistance or dysfunction by the pancreas β- cells result in type I and II diabetes respectively (Kazeem *et al.,* 2013).

Alpha-amylase and glucosidase are gastrointestinal digestive enzymes found in the pancreatic juice and saliva and in the mucosal brush border of the small intestine respectively. While α-amylase catalyzes the initial breakdown of large insoluble starch molecules into absorbable smaller oligosaccharides, α- glucosidase on the other hand, initiates the end of the digestion by further degrading the smaller molecules into glucose which enters the bloodstream on absorption leading to post-prandial hyperglycemia (elevated blood glucose levels) (Kazeem *et al.,* 2013; Mohamed *et al.,* 2012). Therefore, the inhibition of both α- amylase and glucosidase delays digestion of carbohydrate in the small intestine and reduces post-prandial hyperglycemia.

Anti-diabetic mechanisms of action act by lowering the blood glucose levels by inhibiting glucose digestion and/or absorption in/from the small intestine, increment of the secretion of insulin from the pancreas β- cells, elevation of the insulin activities in the peripheral tissues, inhibition of gluconeogenesis by reducing the hepatic glucose production and improvement of glucose utilization by adipose and skeletal muscle tissues (Agnaniet *et al.,* 2016; El-Abhar and Schlaalan, 2014).

Different synthetic anti-diabetic drugs have been developed (Derosa and Maffioli, 2012) and though they are effective, but side effects and cost have been their major concern and setbacks.

Hence, the use of plant-based pharmaceuticals has been sought as alternatives with increased potency, easily available and with less side effects than existing synthetic drugs (Mallare *et al.,* 2005). Although different researchers have reported different anti-diabetic activities in some mistletoes *in-vivo* (Takem *et al.,* 2015; Osadebe *et al.,* 2010, 2004), none has investigated the possible mode of action. Therefore, this present study was aimed at investigating the effect of *Phragmanthera capitata* activities on α- amylase and glucosidase in an attempt to validate its ethnomedicinal usage as an anti-diabetic agent (Ogunmefun *et al.,* 2015; Dibong *et al.,* 2009) and its possible mechanism of action.

Methodology

Plant collection and extraction

Collection and extraction of the sample were carried out as previously described in chapter two.

Alpha-amylase inhibition assay

Assay Principle: Alpha-amylase activity was measured by the amount of starch hydrolyzed into monosaccharides in the presence of the enzyme. The reaction incorporates an iodine reagent which gives a blue colour in the presence of starch. In the presence of an enzyme inhibitor, the intensity of the colour measured spectrophotometrically indicates the amount of starch remaining in the reaction mixture, and hence, the extent of alpha-amylase inhibition.

Reagents

i. *Starch solution* (2%): 0.2 g of starch in 100 mL distilled water. Proper dissolution was initiated by boiling the solution in a glass beaker directly on a stirring plate for about 15 minutes.

ii. Phosphate buffer: 100 mM pH 6.8

iii. 1M Hydrochloric acid (stock solution = 37%): 8.212 mL HCl (1N) was slowly added to 25 mL distilled water and made up to 100 mL with the same.

iv. Iodine reagent: 0.127 g I_2 and 0.083 KI were mixed together in 100 mL of distilled water.

v. Alpha-amylase enzyme: 10 mg porcine pancreatic amylase was dissolved in 100 mL phosphate buffer. The preparation was made just before use and kept in ice pack.

vi. Acarbose stock solution (positive control): A 500 µM solution was initially prepared fresh immediately before the assay by dissolving 3.2 mg acarbose in 10 mL phosphate buffer. Aliquots of 1 mL were made into eppendorf tubes and placed in ice pack. The aliquots were diluted five-fold to give a working concentration of 100 µM which is 64 µg/mL.

vii. Plant extracts: Different concentrations (50, 100, 500 and 1000 µg/mL) of the acetone, methanol, ethanol and aqueous crude extracts were prepared in phosphate buffer.

Procedure:

10 µL of the enzyme solution was pipetted into appropriate wells of a 96-well plate. Thereafter, 30 µL of test samples, phosphate buffer (the control, enzyme without inhibitor) or positive control (acarbose, 64 µg/mL) were added to the enzyme in respective wells. The mixture was pre-incubated for 10 minutes at 37ºC to allow interaction of the enzyme with the different compounds. The reaction was started by the addition of 40 µL starch solution to the wells and was incubated again for 30 minutes at 37ºC. The reaction was terminated by adding 20 µL of 1M HCl to each well, followed by 75 µL iodine reagent. Absorbance was measured at 580 nm using a microplate reader (Diagnostic Automation, Inc, USA, DAR 800). Controls without enzyme and without starch were also included in the assay to be certain that no reaction occurred when one of either the enzyme or substrate was absent. This was done to eliminate false positive results because some plants extracts have been reported to contain traces of α-amylase or starch.

Alpha-amylase inhibition was measured as a percentage of the enzyme control using the formula:

% α-amylase inhibition = [(Abs control – Abs sample)/ (Abs control)] X 100.

Alpha-glucosidase inhibition assay

Assay Principle: The assay is based on the hydrolysis of p-nitrophenyl-α-D-glucopyranoside (PNP-GLUC) specifically by α-glucosidase into a yellow coloured product, p-nitrophenol (PNP) and D-glucose, with an absorbance maximum at 405 nm. Inhibition of α-glucosidase results in reduced formation of PNP.

Reagents

i. Potassium phosphate buffer (100 mM; pH 6.8)

ii. p-Nitrophenyl-α-D-glucopyranoside (PNP-GLUC) solution (10 mM)

iii. Sodium carbonate solution (100 mM)

iv. α-glucosidase solution (50 µg/mL)

v. Epigallocatechin gallate (positive control); EGCG (10 µg/mL)

Procedure

Briefly, 10 µL of plant extract (50, 100, 500 and 1000 µg/mL) or the positive control (EGCG) was mix with 40 µL of α-glucosidase solution. The mixture was pre-incubated at 37ºC for 5 minutes and initial background absorbance was read at 405 nm. Thereafter, 10 µL of PNP-GLUC was added and the reaction mixture was incubated again for 20 minutes at 37ºC. The reaction was terminated by the addition of 50 µL of sodium carbonate solution. The absorbance was measured again at 405 nm using a microplate reader (Diagnostic Automation, Inc, USA, DAR 800). Controls without the enzyme inhibitor (phosphate buffer) and without the substrate (PNP-GLUC) were also included in the assay.

The percentage inhibition of α-glucosidase was calculated as follows:

% α-glucosidase inhibition = [(Abs control – Abs sample)/ (Abs control)] X 100.

Statistical analysis

All data in triplicates were subjected to one-way analysis of variance (ANOVA) using GENSTAT 8 statistical package. Where the data showed significance ($P \leq 0.05$), mean separation was done by Fisher's Least Significant Differences (LSD).

Result

The ability of the crude extracts of *P. capitata* to inhibit α- amylase was shown in Figure 1. All the extracts exhibited weak α- amylase inhibition at the concentrations (50, 100, 500 and 1000 µg/mL) tested in a concentration-dependent manner. While the percentage inhibition of the positive control (acarbose) was 94.58 ± 2.32% at a concentration of 64 µg/mL, it ranged from -0.22 ± 0.39 to 14.05 ± 0.24% in the acetone extract, 1.71± 0.34 to 13.96 ± 0.28% in the methanol extract, 0.80 ± 0.71 to 11.96 ± 1.24% and -2.66 ± 0.54 to 1.18 ± 0.46% for the ethanol and aqueous extracts respectively. The concentration of the extracts that inhibited 50% of α- amylase could not be determined in this study (Table 1).

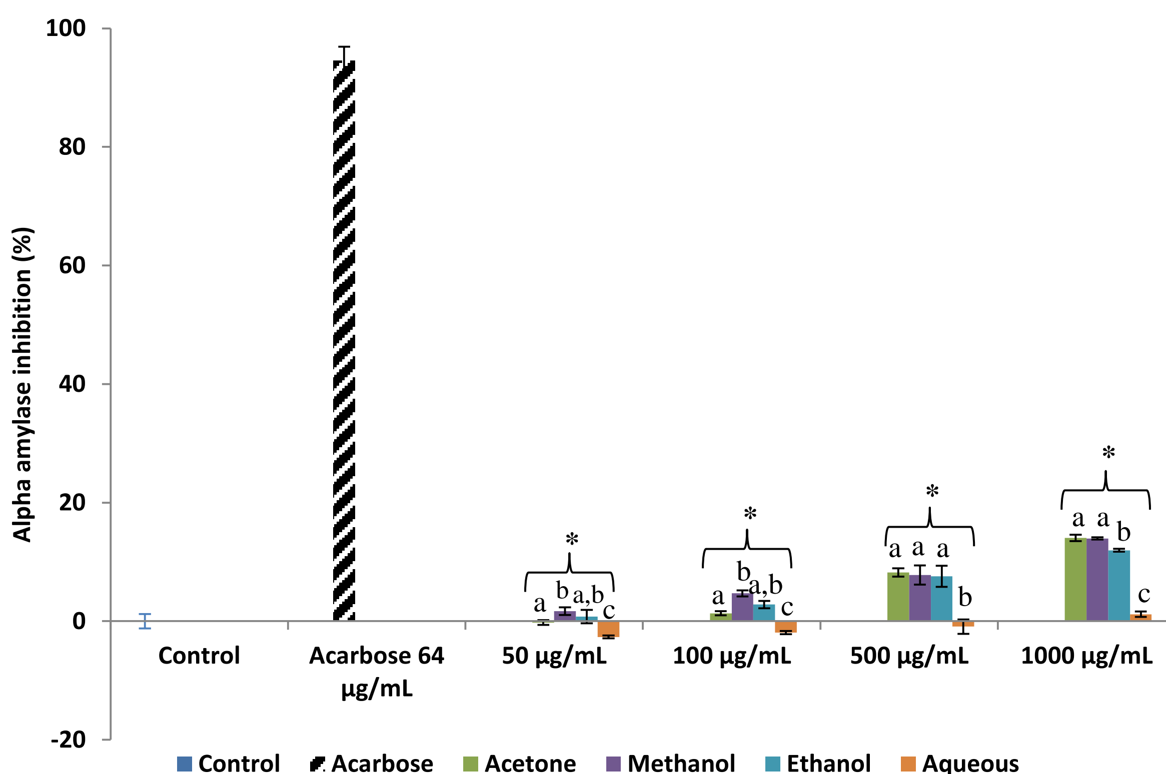


Figure 1: Alpha-amylase inhibitory activity of the different solvent extracts of *Phragmanthera capitata.* Values are mean ± SD (n=3). Set of bars (the same concentration) with different alphabets are significantly different (p <0.05). '*' significantly lower than acarbose (positive control) (p<0.05).

The same concentration range used for the α-amylase assay was also used in evaluating the α-glucosidase inhibition of *P. capitata* and the result was shown in Figure 2. A concentration-

dependent inhibition was also observed in α- glucosidase investigation. In contrast to the low α-amylase inhibition by the different solvent extracts of *P. capitata,* a stronger inhibition was observed with α- glucosidase activity. The percentage α- glucosidase inhibition ranged from 41.44 ± 2.20 to 94.05 ± 0.08% in the acetone extract and from 14.31± 0.69 to 50.96 ± 2.17% in the aqueous extract. The concentration of the extract that inhibited 50% (IC_{50}) of α- glucosidase enzyme was shown in Table 1. The acetone followed by the methanol extract had the best α-glucosidase inhibitory activities with IC_{50} values of 71.89 ± 3.48 µg/mL and 91.98 ± 2.24 µg/mL respectively. The aqueous extract had the least activity with an IC_{50} value of 952.87 ± 9.87 µg/mL. Though the inhibition of α- glucosidase at lower concentrations (50 and 100 µg/mL) assayed for in this study was significantly lower than the positive control (EGCG at 10 µg/mL), higher concentrations of 500 and 1000 µg/mL of all the solvent except the aqueous extract, had significantly higher inhibitions (89.95 ± 7.06 to 94.05 ± 0.08%) than the EGCG with 64.57 ± 1.03% inhibitory activity ($p < 0.05$).

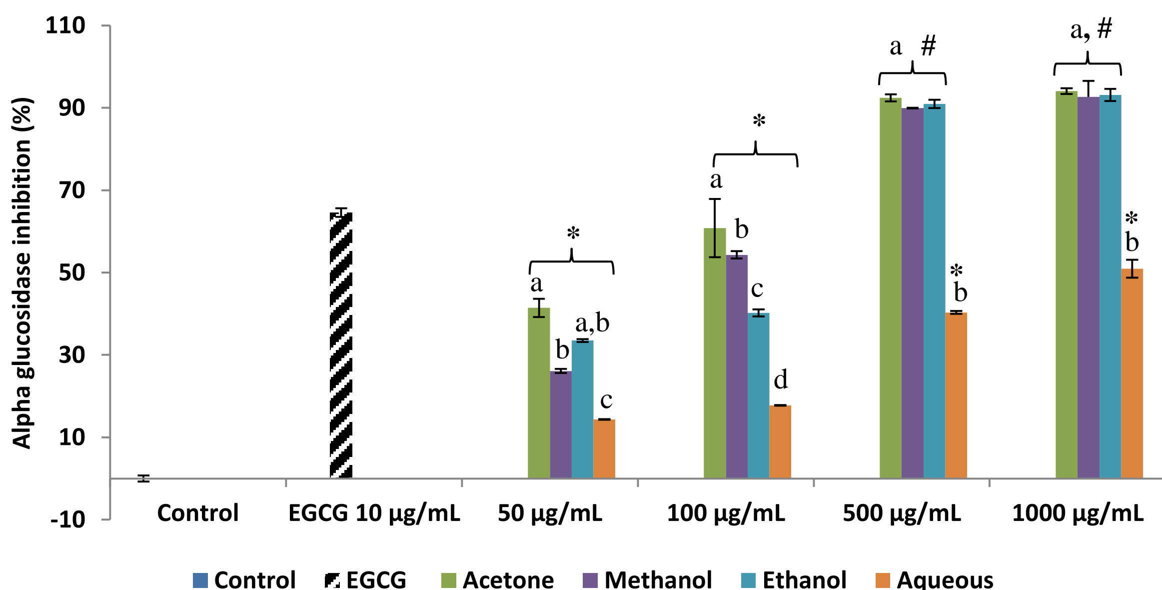


Figure 2: Alpha-glucosidase inhibitory activity of the different solvent extracts of *Phragmanthera capitata.* Values are mean ± SD (n=3). Set of bars (the same concentration) with different alphabets are significantly different ($p < 0.05$). #: significantly higher than the positive control (EGCG: Epigallocatechin gallate) while '*' is significantly lower than EGCG ($p < 0.05$).

Table 1: IC_{50} of α-amylase and α- glucosidase inhibitory activity of the different solvent extracts of *P. capitata*

	IC_{50} µg/mL	
	α- Glucosidase	**α- Amylase**
Acetone	71.89 ± 3.48 [a]	ND
Methanol	91.98 ± 2.24 [a]	ND
Ethanol	178.71 ± 6.82 [b]	ND
Aqueous	953.87 ± 9.87 [c]	ND
Acarbose	-	< 64
Epigallocatechin gallate	< 10 [d]	-

Values are means ± SD (n=3). Figures with different superscript are significantly different at $p < 0.05$. **ND:** not determined.

Discussion

Diabetes had been a global health issue with an estimated 3% of the world's population been diabetic and the prevalence had been postulated to increase to 6.3% by the year 2025. The management of diabetes still posed a challenge in the health sector because of the complications and secondary failure rates associated with the use of synthetic anti-diabetic medications (Kumaresan *et al.,* 20i3; Kwon *et al.,* 2007). This had led to the continuous search for natural anti-diabetic products which are effective, cheap and less toxic. *Phragmanthera capitata* had been used extensively for the management and treatment of a wide range of ailments including diabetes in folklore medicine in Africa (16, 15; 4). This present study was carried out to investigate the anti-diabetic properties of *P. capitata*.

Crude extracts prepared from different solvents (acetone, methanol, ethanol and aqueous) were used for this investigation and the concentration range (50 µg/mL to 1000 µg/mL) for the evaluation was however within the safe limit (LD_{50} > 1000 µg/mL) earlier established in chapter five of this study. From the results of the investigation, all the solvent extracts

exhibited weak α- amylase inhibition (Figure 1) and this may give the impression that the anti-diabetic mechanism of action of *P. capitata* may not probably be by the inhibition of pancreatic α-amylase. The stronger α-glucosidase inhibition (Figure 2) could be one of the best possible mechanisms whereby this plant exerts its antihyperglycemic activity.

Contrary to the results of this study, Channabasava and Sadananda (2013) investigation on *Dendrophthoe falcate*, a mistletoe in the Loranthaceae family that was harvested from neem plant, reported a very high α-amylase inhibition of 84 to 88% in the methanol and aqueous extracts respectively (concentrations used were not well defined). The weak α-amylase and strong α-glucosidase inhibitory activity observed in this study were in consonance with an earlier report by Kwon *et al* (2007) that most phytochemicals are weak α-amylase but stronger α-glucosidase inhibitors. This also agreed with the reports of Oboh *et al* (2012) on *Telfaria occidentalis*, Kazeem *et al* (2013) on *Morinda lucida* and Kazeem and Ashafa (2015) on *Dianthus basuticus* who observed weak α-amylase and stronger α-glucosidase inhibitions. However, weak α-amylase inhibition by medicinal plants confers an advantage over synthetic anti-diabetic medications: with high α- amylase inhibition in the management of postprandial blood glucose. The excessive pancreatic α-amylase inhibition by synthetic anti-diabetic drugs could result in the accumulation of undigested carbohydrates in the colon which may promote abnormal bacterial fermentation on them. This abnormal bacterial fermentation is believed to be responsible for most of the gastrointestinal side effects observed by synthetic anti-diabetic drugs (Apostolidis *et al.,* 2007; Kwon *et al.,* 2007). Therefore, mild α-amylase inhibitory activity is desirable. Hence, the low α-amylase and high α- glucosidase inhibition exhibited by *P. capitata* could serve as a therapeutic pathway for the treatment of postprandial hyperglycemia with little or no side effects. The use of crude extracts for the management of diabetes may confer a further advantage over synthetic drugs because the purer an extract becomes the synergistic interactions of the compounds may diminish and eventually the thinner the safety may be (Osadebe *et al.,* 2010b).

There was no positive correlation of extraction yield to inhibitory activities in this study. Though the methanol and aqueous had more extraction yield, the acetone extract exhibited the best α-glucosidase [Table 1] and α- amylase (500 µg/mL – 1000 µg/mL [Figure 1]) inhibitory activity while the aqueous extract had the least activity. Channabasava and Sadananda (2013) reported a contrary finding with the aqueous extract of *D. falcata* been more potent that the methanol extract. The best activity observed in the acetone extract in this study could be due to the ability of acetone to extract both hydrophilic and lipophilic compounds from the sample (Eloff, 1998). This also supports earlier findings in this study (Chapter two) where the acetone extract had better polyphenolic compounds than the other solvent extracts tested with corresponding higher anti-oxidant activity.

Conclusion

This study is the first *in-vitro* anti-diabetic report of *P. capitata* harvested from rubber trees.. This study suggests that the inhibition of carbohydrate-hydrolyzing enzymes (weak α-amylase and strong α-glucosidase inhibition) could be one of the possible mechanisms of action by which P. *capitata* exerts its anti-diabetic activity hence, supports its reported ethnomedicinal application in the management of diabetes in African folklore.The low α- amylase inhibition observed in this study may suggest that the anti-diabetic mechanism of action of *P. capitata* may not probably be by the inhibition of pancreatic α- amylase. The stronger α- glucosidase inhibition could be one of the best possible mechanisms whereby this plant exerts its anti-

References

Agnaniet, H., Mbot, E.J., Keita, O., Fehrentz, J-A., Ankli, A., Gallud, A., Garcia, M., Gary-Bobo, M., Lebibi, J, Cresteil, T., Menut, C., 2016. Antidiabetic potential of two medicinal plants used in Gabonese folk medicine. BMC Complementary and Alternative Medicine 16, 71. DOI: 10.1186/s12906-016-1052-x.

Apostolidis, E., Kwon, Y.-I., Shetty, K., 2007. Inhibitory potential of herb, fruit, and fungal-enriched cheese against key enzymes linked to type 2 diabetes and hypertension. Innovative Food Science and Emerging Technologies 8 (1), 46–54, 2007.

Channabasava, M.G., Sadananda, T.S. 2013. *In vitro* anti-diabetic activity of parasitic plant, *Dendrophthoe falcata* (L.f) Ettingsh. Natural Products 9(8): 311-318.

Derosa, G., Maffioli, P., 2012. α-Glucosidase inhibitors and their use in clinical practice. Archives of Medical Science 8 (5), 899-906.

Dibong, S.D., EngoneObiang, N.L., Ndongo, D., Priso, R.J., Taffouo, V., Fankem, H., Salle, G., Missoup, A.D., Boussim, I.J., Amougou, A., 2009. An assessment on the uses of Loranthaceae in ethnopharmacology in Cameroon: A case study made in Logbessou, North of Douala. Journal of Medicinal Plants Research 3(8), 592-595.

El-Abhar, H.S., Schaalan, M.F., 2014. Phytotherapy in diabetes: Review on potential mechanistic perspectives. World Journal of Diabetes 5(2), 176–197.

Eloff, J.N. 1998. Which extractant should be used for the screening and isolation of antimicrobial components from plants? Journal of Ethnopharmacology 60(1): 1–8.

Kazeem, M.I., Ashafa, A.O.T., 2015. In-vitro antioxidant and antidiabetic potentials of *Dianthus basuticus* Butt Davy whole plant extracts. Journal of Herbal Medicine 5(3), 158–164.

Kazeem, M.I., Adamson, J.O., Ogunwande, I.A., 2013. Modes of inhibition of α-amylase and α-glucosidase by aqueous extract of *Morinda lucida* Benth leaf. BioMed Research International Article ID 527570, 6 pages. http://dx.doi.org/10.1155/2013/527570.

Kwon, Y.I., Apostolidis, E., Kim, Y.C., Shetty, K., 2007. Health benefits of traditional corn, beans and pumpkin: In vitro studies for hyperglycemia and hypertension management. Jornal of Medicinal Food 10, 266–275.

Kumaresan, P.T., Saravanan, S., Subish, R. 2013. In vitro anti diabetic activity of *Morinda tinctoria* fruits extracts. Asian Journal of Pharmaceutical and Clinical Research 7(1): 90-92.

Lee, S-H., Jeon, Y-J., 2013. Anti-diabetic effects of brown algae derived phlorotannins, marine polyphenols through diverse mechanisms. Fitoterapia 86, 129–136. http://doi.org/10.1016/j.fitote.2013.02.013.

Mallare, J.T., Karabell, A.H., Velasquez-Mieyer, P., Stender, S.R.S., Christensen, M.L., 2005. Current and future treatment of metabolic syndrome and Type 2 diabetes in children and adolescents. Diabetes Spectrum 18 (4), 220-228.

Mohamed, E.A., Siddiqui, M.J., Ang, L.F., Sadikun, A., Chan, S.H., Tan, S.C., Asmawi, M.Z., Yam, M.F., 2012. Potent α-glucosidase and α-amylase inhibitory activities of standardized 50% ethanolic extracts and sinensetin from Orthosiphon stamineus Benth as anti-diabetic mechanism. BMC Complementary and Alternative Medicine 12, 176. DOI: 10.1186/1472-6882-12-176.

Oboh, G., Akinyemi, A.J., Ademiluyi, A.O., 2012. Inhibition of α-amylase and α-glucosidase activities by ethanolic extract of *Telfairia occidentalis* (fluted pumpkin) leaf. Asian Pacific Journal of Tropical Biomedicine 2(9), 733–738. Doi: 10.1016/S2221-1691(12)60219-6.

Ogunmefun, O.T., Olatunji, B.P., Adarabioyo, M.I., 2015. Ethnomedicinal survey on the uses of mistletoe in South-western Nigeria. European Journal of Medicinal Plants. 8 (4), 224-230. DOI: 10.9734/EJMP/2015/11048.

Osadebe, P.O., Okide, G.B., Akabogu, I.C., 2004. Study on anti-diabetic activities of crude methanolic extracts of *Loranthus micranthus* (Linn.) sourced from five different host trees. Journal of Ethnopharmacology 95 (2-3), 133–138.

Osadebe, P.O., Omeje, E.O., Uzor, P.F., David, E.K., Obiorah, D.C., 2010. Seasonal variation for the antidiabetic activity of *Loranthus micranthus* methanol extract. Asian Pacific Journal of Tropical Medicine 3, 196–199.

Rayar, A., Manivannan, R., 2015. In-vitro alpha-amylase and alpha-glucosidase inhibition activity of Umbelliferone and beta-ionone isolated from *Coriandrum sativum* Linn. World Journal of Pharmacy and Pharmaceutical Sciences 5, (1), 1280-1289.

Takem, L.P., Essien, A.D., Udia, P.M., Anele, E.I., 2015. Evaluation of lipogenic property of *Phragmanthera capitata* in diabetic rats. The Journal of Phytopharmacology 4, 299-302.

CHAPTER NINE

GENERAL DISCUSSION AND CONCLUSION

GENERAL DISCUSSION AND CONCLUSION

Discussion

The increase in the use of herbal medicine has simultaneously resulted in a rapid demand for medicinal plants globally (Chen *et al.,* 2016). However, in accordance with the reports of the International Union for Conservation of Nature and the World Wildlife Fund, a vast number of medicinal plants are threatened with extinction due to overexploitation and habitat destruction (Bentley, 2010). Another factor which could lead to the deliberate destruction of medicinal plants is ignorance of importance or use. Many plants are threatened with extinction because their uses are either undocumented and/or unknown and hence, they are considered as weeds and destroyed with prejudice. This is the case with mistletoe. Mistletoes are globally seen as pests of gardens and plantations that reduce the quality of produce or even kill the crops (Adesina *et al.,* 2013). Hence, there is a deliberate attempt by plantation and garden owners to eradicate them; an act which has left some species of mistletoe now at the risk of extinction (IUCN, 2015). It is believed that proper documentation of plants' uses and importance will serve as a key in sustainable conservation for future purposes. This study, therefore, sought to evaluate, validate and document the medicinal potentials of *Phragmanthera capitata* (Sprengel) Balle, a mistletoe commonly found in rubber plantations in Africa (Dibong *et al.,* 2010; EngoneObiang *et al.,* 2009) and faced with the scourge of unwholesome destruction from rubber plantations across Africa.

Studies in phytopharmacology involve multidisciplinary sciences and the research is almost unlimited. In this thesis, the phytochemical, anti-oxidative, nutritive, essential oil and toxicity evaluations of *P. capitata* were reported. Biological activities of the extracts on some human pathogenic micro-organisms most especially *Mycobacterium tuberculosis;* and its effect on diabetes were also discussed.

The presence of polyphenolics like phenols, flavonoids and proanthocyanidins etc have been reported to be responsible for the anti-oxidative properties in medicinal plants extracts. Antioxidants are substances with protective abilities from damages caused by free radical-induced stresses as a result of oxidation (Iloki-Assanga *et al.,* 2015). In this study, different solvents (methanol, acetone, ethanol and water) were used for the extraction of the mistletoe to determine their effects on the total yield and activities. From the result, all the solvent extracts had high polyphenolic content with correlated anti-oxidant activities. Methanol had the highest extract yield while ethanol had the lowest yield. However, the acetone followed by the ethanol extracts had higher amounts of polyphenolic assayed for with corresponding higher antioxidant activities as reported in chapter two of this thesis. Hence, this plant may be effective in the management of oxidative related stresses or inflammations.

This study also evaluated the potential nutritional and mineral compositions of the sample. This research revealed important mineral compositions even higher than those found in some conventional vegetables. It is rich in carbohydrate which is the major source of energy and a substantial amount of fibre, protein and fat. The findings of this work suggest that this species could have the potential to boost the immune system due to its rich mineral and nutrient compositions. The nutritional and mineral potentials of *P. capitata* could be a contributing factor to its wide therapeutic applications in herbal medicine.

The use of essential oils in healthcare is topical in recent times most especially in aromatherapy (Lawal and Ogunwande, 2013; Baser and Buchbaur, 2010; Lee *et al.,* 2010). The essential oils from *P. capitata* revealed the presence of some pharmacological important compounds like Alpha-linolenic acid, Isoeugenol, Methyl hexadecanoic acid, Phytol and D-limonene. The presence of these and other compounds in the plant could also be a contributing factor to its pharmacological activities.

The general perception that herbal remedies are safe and devoid of adverse effects may not be entirely true and misleading. In fact, there had been several reports of toxicity in herbal remedies which had led to serious damages to the consumers (Ekor, 2014). Arising from these contraindications, the different solvent extracts were screened for possible toxicity using the brine shrimp assay. The outcome of the evaluation revealed all extracts of *P. capitata* to be non-toxic based; hence, could be explored for further plant-based pharmaceuticals.

The increase in multidrug resistance (MDR), total drug resistance (TDR) and extensive drug resistance (XDR) in most human pathogenic microorganisms to conventional drugs has necessitated the screening of plant-based products as possible anti-microbial agents (Djeussi *et al.,* 2013; Coates *et al.,* 2002). Hence, the assessment of the extracts of *P. capitata* on some human pathogenic bacteria and fungi was carried out. The outcome of the assessment showed that *P. capitata* has a better potential as an anti-bacterial agent than as an anti-fungi agent except for the aqueous extract which proved otherwise (with better antifungal activity). Most notable of its bacterial activity is the high inhibitory effect on *Mycobacterium tuberculosis*, the major causative agent of tuberculosis. Hence, this plant may be said to have potential to confer some immunity against the dreaded TB and other pathogenic diseases in general.

The anti-diabetic screening of the *P. capitata* revealed that the potential mechanism of action could be by the inhibition of pancreatic α- amylase and α- glucosidase. The use of phytotherapy for the management of diabetes could eradicate the side effects associated with the use of synthetic anti-diabetic medications. This study supports the ethnopharmacological application of *P. capitata* in the management of diabetes (Ogunmefun *et al.,* 2015; Dibong *et al.,* 2009).

Conclusion

The outcome of this study revealed the potential of *P. capitata* in phytotherapeutics. The high polyphenolic contents with correlative antioxidative activities of the extracts are an indication of the possible anti-inflammation activity of *P. capitata* against cellular oxidative stresses

caused by free radicals. The nutrient value and mineral contents in the sample could suggest its immune boosting potentials and even supplement for most nutrient deficiency syndromes. The compounds identified in the essential oils of the sample could have a significant contribution to the total pharmacological activities observed in this study and will also pave way for further studies in this line as it is the first ever report of the chemical constituents of the essential oils of the sample. As one of the WHO criterion for the use of phytotherapy, the non-toxic outcome of this study could alleviate the scare of the reported toxicity of most mistletoe. The outcome of this study further validated some ethnomedicinal uses of *P. capitata* against infectious diseases, diabetes and hence, may serve as an important herb in the management of the infections caused by microorganisms.

From the foregoing, *P. capitata* has exhibited potentials in phytotherapy. Therefore, instead of its total destruction from rubber trees and other crop plantations, a sustainable conservation approach should be implemented to have the full benefits of this plant now and in the future (because the destruction of a plant could result to the permanent loss of a potential tomorrow's lifesaving drug).

References

Adesina, S.K., Illoh, H.C., Imoh, I.J., Imoh, E.J., 2013. African Mistletoes (Loranthaceae); Ethnopharmacology, Chemistry and Medicinal Values: An Update. *African Journal of* Traditional, Complementary and Alternative Medicines 10(4), 161–170. PMCID: PMC3794408.

Baser, K.H.C. Buchbauer, G., 2010. Handbook of Essential Oils: Science, Technology and Applications. CRC Press, Boca Raton, London, New York.

Bentley, R., editor. Medicinal plants. London: Domville-Fife Press; 2010. p. 23–46.

Chen, S−L. , Yu, H., Luo, H-M., Wu, Q., Li, C-F., Steinmetz, A., 2016. Conservation and sustainable use of medicinal plants: problems, progress, and prospects. BMC Chinese Medicine 11, 37. DOI: 10.1186/s13020-016-0108-7.

Coates, A., Hu, Y., Bax, R. Page, C., 2002. The future challenges facing the development of new antimicrobial drugs. Nature Reviews Drug Discovery. 1, 895-910.

Dibong, S.D., EngoneObiang, N.L., Ndongo, D., Priso, R.J., Taffouo, V., Fankem, H., Salle, G., Missoup, A.D., Boussim, I.J., Amougou, A., 2009. An assessment on the uses of Loranthaceae in ethnopharmacology in Cameroon: A case study made in Logbessou, North of Douala. Journal of Medicinal Plants Research 3(8), 592-595.

Dibong, S.D., Mony, R., Ndiang, Z., Ondoua, J.M., Boussim, I.J., Bilong, B., Amougou, A., 2010. The struggle against *Phragmanthera capitata* (Sprengel) S. Balle (Loranthaceae) parasite of agroecosystems' fruit trees in Cameroon. Journal of Agricultural Biotechnology and Sustainable Development 2(5), 76-81.

Djeussi, D.E., Noumedem, J.A., Seukep. J.A., Fankam, A.G., Voukeng, I.K., Tankeo, S.B., Nkuete, A.H., Kuete, V., 2013. Antibacterial activities of selected edible plants extracts against multidrug-resistant Gram-negative bacteria. BMC Complementary and Alternative Medicine 3,164. DOI: 10.1186/1472-6882-13-164

Ekor, M., 2014. The growing use of herbal medicines: issues relating to adverse reactions and challenges in monitoring safety. Frontiers in Pharmacology 4, 177.

EngoneObiang, N.L., Sallé, G., Eschbach, J.M., Salomon, M., 2009. Susceptibility of rubber trees to Loranthaceae in Gabon. Journal of Plant Interactions 4 (3), 233-240. DOI: 10.1080/17429140902962613.

Iloki-Assanga, S.B., Lewis-Lujan, L.M., Lara-Espinoza, C.L., Gill-Salido, A.A., Fernandez-Angulo, D., Rubio-Pino, J.L., Haines, D.D., 2015. Solvent effects on the phytochemical constituent profiles and antioxidant activities, using four different extraction formulations for analysis of *Bucida buceras* L. and *Phoradendron californicum.* BMC Research Notes 8, 396. DOI 10.1186/s13104-015-1388-1.

International Union for the Conservation of Nature. 2015. Red List of Threatened Species. Version 2015.1. <www.iucnredlist.org>. Downloaded on 04 June 2015

Lawal, O.A. Ogunwande, I.A., 2013. Essential Oils from the Medicinal Plants of Africa. In book: Medicinal Plant Research in Afric, 203-224. DOI: 10.1016/B978-0-12-405927-6.00005-9.

Lee, M.S., Choi, J., Posadzki, P., Ernst, E., 2012. Aromatherapy for healthcare: An overview of systematic reviews. Maturitas 71 (3), 257–260. PMID 22285469.

Ogunmefun, O.T., Olatunji, B.P., Adarabioyo, M.I., 2015. Ethnomedicinal survey on the uses of mistletoe in South-western Nigeria. European Journal of Medicinal Plants. 8 (4), 224-230. DOI: 10.9734/EJMP/2015/11048.