EVALUATION OF PHYTOCHEMICAL COMPOSITION IN SELECTED MEDICINAL PLANTS AND POTENTIAL APPLICATION AS ANTIMICROBIAL AGENT

by

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A THESIS

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DEDICATION

I wholeheartedly dedicate this thesis to my deceased parents, a loving and cherishable couple; Mrs. Elizabeth Yassah Konah Smith and Mr. Kpaka Bobo Smith through whose time, energy, blessing, and motivation helped me in this climb. My blessed two, I treasure you now and forever; and always pray for yet an eternal rendevouz.

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Evaluation of Phytochemical Composition in Selected Medicinal Plants and Potential Application as Antimicrobial Agent

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ABSTRACT

Four medicinal plants native to Cameroon (C) and Kenya (K): *Prunus africana* (C) bark and root, *P. africana* (K) bark, *Pausinystalia yohimbe* bark, and *Orthero* root were screened to assess their phytochemical compositions and antioxidant activities. The plant parts were extracted using acetone, methanol, and ethanol (solvent: water, 80:20 v/v). The antioxidant capacity of the extracts was evaluated by their ability to scavenge free radicals using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and their antiradical power (ARP); trolox equivalent antioxidant capacity (TEAC) by utilizing 2,2′-azinobis-(3-ethylbenzothiaziline-6-sulfonate) (ABTS); and ferric reducing antioxidant power (FRAP). The total phenolic content (TPC) and total flavonoid content (TFC) were estimated by the Folin-Ciocalteu (FC) and aluminum-chloride ($AlCl_3$) assays, respectively. Pigment content was determined spectrophotometrically, and phenolic acids were quantified by high performance liquid chromatography (HPLC) using eight extracts with EC_{50} values (±0.125), in comparison with the standard (i.e., ascorbic acid). In addition, the antimicrobial activity was demonstrated using the agar disc diffusion assay followed by the measurement of the average zone of inhibition (ZOI) in which the first five extracts in the order of decreasing antioxidant power were tested against two clinical foodborne bacteria (gram-negative *E. coli* and gram-positive *S. aureus*). The highest DPPH radical scavenging activity was found in *P. africana* (K) bark (95.77%; acetonic extract) though its methanolic extract exhibited the lowest antioxidant power with the highest effective concentration (EC_{50}) and lowest ARP (7.298 mg/mL and 0.137), respectively; and its acetonic extracts also exhibited the lowest TPC and TFC, respectively (166.27

mg GAE/g and 9.60 mg RU/g). However, the highest EC_{50} and ARP (0.093 mg/mL and 10.753 respecively), and highest TPC (1131.70 mg GAE/g) were exhibited by the ethanolic extract of *P. africana* (C) bark while the highest FRAP (11.33 mM Fe^{2+}/g) and $ABTS^{\bullet+}$ radical scavenging activity (126.87 mM TE/g) was exhibited by the acetonic and methanolic extracts of *P. yohimbe* bark, respectively. Moreover, the highest TFC (61.33 mg RU/g) was also exhibited by the acetonic extract of *P. yohimbe* bark. Except for xanthophylls, the highest recorded pigment (61.92 mg/g) in this study was exhibited by the acetonic extract of *Orthero* root; the highest measure of all other pigments (chlorophyll a and b; total chlorophyll, lycopene, β-carotene, carotenoids, and anthocyanin) were exhibited by the acetonic extracts of *P. yohimbe* bark (9.16 and 22.46; 32.21, 2.78, 6.83, 20.08, and 0.53 mg/g, respectively). There was a significant positive correlation between TPC, TFC and DPPH, and a significant negative correlation between TPC and the other two antioxidant assays (FRAP and TEAC). Methyl 4-hydrocybenzoate and protocatechuic acid were detected in all the selected extracts, trans-sinapic acid (SIA) in *P. africana* (C) bark and root, and ferulic acid (FA) in *P. africana* root extract only. *P. africana* (C) bark and root extracts had the highest phenolic acid in all plant parts (i.e., SIA) with recorded values >100 mg/g which agrees with their spectrophotometrically determined high TPC. All solvent controls used for extraction, inhibited the growth of both *E. coli* and *S. aureus* while the sterile blank disc controls showed no inhibitory zone. Of all the tested extracts, both concentrations (50 and 100 mg/mL) of acetonic extracts of *P. africana* (C) bark exhibited no growth inhibition against *E. coli* but an active to highly active inhibitory zone against *S. aureus* (8.0 to 17.33 mm). The highest growth inhibition was exhibited by the ethanolic extract of *P. africana* (C) bark against *S. aureus* (17.33 mm). These results clearly support the potential uses of these plant parts in a wide range of applications such as antimicrobials and antioxidants.

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LIST OF ABBREVIATIONS

AA	Atraric Acid
ABTS	2, 2’ -azinobis-(3 ethylbenxothiazoline-6-sulfonate)
ANOVA	Analysis of Variance
ARP	Anti-Radical Power
ASE	Accelerated Solvent Extraction
CA	Caffeic Acid
CE	Capillary Electrophoresis
CZE	Capillary Zone Electrophoresis
DHBA	Dihydroxybenzoic Acid
DMAPP	Dimethylallyl Diphosphate
DPPH	2, 2 –Diphenyl-1-picrylhydrazyl
DXP	1-Deoxyxylulose 5-phosphate
EAD	Electro-Array Detection
EC_{50}	Effective Concentration
ECD	Electro-Chemical Detection
EO	Essential Oil
FA	Ferulic Acid
FC	Folin-Ciocalteau
FBD	Food-Borne Diseases
FRAP	Ferric Reducing Antioxidant Power
GA	Gallic Acid
GC	Gas Chromatography
HBA	Hydroxybenzoic Acid
HCA	Hydrocycinnamic Acid
HPLC	High Performance Liquid Chromatography
IPP	Isopentenyl Diphosphate
MAE	Microwave-Assisted Extraction
MDR	Multidrug-Resistant
MRSA	Methicillin-Resistant *Staphylococcus aureus*
MIC	Minimum Inhibitory Concentration

MS	Mass Spectrometry
MVA	Mevanolic Acid
NBSS	N-butyl Benzene-sulfonamide
NMR	Nuclear Magnetic Resonance
PA	p-Coumaric Acid
PDA	Photo-Diode Array
PEP	Phosphoenolpyruvate
PFE	Pressurized Fluid Extraction
RP	Reversed-Phase
SA	Salicylic Acid
SAR	Structure-Activity Relationship
SIA	Sinapic Acid
SE	Staphylococcal Enterotoxins
SFE	Supercritical Fluid Extraction
SWE	Subcritical Water Extraction
TEAC	Trolox Equivalent Antioxidant Capacity
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TROLOX	6-hydroxy -2, 5, 7, 8 –tetramethylchroman-2-carboxylic acid
TSA	Tryptic Soy Agar
TSB	Tryticase Soy Broth
UAE	Ultrasound-Assisted Extraction
UV/VIS	Ultraviolet/Visible
WSCP	Water-Soluble Chlorophyll-binding Protein
ZOI	Zone of Inhibition

CHAPTER 1: INTRODUCTION

1.1 Background and Significance

The use of medicinal plants in traditional medicine is widely used in many developing countries (Mwitari, Ayeka, Ondicho, Matu, & Bii, 2013), suggesting a dependency of over three-fourth of the earth's population on these natural products (Petrovska, 2012). According to the World Health Organization, the functional and structural units of such plants contain chemical substances that are utilized for therapeutic purposes or employed in the control/treatment of a disease condition (Hamuel, 2012). The chemical/bioactive components, also known as phytochemicals include, but are not limited to alkaloids, terpenoids, essential oils, and phenolic compounds such as flavonoids and phenolic acids. Phytochemicals have exhibited antioxidant, anticancer (Dudonné, Vitrac, Coutière, Woillez, & Mérillon, 2009), antimicrobial, anti-inflammatory, anti-allergic, antimutagenic, antiviral, antithrombotic, and vasodilatory effects (Proestos, Lytoudi, Mavromelanidou, Zoumpoulakis, & Sinanoglou, 2013). Details of their structures and sources and evidence of their functionality are continuously compiled (Copping & Duke, 2007; Cowan, 1999; Dorman & Deans, 2000; Gibbons, Moser, & Kaatz, 2004; Newman, Cragg, & Snader, 2000; Stavri, Piddock, & Gibbons, 2007). These antimicrobial properties of medicinal plants are so important because of their potential ability to counteract emerging resistive mechanisms of a variety of microbial strains (Dantas, Sommer, Oluwasegun, & Church, 2008).

Two medicinal plants, *Prunus africana*, commonly known as *Pygeum africanum* or African cherry, belonging to the *Rosaceae* family (Bii, Korir, Rugutt, & Mutai, 2010; Das, 2017; Medicines Agency, 2015; Stewart, 2003), and *Pausinystalia yohimbe*, commonly called *Yocon*/ *Dankamaru* and belonging to the *Rubiaceae* family (Igwe & Madubuike, A. J., Ikenga, Chika, Otuokere, I. E., Amaku, 2016), are employed by traditional healers. The former contains bioactive

phenolic compounds including atraric acid (AA) (Papaioannou et al., 2010; Roell & Baniahmad, 2011), ferulic acid (FA) (Ngule, Ndiku, & Ramesh, 2014; Nyamai DW, Mawia AM, Wambua FK, Njoroge A, Matheri F, Lagat R, Kiambi J, Ogola P, Arika W, Cheseto X, King'ori E, Ramni J, Ngugi MP, Muchugi A, Ng'ang'a M, 2015; G.-W. Yang, Jiang, & Lu, 2015) and N-butyl benzene-sulfonamide (NBBS) (Papaioannou et al., 2010; Rider et al., 2012; Roell & Baniahmad, 2011). Other phytochemicals identified include, the abundant sterols: β-sitosterol (Hass, Nowak, Leonova, Levin, & Longhurst, 1999; Shenouda et al., 2007), β-sitosterol-3-O-glucoside (Maiyo, Moodley, & Singh, 2016); pentacyclic terpenoid saponins: ursolic acid (Bishayee, Ahmed, Brankov, & Perloff, 2011; Steenkamp, 2003); and oleanolic acid (Bishayee et al., 2011; Fourneau, Hocquemiller, & Cavé, 1996; Hass et al., 1999; Steenkamp, 2003); triterpene: β-amyrin (Maiyo et al., 2016); and the medium chain fatty acid lauric acid (Nyamai DW, Mawia AM, Wambua FK, Njoroge A, Matheri F, Lagat R, Kiambi J, Ogola P, Arika W, Cheseto X, King'ori E, Ramni J, Ngugi MP, Muchugi A, Ng'ang'a M, 2015). Tested methanol and ethanol extracts of the bark of *P. africana* from diverse geographical locations exhibited moderate to strong antimicrobial properties on a variety of clinical strains including gram (+) *S. aureus*, methicillin resistant *S. aureus*, *B. subtilis*, gram-negative *P. aeruginosa*, *E. coli*, *C. albicans*, *C. koseri*, *E. aerogenes*, and *E. cloaceae* (Bii et al., 2010; Gashe & Zeleke, 2017; Madivoli et al., 2012; Mwitari et al., 2013). In addition, the bark extract of *Pausinystalia yohimbe* contains up to 5.9% of the total indole alkaloid yohimbine, depending on age of the plant and height at which the bark has been collected since it increases by about 1% during the rainy season (Zanolari, 2003).

This study seeks to identify potent phytochemicals with high efficacy to combat newly evolved drug resistant strains like the Hepatitis A virus, *Salmonella typhi*, and *E. coli* O157:H7.

1.2 Problem Statement

With increasing interest in natural medicinal products which may have less toxic and carcinogenic side effects than synthetic ones, researchers seek to assess and identify natural compounds as green alternatives (Johnston, Sepe, Miano, Brannan, & Alderton, 2005; Karre, Lopez, & Getty, 2013; Nikmaram et al., 2018; Rather et al., 2016; Roohinejad et al., 2017). The antimicrobial activities of numerous medicinal plants are not yet well defined although they have been traditionally used by local communities (Ashraf, Sarfraz, Mahmood, & Din, 2015). In addition, global health is threatened by the emergence of multidrug-resistant (MDR) microorganisms, particularly with regards to antibiotic treatments (Davies & Davies, 2010; Ventola, 2015). Therefore, a search for new plant-derived antimicrobial agents is crucial (Hughes & Karlén, 2014) to mitigate the increasing impotence of current synthetic antibiotics.

1.3 Hypotheses

i. The content and potence of phytochemicals and antioxidant activities in plant parts extracted with various solvents will vary.
ii. Selected extracts with low effective concentration (EC_{50}) will inhibit microbial growth.

1.4 Objectives

The purpose of this study is to assess the phytochemical compositions and antioxidant power of the extracts of the selected medicinal plant part(s); and to evaluate the antimicrobial activity of selected extracts. The specific objectives include:

i. To identify specific phenolic acids and quantify phenolic compounds (flavonoids and phenols) of *P. africana* (C) bark and root, *P. africana* (K) bark, *P. yohimbe* (C) bark and *Orthero* (C) root extracts.

ii. To compare the effects of solvent types used for extraction and of plant parts (bark vs. root) used on the antioxidant properties/activities

iii. To evaluate the antimicrobial properties (growth inhibition) of the five (5) selected extracts on two foodborne microorganisms (gram-negative *E. coli* and gram-positive *S. aureus*).

CHAPTER 2: LITERATURE REVIEW

2.1 Introduction

Medicinal plants (otherwise referred to as herbs, herbal medicines, pharmacologically active plants or phytomedicinals) are the dominant form of medicine in most countries. Such a plant will have its parts including leaves, roots, rhizomes, stems, barks, flowers, fruits, grains or seeds, employed in the control or treatment of a disease condition and therefore contains chemical components that are medically active (Hamuel, 2012). These non-nutrient plant chemical compounds, secondary metabolites, or bioactive components are often referred to as phytochemicals (phyto- from Greek meaning 'plant') or phytoconstituents (Abo, Ogunleye, & Ashidi, 1999; J. H. Doughari, Human, Bennade, & Ndakidemi, 2009; R. H. Liu, 2004). Their chemical and/or structural compositions, for example, phenolics, alkaloids, saponins and terpenoids (Patra, 2012), have various identifiable characteristic features, although a benzene ring with one or more hydroxyl groups is common (Oz & Kafkas, 2017).

About 200,000 phytochemicals are known so far and 20,000 of them have been identified as originating from fruits, vegetables, grains (Patra, 2012), spices and beverages such as green tea and red wine (J. H. Doughari et al., 2009; James Hamuel Doughari & Obidah, 2008). Plant part extracts containing complex mixtures of sugars and acids (that is, phenolic and non-phenolic compounds) are important sources of phytochemicals, which have many health effects as antioxidants, antibacterial, antifungal, antiviral, cholesterol-lowering, antithrombotic, or anti-inflammatory (Schreiner & Huyskens-Keil, 2007). Furthermore, phytochemicals are also used as pharmaceuticals, agrochemicals, flavors, fragrances, coloring agents, biopesticides and food additives (Patra, 2012). Current research on plant antimicrobial effects suggests that phytochemical structures can reduce or inhibit the growth of pathogenic microorganism and in

some cases preserve the overall quality of food products, thereby extending their shelf life (Patra, 2012). In spite of their differing structures, these secondary metabolites may sometimes work synergistically in combating metabolic processes of resistant pathogens (Oz & Kafkas, 2017). Moreover, plant-defensive metabolites which include phytoalexins are biosynthesized to respond to biotic and abiotic stresses, thus protecting the plant and controlling pathogenic growth (Oz & Kafkas, 2017). Furthermore, most secondary metabolites are responsible for the organoleptic and qualitative properties of foods originating from such plants (Oz & Kafkas, 2017), as exhibited by anthocyanins constituting a pigment group responsible for the coloration of a great variety of fruits, flowers and leaves (Harborne & Williams, 2000); and flavan-3-ols involved in the bitterness and astringency of tea, grapes and wine (Halsam & Lilley, 1988; Noble, 1994). In addition, these compounds are unique sources of industrial material in the form of food additives, pharmaceuticals and flavors (Zhao, Davis, & Verpoorte, 2005). Therefore, food scientists and nutrition specialists suggest that phytochemicals can offer many health benefits when consumed as part of the usual human diet (Tlili et al., 2014).

2.2 Phytochemicals and Secondary Metabolites

Phytochemicals constitute a heterogeneous group of secondary plant metabolites (that is, derivatives of primary metabolism), with a vast variation in biological properties such as; antioxidant activity, antimicrobial and stimulant effects, and anticancer property (Altiok, 2010). These secondary metabolites are compounds involved in various processes of secondary metabolism that is, metabolic pathways that provide most of the pharmacological active natural products (Dewick, 2002). They have much more limited distributions and unlike primary metabolites, are found in only specific plant families or groups of species while expressing

diversity and complexity. Furthermore, these compounds are not produced under all conditions as in the case of products of primary metabolism, and in vast number of cases have potential benefits that need exploration. However, secondary metabolites play vital roles in the plant's wellbeing which include, synthesizing toxic chemicals as defense mechanisms against predators; as volatile or aromatic detractants for predators and attractants for other unharmful species (Dewick, 2002).

Plants have an almost limitless ability to synthesize these aromatic substances, most of which are phenols or their oxygen-substituted derivatives (Geissman, 1963), that are deposited in specific parts or in all parts of the plant (Joseph & Raj, 2010). These secondary metabolites have up to 12,000 presently been isolated which is thought to be less than 10% of the estimated total number of metabolites (Ponman & Bussmann, 2012). These phytochemicals are used as the basis for the production of valuable synthetic compounds such as pharmaceuticals, cosmetics, or more recently nutraceuticals (Bourgaud, Gravot, Milesi, & Gontier, 2001). Furthermore, they are largely viewed as potential sources of new drugs, antibiotics, insecticides and herbicides (Crozier, Clifford, & Ashihara, 2006) and may also produce beneficial medicinal effects in combination (Ciocan, Ionela D. & Bara, 2007). Recently, their roles in the protection of human health, and their significance in dietary intake has been reported (Altiok, 2010; Oz & Kafkas, 2017).

Meanwhile, these natural products may not participate directly in plant growth and development but play an important role in ecological interactions with other organisms (Civjan, 2012). Some, such as terpenoids, give plants their odors; others (quinones and tannins) (Cowan, 1999) and carotenoids are responsible for plant pigment (Delgado-Vargas, Jiménez, & Paredes-López, 2000; Mitchell & Koh, 2008). Many other compounds are responsible for plant flavor (e.g., the terpenoid capsaicin from chili peppers), and some of the same herbs and spices used by humans to season food yield useful medicinal compounds (Cowan, 1999). Furthermore, these plant

chemicals protect plant cells from environmental hazards such as pollution, stress, drought, and UV exposure (Altiok, 2010).

Since there are a wide variety of phytochemicals, precisely classifying them is challenging. Notwithstanding, secondary metabolites can be classified on the basis of chemical structure (for example, having rings, containing a sugar; acyclic, bicyclic, heterocyclic); composition (e.g., nitrogenous, non-nitrogenous, oxygenated, non-oxygenated), their solubility in various solvents (for example, polar/non-polar), or their (biosynthetic pathways (Walton & Brown, 1999)); (such as, phenyl propanoid, which produces tannins) (Tiwari & Rana, 2015).

Building blocks for these secondary metabolites are derived from primary metabolism or biosynthetic processes such as photosynthesis, glycolysis, and the citric acid /Krebs cycle (Dewick, 2002). These basic building blocks undergo a variety of biosynthetic transformations and combinations that lead to numerous classes of plant natural products including, but not limited to, aromatic polyketides (phenols and quinones), terpenoids and steroids, phenyl propanoids (lignans and lignin, coumarins, flavonoids, and isoflavonoids), and alkaloids (Civjan, 2012). Therefore, pathways that are utilized for the synthesis of these chemical compounds include: acetate (acetyl coenzyme A/acetyl-CoA) pathway; shikimate (shikimic acid) pathway; mevalonate (mevalonic acid or MVA) pathway; and deoxyxylulose (1-deoxyxylulose 5-phosphate or DXP) pathway (Civjan, 2012; Dewick, 2002).

The precursor, acetyl coenzyme A (acetyl-CoA), is the initial substrate for synthesis of the C_2 (two-carbon unit backbone) of plant polyketides (that is, naturally occurring polymers of ketene; CH_2CO), also known as the polyketide pathway (Civjan, 2012). Aromatic natural products of polyketide origin are less prevalent in plants with the majority of these plant constituents containing aromatic structures arising from the shikimate pathway (Civjan, 2012). Unlike those

derived from the shikimate pathway, aromatic products of the polyketide pathway invariably contain a meta oxygenation pattern because of their origin from the cyclization of polyketides (Civjan, 2012). Phenolic compounds such as the anthraquinones, (for example, aloe-emodin) and hypericin (*Hypericum perforatum*, Hypericaceae), a constituent of the antidepressant herbal supplement, St. John's wort; are products of the polyketide pathway (Miskovsky, 2002) (Fig. 1).

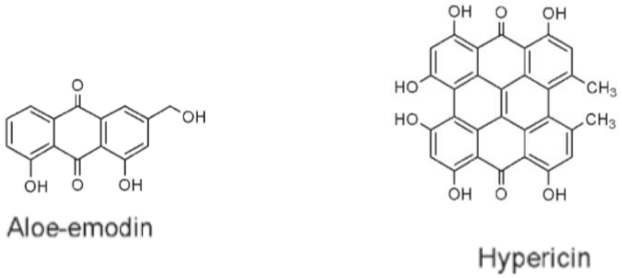

Figure 1: Examples of Aromatic Polyketides in plants

Source: Civjan, N. (2012)

The shikimate pathway with its central intermediate shikimic acid (Fig. 2), a compound isolated from plants of *Illicium* species (Japanese 'shikimi'); is found only in plants and microorganisms and provides an alternative route to aromatic and phenolic compounds, particularly the aromatic amino acids *L*-phenylalanine, *L*-tyrosine and *L*-tryptophan (Civjan, 2012; Dewick, 2002). Generally, all structural building blocks and their subsequent derivatives arising from the shikimate pathway are produced from a combination of the glycolytic and pentose phosphate pathways intermediates (phosphoenolpyruvate (PEP) and erythrose 4-phosphate), respectively (Dewick, 2002). Phenylalanine and tyrosine form the basis of C_6C_3 phenylpropyl unit (along with their modified C_6C_2 and C_6C_1 units) found in phenylpropanoids; and supplies the carbon-nitrogen skeleton of the C_6C_2N system. Furthermore, the indole-containing C_2N system which is derived from tryptophan, along with the C_6C_2N form a vast array of alkaloids produced by means of this pathway. In addition, it is found that the simple phenolic acids protocatechuic

acid (3, 4-dihydroxybenzoic acid or DHBA), GA (3, 4, 5-trihydroxybenzoic acid), and 4-hydroxybenzoic acid or HBA; demonstrate some of the hydroxylation patterns characteristic of shikimic acid-derived metabolites that is, single para-hydroxylation and the ortho-polyhydroxylation patterns which contrast with the typical meta-hydroxylation patterns characteristic of phenols derived via the acetate pathway (Fig. 2) Protocatehuic acid and gallic acid (GA) are formed via branchpoint reactions from 3-dehydroshikimic acid in the shikimate pathway, while 4-HBA is derived via side-chain degradation of chorismic acid which is a cinnamic acid derivative (Dewick, 2002) (Fig. 2).

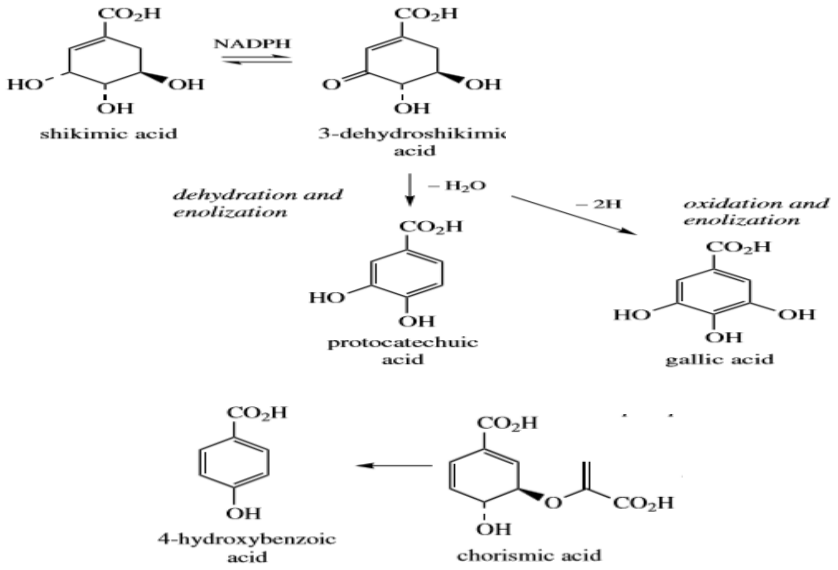

Figure 2: Characteristic Phenols derived from the Shikimate pathway

Source: Dewick, P. M. (2002)

The mevalonic acid (MVA), a six-carbon building block, utilizes and directs acetate (that is, three molecules of acetyl-CoA) into a series of natural products different from those derived directly from the acetate pathway (Civjan, 2012; Dewick, 2002). Meanwhile, the deoxyxylulose phosphate (DXP) pathway arises from a combination of two glycolytic pathway intermediates, namely; pyruvic acid and glyceraldehyde 3-phosphate (Dewick, 2002). The branched-chain C_5

"isoprene unit" is a feature of compounds formed from mevalonate (that is, utilizing 5 of mevalonate 6 carbons), or DXP (an alternative precursor derived from a straight-chain sugar undergoing rearrangement) (Dewick, 2002). Isoprene alkaloids (lysine derivatives occurring as a piperidine ring system) are metabolites of the MVA and DXP pathways with a characteristic carbon-nitrogen skeleton (C_5N unit) as a structural feature. Furthermore, both pathways are together responsible for the biosynthesis of a vast array of terpenoid and steroid metabolites (Dewick, 2002).

Moreover, secondary metabolites can also be synthesized by combining or using a mixture of several elements or building blocks of the same type from the acetate, shikimate, mevanolate and/or deoxyxylulose phosphate pathways; with some containing one or more sugar units in their structure, such as glucose or ribose, or other alternatively modified and unusual sugars. These combinations of complex mixtures of compounds lead to structural diversity of secondary metabolites, and consequently establishing biosynthesis as the basis of classification (Dewick, 2002). Notwithstanding, phytochemicals can be classified into alkaloids, phenolic compounds (which include flavonoid and non-flavonoid phenylpropanoids and their derivatives); essential/volatile oils and their constituents (terpenes and terpenoids); glycosides (glucose and aglycone/genin), and other derivatives (e.g., steroids and saponins).

2.2.1 Alkaloids

Alkaloids form a class of the best known and widely distributed (Egamberdieva, Mamedov, Ovidi, Tiezzi, & Craker, 2017) heterocyclic and non-heterocyclic nitrogenous compounds which are biosynthetically derived from amino acids building blocks (Springob & Kutchan, 2009). Alkaloids commonly originate from the amino acids, *L*-ornithine, lysine, nicotinic acid, tyrosine,

phenylalanine, tryptophan, arginine, anthranilic acid, and histidine, and thus contain pyrrolidine, pyrrolizidine, piperidine, quinolizidine, indolizidine, pyridine, quinoline, isoquinoline, indole, and imidazole ring systems (Civjan, 2012). Alkaloids are also known to originate from mixed biosynthetic pathways, the most important of which include terpenoid and steroidal alkaloids (Civjan, 2012). A limited number of alkaloids that contain a purine ring (e.g., caffeine) also occur in plants (Civjan, 2012; Dewick, 2002).

Moreover, these compounds have basic properties as a result of nitrogenous atoms occurring typically as primary, secondary, tertiary or quartenary amines; and are also alkaline in reactions (Yalavarthi & Thiruvengadarajan, 2013). Their reaction with acids yields crystalline salts formation with non-water production with a majority existing in solid forms such as atropine, and some as liquids containing carbon, hydrogen, and nitrogen. Most alkaloids are readily soluble in alcohol and sparingly soluble in water, even though their salts are usually soluble (Yalavarthi & Thiruvengadarajan, 2013). The solutions of alkaloids are intensely bitter.

There are currently more than 12,000 known alkaloid structures discovered (Civjan, 2012; Yalavarthi & Thiruvengadarajan, 2013) in about 20% of flowering plant species (Croteau, R; Kutchan, T.M.; Lewis, 2000; Yalavarthi & Thiruvengadarajan, 2013); and only a few have been exploited for medicinal purposes (Yalavarthi & Thiruvengadarajan, 2013). Alkaloids can originate from animal, microbial, and plant sources (Cushnie, Cushnie, & Lamb, 2014). In the plant kingdom, they are commonly found in angiosperms and rarely in gymnosperms (Chandra et al., 2017). Although the function(s) of some alkaloids are still unknown, these compounds play fundamental roles in plants as deterrents for herbivores, protection from pathogens, and inhibitions of competitors (Cushnie et al., 2014). They are widely exploited as pharmaceuticals, stimulants, narcotics, and poisons due to their potent biological activities (Yalavarthi & Thiruvengadarajan,

2013). Plant alkaloids have also been investigated and used in traditional medicine in different regions of various countries with possible health improvement being the main focus of the research in plant pharmacology and pharmaceutical applications (Egamberdieva et al., 2017).

With such large number and high structural diversity of plant alkaloids, it is impossible to give a comprehensive summary of all the different types (Springob & Kutchan, 2009). In so doing, only representatives of some classes are tabulated and figured here based primarily on their origin (amino acid precursors and/or carbon building blocks); ring systems (that is, basic organic skeleton from which they are also derived); and the nature of the nitrogen-containing moiety (e.g.; as free N-heterocyclic or other N-containing bi- tri- or tetracyclic side chain) (Civjan, 2012; Dewick, 2002; Springob & Kutchan, 2009) (Table 1). Representative structures of a variety of alkaloid types are shown in Figure 3.

Table 1: Major Alkaloids and Chemical groups

Alkaloid type	Precursor	Chemical group, ring system, or parent compounds	Examples	Taxa
True alkaloids	L-ornithine and L-phenylalanine	Tropane alkaloids; pyrollidine ring system	cocaine, hyoscyamine, scopolamine	*Solanaceae Brassicaceae*, *Euphorbiaceae*, *Erythroxylaceae*, *Proteaeceae*, and *Rhizophoraceae*
	Arginine and L-ornithine	Pyrrolizidine alkaloids	Senecionine, lycopsamine	
	L-lysine	Piperidine alkaloids	Piperine (black pepper); pseudopelletierine (pomegranate)	*Asteraceae Piperaceae Asteraceae*, *Boraginaceae*, *Fabaceae*, and *Orchidaceae*
		Quinolizidine alkaloids	Lupinine, sparteime (broom plant), cytisine, lupanine	*Leguminosae Fabaceae Berberidaceae*, *Chenopodiacae*, *Ranunculaceae*, *Rubiaceae*, *and Solanaceae*
	L-tyrosine	Simple tetrahydro-isoquinoline alkaloids; Benzylisoquinoline (S-Retuculine)	Codeine, morphine, papaverine, thebaine, tubocurarine, noscapine, narceine, berberine, sanguinarine	*Papaveraceae*, *Berberidaceae*, *Fumariaceae*, *Papaveraceae*, *Menispermaceae*, *and Ranunculaceae*
	L-tyrosine or L-phenylalanine	Phenethylisoquinoline alkaloids; Amaryllidaceae (Norbelladine)	Galanthamine, lycorine, narciclasine, pancratistatin	*Crinum*, *Clivia*, *Galanthus*, *Narcissus*, *Leucojum*, *Hymenocallis*
Protoalkaloids	L-trytophan	Terpenoid indole alkaloids, Indole ring system	Yohimbine	*Rubiaceae*
Pseudo-alkaloids	Acetate	Piperidine alkaloids	Conine	*Apiaceae*
	Ferulic acid	Aromatic alkaloids; Phenyl	Capsaicin	*Solanaceae*
	Saponins	Steroid alkaloids	Cholestane	*Brassica*
	Adenine or Guanine	Purine alkaloids	Caffeine, theobromine, theophylline and paraxanthine	*Rubiaceae*, *Sterculiaceae*, *Sapindaceae*, *Sterculiaceae*

Source: Seneca. (2007); and Springob, K., & Kutchan, T. M. (2009)

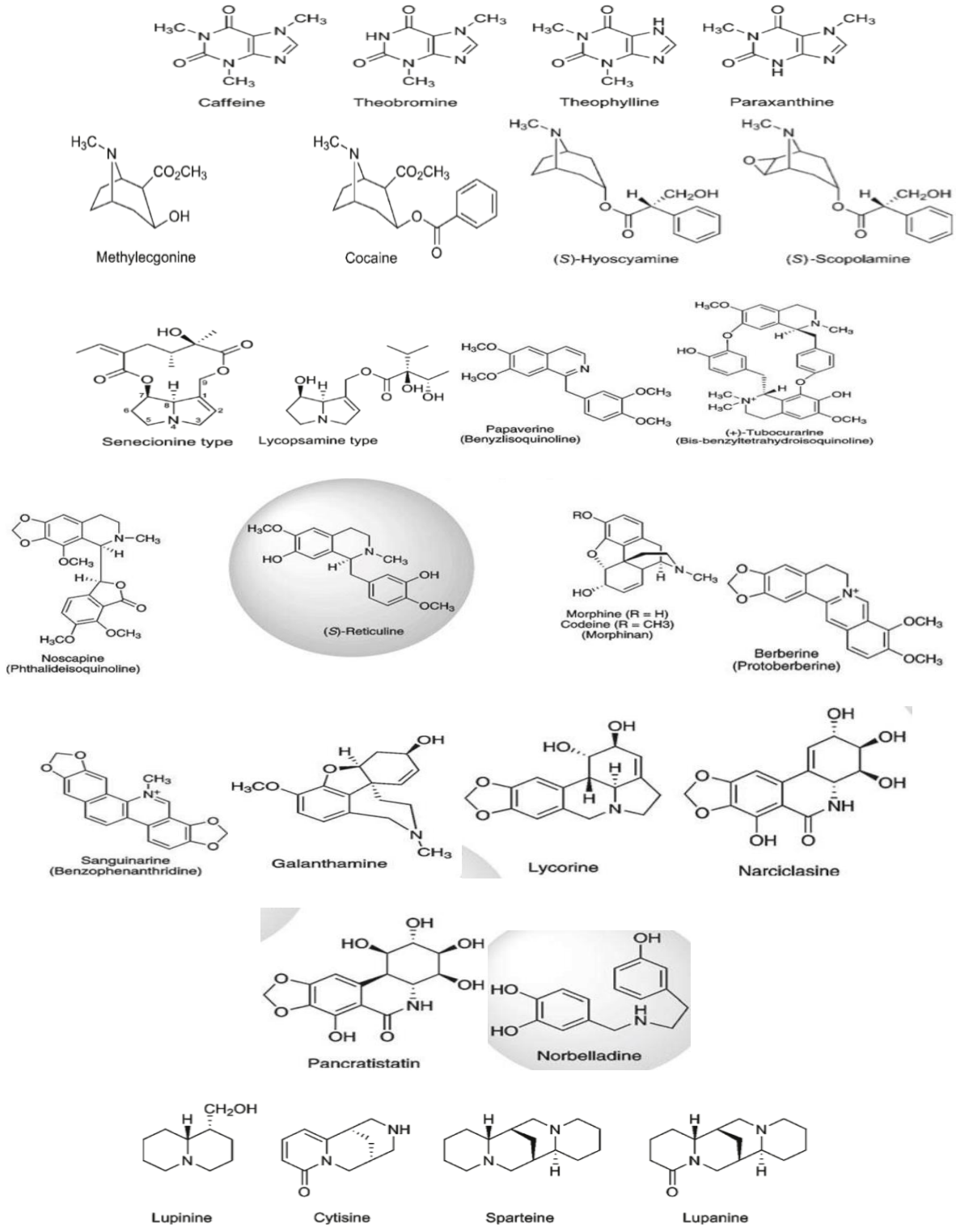

Figure 3: Structures of Representative Alkaloids

Source: Springob, K., & Kutchan, T. M. (2009)

2.2.2 Phenolic Compounds

Most plant phenolics are derived directly from the shikimic acid (simple benzoic acids), shikimate (phenylpropanoid) pathway, or a combination of shikimate and acetate (phenylpropanoid-acetate) pathways (Civjan, 2012). Products of each of these pathways undergo additional structural elaborations that result in a vast array of plant phenolics such as simple benzoic acid and cinnamic acid derivatives, monolignols, lignans and lignin, phenylpropenes, coumarins, stilbenes, flavonoids, anthocyanidins, and isoflavonoids (Civjan, 2012). Approximately 8,000 naturally occurring compounds belong to the category of phenolics, all of which share a common structural feature: an aromatic ring bearing at least one hydroxyl substituent, that is, a phenol (Goleniowski, Bonfill, Cusido, & Palazón, 2013). The hydroxyl group(s) can be free or engaged in another function as ethers, esters, or glycosides (Herrmann & Weaver, 1999). Moreover, phenolic compounds are potent antioxidants and free radical scavengers which can act as hydrogen donors, reducing agents, metal chelators and singlet oxygen quenchers (Chew, Goh, & Lim, 2009). Meanwhile, phenolics, phenols or polyphenolics are chemical components that occur pervasively as natural color pigments responsible for the color of fruits of plants (Yalavarthi & Thiruvengadarajan, 2013). With much significance to plants and having multiple functions (Yalavarthi & Thiruvengadarajan, 2013), they play a major role in plant defense against pathogens and predation by herbivores, and so are used as control measures for human pathogenic infections (R. H. Liu, 2004).

A straightforward classification attempts to divide the broad category of phenolics into simple phenols and polyphenols, based exclusively on the number of phenol subunits present, but many plant phenolic compounds are polymerized into larger molecules (Goleniowski et al., 2013). Thus, the term “plant phenolics” encompasses simple phenols, phenolic acids, coumarins,

flavonoids, stilbenes, hydrolyzable and condensed tannins, lignans, and lignins (Clifford, 1999). Notwithstanding, their classification includes but not limited to two major classes: non-flavonoid simple phenols (Altiok, 2010) and phenylpropanoids (benzoic and cinnamic acid derivatives) (Altiok, 2010; Civjan, 2012); and flavonoid phenylpropanoids and phenylpropanoid-acetate pathway derivatives (Civjan, 2012; Yalavarthi & Thiruvengadarajan, 2013) (Table 2).

Table 2: Classification of Phenolic Compounds in Plants

Phenolic group	Phenolic compounds	Pathway	Precursor	C-skeleton
Aromatic polyketides	Simple phenols and quinones (benzoquinones)	Acetate and/or Polyketide	Acetyl-CoA	C_6
Phenyl propanoids (flavonoids and non-flavonoids)	Polyphenols (cinnamic acid derivatives: phenylpropenes, coumarins, lignins, lignans, benzenoids (benzoic acids); flavonoids, flavan derivatives (xanthones and tannins), and isoflavonoids); acetophenones, phenylacetic acids)	Shikimate	Shikimic acid (L-phenylalanine, L-tyrosine and L-tryptophan)	Phenyl propane unit (C_6-C_3) and modified forms C_6-C_2; C_6-C_1; $(C_6$-$C_3)_2$; C_6-C_1-C_6; $(C_6$-C_3-$C_6)_n$
Phenyl propanoid-acetate derivatives	Stilbenes and Flavonoids	Shikimate and Acetate (Phenyl propanoid-acetate)	Shikimic acid (L-phenylalanine); Acetate (acetyl-CoA)	Diphenyl propane unit (C_6-C_2-C_6)

2.2.2.1 Non-flavonoid Phenylpropanoids

Catechol which has two OH groups and pyrogallol three, are both hydroxylated simple phenols, shown to be toxic to microorganisms (Ciocan, Ionela D. & Bara, 2007; Cowan, 1999; Godstime, Felix, Augustina, & Christopher, 2014) (Fig. 4). The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence

that increased hydroxylation results in increased toxicity (Florkin & Stotz, 1963). In addition, some authors have found that more highly oxidized phenols are more inhibitory (Rama Raje Urs, N. V.; Dunleavy, 1975; Scalbert, 1991). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason & Wasserman, 1987).

Phenylpropenes constitute a subfamily of phenylpropanoids that are synthesized from the amino acid phenylalanine and *L*-tyrosine via the shikimic acid pathway (Vogt, 2010), and are constituents of essential oils (EOs) (Morsy, 2017; Schmidt, 2009). Examples of this group include anethole (the main constituent of oils from aniseed and fennel; *Pimpinella anisum* and *Foeniculum vulgare*, respectively, (Umbelliferae); and star anise; *Illicium varum*, Illiciaceae (Civjan, 2012)) (Hyldgaard, Mygind, & Meyer, 2012; Kfoury et al., 2016). Others include, methyl chavicol, eugenol (i.e., clove oil (Cowan, 1999); and cinnamon leaf (Civjan, 2012)); isoeugenol, vanillin, safrole, myristicin (nutmeg; *Myristica fragrans*, Myristicaceae) (Civjan, 2012)) and cinnamaldehyde (bark of cinnamon; *Cinnamomum zeylanicum*, Lauraceae (Civjan, 2012)) (Hyldgaard et al., 2012; Kfoury et al., 2016) (Fig. 4). Phenylpropenes are derived from cinnamic acid and its derivatives by a series of reductions and transformations (for example; cinnamaldehyde, the first product of the reduction of cinnamic acid); coupled with several hydrocarbon analogs which are also known to occur in plants (Civjan, 2012).

Phenolic acids (phenolcarboxylic acids) are phenols that include substances containing a phenolic ring and at least one organic carboxylic acid function (Goleniowski et al., 2013). Depending on the carbon units of the lateral chain attached to the phenolic ring, the phenolic acids can be divided into C_6-C_3, C_6-C_2, and C_6-C_1 compounds, the most important being C_6-C_3 (derived

from the hydroxycinnamic acid) and C_6-C_1 (compounds with a hydroxybenzoic structure) (Goleniowski et al., 2013). Although the basic skeleton remains the same, phenolic acids differ in the number and position of the hydroxyl groups on the aromatic ring (Goleniowski et al., 2013). Moreover, the numbers of hydroxyl groups connected with the aromatic ring, in ortho or para position relative to each other, enhance the antioxidative and antiradical activity of phenolic acids (Tapas, Sakarkar, & Kakde, 2008).

Hydroxybenzoic acids (HBAs) are direct derivatives of benzoic acid, for example, p-hydroxybenzoic acid (p-HBA); salicylic acid (SA); 2,3-dihydroxybenzoic acid (2,3-DHBA); 2,5-dihydroxybenzoic acid (2,5-DHBA); 3,4-dihydroxybenzoic acid (3,4-DHBA); 3,5-dihydroxybenzoic acid (3,5-DHBA); gallic acid (GA); and vanillic acid (Goleniowski et al., 2013) (Fig. 4). As apparent from their structures, many benzoic acid derivatives are directly formed from shikimic acid by dehydration, dehydrogenation, and enolization reactions (Civjan, 2012). Variations in the structures of individual HBAs lie in the hydroxylations and methylations of the aromatic ring (Goleniowski et al., 2013). Other benzoic acid derivatives in plants commonly occur: protocatechuic acid and syringic acid (Civjan, 2012; Goleniowski et al., 2013) (Fig. 4). They may be present in soluble form conjugated with sugars or organic acids as well as bound to cell wall fractions, for example, lignin (Goleniowski et al., 2013). Gallic acid for example, is a component of gallotannins common in some plants that are used in the tanning of animal hides to make leather (Civjan, 2012). Astringency of some foods and beverages, especially coffee, tea, and wines, is because of their constituent tannins (Civjan, 2012).

Nonetheless, some of the simple HBAs (C_6C_1 compounds) such as 4-HBA and gallic acid can be formed directly from intermediates early in the shikimate pathway, (e.g.; 3-dehydroshikimic acid or chorismic acid) (Dewick, 2002). Furthermore, alternative routes exist in which cinnamic

acid derivatives (C_6C_3 compounds) are cleaved at the double bond and lose two carbons from the side-chain. Thus, 4-coumaric acid may act as a precursor of 4-HBA, and likewise ferulic acid (FA) may give rise to vanillic acid (Dewick, 2002).

L-Phenylalanine and *L*-tyrosine, as C_6C_3 building blocks, are precursors for a wide range of plant natural products such as cinnamic acids by first eliminating ammonia from the side-chain (Civjan, 2012; Dewick, 2002). In the case of phenylalanine, this would give cinnamic acid, whilst tyrosine could yield 4-coumaric acid (p-coumaric acid) (Dewick, 2002) (Fig. 4). Some common natural hydroxycinnamic acids (HCAs) or cinnamic acid derivatives include p-coumaric acid (PA), caffeic acid (CA), ferulic acid (FA), sinapic acid (SIA) (Civjan, 2012) (Fig. 4), and their esterified/ etherified conjugates such as chlorogenic acids (Goleniowski et al., 2013). However, the most commonly found naturally occurring HCAs is chlorogenic acid, which is caffeic acid esterified with quinic acid (Goleniowski et al., 2013). Cinnamic and caffeic acids are common representatives of this wide group of phenylpropane-derived compounds which are in the highest oxidation state (Cowan, 1999). Others include, ellagic acid (Altiok, 2010) o- and m-coumaric acids, gentistic acid, and veratric acid (Goleniowski et al., 2013) (Fig. 4).

Lignans are a group of dimericphenylpropanoids with two C_6-C_3 attached to a central carbon (C_8) (Cunha, W. R.; Andrade e Silva, M. L.; Sola Veneziani & Ambrosio, S. R.; Bastos, 2012) (Figure 4) They were first introduced in 1948 by Howarth; and can be formed by the condensation process of two cinnamyl alcohol/cinnamic acids through the β-carbon of the aliphatic chain (Chandra et al., 2017). The term Lignan was first introduced by Haworth (1948) to describe a group of dimeric phenylpropanoids where two C_6-C_3 are attached by its central carbon (Cunha, W. R.; Andrade e Silva, M. L.; Sola Veneziani & Ambrosio, S. R.; Bastos, 2012) (Figure 4). More recently, Gotlieb (1978) proposed that micromolecules with two phenylpropanoid units coupled

in other manners, like C_5-C_5 for example should be named neolignans (Umezawa, 2003). Lignans can be found in more than 60 families of vascular plants and have been isolated from different plant parts, exudates and resins (Cunha, W. R.; Andrade e Silva, M. L.; Sola Veneziani & Ambrosio, S. R.; Bastos, 2012).

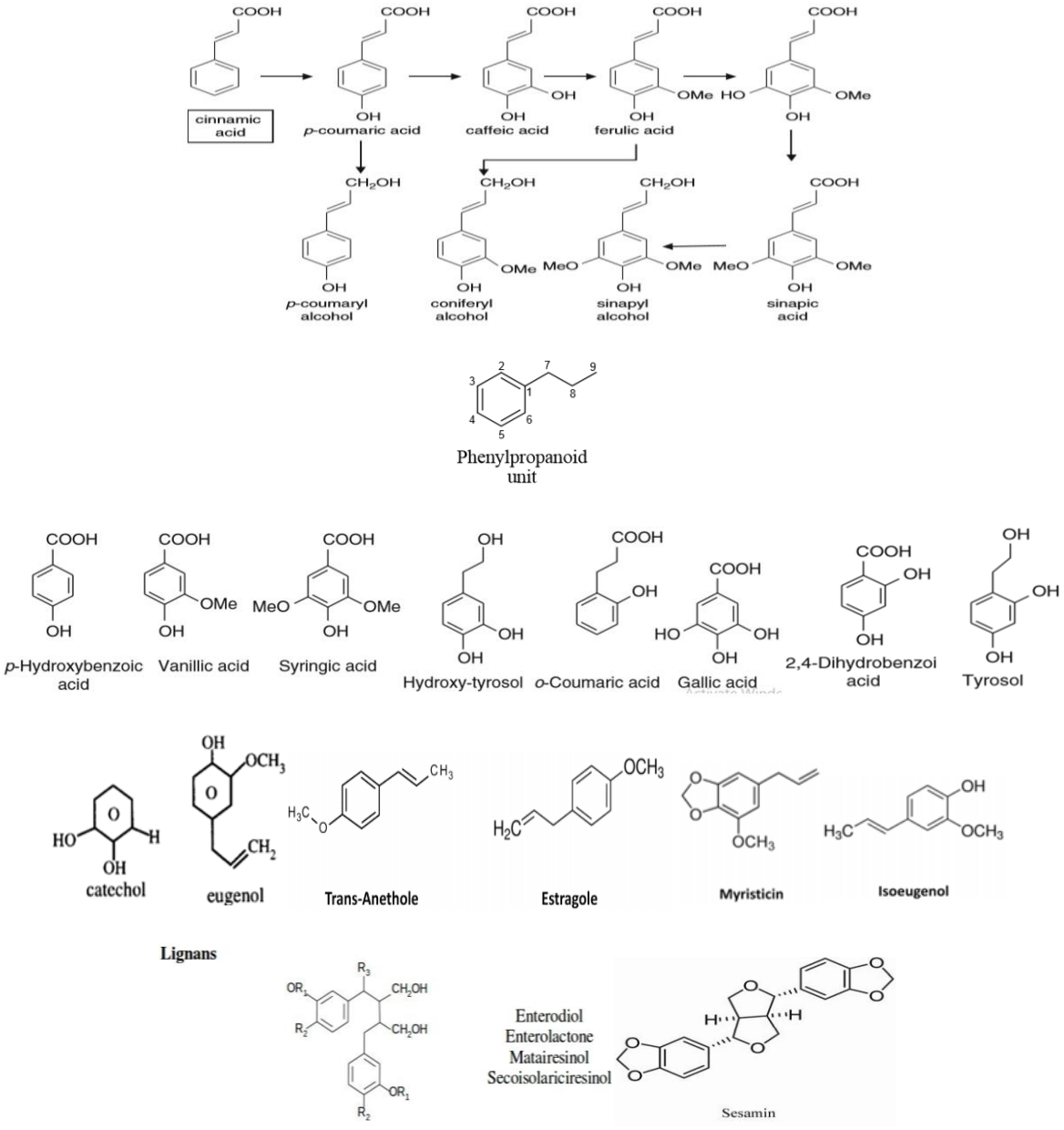

Figure 4: Structures of Non-flavonoid Phenylpropanoids

Source: Goleniowski et al. (2013); Cowan, 1999

2.2.2.2 Flavonoids Phenylpropanoids and Stilbenes

Flavonoids are products of both the shikimate (phenylpropanoid) and acetate pathways (Aherne & O'Brien, 2002; Civjan, 2012; Dewick, 2002); and so are commonly known as phenylpropanoid-acetate derivatives (Civjan, 2012), with most sharing a diphenylpropane (C_6-C_3-C_6) structure of three phenolic rings (Chen & Blumberg, 2008) (Fig. 5). They are termed phenylpropanoid derivatives based on the characteristic C_6-C_3-C_6 flavan nucleus (Altiok, 2010; Mitchell & Koh, 2008) (Fig. 5). These hydroxylated phenolic compounds occur as a C_6-C_3 (that is, a C3 unit or central pyran ring) linked/bonded to two aromatic rings (Cowan, 1999; Delgado-Vargas et al., 2000). The six-membered ring condensed with the benzene ring is either -pyrone (flavones and flavonols) or its dihydroderivative (flavanones and flavan-3-ols) (Altiok, 2010). The position of the benzenoid substituent divides the flavonoids into two classes: flavonoids (2-position) and isoflavonoids (3-position) (Altiok, 2010).

Most flavonoids occur naturally associated with sugar in conjugated forms (Altiok, 2010) as O- glycosides or, less frequently, C-glycosides; although aglycones (flavonoids without attached sugar), are sometimes found in edible plants as well (for example, the catechin and epicatechin aglycones present in cocoa) (Mitchell & Koh, 2008). The sugars of the O-glycosides are generally bound to the hydroxyl groups of the flavonoid nucleus at C-3 and/or C-7 positions and the carbohydrate unit can be *L*-rhamnose, *D*-glucose, glucorhamnose, galactose or arabinose (Tapas et al., 2008); whereas C-glycosides are attached to either the C-6 or C-8 positions (Mitchell & Koh, 2008). Unlike the O-glycosides, the C-glycosides are not cleaved by acid hydrolysis (Mitchell & Koh, 2008). In numerous plants, the sugars are often further substituted by acetyl, malonyl, or organic acid residues (for example, the isoflavones in soybeans and anthocyanidins in wine) (Mitchell & Koh, 2008). Hence, the majority of flavonoids can be modified by

hydroxylation, methylation, acylation, glycosylation (Delgado-Vargas et al., 2000), malonylation, and/or sulfation (Bravo, 2009) to obtain a great natural diversity of compounds (Delgado-Vargas et al., 2000) with glucoside as the most frequently occurring conjugate in plant foods (Bravo, 2009).

Flavonoids also represent the largest, most commonly studied and widely distributed class of polyphenols (plant phenols) (Balasundram, Sundram, & Samman, 2006; Delgado-Vargas et al., 2000; Harborne & Williams, 2000) with more than 5000 (Koes, Quattrocchio, & Mol, 1994; Pretorius, 2003), or about 6500 that have been chemically characterized or identified to date (Harborne & Williams, 2000). They are water soluble and show a wide distribution in vascular plants where they are present in each part of the plant (Delgado-Vargas et al., 2000). Some are pigments in higher plants (Hamuel, 2012) such as the anthocyanins and anthoxanthins (Mitchell & Koh, 2008). The most abundant flavonoids in fruits, vegetables and herbs are shown in Table 3.

Flavonoids can be placed into subclasses based upon the degree of saturation and oxidation present in the heterocyclic ring (C-ring or pyran ring) of the flavan nucleus (Koes et al., 1994; Mitchell & Koh, 2008). Subclasses include the flavones, flavonols, flavanones, flavan-3-ols, anthocyanins, and isoflavones (Mitchell & Koh, 2008) (Fig. 5 and Table 3). However, they are sometimes divided in 13 subclasses: anthocyanins, aurons, chalcones, yellow flavonols, flavones, uncolored flavonols, flavanones, dihydroflavonols, dihydrochalcones, leucoanthocyanidins, catechins (flavan-3-ols), flavans, and isoflavonoids based on the oxidation state of the pyran ring and on the characteristic color (Delgado-Vargas et al., 2000). Therefore, flavonoids include, flavanones (naringenin, hesperedin, and eriodictyol; isoflavones like genistein, daidzein, and glycitein); flavan-3-ols (catechin and epicatechin); and anthocyanins (cyanidin and malvidin) (Fig.

5) which are widespread in plant foods (Manach, Scalbert, Morand, Rémésy, & Jiménez, 2004) (Table 3).

Table 3: Common Flavonoids and their Natural Sources

Subclass	**Examples (Type of flavonoid)**	**Foods, oils and beverages**
Flavonols	Quercetin, kaempferol, myricetin	Onions, red apple, lettuce, broccoli, berries, olive oil, tea, red wine, kale, leeks, endive, radish, grapefruit, grapes
Flavones	Apigenin and luteolin	Parsley, celery, cereals, apple skins
Flavanones	Naringenin, hesperedin and eriodictyol	Tomatoes, mint, grapefruit, citrus fruits (e.g., oranges, lemons etc.);
Isoflavones	Genistein, dadzein and glycitein	Soy foods
Flavan-3-ols	Catechin, epicatechin, and their gallate forms	Green and black tea, grapes, red wine, cocoa
Anthocyanins	Cyanidin and malvidin	Red wine, grapes, beans, onions, berries, cherries
Proanthocyanidin (or condensed tannins)	Procyanidin	Red wine, red grapes

Source: (Altiok, 2010)

Catechins and epicatechins are among the commonest flavonoids known, sharing a distribution almost as widespread as quercetin in the dicotyledoneae (Delgado-Vargas et al., 2000). The 3,4,5-trihydroxy, B-ring flavan-3-ols gallocatechin and epigallocatechin; have also a wide distribution (paralleling myricetin), especially in more primitive plants (the Coniferae being outstanding). Many flavans are lipid soluble and appear to be leaf-surface constituents (Delgado-Vargas et al., 2000).

Isoflavones differ from the other flavonoid subclasses, as they have the B-ring attached to the C-3 instead of the C-2 of the heterocyclic C-ring (Delgado-Vargas et al., 2000; Mitchell &

Koh, 2008) (Fig. 5). They include plant natural products such as, rotenoids, pterocarps and coumestans (Delgado-Vargas et al., 2000) and genistein, which is referred to as a phyto-oestrogen due to its oestrogenic activity (Civjan, 2012). They are the most common in legumes, especially in Lotoideae, and have also been reported in Amaranthaceae, Iridicaceae, Miristicaceae, and Rosaceae (Francis & Markakis, 1989; Harborne & Mabry, 1982; Koes et al., 1994). Flavones are hydroxylated phenolic structures containing one carbonyl group (in contrast to two in quinones) (Cowan, 1999), while the addition of a 3-hydroxyl group yields a flavonol (Chandra et al., 2017; Fessenden & Fessenden, 1982) (Fig 5). Of the more than 5000 flavonoids being described so far within the parts of plants normally consumed by humans, approximately 650 flavones and 1030 flavanols are known (Pretorius, 2003). Common examples include flavones like luteolin and apigenin (from German chamomile *Matricaria recuitita*, Asteraceae) (Civjan, 2012). Flavonols include quercetin, kaempferol, myricetin (Manach et al., 2004) and rutin, a flavonol glycoside found in several plants including hawthorn (Crataegus spp., Rosaceae) (Civjan, 2012) (Fig. 5). Kaempferol, quercetin and its glycosidic derivative quercitrin are common flavonols present in nearly 70% of plants (Yalavarthi & Thiruvengadarajan, 2013). Flavonols are widely distributed in woody angiosperms and common in shrubby angiosperms, whereas flavones and flavanones are found free or glycosylated in leaves of angiosperms (Delgado-Vargas et al., 2000). Flavanones are especially common in Rosaceae, Rutaceae, legumes and Compositae (Delgado-Vargas et al., 2000) and thus include naringenin (from Heliotropium and Nonea spp.; Boraginaceae) (Civjan, 2012).

The number of hydroxyl groups and the presence of a 2, 3-double bond and orthodiphenolic structure enhance the antiradical and antioxidative activity of flavonoids (Tapas et al., 2008). The glycosylation, blocking the 3-OH group on the C-ring, lack of a hydroxyl group or the presence of only a methoxy group on the B-ring has a decreasing effect on antiradical or antioxidative activity

of these compounds (Tapas et al., 2008). Moreover, flavonoids are also known to be synthesized by plants in response to microbial infection, and they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms (Cowan, 1999; Dixon, 2001). Their activity is probably due to their ability to complex with extracellular and soluble proteins and with bacterial cell walls (Cowan, 1999). Studies have shown that phenolic compounds such as catechin and quercetin are very efficient in stabilizing phospholipid bilayers against peroxidation induced by reactive oxygen species (Gülçin, 2010; Gülçin, Bursal, Şehitoğlu, Bilsel, & Gören, 2010).

Coumarins are phenolic substances made of fused benzene and α-pyrone rings (O'Kennedy & Thornes, 1997) (Fig. 5). Deriving their name from their precursor, o-coumaric acid (Civjan, 2012), they occur widely in plants both in the free form and as glycosides commonly found in Umbelliferae and Rutaceae (Civjan, 2012); and are responsible for the characteristic odor of hay (Cowan, 1999). The parent compound coumarin is found in sweet clover (*Melilotus alba*, Leguminosae) and its hydroxyl derivative umbelliferone has been isolated from several Ferula spp. (Umbelliferae) (Civjan, 2012). Coumarins are well-known for their antithrombotic (Thastrup, Knudsen, Lemmich, & Winther, 1985), and anti-inflammatory activities (Piller, 1975). The coumarin warfarin (Fig. 5) is utilized both as an oral anticoagulant, and interestingly, as a rodenticide (King & Tempesta, 1994).

Tannins are flavan-derived phenolic polymers or polymeric phenolic substances widely distributed in the plant flora (Dai & Mumper, 2010; Yalavarthi & Thiruvengadarajan, 2013). They have high molecular weights ranging from 2,000 and 5,000 Daltons (Khanbabaee & Ree, 2001) and form two classes: hydrolyzable and condensed tannins (Dai & Mumper, 2010; Yalavarthi & Thiruvengadarajan, 2013) (Fig. 5). Hydrolysable tannins have a central core of glucose or another

polyol esterified with gallic acid (gallotannins), or with hexahydroxydiphenic acid (ellagitannins) (Dai & Mumper, 2010) (Fig. 5). They also form pyrogallic acid upon heating under pressure (Yalavarthi & Thiruvengadarajan, 2013). Their acidic nature in reactions and antiseptic activity are attributed to the presence of a phenolic or carboxylic group attachment (Yalavarthi & Thiruvengadarajan, 2013). Condensed tannins are oligomers or polymers of flavan-3-ol linked through an interflavan carbon bond; and because they are decomposed to anthocyanidins through acid-catalyzed oxidation reaction upon heating in acidic alcohol solutions they can also be referred to as proanthocyanidins (Dai & Mumper, 2010).

Tannins, soluble in water and alcohol, are found in the root, bark, stem and outer layers of plant tissue where they form complexes with proteins, carbohydrates, gelatin and alkaloids (Yalavarthi & Thiruvengadarajan, 2013). Due to the high number of OH-groups, tannins show strong antioxidative properties, as demonstrated by the *in vivo* antioxidative effect of some others in red wine or gallate esters (Altiok, 2010). Tannins find application in the tanning industry where they convert hide into leather (Yalavarthi & Thiruvengadarajan, 2013). Tannins and flavonoids are thought to display antidiarrheal activity by increasing colonic water and electrolyte reabsorption (Palombo, 2006). Theaflavins (from tea), and daidezein, genistein and glycitein (from soy bean/soy products) are common examples of hydrolysable tannins (Yalavarthi & Thiruvengadarajan, 2013). Tannin-rich medicinal plants and their formulations (such as the Ayurveda formulation) are used as healing agents in diseases like leucorrhoea, rhinnorhoea and diarrhea (Yalavarthi & Thiruvengadarajan, 2013).

In contrast to other plant phenolics, the basic carbon skeleton of stilbenes like flavonoids, anthocyanidins, and isoflavonoids, incorporates elements of both the shikimate (phenylpropanoid) and acetate pathways (Civjan, 2012). Plant phenolics derived from this mixed biosynthetic

pathway include the well-known cancer chemopreventative stilbene, resveratrol, present in wine, especially red wine (Civjan, 2012) (Fig. 5).

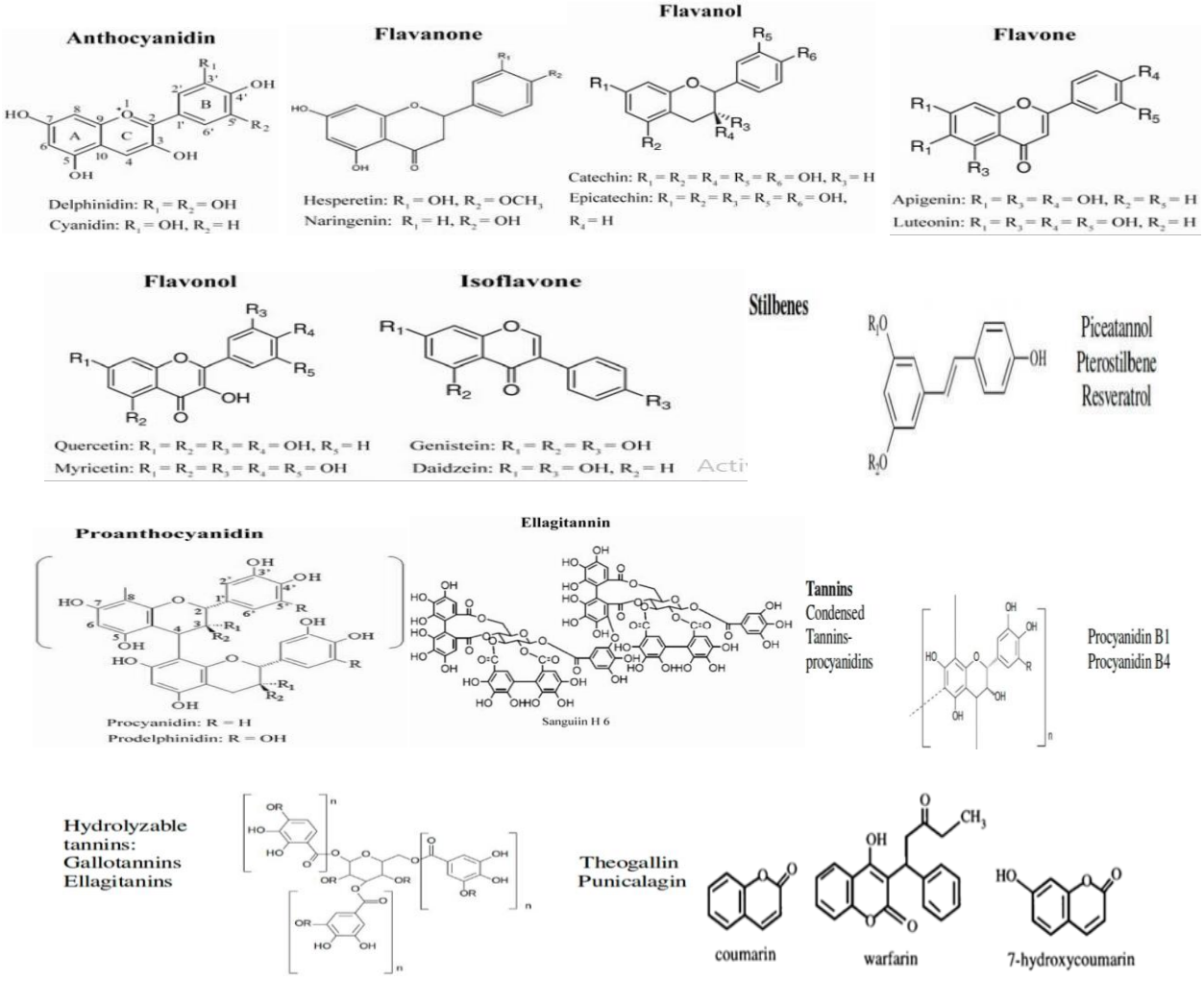

Figure 5: Chemical Structures of Representative Flavonoids and Stilbenes

Source: Dai, J., & Mumper, R. J. (2010); Cowan, 1999

2.2.3 Essential Oils and their Constituents (Terpenes and/or Terpenoids)

Essential oils (EOs), also referred to as volatile oils or ethereal oils (Yalavarthi & Thiruvengadarajan, 2013), are examples of a complex mixture of polar and non-polar plant secondary compounds (Masango, 2005) with isoprene (terpene) structures and the structural

formula $C_{10}H_{16}$ (Chandra et al., 2017). The essential oil composition in a plant depends on the species, its geographic location, harvest time, and extraction technique (Dima & Dima, 2015). Essential oils and their constituents (terpenoids, carvacrol and thymol) (Figure 6) occur widely in nature contributing to the characteristic plant flavors and aromas (Simões, Bennett, & Rosa, 2014). They are secreted either directly by the plant protoplasm or by hydrolysis of some glycosides and other plant structures, such as glandular hairs (Lamiaceae e.g., *Lavandula angustifolia*), oil tubes (vittae) (Apiaceae e.g. *Foeniculum vulgare*, and *Pimpinella anisum* or aniseed), modified parenchymal cells (Piperaceae e.g. *Piper nigrum* - black pepper), schizogenous or lysigenum passages (Rutaceae e.g. *Pinus palustris* - pine oil) (Yalavarthi & Thiruvengadarajan, 2013). These oils are obtainable from leaves, rhizomes, flowers, roots, bark, seeds, peel, fruits, wood or the whole plants (Hyldgaard et al., 2012). Chemically, a single volatile oil contains more than 200 different components, with the trace constituents mostly or solely responsible for characteristic attributes such as flavor and odor (Yalavarthi & Thiruvengadarajan, 2013). Essential oils can be classified into the four main groups: terpenes (related to isoprene); straightchain compounds without any side chain; phenylpropanoids (benzene derivatives); and a miscellaneous group with varied structures not included in first three groups (that is, sulfur- or nitrogen-containing compounds) (Morsy, 2017; Schmidt, 2009). Examples of volatile components of EOs include thymol and carvacrol in oregano (*Origanum vulgare*) and thyme plant (*Thymus vulgaris*); limonene and sabinene in black pepper oil (Morsy, 2017) and the black pepper plant (*Piper nigrum*) (Bhattacharya, 2016); menthol and menthone in peppermint oil (Barceloux, 2008) and the peppermint plant (*Mentha piperita*) (Raghavan, 2007); zingiberene in ginger oil (*Zingiber officinale*); and carvone in caraway oil (*Carum carvi*) (Bhattacharya, 2016). The radical-

scavenging ability of essential oils is as follows: clove > cinnamon > nutmeg > basil > oregano > thyme (Tomaino et al., 2005).

Terpenes present the most common and chemically diverse class of chemical compounds found in EOs (Chizzola, 2013; Yalavarthi & Thiruvengadarajan, 2013); they are synthesized in the cytoplasm of plant cells through the MVA pathway (Hyldgaard et al., 2012). Regarded as polymers of isoprene (C_5H_8) joined in a repetitive head- to-tail manner (Croteau, R; Kutchan, T.M.; Lewis, 2000) as shown in Figure 6, they can be divided into two major groups: flammable unsaturated terpene hydrocarbons (Yalavarthi & Thiruvengadarajan, 2013) and oxygenated compounds (Mohamed, El-Emary, & Ali, 2010). The structural diversity and complexity of terpenes generates an enormous potential for mediating plant–environment interactions (Tholl, 2006). When terpenes contain oxygen, they are called terpenoids (Chandra et al., 2017) with the general formula C_5H_8 (Yalavarthi & Thiruvengadarajan, 2013). Representing a big family of natural compounds, they are found in all plant kingdoms where they function as hormones, pigments, and phytoalexins (Delgado-Vargas et al., 2000). Over 40,000 different terpenoids have been isolated and characterized from various natural sources including plants (Rohdich, Bacher, & Eisenreich, 2005; Withers & Keasling, 2006), and many new structures are identified each year (Delgado-Vargas et al., 2000).

Just as terpenes, most terpenoids are also derived from the MVA pathway through the universal precursor isopentenyl diphosphate (IPP) and its allylic isomer dimethylallyl diphosphate (DMAPP) (Civjan, 2012). Thus, the vast majority of terpenoids contain the basic structural residue 2-methylbutane, often less precisely referred to as a isoprenoid unit (Civjan, 2012; Yalavarthi & Thiruvengadarajan, 2013). According to the number of fused isoprene units or rings (Chizzola, 2013), they are classified into hemiterpenes (C_5; 1 unit); monoterpenes (C_{10}; 2 units);

sesquiterpenes (C_{15}; 3 units); diterpenes (C_{20}; 4 units); sesterterpenes (C_{25}; 5 units), triterpenes (C_{30}; 6 units) tetraterpenes (C_{40}; 8 units), and polyterpenes (C_{5n}, n = >8; many units) (Civjan, 2012; Zuzarte & Salgueiro, 2015). A "terpene unit" results when two isoprene units are combined (Morsy, 2017). Primary products of condensation undergo further reactions (reduction, oxidation, derivatization, etc.) and "decorations" that result in the terpenoid hydrocarbons, their oxygenated forms (alcohols and their glycosides), ethers, aldehydes, ketones, and carboxylic acids and their esters, which are indicative of their vast diversity (Civjan, 2012) (Fig. 6).

Compared to other terpenoids, there are only a few true hemiterpenoids in nature, and over 90 of these occur as glycosides (Dembitsky, 2006), with isoprene (a volatile hemiterpenoid released by many trees) being the most noteworthy example (Civjan, 2012). Prenols in the flowers of *Cananga odorata* (Annonaceae) and hops (*Humulus lupulus*, Cannabaceae), constituents of the essential oils of grapefruit, hops, and oranges, and the characteristic flavor of blackcurrant (*Ribes nigrum*, Saxifragaceae) are all characteristic features of hemiterpenes (Civjan, 2012).

The perfume and spice industries value monoterpenoids ($C_{10}H_{16}$) for their fragrances and/or flavors (Civjan, 2012). Constituting over 1,500 known compounds, they are classified according to their number of ring of atoms such as monocyclic monoterpenes, bicyclic monoterpenes and tricyclic monoterpenes (Connolly, J.D.; Hill, 1991; George, Alonso-Gutierrez, Keasling, & Lee, 2015) (Fig. 6). These compounds are oxidized easily by reacting withair and heat sources (Hunter, 2009). Several acyclic monoterpenoid hydrocarbons form constituents such as EOs in basil (*Ocimum basilicum*, Labiatae) and bay leaf (*Pimenta acris*, Myrtaceae), pettitgrain leaves (*Citrus vulgaris*, Rutaceae), strobiles of hops (*Humulus lupulus*, Cannabaceae), and others (Civjan, 2012). Commonly known alcoholic monoterpenes include camphor, eugenol and menthol (Yalavarthi & Thiruvengadarajan, 2013) (Fig. 6).

There are over 5,000 naturally occurring diterpenoids, many located in the plant families Araliaceae, Asteraceae, Cistaceae, Cupressaceae, Euphorbiaceae, Leguminosae, Labiatae, and Pinaceae (Connolly, J.D.; Hill, 1991). Resins and taxol are commonly known diterpenes (Yalavarthi & Thiruvengadarajan, 2013) (Fig. 6).

The C_{15} terpenoids known as sesquiterpenoids are one of the most chemically diverse and dominant group of terpenoids in nature, and like monoterpenoids, many contribute to the flavor and/or fragrance of a variety of plant products (Civjan, 2012). To date about 10,000 sesquiterpenoids have been identified (Connolly, J.D.; Hill, 1991). In plants, they typically occur as hydrocarbons, alcohols, aldehydes, ketones, carboxylic acids, lactones, and oxiranes (Civjan, 2012).

The triterpenes include steroids, sterols, and cardiac glycosides with anti-inflammatory, sedative, insecticidal, or cytotoxic activity (Yalavarthi & Thiruvengadarajan, 2013). Amyrins, ursolic acid, and oleanic acid are common examples of triterpenes (Yalavarthi & Thiruvengadarajan, 2013). Triterpenoids and steroids contain derivatives of the C_{30} precursor (squalene) which impacts their chemical diversity (Civjan, 2012) (Fig. 6). Triterpenoids originate mostly in plants, whereas steroids can be found in plants, animals, or microorganisms. Plant triterpenoids belong to two main groups, the tetracyclic triterpenoids, also called methylated steroids, and very diverse pentacyclic triterpenoids. Steroids are modified triterpenoids that contain the tetracyclic ring system present in lanosterol, with chemically diverse side chains attached to the steroid nucleus. Stiamastane and cycloartane are the most prevalent classes of plant steroids (Civjan, 2012).

Triterpenoids and steroids frequently occur as glycosides called saponins (Vincken, Heng, de Groot, & Gruppen, 2007), first derived from *Saponaria vaccaria* (*Quillaja saponaria*) and

utilized for soap making (Yalavarthi & Thiruvengadarajan, 2013). The chemical diversity of saponins is dependant on both the nature of the 30-carbon moiety and the carbohydrate residue (Civjan, 2012), resulting in high molecular weight compounds (Yalavarthi & Thiruvengadarajan, 2013). Some saponins contain carbohydrate (sugar) residues attached to several different positions of the aglycone (triterpenoid/steroid) skeleton (Civjan, 2012; Yalavarthi & Thiruvengadarajan, 2013). The two main groups of saponins are steroid saponins and triterpene saponins (Yalavarthi & Thiruvengadarajan, 2013), which are further subdivided into 11 main structural classes based on the carbon skeletons of their aglycone moiety (Vincken et al., 2007). In addition to their soap-like behavior in aqueous solution caused by their polar (carbohydrate) and non-polar (aglycone) structural elements, saponins exhibit pharmacological and medicinal properties (Civjan, 2012), such as hypolipidemic and anticarcinogenic effects (Yalavarthi & Thiruvengadarajan, 2013). Plant saponins are present in two major taxonomic classes, Magneliopsida (dicots) and Liliopsida (monocots) (Vincken et al., 2007). Some important examples of saponins include the triterpene saponin/glycoside, glycyrrhizic acid, from licorice and the steroid saponin/cardiac glycoside, digitoxin, from foxglove (*Digitalis purpurea*) (Civjan, 2012).

Quinones are triterpenoids consisting of two isomers of cyclohexadienedione with the molecular formula $C_6H_4O_2$, and are responsible for the enzymatic browning reaction in bruised or cut fruits and vegetables (Chandra et al., 2017; Ciocan, Ionela D. & Bara, 2007; Cowan, 1999; Khullar, 2016). Depending on their chemical structure, quinones are subdivided into benzoquinones, naphthoquinones (e.g.; vitamin K, a complex naphthoquinone (Ciocan, Ionela D. & Bara, 2007; Cowan, 1999)), anthraquinones (derivatives of phenolic and glycosidic compounds anthracene (Yalavarthi & Thiruvengadarajan, 2013)); and hydroquinones (diphenol to diketone) and other miscellaneous quinones (dibenzoquinones, dianthraquinones, dinaphthoquinones

(Delgado-Vargas et al., 2000)) (Figure 6). Due to their variability in kind and structure of substituents, a large number of other subclasses of quinones are derived: plastoquinones (found in the chloroplasts of higher plants and algae); ubiquinones (widespread in living organisms); and anthraquinones (prevalent in flowering plants) (Delgado-Vargas et al., 2000). In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins, often inactivating the protein and resulting in the loss of function (Ciocan, Ionela D. & Bara, 2007; Khullar, 2016). For this reason, quinone have a significant antimicrobial potential, proposed to be targeting surface-exposed adhesins, cell wall polypeptides, membrane-bound enzymes, and also interfering with microbial substrate availability (Pandey, 2013).

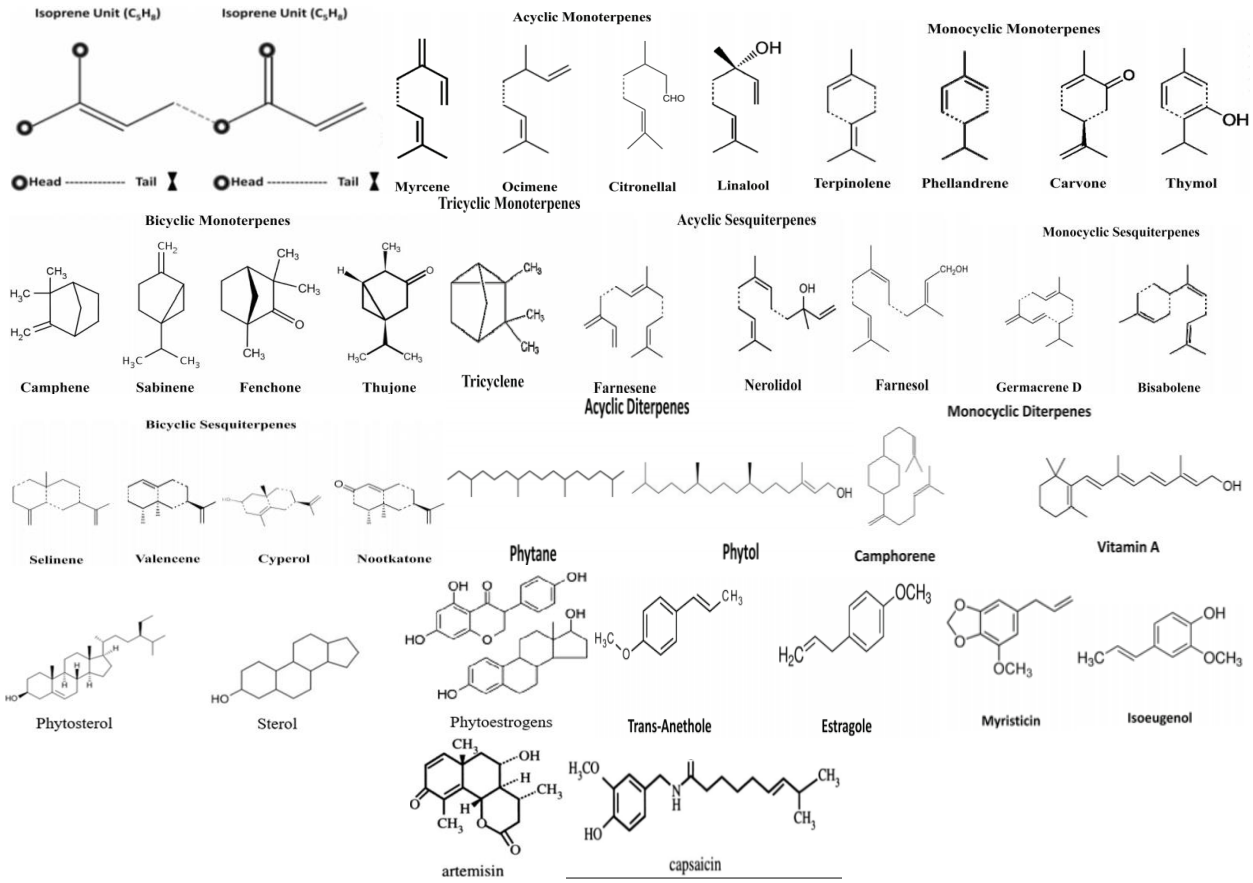

Figure 6: Chemical Structures of Essential Oils Constituents and Derivatives

Source: Morsy, N. F. S., 2017; Cowan, 1999

2.2.4 Glycosides

Glycosides contain a hydrophilic glycone unit (primarily D-glucose, but occasionally L-rhamnose and L-fructose) and the non-carbohydrate aglycone or genin unit (Egamberdieva et al., 2017; Yalavarthi & Thiruvengadarajan, 2013) which is mainly lipophilic (Bartnik & Facey, 2017). Flavonoids, terpenes (Tian et al., 2014; Zahmanov et al., 2015) or glycerol serve as the aglycone unit (Yalavarthi & Thiruvengadarajan, 2013). Thus, glycosides are condensation products of sugars attached to various organic hydroxyl (occasionally thiol) compounds with monohydrate character (Yalavarthi & Thiruvengadarajan, 2013). They are colorless water-soluble of the cell sap, containing cryastalline carbon, hydrogen and oxygen and sometimes nitrogen and sulfur. Glycosides are neutral in reactions and readily hydrolyze into their components when reacting with ferments or mineral acids. They are classified based on the chemical nature of the aglycone, pharmacological action or type of sugar component as indicated by replacing the suffix of the parent sugar "ose" with "oside". This pattern contrasts the archaic system utilizing the suffix 'in' with names essentially indicative of glycosidic source, (for instance: strophanthidin from *Strophanthus*, digitoxin from *Digitalis*, barbaloin from *Aloes*, salicin from *Salix*, cantharidin from *Cantharides*, and prunasin from *Prunus*) (Yalavarthi & Thiruvengadarajan, 2013). In so doing, glycosidic compounds currently used in medicine include glycosides of vitamins, polyphenolic glycosides (flavonoids), alkaloid glycosides, glycoside antibiotics, glycopeptides, cardiac glycosides, and steroid and terpenoid glycosides (Kren & Martinkova, 2001).

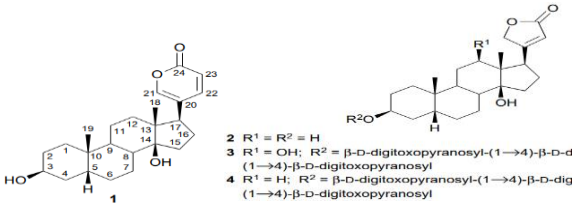

Figure 7: Chemical Structures of Representative Glycosides

Source: (S. Steyn & R. van Heerden, 1998)

2.2.5 Pigments

Pigments are chemical compounds that absorb light in the wavelength range of the UV-visible region (Delgado-Vargas et al., 2000), and are present in the form of porphyrin pigments (chlorophyll a, b and c), carotenoids, anthocyanins and flavones (Britton, 2009; Costache, Campeanu, & Neata, 2012). The resulting color is due to a molecule-specific structure (chromophore) which captures energy causing the excitation of an electron from an external orbital to a higher orbital (Hari, Patel, & Martin, 1994). Natural plant pigments are organic compounds (Delgado-Vargas et al., 2000) and can be classified by their chromophore chemical structure (Wong, 2018) as either chromophores with conjugated systems such as carotenoids, anthocyanins, betalains, or as metal-coordinated porphyrins such as chlorophylls and their derivatives (Delgado-Vargas et al., 2000). In addition, natural pigments can also be classified by their structural characteristics (Hari et al., 1994) as tetrapyrrole derivatives (e.g., chlorophylls); isoprenoid derivatives (e.g., carotenoids and iridoids); N-heterocyclic compounds (flavins and betalains); benzopyran derivatives (oxygenated heterocyclic compounds): anthocyanins and other flavonoid pigments; and quinones (e.g., benzoquinone, naphthoquinone, anthraquinone) (Delgado-Vargas et al., 2000).

The plant tetrapyrrole derivatives (chlorophylls) are composed of pyrrole rings in linear or cyclic arrays (Delgado-Vargas et al., 2000) (Fig. 8). Chlorophylls constitute the most important subgroup of plant pigmented compounds within this group and are mainly present as photosynthetic pigments in thylakoid membranes inside the chloroplast of higher plants (Delgado-Vargas et al., 2000) held in place by a water-soluble chlorophyll-binding protein (WSCP) (N. Sumanta, C. I. Haque, 2014). Chlorophylls, porphyrins with a phytyl group conferring hydrophobic characteristics (Delgado-Vargas et al., 2000), have a magnesium ion (Mg^{2+}) at its

center which makes it ionic and hydrophilic as indicative of its polarity and a carbonyl group at the tail of the hydrophobic ring (N. Sumanta, C. I. Haque, 2014). Only chlorophyll a and b are present in higher plants, while others have been found in groups such as algae and bacteria (Delgado-Vargas et al., 2000; Hari et al., 1994). Chlorophyll a is the main pigment which converts light energy into chemical energy while chlorophyll b serves as accessory pigment, transferring the light it absorbs to chlorophyll a (N. Sumanta, C. I. Haque, 2014). Chlorophyll a differs from chlorophyll b only in the functional substitution of a methyl group for a CHO group on the porphyrin ring (N. Sumanta, C. I. Haque, 2014).

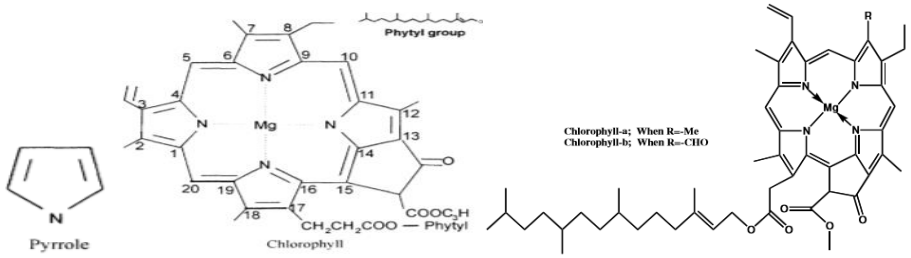

Figure 8: Basic Structures of Chlorophyll

Source: Delgado-Vargas et al., (2000); and Sumanta et al., (2014)

The ratio of chlorophyll-a and chlorophyll-b in terrestrial plants has been used as an indicator of response to light shade conditions (Ioana Vicas, Laslo, Pantea, Emil Bandici, & Vicas, 2010; Porra, 1991). In addition, the small ratio of chlorophyll a to b is considered a sensitive biomarker of pollution and environmental stress (Tripathi & Gautam, 2007).

Isoprenoid or terpenoid pigment derivatives are categorized into quinones, carotenoids; and recently iridoids (Delgado-Vargas et al., 2000). However, not all quinones are included since they are not all produced by this biosynthetic pathway (Delgado-Vargas et al., 2000). Therefore, carotenoids are a focus of this review and are described in-depth. In relation to iridoids, carotenoids are present in about 70 plant families (Capriofilaceae, Rubiaceae, Cornaceae, among others)

grouped into 13 orders (Delgado-Vargas et al., 2000). Saffron (*Crocus sativus* L.) and cape jasmine fruit (*Gardenia jasminoids* Ellis) are the best-known iridoid-containing plants, but their colors are more significantly affected by carotenoids (Sacchettini & Poulter, 1997).

Generally, carotenoids are free, non-esterified compounds and a class of fat-soluble, light-harvesting pigments (Mitchell & Koh, 2008). Present in green leaves, they have an inverted molecular center with eight isoprenoid units (Delgado-Vargas et al., 2000) (Fig 9). Carotenoids as phytochemicals have primarily a 40 carbon backbone since they are derived from a 40-carbon polyene chain (Delgado-Vargas et al., 2000). All are lycopene (C_{40} H_{56}) derivatives that are formed by hydrogenation, dehydrogenation, cyclization, oxygen insertion, double bond migration, methyl migration, chain elongation, or chain shortening (Goodwin, 1980). Classified on the basis of their chemical structure, there are non-polar hydrocarbon carotenes (with carbon and hydrogen constituents) and more polar oxycarotenoids (oxygenated derivatives (Mitchell & Koh, 2008)), also called xanthophylls, which contain carbon, hydrogen, and an added oxygen (Delgado-Vargas et al., 2000) as shown in representatives such as zeaxanthin, lutein, violaxanthin, β-cryptoxanthin, and capsanthin (Mitchell & Koh, 2008) (Fig. 9). On the basis of their source or origin, carotenoids can also be classified as either primary or secondary (Delgado-Vargas et al., 2000), with primary carotenoids required in photosynthesis (β-carotene, violaxanthin, and neoxanthin), and secondary carotenoids localized in fruits and flowers (α-carotene, β-cryptoxanthin, zeaxanthin, antheraxanthin, capsanthin, capsorubin) (Lichtenthaler, 1987) (Fig. 9).

Approximately 600 carotenoids have been identified to date (Mitchell & Koh, 2008)and more than 70 characteristic carotenoids have been described on the basis of quantity (minimal/maximal) and specificity; for example, capsanthin and capsorubin in pepper fruits (Goodwin, 1993; Lichtenthaler, 1987). Moreover, carotenoids are the most widely distributed

group of pigments, having been identified in photosynthetic and non-photosynthetic organisms: in higher plants, algae, fungi, bacteria, and at least in one species of each form of animal life (Delgado-Vargas et al., 2000). They play a critical role in photosynthesis by protecting the photosynthetic apparatus from damage associated with the production of chlorophyll triplet states and singlet oxygen (Mitchell & Koh, 2008). Research suggests that carotene pigments are the most significant photosynthetic pigments, protecting chlorophyll and thylakoid membranes from peroxidation damage by absorbed energy (N. Sumanta, C. I. Haque, 2014). Carotenoids are located in chromoplasts contributing to color in vegetables/fruits; and also in chloroplasts where they are involved with chlorophyll in the two photosystems (N. Sumanta, C. I. Haque, 2014). They are responsible for many of the brilliant red, orange, and yellow colors of fruits, vegetables and flowers (Goodwin, 1980; Hari et al., 1994; Wong, 2018) (Table 4). Animals have to consume carotenoids through their diet (e.g., β-carotene, which is a precursor of vitamin A) (Mitchell & Koh, 2008) since only plants and microorganisms can synthesize them; carotenoids in animal products come from these two sources, although they can be modified during their metabolism prior to tissue storage (Goodwin, 1980).

In the chloroplasts of all green plants, carotenoids accumulate in a mixture of α- and β-carotene, β-cryptoxanthin, lutein, zeaxanthin, violaxanthin, and neoxanthin, forming complexes with proteins through noncovalent bonding (Delgado-Vargas et al., 2000). Flowers that synthesize highly oxygenated carotenoids (frequently 5,8-epoxydes) have been identified, producing species specific carotenoids such as eschscholzxanthin in poppies. Nonetheless, the synthetic ability of fruits is significantly higher than of flowers. Interestingly, carotenoids have also been identified in wood samples, for example in oak (*Quercus robur* L., *Quercus petrae* Liebl., and *Quercus alba* L.), chestnut (*Castanea sativa* Mill.), and beech (*Fagus silvatica* L.) (Delgado-Vargas et al., 2000).

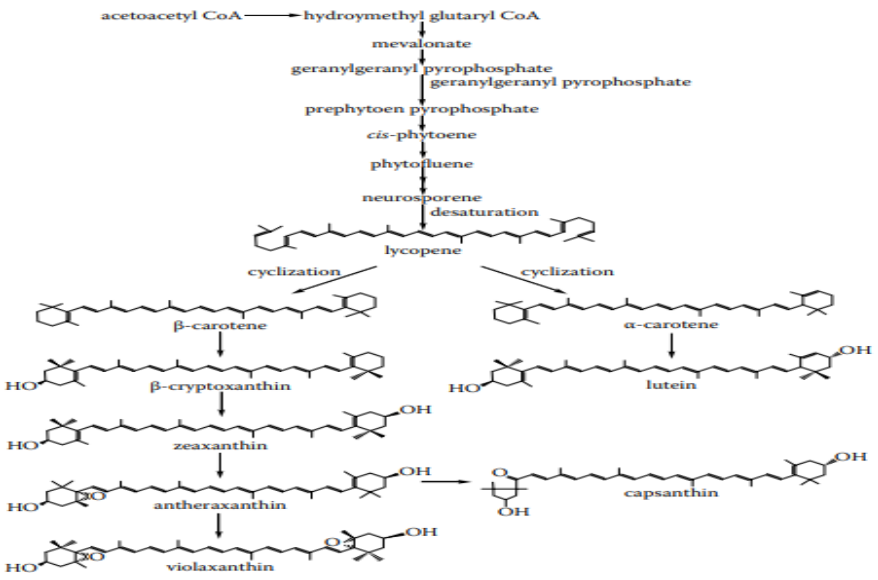

Figure 9: The Biosynthetic Pathway of Carotenoid Structures

Source: Mitchell, A., & Koh, E. (2008)

N-heterocyclic derived flavins contain a pteridin and a benzene ring. Riboflavin, also known as vitamin B_2 and the main compound of this group, is synthesized in all live plant and microbial cells (Delgado-Vargas et al., 2000).

Among the flavonoids, anthocyanins, benzopyran derivatives that produce colors ranging from orange to blue in petals, fruits, leaves, and roots, are the most important pigments (Delgado-Vargas et al., 2000) (Fig. 10). Anthocyanins also contribute to the yellow color of flowers, where they are present with carotenoids or alone in 15% of the plant species (Koes et al., 1994) (Table 4). Other benzopyran derivatives include the aurones, present in flowers (Bidens sp., Cosmos sp., Dahlia sp.), wood (Rhus sp., Schinopsis sp.), bryophytes (*Funaria hygrometrica*), and in Cypreceae plants. They are more common in inflorescences, seeds, and leaves. Also, the chalcones are common in mixtures with aurones to generate the anthclor pigment in the Compositae (Delgado-Vargas et al., 2000) (Fig. 10).

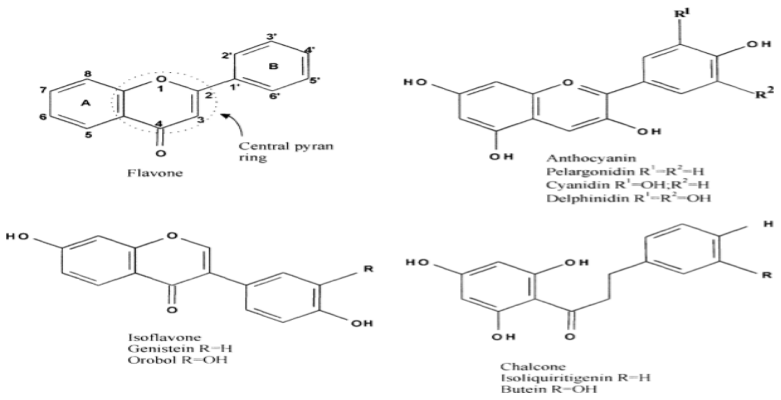

Figure 10: Benzopyran Derivatives

Source: Delgado-Vargas et al., (2000)

Table 4: Categories of Pigments with Characteristic Color Range and Sources

Pigment	**Color**	**Sources**
Anthocyanins (flavonoids)	blue/purple/red	Berries, grapes, red peppers, beets, eggplant, plums
Anthoxanthins (flavonoids)	yellow - ivory	Cauliflower, onions, white potatoes and turnips
Carotenoids (triterpenoids)	yellow - red	Fruits and dark green leafy vegetables
Chlorophylls	Greens	Fruit bearing green plants and dark green vegetables
Xanthophylls (carotenoids)	ivory - yellow	Kale, spinach, broccoli, peas and lettuce
β-carotene	orange/yellow/green	Acerola, mango, pumpkin, carrots, nuts, citrus, papaya, melon, squash, oil palm
Curcumin	Yellow	Turmeric
Lutein (xanthophyll)	yellow/orange	Collards, spinach, corn, citrus
Lycopene (carotenoid)	red/pink	Tomatoes, watermelon, red grapefruits
Zeaxanthin (xanthophyll)	Yellow	Corn

Quinones, the largest one in number and structural variation, consist of a significant number of coloring compounds and are more widely distributed than other natural plant pigments with the exception of carotenoids (Delgado-Vargas et al., 2000). While quinones are generally

yellow, red, or brown colorations, quinone salts can be purple, blue, or green (Koes et al., 1994; Thomson, 1991).

2.3 Methods of Extraction, Isolation and Characterization of Secondary Metabolites

For the analysis of phytochemicals (phenolic compounds), the following steps are required: sample preparation, extraction of phytochemicals, and quantification using spectrophotometry, gas chromatography (GC), high performance liquid chromatography (HPLC) or capillary electrophoresis (CE) methods (Khoddami, Wilkes, & Roberts, 2013). This preliminary work precedes the preparation of dietary supplements or nutraceuticals, food ingredients, pharmaceutical, and cosmetic products (Dai & Mumper, 2010) (Fig. 11). Phenolics, extractable from fresh, frozen or dried plant samples (Dai & Mumper, 2010), are typically milled, ground, and homogenized first; these steps may be preceded by air-drying or freeze-drying (Dai & Mumper, 2010). Generally, freeze-drying retains a greated quantity of phenolics than air-drying (Abascal, Ganora, & Yarnell, 2005; Asami, Hong, Barrett, & Mitchell, 2003), but drying, including freeze-drying, can affect the constituent profile of plant samples. Therefore caution must be taken when planning and analyzing properties of medicinal plants prepared by drying (Abascal et al., 2005).

Solvent extraction is the preferred preparation method for plant materials due to its ease of use, efficiency, and wide applicability (Dai & Mumper, 2010). Yield of chemical extraction depends on the type and polarity of solvents, extraction time and temperature, sample-to-solvent ratio, and chemical composition and physical characteristics of the samples (Dai & Mumper, 2010). The solubility of phenolics specifically is governed by the chemical nature of the plant sample and the polarity of the solvents used. Plant materials may contain different quantities of phenolics ranging from simple (e.g., phenolic acids, anthocyanins) to highly polymerized

substances (e.g., tannins). that may also be associated with other plant components such as carbohydrates and proteins. Therefore, no universal extraction procedure suitable for extraction of all plant phenolics has been identified. Depending on the solvent system used during exaction, differing mixtures of phenolics soluble in the solvent will be extracted from plant materials in addition to non-phenolic substances such as sugar, organic acids and fats. As a result, additional steps may be required to remove those unwanted components (Dai & Mumper, 2010).

Methanol, ethanol, acetone, ethyl acetate, and combinations of those with different proportions of water have been used for the extraction of phenolics from plant materials (Dai & Mumper, 2010). Solvent selection affects the amount and rate of polyphenol extraction (B. J. Xu & Chang, 2007). Methanol tends to be more efficient in extraction of lower molecular weight polyphenols while aqueous acetone extracts the higher molecular weight flavanols more efficiently (Guyot, Marnet, & Drilleau, 2001; Labarbe, Cheynier, Brossaud, Souquet, & Moutounet, 1999; Metivier, Francis, & Clydesdale, 1980; Prior, Lazarus, Cao, Muccitelli, & Hammerstone, 2001). Ethanol, another good solvent for polyphenol extraction, has the benefit of being sFafe for human consumption (Shi et al., 2005).

The recovery of phenolic compounds from plant materials is also influenced by the extraction time and temperature, as there is a fine balance between effective solubilization and analytic degradation by oxidation (Robards, 2003). An increase in the extraction temperature promotes higher analyte solubility by increasing both solubility and mass transfer rate and decreasing the viscosity and surface tension of the solvents, which helps the solvents to access sample matrices and thereby improving the extraction rate (Dai & Mumper, 2010). However, many phenolic compounds are easily hydrolyzed and oxidized. Long extraction times and higher temperatures increase the likelihood of phenolic oxidation, and decrease the yield of phenolics in

the extracts (Dai & Mumper, 2010; Havlikova & Mikova, 1985; Jackman, Yada, Tung, & Speers, 1987). Therefore, it is of critical importance to select efficient extraction procedure/method while maintaining the stability of phenolic compounds. Conventional extraction methods such as maceration and Soxhlet extraction are inefficient with long extraction times and pollute the environment by requiring large volumes of organic solvents (Dai & Mumper, 2010).

Newer methods including microwave and ultrasound-assisted extractions, and the use of compressed fluids as extracting agents, as in subcritical water extraction (SWE), supercritical fluid extraction (SFE), pressurized fluid extraction (PFE,) or accelerated solvent extraction (ASE) have potential in the extraction of phenolic compounds from plant materials (Dai & Mumper, 2010). On the other hand, some of these methods may have drawbacks which include, the production of toxic wastes, extracts post-processing to solvent removal, and thermal degradation of thermolabile compounds (Mezzomo & Ferreira, 2016). However most these methods are simple, produce high extraction yield, require low solvent volume as well as moderate/reduced extraction time (Dai & Mumper, 2010; Khoddami et al., 2013; D. Xu et al., 2017).

Quantification of phenolic compounds in plant extracts is influenced by the chemical nature of the analyte, the assay method, selection of standards, and presence of interfering substances (Naczk & Shahidi, 2006). Traditional spectrophotometric assays provide simple and fast screening methods to quantify classes of phenolic compounds in crude plant samples. However, due to the complexity of plant phenolics and the differences in reactivity of phenols and assay reagents, the specific method selected for the assay of constituents affects the results and may not be reproducible by another method. In addition, many methods are prone to interferences such as complexation of phytoconstituents and thermal degradation, and consequently frequently result in over- or underestimation of the contents. Modern high-performance chromatographic

techniques combined with instrumental analysis are used for the profiling and quantification of phenolic compounds. Gas chromatographic (GC) techniques have been widely used especially for separation and quantification of phenolic acids and flavonoids although the low volatility of phenolic compounds is a major concern with this technique. Therefore, phenolics are usually transformed into more volatile derivatives by methylation, conversion into trimethylsilyl derivatives, etc. prior to chromatography (Dai & Mumper, 2010). A detailed discussion on application of GC on analysis of phenolic acids and flavonoids was provided by Stalikas (2007).

HPLC currently represents the most popular and reliable technique for analysis of phenolic compounds (Dai & Mumper, 2010) with various supports and mobile phases available for the analysis including anthocyanins, proanthocyanidins, hydrolysable tannins, flavonols, flavan-3-ols, flavanones, flavones, and phenolic acids (Anttonen & Karjalainen, 2006; Harris et al., 2007; McCallum, Yang, Young, Strommer, & Tsao, 2007; Mertz, Cheynier, Günata, & Brat, 2007; Pawlowska, Oleszek, & Braca, 2008; Prior et al., 2001; Rodríguez-Bernaldo de Quirós, López-Hernández, Ferraces-Casais, & Lage-Yusty, 2007; Ruiz, Egea, Gil, & Tomás-Barberán, 2005; Vrhovsek, Rigo, Tonon, & Mattivi, 2004; Yanagida, Shoji, & Kanda, 2002; K.-Y. Yang, Lin, Tseng, Wang, & Tsai, 2007). In addition, HPLC techniques offer a unique opportunity to analyze all components of interest, their derivatives, or degradation products simultaneously (Downey & Rochfort, 2008; Hiroyuki Sakakibara, Yoshinori Honda, Satoshi Nakagawa, Hitoshi Ashida, & Kanazawa, 2002). The introduction of reversed-phase (RP) columns has considerably enhanced separation of different classes of phenolic compounds; RP C_{18} columns are almost exclusively employed (Dai & Mumper, 2010). Since column temperature potentially affects the separation of phenolics such as individual anthocyanin (Oh et al., 2008), constant column temperature is recommended for reproducibility (Stalikas, 2007). The most frequently used organic modifiers are

acetonitrile and methanol. Often, the mobile phase is acidified with a modifier such as acetic, formic, or phosphoric acid to minimize peak tailing. Isocratic or gradient elution are applied to separate phenolic compounds and are selected based on the number and type of the analyte and the nature of the matrix (Dai & Mumper, 2010). Several reviews discuss the application of HPLC methodologies for the analysis of phenolics in detail (Merken & Beecher, 2000; Naczk & Shahidi, 2004; Robbins, 2003; Stalikas, 2007).

Depending on the intrinsic existence of conjugated double and aromatic bonds, phenolics exhibit differing absorption rates in ultraviolet (UV) or ultraviolet/visible (UV/VIS) regions. For identification, UV/VIS, photodiode array (PDA), and UV fluorescence detectors are coupled to LC. PDA is the most prevalent method since it allows for scanning real time UV/VIS spectra of all solutes passing through the detector, providing more information about compounds in complex mixtures such as a plant crude extract (Dai & Mumper, 2010). Other methods include electrochemical detection (ECD) (Novak, Janeiro, Seruga, & Oliveira-Brett, 2008), the voltammetry technique (Woodring, Edwards, & Chisholm, 1990), on-line connected PDA and electro-array detection (EAD) (Mattila, Astola, & Kumpulainen, 2000), chemical reaction detection techniques (Pascual-Teresa, Treutter, Rivas-Gonzalo, J. C., & Santos-Buelga, 1998), mass spectrometric resonance (MS) (Cavaliere et al., 2008; Harris et al., 2007; Oh et al., 2008), and nuclear magnetic resonance (NMR) detection (Christophoridou & Dais, 2009; Pawlowska et al., 2008). MS and NMR detections are more useful for the identification of structure than for quantification of phenolic compounds (Dai & Mumper, 2010). Electromigration techniques including capillary electrophoresis (CE), capillary zone electrophoresis (CZE), micellar electrokinetic chromatography coupled with UV, and to a lesser extent EC and MS detection can also be employed for phenolics analysis (Jáč, Polášek, & Pospíšilová, 2006).

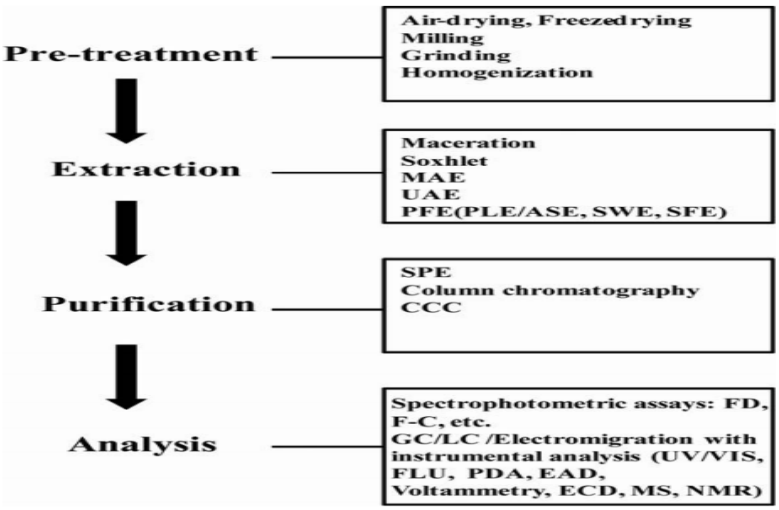

Figure 11: Steps in Extraction and Analysis of Phytoconstituents

Source: Dai, J., & Mumper, R. J. (2010)

2.4 Antimicrobial Properties of Plant Extracts

Plants all over the world are screened as potential alternative antimicrobial medicines (Chandra et al., 2017) since plant ‘‘immune systems’’ effectively prevent infections caused by numerous phytopathogens (Tierens et al., 2001). Bacterial pathogens have similar strategies to infect and colonize plant and animal hosts with a mechanistic ability to deliver effector proteins into host cells to mimic, suppress, or modulate host defense signaling pathways and to enhance pathogen fitness (Simões et al., 2014). On the host side, plants have developed sophisticated surveillance mechanisms to recognize distinct effectors from various pathogenic bacteria (Simões et al., 2014; Staskawicz, Mudgett, Dangl, & Galan, 2001). Some bacteria such as *E. coli* and *Salmonella* species are commonly associated with animal hosts, but there is evidence that these bacteria can also colonize plants (Aruscavage, Lee, Miller, & LeJeune, 2006). Research suggests that the phytochemical susceptibility of bacteria typically pathogenic in animals similar to that of bacteria that are pathogenic in plants (Simões et al., 2014).

The bacterial response to various stress factors that create an unfavorable growth environment is a key to their survival in different environmental conditions (Simões et al., 2014). The exposure to antimicrobial plant substrates produces such stress on their resistance mechanism (Dantas et al., 2008). To prevent the evolution of resistant microorganisms, substantial resources have been invested in the research of new antimicrobials (Butler, 2005; Simões et al., 2014). While microbial products have provided important substrates for the development of effective antimicrobials to the pharmaceutical industry, especially considering the emergence of antibiotic resistant species (Clardy, Fischbach, & Walsh, 2006) the effectiveness of plant antimicrobials should be reconsidered, as it has been demonstrated in traditional medicine over centuries (Heinrich & Gibbons, 2001). An estimated 14 - 28% of higher plant species are used medicinally, and ~74% of pharmacologically active derived components from these plants have been discovered after a series of plant ethnomedical evaluations (Eloff, 1998; Simões et al., 2014). The inhibitory potential of numerous plant species has been evaluated *in vitro* for hundreds of bacteria, since many medicinal plants produce phytochemicals with broad spectrum effect even on fungi and yeasts (Madivoli et al., 2012; Mwitari et al., 2013; Simões et al., 2014; Singh et al., 2016). This success is largely due to their chemical diversity and structural complexity (Gibbons et al., 2004; Simões et al., 2014). Moreover, these studies have also confirmed that medicinal plants exhibit a broad range of biological activities and that plant species often contain a diverse range of bioactive molecules responsible for their antimicrobial properties (Pandey, 2013) (Table 5).

2.4.1 Potentials of Antimicrobials from Plants to Microbial Resistance

During the advent of antibiotics in the 1950s, plant applied antimicrobials included antibiotics that inhibit microbial growth, and were either microbial products, synthetic derivatives (Cowan, 1999);

or antimicrobial peptides produced by complex organisms (Jenssen, Hamill, & Hancock, 2006) while plant antimicrobials were virtually unknown (Simões et al., 2014). Interest in phytochemical derived antimicrobials increased in the late 1990s with the increased inefficacy of conventional antibiotics, due in part to their sometimes excessive or inappropriate use in mammalian infections (Cowan, 1999; Simões et al., 2014). However, phytochemicals have not been used as systemic antibiotics so far (Gibbons, 2004; Lewis & Ausubel, 2006) although many have demonstrated antibacterial potential (Simões et al., 2014), some of which are described in Table 5. In recent years, phytochemicals such as the plant alkaloid reserpine, capable of potentiating the activity of antibacterial agents, have been identified, but none have reached clinical application (Aeschlimann, Dresser, Kaatz, & Rybak, 1999; Gibbons et al., 2004; Schmitz et al., 1998). Phytochemicals are routinely classified as antimicrobials based on susceptibility tests that produce a minimum inhibitory concentration (MIC) ranging from 100 to 1000 mg/mL (Simões et al., 2014). Comparatively, typical antibiotics produced by bacteria and fungi have a significantly lower MIC of 0.01 to 10 mg/mL (Tegos, Stermitz, Lomovskaya, & Lewis, 2002). In spite of the differences in potency, the rapid development of bacterial (cross)-resistance to many of these classes of microbial antibiotics (Simões et al., 2014) requires alternative treatment modalities. In addition, several studies suggest that phytochemicals can act as antiviral (Jassim & Naji, 2003; Mukhtar et al., 2008; Naithani et al., 2008), anti-parasitic (Atawodi & Alafiatayo, 2007; Chan-Bacab & Peña-Rodríguez, 2001; Simões et al., 2014), and antifungal agents (Morel et al., 2002; Simões et al., 2014), and may be cytotoxic to tumor cells (Agnes Rimando & Suh, 2008; Suffredini, Varella, & Younes, 2006; Udenigwe, Ramprasath, Aluko, & Jones, 2008). Therefore, the potential of medicinal plant products as pharmacological agents has received increased attention in recent years (Cowan, 1999).

Table 5: Medicinal Plants as Antimicrobial Agents

Plant	Part used	Solvent	Analysis	Results/Comments	References
P. africana (Ethiopia)	Bark (stem bark)	Absolute (98%) ethanol	Agar disc diffusion assay and minimal inhibitory concentration (MIC) assay	Effective antibacterial agent against *Shigella* spp (*Enterbacteriaceae*); Zone of inhibition (ZI in mm) against *S. boydii* and uniform MIC (2.625 mg/mL)	Das, 2017
P. africana (Kenya)	Bark (stem bark)	Dichloromethane, ethyl acetate and methanol	MIC	Methanol extract very active against *S. aureus* (SA) and Methicillin Resistant *S. aureus* (MRSA) with ethyl acetate being moderately active	Mwitari et al., 2013
P. africana (Kenya)	Bark	Methanol	Disc diffusion method prepared against Streptomycin	Moderate activity against *S. aureus*, *B. subtilis*, *P. aeruginosa*, *E. coli* and *C. albicans*	Madivoli et al., 2012
P. africana (Ethiopia)	Bark	Petroleum ether, chloroform, acetone and methanol	Agar dilution method	Methanol extract (strongest activities against *E. coli*, *C. koseri*, *E. aerogenes*, *S. aureus* and *E. cloacae* clinical strains); acetone extract the most effective with MIC of 0.65 mg/mL^{-1} against *Citrobacter fruindi* and *S. pyogenes*	Gashe & Zeleke, 2017
P. africana (Kenya)	Bark (stem bark)	Methanol and hexane	Disc diffusion method	Methanol extract active against gram (+) *S. aureus* and gram (-) bacteria *P. aeruginosa*; also, highest activity with a mean zone diameter of 21 mm against *T. mentagrophytes* and 20 mm against *M. gypseum* with MIC values of 0.0395 and 0.078 mg/mL, respectively; but both extracts had no activity against the test yeast (*C. albicans* and *C. neoformans*	Bii et al., 2010
Punica granatum	Fruit	Ethanol and fractions (n-hexane, water, chloroform, dichloromethane, ethyl acetate, n-butanol	MIC	Greatest inhibition activities found in the ethyl acetate fraction against MRSA	Machado et al., 2003
Mikania glomerata, *Psidium guajava*, *Mentha, piperita*, *Syzgium aromaticum*, *Zingiber officinale*	Leaves	70% methanol	MIC; Mueller-Hinton Agar (MHA)	All extracts showed some activity against *S. aureus*, and the most effective were those from clove at concentration of 0.36 mg/mL and guava at 0.56 mg/mL.	Betoni, Mantovani, Barbosa, Di Stasi, & Fernandes Junior, 2006
Croton urucurana	Leaf, phloem, and latex	Hexane, dichloromethane, hydroalcoholic	Agar disc diffusion method	Latex inhibited all tested bacteria (Enterococcus facalis, S. aureus, Staphylococcus epidermidis, Streptococcus pyogenes, Klebsiella pneumoniae, Salmonella), except E. coli	Oliveira, Lima, Silva, & Martins, 2008

Plants produce phytochemicals with demonstrated potential as antibacterials when used alone, as synergists, or as potentiators of other antibacterial agents. The phytochemical mechanisms differ from conventional antibiotics and could therefore be of use in the treatment of resistant bacteria as described in Table 6 (Abreu, McBain, & Simões, 2012). Many researchers have screened plants for antibacterial properties and mode of action and indicated that these antimicrobials can act on multiple biochemical targets of the bacterial cell. The site of action of the phytochemicals are proposed to depend on their chemical structure and properties (Simões et al., 2014) as discussed in more detail below. The dyeing properties of henna (*Lawsonia inermis*) are due to the presence of quinones which possesses antimicrobial activity against *Pseudomonas aeruginosa* (Habbal et al., 2011). Hypericin, an anthraquinone from *Hypericum perforatum*, has general antimicrobial properties and also specifically inhibits methicillin-resistant and methicillin-sensitive *Staphylococcus* (Dadgar et al., 2006)

The value of the medicinal properties of alkaloids was first recognized when morphine, a potent narcotic analgesic, was isolated from *Papaver somniferum* (Chandra et al., 2017). Alkaloids' antimicrobial properties reduce the infectivity of *Giardia* and *Entamoeba* species, and serve as antidiarrheals due to their probable effects on transit time in the small intestine (Cowan, 1999). The main disadvantage of alkaloids is their toxicity, the cause of their marked therapeutic effect in small quantities; therefore, alkaloids-based herbal preparation have not been frequently used in folklore medicine (Chandra et al., 2017).

Plant alkaloids, including berberine, found in *Berberis* species, and piperine, found in *Piper* species, can interact with the bacterial cytoplasmic membrane, intercalate with DNA, and inhibit efflux pumps in *Staphylococcus aureus* (Jennings & Ridler, 1983; Khan, Mirza, Kumar, Verma, & Qazi, 2006). Hasubanalactam alkaloids, isolated from the tubers of *Stephania glabra*, have

antimicrobial activity against *Staphylococcus aureus*, *S. mutans*, *Microsporum gypseum*, *M. canis* and *Trichophyton rubrum* (Semwal & Rawat, 2009).

Flavonoids have antimicrobial, antiviral, anti-allergic and anti-inflammatory properties (Gábor, 1986). Quercetin has antimicrobial potency against many microorganisms. Antimicrobial activity of six flavonoids isolated from *Galium fissurense*, *Viscum album* ssp. album and *Cirsium hypoleucum* was effective against extended-spectrum β-lactamase-producing multidrug-resistant bacteria *Klebsiella pneumoniae* (Özçelik, Orhan, Özgen, & Ergun, 2008). Other polyphenols, such as flavonoids (robinetin, myricetin and epigallocatechin gallate) from *Elaegnus glabra*, can inhibit the synthesis of nucleic acids of both Gram negative and Gram-positive bacteria (Cushnie & Lamb, 2005; Mori, Nishino, Enoki, & Tawata, 1987). Researchers suggested that the B ring of the flavonoids may play a role in intercalation or hydrogen bonding with the stacking of nucleic acid bases which may explain the inhibitory action on DNA and RNA synthesis (Simões et al., 2014). Quercetin, a component of propolis, binds to the GyrB subunit of *E. coli* DNA gyrase and inhibits the ATPase activity (Plaper et al., 2003). This flavonoid was also reported to cause an increases permeability of the inner bacterial membrane and a dissipation of the membrane potential (Mirzoeva, Grishanin, & Calder, 1997). Epicatechin gallate and epigallocatechin gallate, two main flavonoid constituents in green tea, inhibited antibiotic efflux pumps in MRSA (Gibbons et al., 2004). Epigallocatechin gallate is also a potent inhibitor of both the b-ketoacyl-ACP reductase (FabG) and the trans-2-enoyl-ACP reductase (FabI) components in the bacterial type II fatty-acid synthase system. This property is common to a broad range of plant polyphenols (Y.-M. Zhang & Rock, 2004).

Flavonoids such as kaempferol, rutin, and quercetin have antifungal properties (Chandra et al., 2017; Soto-Hernandez, Palma-Tenango, & Garcia-Mateos, 2017). A leguminous plant

Lupinus spp. contains dihydrofuranoisoflavones which demonstrated antifungal activity against *Botrytis cinerea* and *Aspergillus flavus* (Tahara, Ingham, Nakahara, Mizutani, & Harborne, 1984).

Coumarins isolated from *Angelica lucida* L. inhibit oral pathogens *Streptococcus mutans* and *S. viridians* (Widelski, Popova, Graikou, Glowniak, & Chinou, 2009). Pyranocoumarins isolated from *Ferulago campestris* showed antibacterial activity against nine clinically isolated gram-positive and gram-negative bacterial strains (Basile et al., 2009).

The mechanism of action of essential oils and their constituents (terpenoids, carvacrol and thymol) against bacteria is yet not fully understood, but it is speculated to involve membrane disruption by lipophilic products (Griffin, Wyllie, Markham, & Leach, 1999; Mendoza, Wilkens, & Urzúa, 1997). This antibacterial action can result in membrane expansion, increase of membrane fluidity and permeability, disturbance of membrane embedded proteins, inhibition of respiration, and alteration of ion transport processes in both Gram-positive and Gram-negative bacteria (Brehm-Stecher & Johnson, 2003; Carson, Mee, & Riley, 2002; Cox et al., 2000; Trombetta et al., 2005).

Phenols and phenolic acids can cause the disruption of energy production due to enzyme inhibition by oxidized products, reaction with sulfhydyl groups, or more nonspecific interactions with proteins (Mason & Wasserman, 1987). Phenolic extracts from *Origanum vulgare* and *Vaccinum macrocarpon* caused urease inhibition and the disruption of energy production by inhibiting proline dehydrogenase at the plasma membrane of the Gram-negative human gastric pathogen *Helicobacter pylori* (Y. T. Lin, Kwon, Labbe, & Shetty, 2005). Glycoside saponins might induce pore-like structures which change the membrane permeability, resulting in alterations in the ionic homeostasis between intracellular and extracellular compartments (Melzig, Bader, & Loose, 2001). They can also interfere with the energy metabolism through interaction

with catabolic enzymes and the electron transport chain (Mandal, Sinha Babu, & Mandal, 2005; Simões et al., 2014). The diallyl thiosulfinate allicin, a phytochemical commonly obtained from *Allium sativum* (garlic), has potent antimicrobial activity and can interact with intracellular thiols and thiol containing proteins (Miron, Rabinkov, Mirelman, Wilchek, & Weiner, 2000; Rabinkov et al., 1998). Others proposed that inhibition of RNA synthesis is the primary target of allicin action against *Salmonella typhimurium* (Feldberg et al., 1988). Plant peptides can act on bacterial cells by forming ion channels in the membrane and inhibiting adhesion of microbial proteins to host polysaccharide receptors (Suarez et al., 2005; Y. Zhang & Lewis, 2006). Peptides from *Moringa oleifera* caused membrane permeabilization and disruption of pathogenic Gram-negative and -positive bacteria including MRSA (Suarez et al., 2005).

While the predictive action sites and aspects of the mode of action of several phytochemicals have been studied, other factors such as the SAR are not well understood (Griffin et al., 1999; Simões et al., 2014). Further research into the inhibitory mechanisms on cell function and occasionally lysis by phytochemicals is necessary to understand and hence exploit these mechanisms in the development of therapeutic or biocontrol strategies (Simões et al., 2014). The resulting structurally complex libraries of natural products including phytochemicals might provide information onpotential antibacterials. However, since bacteria are aquiring antimicrobial resistance more rapidly, phytochemicals with antibacterial potential may have only a limited period of practical utility (Simões et al., 2014).

Lignans isolated from *Pseudolarix kaempferi* have antimicrobial activity against *Candida albican* and *S. aureus* (He et al., 2011). Dibenzocyclooctadiene lignin isolated from Schissandra chinensis inhibits the growth of Chlamydia trachomatis and *C. pneumonia* (Hakala et al., 2015).

Table 6: Antimicrobials Mechanism of Action against Microorganisms

Class	Subclass	Examples	Mechanism
Phenolics	Simple phenols	Catechol	Substrate deprivation
		Epicatechin	Membrane disruption
	Phenolic acid	Cinnamic acid	-
	Quinones	Hypericin	Adhesin binding, complex with cell wall, enzyme inactivation
	Flavonoids	Chrysin	Adhesin binding
	Flavones	-	Complex with cell wall
		Abyssinone	Enzyme inactivation; HIV reverse trancriptase inhibition
	Flavonols	Totarol	-
	Tannins	Ellagitannin	Protein and adhesin bindings, enzyme inhibition, substrate deprivation, complex with cell wall, membrane disruption, metal ion complexation
	Coumarins	Warfarin	Interaction with eukaryotic DNA (antiviral activity)
Terpenoids, Essential oils	-	Capsaicin	Membrane disruption
Alkaloids	-	Berberine	Intercalation into cell wall and/or DNA
		Piperine	

Source: (Cowan, 1999 and Pandey, 2013)

2.4.2 Antimicrobials from Plants to Improve Food Quality and Safety

Food quality can be adversely affected by spoilage as a result of microbial contaminations which are commercially undesirable (Mohapatra, Mishra, Giri, & Kar, 2013) and affect the quality of final product (Lee, 2004; Mohapatra et al., 2013). Since many natural food products are perishable they must be protected from spoilage during the post-harvest, preparation, storage, and distribution phases to extend the shelf life. Specifically, minimally processed foods, ready-to-eat fresh food products, globalization of food trade, and distribution from centralized processing units pose major challenges for maintenance of food safety and quality (Delesa, 2018). Especially meat, fish, fruits, and vegetables are prone to bacterial and fungal contamination that deteriorate flavor, odor, color, sensory, and textural properties. In addition, the potential carcinogenicity of synthetic preservatives has prompted research into finding some natural plant-based alternatives (Nikmaram et al., 2018). Microbial spoilage is currently prevented or delayed by various preservation

techniques: Chilling; freezing (G. W. Gould, 1996; Negi, 2012); vacuum packing, modified atmosphere packing; synthetic preservatives (G. W. Gould, 1996); fermentation, acidification; nutrient restriction; water activity reduction; synthetic antimicrobials; pasteurization (Negi, 2012); and other inactivation techniques (e.g., pulse electric field, irradiation) (Mohapatra et al., 2013).

Nevertheless, the food industry has demonstrated interest in the potential of plant antimicrobials and antioxidants since some have been assessed and found 'Generally Recognized as Safe' (GRAS) (Kim, Cho, & Han, 2013; Vinceković et al., 2017). They did not affect sensory properties negatively (e.g. color, odor or flavor); were efficient at low concentrations (0.001 - 0.01%); compatible with foods and easy to use while demonstrating significant stability during processing and extending the shelf-life (Nikmaram et al., 2018). Most importantly, these components and their metabolites were nontoxic at larger doses than needed for application. These phytochemicals were mixtures including phytophenolics in herbs and spices, phenolics, flavonoids and acids in fruits and glucosinolates in cruciferous vegetables (Patra, 2012).

The efficiency of an antimicrobial treatment depends on many species-specific factors, including type, genus, species, and strain of the main microorganism, in addition to environmental factors such as pH, water activity, temperature, atmospheric composition and the initial microbial load on the food materials (Negi, 2012). Therefore, the type of the microorganism(s) should be assessed since antimicrobial cocktails are often more effective than individual ones (Oz & Kafkas, 2017). The interaction between phytochemicals and the growth of microorganisms is one of the most important factors when evaluating their antimicrobial preservative potential although there are other influencing factors (Negi, 2012).

Limited information is available on the use of plant extracts and their essential oils (EOs) in the food industry, but research has demonstrated that these extracts are effective against

foodborne pathogens (R. Gyawali, Hayek, & Ibrahim, 2014). Antimicrobial agents from plants have been mostly used in the form of biofilm and edible coatings in food systems. These antimicrobial films and coatings slowly diffuse inside the food package, thereby extending the duration of the antimicrobial activity (R. Gyawali et al., 2014). The shelf life for shrimp increased when thyme oil and trans-cinnamaldehyde were added to the shrimp's soy and whey protein derived coating (Ouattara, Simard, Piette, Begin, & Holley, 2000). A 20 - 21-day increase in shrimp shelf life was also observed by using a combination of a protein-based coating containing consisting of 0.9% thyme oil and 1.8% trans-cinnamaldehyde combined with 3.0 kGy gamma irradiation (Ouattara, B., & Mafu, 2003). Shen et al. (2009) reported a significant reduction of *L. monocytogenes* on frankfurters (simulated home storage) dipped in salad dressing in combination with oil and lemon juice or vinegar as compared to those dipped in distilled water. *L. monocytogenes* populations were also reduced when chicken frankfurters were treated with clove oil at 1 or 2% and stored at 5°C for 2 weeks or 15°C for 1 week, but they survived and multiplied on control frankfurters (Mytle, Anderson, Doyle, & Smith, 2006). Lime extract alone or in combination with other edible fruit extracts exhibited antimicrobial properties by reducing *C. jejuni* and *Campylobacter coli* in chicken skin by >4.0 log cycles (Valtierra-Rodríguez, Heredia, García, & Sánchez, 2010). Bell pepper (*Capsicum annum*) extract was effective in inhibiting the growth of *Salmonella typhimurium* in raw beef, requiring a minimum of 1.5 mL/100 g of meat (Careaga et al., 2003).

Green tea extract has been used as an antimicrobial in various foods such as extra virgin olive oil (Rosenblat, Volkova, Coleman, Almagor, & Aviram, 2008), meat, fish (Alghazeer, Saeed, & Howell, 2008), dehydrated apple products (Lavelli, Vantaggi, Corey, & Kerr, 2010), rice starch products (Y. Wu, Chen, Li, & Li, 2009), and biscuits (Mildner-Szkudlarz, Zawirska-Wojtasiak,

Obuchowski, & Gośliński, 2009). Grape seed extract demonstrated antimicrobial activities alone or in combination with other hurdle technologies in various food applications such as tomatoes, frankfurters, raw and cooked meat, poultry products, and fish (Bisha, Weinsetel, Brehm-Stecher, & Mendonca, 2010; Brannan, 2009; Perumalla & Hettiarachchy, 2011). The combined effect of polyphenol-rich cocoa powder with pulsed electric field application significantly inactivated the level of *Cronobacter sakazakii* in infant milk formula over 12 h at 8°C storage (R. Gyawali et al., 2014). In this study, a reduction of 4.41 log cycles was achieved when 12% of cocoa powder was added 4 h after the pulsed electric field application (Pina-Pérez, Martínez-López, & Rodrigo, 2013). Recently, the antimicrobial effect of caffeine was demonstrated against *E. coli* O157:H7 in skim milk with other researchers reporting its potential application as a preservative in the beverage industry (Rabin Gyawali, Adkins, C. Minor, & Ibrahim, 2014).

CHAPTER 3: Evaluation of Phytochemical Content and Activities

3.1 Materials

Dried plant samples were obtained from Kenya (K) and Cameroon (C). Analytical reagent grade chemicals and solvents were obtained from Sigma-Aldrich Co., St. Louis, MO., and Fisher Scientific Co. Fair Lawn, NJ.

3.2 Methods

3.2.1 Sample Preparation and Extraction

The dried root and bark samples were ground using an Oster 16-speed blender plus food chopper (Oster, Owosso, MI) and a blade coffee grinder (KitchenAid, St. Joseph, MI). Ten grams of plant samples were weighed and placed in 250 mL Erlenmeyer flasks containing 100 mL of the extraction solvents (1:10, w/v); that is, 80% acetone, methanol, and ethanol (organic solvent: water, 80:20 v/v). Frequent agitation for thorough mixing was required on an orbital shaker (Benchmark, Edmonton, AB) for 24 h at room temperature (Bandiola, 2018). The mixtures were then strained, marcs were pressed, and the combined liquids were clarified by filtration using Whatman No. 1 filter paper (Whatman international, England, UK) (Bandiola, 2018). The concentrated extracts were then left to dry in the fume hood and stored at 4°C, until used for analysis. The resulting dried extracts from the acetonic, methanolic and ethanolic extraction were labeled: *P. africana* (C) bark (Pru-B-C-Ace, Met, EtOH); *P. africana* (C) root (Pru-R-C-Ace, Met, EtOH); *P. africana* (K) bark (Pru-B-K-Ace, Met, EtOH); *P. yohimbe* bark (Yom-B-Ace, Met, EtOH); and *Orthero* root (Orth-R-Ace, Met, EtOH).

3.2.2 Determination of Total Phenolic Content (TPC)

The total phenolic content (TPC) was measured using the Folin-Ciocalteu (F-C) assay following the method published by Taga et al. (1984): The absorption at 750 nm was compared and calculated using gallic acid as standard; results were expressed as milligrams of gallic acid equivalents (GAE) per gram of dried sample (mg GAE/g DW) (Taga et al., 1984).

3.2.3 Determination of Total Flavonoid Content (TFC)

The total flavonoid content in the extracts was determined following the procedure described by Stankovic (2011) using aluminum chloride ($AlCl_3$) and rutin as standard. The absorbance of the samples and the rutin standard of concentration (range = 0.005 - 0.03 mg/mL) were measured at 415 nm using a DU 720 general-purpose spectrophotometer (Beckman Coulter Inc., Brea, CA) and expressed as milligram of rutin equivalent (RU) per gram of dried sample (mg RU/g W).

3.2.4 Determination of Pigment Content

Chlorophyll, xanthophyll, carotenoid, anthocyanin, β-carotene and lycopene contents were calculated after measuring the absorbance of the dried extracts in 10 mL of 80% ethanol at various wavelengths (453, 470, 480, 485, 505, 537, 645, 647, and 663 nm) in duplicate (Owayss, Rady, & Gadallah, 2004). Calculations were done in accordance with those described by Nagata & Yamashita (1992):

- Lycopene = $- 0.0458 \times Abs_{663} + 0.372 \times Abs_{505} - 0.0806 \times Abs_{453;}$
- β - carotene = $- 0.216 \times Abs_{663} + 0.304 \times Abs_{505} - 0.452 \times Abs_{453}$;
- Carotenoids = $Abs_{480} + (0.114 \times Abs_{663} - 0.638 \times Abs_{644})$;

- Chlorophyll A = 12.7 x Abs_{663} – 2.69 x Abs_{645};
- Chlorophyll B = 22.4 x Abs_{645} – 4.68 x Abs_{663};
- Total chlorophyll = 8.02 x Abs_{663} + 20.2 x Abs_{645};
- Xanthophylls = 2026.1 x Abs_{470} – 2288.6 x Abs_{485} (A) – 0.06518 (B);
- Anthocyanin = - 0.08173 x Abs_{537} + 0.00697 x Abs_{647} – 0.002228 x Abs_{663}

Results were expressed in milligram of pigment per gram of dried extract (mg/g DW).

3.2.5 Antioxidant Activity Assays

Apak et al. (2007) suggested that antioxidant activity cannot be estimated by using a single test. Three different tests were conducted to strengthen this investigative analysis. To determine the antioxidant activities of plants the free radical scavenging ability using DPPH, $ABTS^{+}$, and Ferric reducing antioxidant power were assessed. The DPPH assay mainly tests the hydrogen donating capacity of an antioxidant to scavenge DPPH radicals (Singh et al., 2016). The antioxidant activity is measured by the hydrogen donating or radical scavenging ability using the stable radical DPPH (Proestos et al., 2013), with the reduction of the radical followed by a decrease in the absorbance at 515 nm which is measured spectrophotometrically. The antioxidant activity of the plant extracts was compared to an ascorbic acid calibration curve, and results were expressed as milligram of ascorbic acid per gram of dried sample weight (mg ascorbic acid/g DW). Percentage of inhibition of the DPPH radical (I) was calculated using the below equation:

$$I\ (\%) = \frac{(A_0 - A)}{A_0} \times 100$$

Where I = DPPH inhibition (%), A_0 = absorbance of the control sample (t = 0 min) and A = absorbance of a tested sample at the end of the reaction (t = 30 min).

Fifty percent (50%) effective concentration (EC_{50}) (Madivoli et al., 2012) was calculated using the formula described by Oussou et al. (2016): EC_{50} (mg/mL DPPH). The EC_{50} is defined as the amount of antioxidant (Mishra, Ojha, & Chaudhury, 2012) or the moles of phenolic compound divided by moles of DPPH necessary to decrease the absorbance of DPPH by 50% (Tabbikha, 2017) of the initial absorbance (Mishra et al., 2012). The lower the EC_{50}, the higher is the antioxidant power (Dajanta, Apichartsrangkoon, & Chukeatirote, 2011; Tabbikha, 2017) of the examined plant extract (Madivoli et al., 2012).

The antiradical power (ARP) defines the antioxidant potential of an antioxidant (Mishra et al., 2012). The higher the ARP, the higher the free radical scavenging activity (Dajanta et al., 2011; Tabbikha, 2017). The antiradical power (ARP) was also calculated using a formula described by Oussou et al. (2016): $ARP = 1/EC_{50}$.

The Trolox equivalent antioxidant activity (TEAC) was determined using the $ABTS^+$ (2,2'-azino-bis 3-ethylbenzothiazoline-6-sulphonic acid) radical cation decolorization method of Benzie and Strain (1999) and Re et al. (1999). This assay measures the reduction of $ABTS^+$ radicals by electron-donating ability of the antioxidants of the tested plant extracts; the absorbance is measured spectrophotometrically at 734 nm and at 0, 5, and 10 min after initial mixing. A Trolox (6-hydroxy-2,5,7,8 tetramethylchroman -2-carboxylic acid) standard curve (0 - 0.330 mM) was compared with the absorbance, and results of the decolorization of the radicals is expressed in millimolar of Trolox equivalent per gram of dried sample (mM TE/g) (Benzie & Strain, 1999; Re et al., 1999). The percentage inhibition of $ABTS^+$ was calculated using the following formula:

$$I\ \% = \frac{At = {}_0 - At}{At = {}_0} \times 100$$

Where I = $ABTS^+$ inhibition (%), $At = {}_0$ = absorbance of the blank (control) sample (t = 0 min) and At = absorbance of a tested sample in 5 or 10 min.

The reducing power of the plant extracts was estimated spectrophotometrically following the procedure described by Benzie and Strain (Benzie & Strain, 1999; Dudonné et al., 2009). The method is based on the reduction of ferric (III) ions (Fe^{3+}) tripyridyltriazine (TPTZ: a colorless complex) to ferric (II) ion (Fe^{2+}- TPTZ: (blue colored complex) formed by the action of electron donating antioxidants at low pH. This reaction was monitored by measuring the change in absorbance at 593 nm. The calibration curve was prepared by plotting the absorbance of different concentrations of the standard (i.e., ferrous sulfate heptahydrate; $FeSO_4.\ 7H_2O$) (0 - 100 µM) at 593 nm. The FRAP values were obtained by comparing the absorbance change in the test mixture with those obtained from increasing concentrations of Fe^{3+} ions and expressed as millimolar Fe^{2+} ions per gram of sample (mM Fe^{2+}/g).

3.2.6 HPLC Analysis

Eight extracts from *P. africana* (C) and *P. yohimbe* (Yom-B-EtOH, Pru-R-C-Ace, Yom-B-Ace, Pru-B-C-Ace, Pru-B-C-Met, Pru-B-C-EtOH, Pru-R-C-EtOH, and Pru-R-C-Met) with EC_{50} values (±0.125) comparable to standard ascorbic acid were chosen for analysis of phenolic acids. For sample identification and quantification, a Shimadzu LC-20AB HPLC equipped with an SPD-M20A multi-wavelength UV/VIS photodiode array detector (PDA; Kyoto, Japan), and a Phenomenex LC C_{18} column (250 x 4.6 mm, 5 µm) (Phenomenex; Torrance, CA) was used at 25°C. The Lab-Solutions software (Kyoto, Kyoto prefecture, Japan) was used to process data from chromatographic separations. After injecting 10 µL of extract, elution was performed using the mobile phase consisting of two solvents, with solvent A being 2.5% (acetic acid: water v/v) and solvent B - 100% acetonitrile. The solvent gradient was started at 0.3% B and held for 0-10 min; next it was programmed for 3 - 10% B for 10 - 12 min; 10 - 15% B for 12 - 64 min; 15 - 24% B

for 64 - 69 min; 24 - 50% B for 69 - 74 min; and then to 95% B for 74 - 75 min. Phenolic acid standards were prepared as 10 mg/mL in methanol stock solutions. Identification of compounds was accomplished by comparison of retention time and spectral patterns of eluting peaks and standard compounds at λ = 250 - 350 nm. Quantification was based on the response ratio of the compounds of interest to the internal standard and was calculated by injecting a known concentration of similar reference standards (Zanolari, 2003).

3.3 Statistical Analysis

Data were analyzed using SPSS, version 25.0 (SPSS Inc., Chicago, IL). Following one-way analysis of variance (ANOVA), $p \leq 0.05$ was considered significant, and treatment means were compared using Tukey's post hoc comparisons tests. Each assay was repeated in triplicates unless otherwise stated.

3.4 Results and Discussions

3.4.1 Total Phenolic Content (TPC)

Total phenolic content varied significantly ($p \leq 0.05$) from 166.27 to 1131.70 mg GAE/g among samples as shown in Fig. 12 and Table 7. Similarly, phenolic content of extracts also varied significantly from 6.86 to 397.03 mg GAE/g in a study by Dudonné et al. (2009). In this study, the highest TPC (1131.70 mg GAE/g) of all analyzed samples was found in the ethanolic extract of *P. africana* (C) bark in contrast to a previous study by Asuzu et al. (unpublished), using absolute (100%) solvents, and reporting the methanolic extract (1397.33 mg GAE/g) of *P. africana* (C) bark as the highest. However, TPC results as shown in the present study using aqueous solvents were much higher than most reported for similar plant extracts in the previous study (122.67 to

913.33 mg GAE/g) (Asuzu et al., unpublished), except for the absolute acetonic and methanolic extracts of *P. africana* (C) bark (1348 and 1397.33 mg GAE/g) respectively and the ethanolic extract (584 mg GAE/g) of the same *P. africana* (C) root.

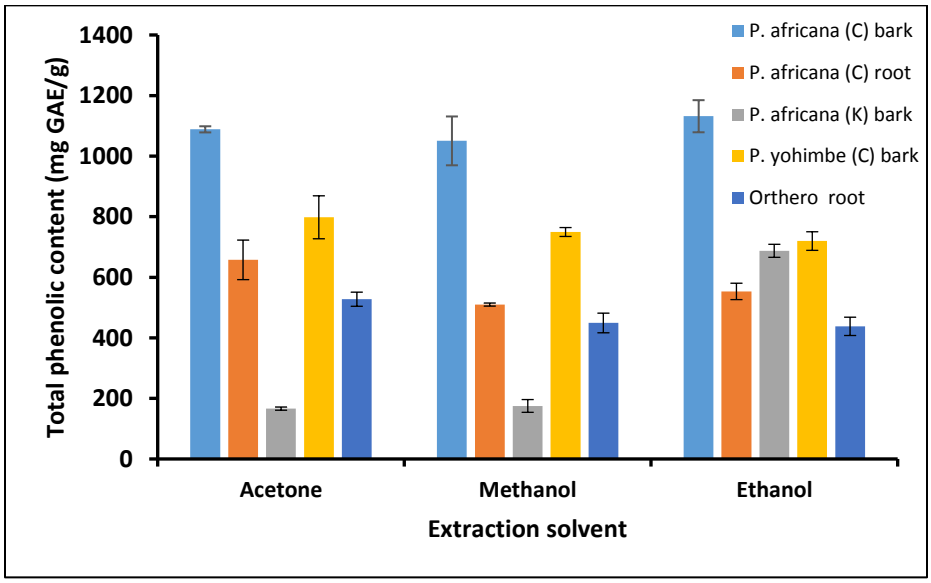

Figure 12: Total Phenolic Content of Plants Extracts

The results in this study were in agreement with findings by Sultana et al. (2009). This may be due to the fact that phenolics are often extracted in higher amounts in polar solvents such as methanol and ethanol as compared with those having absolute polarity (Anwar, Jamil, Iqbal, & Sheikh, 2006; Siddhuraju & Becker, 2003; Sultana, Anwar, & Przybylski, 2007). Other studies also reported that methanol and ethanol are effective solubilizing agents for phenolic compounds from different plant matrices (Anwar, Abdul Qayyum, Ijaz Hussain, Iqbal, & Iqbal, 2010; Siddhuraju & Becker, 2003; Sultana et al., 2009, 2008).

Except for the acetonic and methanolic extracts of *P. africana* (K) bark (166.27 and 175.05 mg GAE/g of sample, respectively), all other plant part extracts in this study showed TPC greater

than three hundred milligrams GAE per gram (>300 mg GAE/g) (Fig. 12 and Table 7). Dudonne et al. (2009) in a parallel study on plant extracts reported four extracts showing TPC greater than 300 mg GAE/g. For the comparative assessment of plant bark in this study, the highest TPC was extracted from *P. africana* bark (C) independent of type of solvent compared to *P. yohimbe* and *P. africana* (K) (Pru-C-B > Yom-B > Pru-K-B) (Table 7). Likewise, the TPC in *P. africana* roots (C) was also the highest ranging from 509.94 mg GAE/g (methanolic extract) to 657.47 mg GAE/g (acetonic extract) in comparison with *Orthero*; 437.93 (ethanolic extract) to 527.50 mg GAE/g, (acetonic extract). Bark extracts exhibited a higher TPC than root extracts; Pru-B-C-EtOH (1131.70 mg GAE/g) > Pru-R-C-Ace (657.47 mg GAE/g) (Fig. 12 and Table 7) which has also been reported by Madivoli et al. (2012) who found a higher phenolic content in the methanolic extract of *P. africana* (C) bark than *H. abyssinica* root (Madivoli et al., 2012). In all the tested plant part extracts, TPC was higher than the TFC, supporting the suggestion that most flavonoids are also phenolics as reported by Ahmed et al. (2015) (Fig. 12 and Table 7).

Sultana et al. (2009) also found that the extracts exhibiting greater TPC can exhibit a good reducing power. The reducing potential of antioxidant components is very much associated with the TPC of the extracts (Sultana et al., 2009), and thus the extracts with high phenolics often exhibit a high reducing power (Z. Cheng et al., 2006; Siddhuraju & Becker, 2003; Sultana et al., 2007).

3.4.2 Total Flavonoid Content (TFC)

Total flavonoid contents in the different extracts are reported in Fig. 13 and Table 7. In-group comparison between extracts of bark showed that the ethanolic extracts of *P. yohimbe* and *P. africana* (K) bark (54.21 and 54.80 mg RU/g, respectively), showed TFC comparable to acetonic extract of *P. yohimbe* bark (61.33 mg RU/g), the highest TFC in the present study.

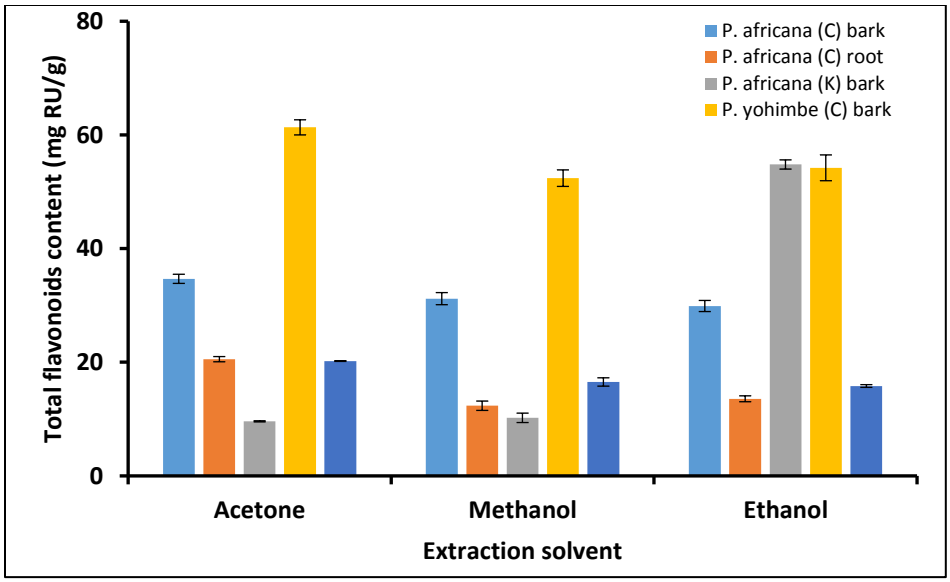

Figure 13: Total Flavonoid Content of Plants Extracts

A previous study by Tan et al. (2014) demonstrated that acetone is the best of five solvents (including water) for extracting flavonoids. The acetonic extract of *P. africana* (C) root as observed in this study had the highest flavonoid content (20.54 mg RU/g) of all tested root extracts. Ferreira & Pinho (2012) also found that the flavonoid hesperidin was more soluble in acetone than in methanol, followed by ethanol, ethyl acetate and acetonitrile. The highest and lowest flavonoid contents (61.33 and 9.60 mg RU/g, from the acetonic extracts of *P. yohimbe* and *P. africana* (K) barks, respectively) in the present study were lower than findings by Asuzu et al. (unpublished) in which the absolute ethanolic and acetonic extracts of *P. yohimbe* and *P. africana* (K) barks (111.61 and 17.78 mg RU/g, respectively) were reported.

The range of TFC (9.60 to 61.33 mg RU/g) in the samples of this study was significantly ($p \leq 0.05$) lower than the large variation in TFC (6.44 to 111.61 mg RU/g) from similar plant parts tested by Asuzu et al. (unpublished). The highest and lowest TFC in root extracts (20.54 and 12.35 mg RU/g) obtained in the acetonic and methanolic extracts of *P. africana* (C), respectively were

higher than those reported by Asuzu et al. (unpublished) (methanolic and acetonic extracts; 18.31 and 6.44 mg RU/g, respectively). TFC obtained in this study were also higher than those from the methanol extracts of *P. africana* bark and *H. abyssinica* root reported by Madivoli et al. (2012), but comparable with those reported by Karan et al. (2017) on alcohol-soluble bark extracts of *P. africana* (Hook.f.) Kalkman (36.4 mg RU/g); and other *Prunus* species (47.0, 28.0, 49.82, 32.8, and 35.1 mg RU/g) (Karan et al., 2017). As phenolics (including many flavonoids) contain polar phenolic hydroxyl group(s), their high extraction by more polar solvents used in this study is quite reasonable, which has previously been suggested by Ahmed et al. (2015).

3.4.3 Antioxidant Activity

Fig. 14 and Table 7 show the DPPH radical scavenging activities of the extracts. All extracts exhibited scavenging properties in the following decreasing order of percentage inhibition of DPPH radical: Pru-B-K-Ace > Pru-R-C-EtOH > Pru-R-C-Met > Ort-R-Ace > Ort-R-Met > Pru-B-K-Ace > Ort-R-EtOH > Pru-R-C-Ace > Pru-B-C-Ace > Yom-B-Met > Pru-B-C-EtOH > Yom-B-EtOH > Yom-B-Ace. Some extracts demonstrated potent (>90%) activity, indicative of the effectiveness of phenolics as hydrogen donors or electron acceptors in scavenging the free DPPH radical, which was also reported by Tupe et al. (2013). However, antioxidant inhibitory activities is not always synonymous with a high TPC (Tupe, R. S., Kemse, N. G. and Khaire, 2013). Sultana et al. (2009) suggested that a higher percentage DPPH scavenging capacity correlates significantly with a high antioxidant activity of extracts. In this study, the greatest inhibition was found in *P. africana* (K) bark (95.77%, acetonic extract) and was comparable to the highest found in *P. africana* (C) root (94.80%, ethanolic extract) (Fig. 14 and Table 7). Madivoli et al. (2012) found that *P. africana* bark exhibited higher radical scavenging activity than *H. abyssinica* root while the

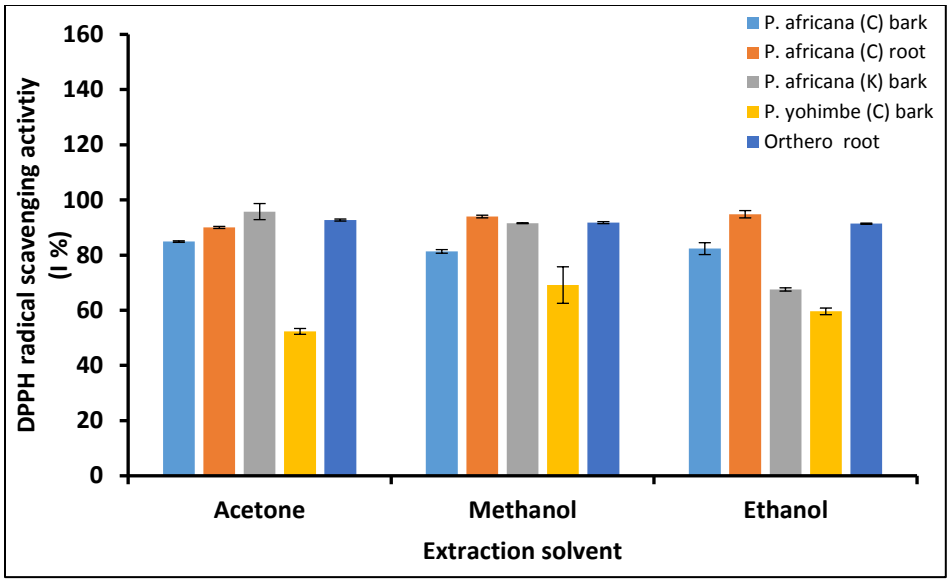

Figure 14: DPPH Radical Scavenging Activity of Plants Extracts

present study reported that *P. africana* (C) bark demonstrated lower radical scavenging activities compared to *P. africana* (C) root and *Orthero*. The results of the present study were comparable to Sarwar et al. (2012) which indicated DPPH scavenging activity as much as 76.25 - 82.80% and 58.20 - 73.25% in methanolic and ethanolic extracts, respectively.

The acetonic extract of *P. africana* (K) (Pru-B-K-Ace) was most effective in inhibiting DPPH (95.77%) but demonstrated a less antioxidant power (EC_{50} of 1.69 mg/mL), which was also indicated by its radical scavenging effect (ARP = 0.59) (Table 7 and 8). The ethanolic extract of *P. africana* (C) root (Pru-R-C-EtOH was also very effective with an inhibition of 94.8%, a high antioxidant power (EC_{50} = 0.26 mg/mL), and a potential radical scavenging effect (ARP = 3.92). However, the acetonic extract of *P. yohimbe* bark (Yom-B-Ace) showed the lowest inhibition of 52.34% but had a significant potential radical scavenging effect (ARP = 5.16) and a high antioxidant power (EC_{50} = 0.19 mg/mL). The ethanolic extract of *P. africana* (C) bark, had the highest antioxidant power (EC_{50} = 0.09 mg/mL) and free radical scavenging effect (ARP = 10.75),

and a potential inhibition of DPPH of 82.34%. At concentrations ranging from 0.09 to 7.30 mg/mL, some solvent extracts were better scavengers than ascorbic acid (EC_{50} = 0.22 mg/mL and ARP = 4.59), specifically Pru-B-C-EtOH (EC_{50} = 0.09 mg/mL and ARP = 10.75), Pru-B-C-Met (EC_{50} = 0.10 mg/mL and ARP = 10.20), Pru-B-C-Ace (EC_{50} = 0.11 mg/mL and ARP = 9.52), Yom-B-Ace (EC_{50} = 0.19 mg/mL and ARP = 5.16), Pru-R-C-Ace (EC_{50} = 0.21 mg/mL and ARP = 4.67), and Yom-B-EtOH (EC_{50} = 0.22 mg/mL and ARP = 4.61) (Table 8). Oussou et al. (2016) also reported that the scavenging activities of some methanol extract and fractions of *Lophira lanceolata* leaves exhibited better scavenging activities than ascorbic acid.

The antioxidant power was assessed determining EC_{50} and ARP (Table 8), with a lower EC_{50} indicating a higher antioxidant capacity (Dajanta et al., 2011) or power (Tabbikha, 2017). The ARP for the extracts and standard ascorbic acid were calculated from the EC_{50}; the higher the ARP indicates the more efficient the antioxidant activity (Dajanta et al., 2011). Two extracts used in this study (acetonic extract of *P. africana* (C) root with ARP = 4.67 and ethanolic extract of *P. yohimbe* bark with ARP = 4.61) exhibited ARP comparable to the standard ascorbic acid (ARP = 4.59). These results are parallel to similar plant part study reported by Madivoli et al. (2012) in which both *P. africana* bark and *H. abyssinica* root exhibited antioxidant activity comparable to ascorbic acid (Madivoli et al., 2012). The antioxidant activities of all extracts, measured as ARP and listed in decreasing order of effectiveness, were: Pru-B-C-EtOH > Pru-B-C-Met > Pru-B-C-Ace > Yom-B-Ace > Pru-R-C-Ace > Pru-R-C-EtOH > Pru-R-C-Met > Pru-B-K-EtOH > Yoh-B-Met > Pru-B-K-Ace > Orth-R-Ace > Orth-R-Met > Orth-R-EtOH > Pru-B-K-Met (Table 8). The type of solvent and plant part used resulted in different ARP.

The reducing capacity of the extracts, another significant indicator of antioxidant activity (Madjid, Amoussa, Sanni, & Lagnika, 2015), is measured by the reduction of Fe^{3+} to Fe^{2+} through

donation of an electron, which is an important mechanism of phenolic antioxidant action (Sumathy et al., 2013). Ferric reducing antioxidant power (FRAP) of the extracts varied from 2.08 to 11.33 mM Fe^{2+}/g (Fig. 15 and Table 7). The acetonic extract of *P. yohimbe* bark had the strongest FRAP with 11.33 mM Fe^{2+}/g followed by the ethanolic extracts of *P. africana* (K) and *P. yohimbe* bark with FRAP of 10.17 and 9.83 mM Fe^{2+}/g, respectively. The lowest FRAP activity was measured in the methanolic extracts of *P. africana* (C) root (FRAP = 2.08 mM Fe^{2+}/g).

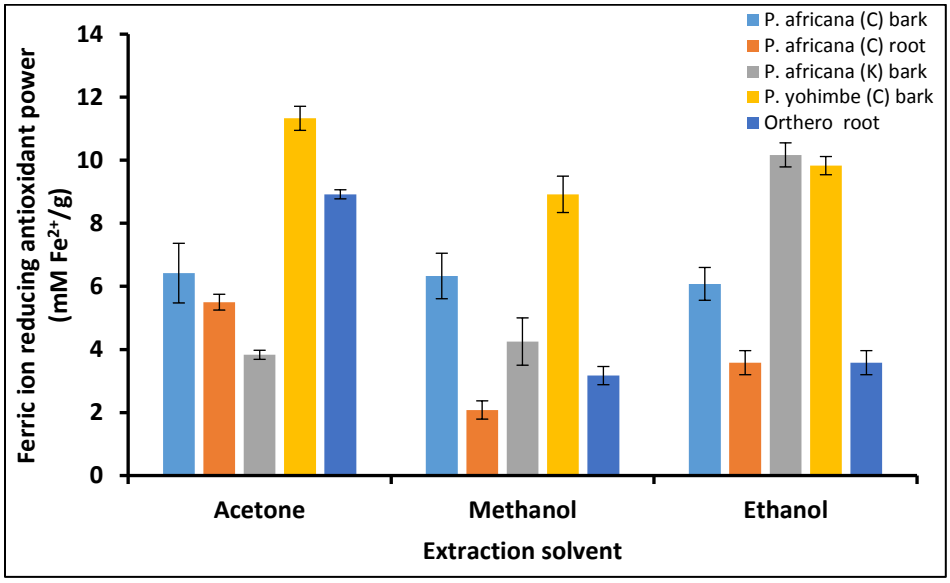

Figure 15: FRAP of Plants Extracts

The antioxidant activity of the extracts, measured by the $ABTS^{\bullet+}$ radical decolorization assay, is shown in Fig. 16 and Table 7. Extracts from *P. yohimbe* bark showed the highest $ABTS^{\bullet+}$ radical activities followed by *Orthero* root extracts. Of all *P. yohimbe* bark extracts analyzed, the methanolic extract (126.87 mM TE/g) exhibited the greatest antioxidant capacity using this assay (Table 7), followed closely by the ethanolic extract of *P. yohimbe* bark (124.66 mM TE/g) and the acetonic extract of *Orthero* root (124.40 mM TE/g). There was no significant difference ($p \leq 0.05$) in $ABTS^{\bullet+}$ radical activity among the acetonic, methanolic and ethanolic extracts of *P. africana*

(C) bark and root and *P. africana* (K) bark, which were less effective in scavenging the radical $ABTS^{\bullet+}$ than the extracts of *P. yohimbe* bark and *Orthero* root.

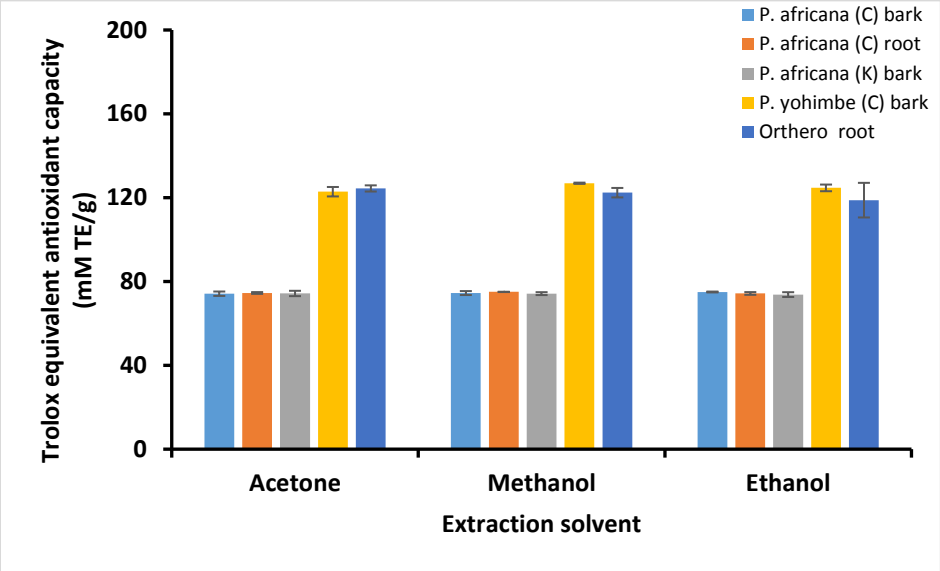

Figure 16: ABTS Radical Scavenging Activity of Plant Extracts

Table 7: Phytochemical Screening and Antioxidant Activity of Plant Extracts

Plants	Part	Extraction solvent	TPC (mg GAE/g)	TFC (mg RU/g)	FRAP (mM Fe^{2+}/g)	DPPH (%)	TEAC (mM TE/g)
Prunus africana (C)	Bark	Acetone	1088.38±10.14^{f}	34.68±0.81^{f}	6.42±0.95^{d}	84.89±0.27^{de}	74.17±1.05^{a}
		Methanol	1050.32±80.62^{f}	31.19±1.07^{e}	6.33±0.72^{d}	81.34±0.65^{d}	74.48±0.94^{a}
		Ethanol	1131.70±52.99^{f}	29.90±0.98^{e}	6.08±0.52^{d}	82.34±2.15^{d}	74.99±0.20^{a}
	Root	Acetone	657.47±65.37^{cd}	20.54±0.46^{d}	5.50±0.25^{cd}	90.03±0.37^{ef}	74.52±0.42^{a}
		Methanol	509.94±5.07^{b}	12.35±.082^{ab}	2.08±0.29^{a}	93.98±0.49^{f}	75.03±0.07^{a}
		Ethanol	553.26±27.04^{bc}	13.57±0.52^{bc}	3.58±0.38^{b}	94.80±1.33^{f}	74.29±0.65^{a}
Prunus africana (K)	Bark	Acetone	166.27±5.37^{a}	9.60±0.10^{a}	3.83±0.14^{b}	95.77±2.91^{f}	74.29±1.27^{a}
		Methanol	175.05±21.10^{b}	10.22±0.83^{a}	4.25±0.75^{bc}	91.56±0.16^{f}	74.21±0.69^{a}
		Ethanol	687.33±21.46^{de}	54.80±0.81^{g}	10.17±0.38^{ef}	67.44±0.58^{c}	73.74±1.15^{a}
Pausinystalia yohimbe	Bark	Acetone	797.99±70.92^{e}	61.33±1.33^{h}	11.33±0.38^{f}	52.34±1.06^{a}	122.84±2.25^{bc}
		Methanol	749.39±14.73^{de}	52.39±1.45^{g}	8.92±0.58^{e}	69.14±6.62^{c}	126.87±0.30^{c}
		Ethanol	719.53±30.69^{de}	54.21±2.27^{g}	9.83±0.29^{e}	59.61±1.21^{b}	124.66±1.59^{bc}
Orthero	Root	Acetone	527.50±23.32^{b}	20.21±0.07^{d}	8.92±0.14^{e}	92.72±0.37^{f}	124.40±1.47^{bc}
		Methanol	449.05±32.50^{b}	16.53±0.73^{c}	3.17±0.29^{ab}	91.77±0.36^{f}	122.38±2.26^{bc}
		Ethanol	437.93±30.24^{b}	15.82±0.24^{c}	3.58±0.38^{b}	91.38±0.22^{f}	118.81±8.26^{b}

C - Cameron; K - Kenya. Values are mean of three determinations ± standard deviation. Values followed by the same superscript in the same column are not significantly different ($p \leq 0.05$).

Table 8: Half-Maximal EC_{50} and Antiradical Power (ARP) of Plant Extracts

Plants & Standard	Parts	Extraction solvent	EC_{50} (mg/mL of DPPH)	ARP ($1/EC_{50}$)
Prunus africana (C)	Bark	Acetone	0.105	9.524
		Methanol	0.098	10.204
		Ethanol	0.093	10.753
	Root	Acetone	0.214	4.673
		Methanol	0.303	3.300
		Ethanol	0.255	3.922
Prunus africana (K)	Bark	Acetone	1.689	0.592
		Methanol	7.298	0.137
		Ethanol	0.349	2.865
Pausinystalia yohimbe	Bark	Acetone	0.194	5.155
		Methanol	0.376	2.660
		Ethanol	0.217	4.608
Orthero	Root	Acetone	2.186	0.457
		Methanol	2.374	0.421
		Ethanol	4.291	0.233
Ascorbic acid	-	-	0.218	4.587

3.4.4 Pigment Content

Color contributes significantly to consumer's acceptance of food since it is the first indicator of food quality (Abou-Arab et al., 2011). Many food products such as confectionery, gelatin desserts, snacks, cake, pudding, ice cream, and beverages would be colorless and thus appear undesirable without the inclusion of colorants (Hirunpanich et al., 2006). The spectrophotometric analysis of the pigments in the extract is presented in Table 9. The highest pigment concentrations were measured mostly in the acetonic and methanolic extracts of *P. yohimbe* bark. Non-polar pigment such as lycopene and β-carotene were more effectively extracted using acetone, as also noted by Sumanta and Haque (2014) who reported the highest extraction of carotenoids with acetone in *Adiantum* sp. Of all pigments in this study, xanthophyll concentration

Table 9: Pigment Content of Plant Extracts

Plants	Part	Extraction solvent	Lycopene (mg/g)	β-carotene (mg/g)	Carotenoids (mg/g)	Chlorophyll a (mg/g)	Chlorophyll b (mg/g)	Total chlorophyll (mg/g)	Xanthophyll a (mg/g)	Anthocyanin (mg/g)
Prunus africana (C)	Bark	Acetone	0.14±0.03^{a}	0.17±0.08^{a}	0.75±0.14^{a}	0.64±0.19^{a}	1.43±0.44^{a}	2.11±0.65^{a}	-	0.30±0.01^{a}
		Methanol	0.16±0.01^{a}	0.29±0.01^{a}	0.92±0.03^{a}	0.63±0.02^{a}	1.45±0.05^{a}	2.12±0.06^{a}	-	0.30±0.00^{a}
		Ethanol	0.16±0.02^{a}	0.28±0.03^{a}	0.89±0.10^{a}	0.64±0.06^{a}	1.45±0.14^{a}	2.12±0.21^{a}	-	0.33±0.00^{a}
	Root	Acetone	0.53±0.00^{a}	0.93±0.01^{a}	0.25±0.02^{a}	0.55±0.03^{a}	1.00±0.04^{a}	1.58±0.07^{a}	-	0.10±0.00^{a}
		Methanol	0.40±0.00^{a}	0.67±0.00^{a}	0.18±0.00^{a}	0.44±0.01^{a}	0.82±0.02^{a}	1.27±1.27^{a}	-	0.10±0.00^{a}
		Ethanol	0.37±0.01^{a}	0.63±0.01^{a}	0.17±0.04^{a}	0.47±0.03^{a}	0.82±0.04^{a}	1.31±0.07^{a}	-	0.10±0.00^{a}
Prunus africana (K)	Bark	Acetone	0.90±0.02^{a}	0.40±0.08^{a}	1.03±0.19^{a}	2.98±0.64^{b}	1.17±0.12^{a}	4.20±0.76^{a}	-	0.20±0.00^{a}
		Methanol	0.40±0.00^{a}	0.16±0.02^{a}	0.37±0.06^{a}	1.65±0.13^{a}	1.03±0.09^{a}	2.72±0.15^{a}	-	0.10±0.00^{a}
		Ethanol	0.19±0.02^{a}	0.48±0.04^{a}	1.41±0.11^{a}	0.85±0.05^{a}	2.05±0.15^{a}	2.96±0.20^{a}	-	0.40±0.00^{a}
Pausinystalia yohimbe	Bark	Acetone	2.78±0.45^{c}	6.83±1.23^{c}	20.80±3.66^{c}	9.16±1.17^{c}	22.46±2.92^{c}	32.21±4.16^{b}	-	0.53±0.09^{c}
		Methanol	2.10±0.10^{b}	5.49±0.27^{b}	16.31±0.80^{b}	8.40±0.42^{c}	20.03±0.96^{bc}	28.95±1.40^{b}	-	0.43±0.02^{b}
		Ethanol	2.35±0.39^{bc}	5.61±0.92^{b}	17.15±2.82^{bc}	8.15±0.88^{c}	19.41±2.39^{b}	28.07±3.33^{b}	-	0.45±0.07^{bc}
Orthero	Root	Acetone	0.30±0.00^{a}	0.13±0.01^{a}	0.20±0.02^{a}	0.57±0.01^{a}	1.09±0.03^{a}	1.69±0.03^{a}	61.92±7.57^{b}	0.10±0.00^{a}
		Methanol	0.30±0.01^{a}	0.11±0.03^{a}	0.18±0.05^{a}	0.52±0.06^{a}	1.03±0.13^{a}	1.58±0.19^{a}	50.29±17.83^{b}	0.10±0.00^{a}
		Ethanol	0.30±0.00^{a}	0.13±0.01^{a}	0.20±0.02^{a}	0.57±0.03^{a}	1.09±0.04^{a}	1.69±0.07^{a}	61.21±9.32^{b}	0.10±0.00^{a}

C - Cameron; K - Kenya. Values are mean of three determinations (n = 3) ± standard deviation (SD). Values followed by the same superscript in the same columns are not significantly different ($p \leq 0.05$).

in the acetonic extract of *Orthero* root was highest with 61.92 mg/g while anthocyanin concentrations in some extracts of P. *africana* (C and K) bark and *P. africana* and *Orthero* root were lowest (0.10 mg/g) (Table 9). Xanthophylls are oxycarotenoids and therefore are more polar pigments than the non-polar β-carotenes and lycopenes which are not oxygenated carotenoids (Delgado-Vargas et al., 2000). Chlorophyll b is more soluble than chlorophyll a in polar solvents because of its carbonyl group (N. Sumanta, C. I. Haque, 2014), which explains the usually higher chlorophyll b in plant extracts in this study compared chrophyll a. Two exceptions were the acetonic and methanolic extracts of *P. africana* (K) bark (Table 9). This may be an indication of the synergistic effects involved in the complexation

of other pigments such as polar flavonoids and quinones and non-polar carotenoids, which may have the ability of increasing the content of chlorophyll a in these extracts through solvent solubility. The chlorophyll a and b and/or total chlorophyll content was higher than other plant pigments (carotenoids) apart from xanthophylls (Table 9). Since chlorophyll molecules are polar in nature, they tend to be soluble in polar solvents such as the solvents used in this study as also reported by Sumanta and Haque (2014), who recorded rapid extraction/solubility of chlorophylls by acetone for chlorophyll a and chlorophyll b at 24 h . Methanol was least effective in extracting plant pigments in this study (Table 9). The non-polar carotenoids, are more effectively extracted by non-polar extracts such as acetone as documented (N. Sumanta and C. I. Haque, 2014). In the present study carotenoid levels were lowest in in *P. africana* (C) root extracts (acetone; 0.25 mg/g and methanol; 0.18 mg/g); and highest in *P. yohimbe* bark extracts (acetone; 20.8 mg/g and then ethanol; 17.15 mg/g) (Table 9). There were significant amounts of pigments ($p \leq 0.05$) in most plant parts in this study. In addition to the well-known antioxidant capacity of polyphenols, other plant metabolites, especially carotenoids and chlorophylls, were widely distributed in the plants, contributing to its antioxidant potential (Komes, Belščak-Cvitanović, Horžić, Marković, & Ganić, 2011).

Using ethanol for extracting natural compounds especially polyphenols such as anthocyanins from plant tissues is more difficult due to its lower extraction efficiency and challenging separation from the media (Yılmaz, Karaaslan, & Vardin, 2015). However, ethanol is preferred in food processing systems because it is less toxic (Abou-Arab et al., 2011; K. . Gould, Davies, & Winefield, 2009; Nakamiya, Furuichi, & Ishii, 2003) than methanol (Abou-Arab et al., 2011) and considered natural (Ferreira-Dias, Valente, Abreu, & Abreu, 2003) and safe for human consumption (Dai & Mumper, 2010; Pompeu, Silva, & Rogez, 2009).

3.4.5 HPLC Analysis

Methyl 4-hydrocybenzoate and protocatechuic acid were measured in all selected extracts, trans-sinapic acid in *P. africana* (C) bark and root, and ferulic acid in *P. africana* root extract only. The trans-sinapic acid content was higher in all plant parts than any other phenolic acid with HPLC values exceeding100 mg/g in *P. africana* (C) bark and root extracts (Table 10). This finding is supported by the high TPC values determined spectrophotometrically.

Table 10: Phenolic Acid Content of Selected Plant Extracts

Plants	Part	Extraction solvent	Methyl 4-hydroxy benzoate (mg/g)	Protocatechuic acid ethyl ester (mg/g)	Trans sinapic acid (mg/g)	Trans ferulic acid (mg/g)
Prunus africana (C)	Bark	Acetone	0.65 ± 0.18^{a}	0.43 ± 0.00^{a}	128.62 ± 5.90^{b}	ND
		Methanol	0.57 ± 0.11^{a}	0.50 ± 0.00^{a}	388.71 ± 27.11^{d}	ND
		Ethanol	0.62 ± 0.09^{a}	0.50 ± 0.00^{a}	346.65 ± 15.26^{cd}	ND
	Root	Acetone	0.34 ± 0.04^{a}	0.40 ± 0.00^{a}	199.77 ± 22.84^{b}	0.74 ± 0.02^{b}
		Methanol	0.48 ± 0.15^{a}	0.30 ± 0.00^{a}	230.04 ± 68.17^{bc}	0.62 ± 0.05^{a}
		Ethanol	0.44 ± 0.24^{a}	0.43 ± 0.00^{a}	228.95 ± 90.33^{bc}	0.89 ± 0.10^{c}
Pausinystalia yohimbe	Bark	Acetone	1.45 ± 0.18^{b}	1.05 ± 0.13^{b}	ND	ND
		Ethanol	1.53 ± 0.04^{b}	1.13 ± 0.21^{b}	ND	ND

C - Cameron; K - Kenya; ND - Not detected. Values are mean of three determinations ± SD. Values followed by the same superscript in same column are not significantly different ($p \leq 0.05$).

3.4.6 Correlation

Pearson's correlation coefficients between total phenolics, total flavonoids and antioxidant activity is shown in Table 11 and 12. As shown in Table 11, although most extracts in this study exhibited a moderately high negative correlation between FRAP and DPPH and TEAC, two FRAP values, Pru-R-Ace ($p = 0.99$ and Pru-R-C-EtOH ($p = 0.95$), correlated strongly with DPPH; and Pru-B-C-Ace ($p = 0.92$) with TEAC. There was also a strong negative correlation between TPC and other antioxidant activity assays (FRAP and TEAC), but a strong positive correlation between

Table 11: Correlation between Antioxidant Assays

FRAP		
Extracts	**% DPPH**	**TEAC**
Pru-B-C-Ace	0.84	0.92
Pru-B-C-Met	-0.24	0.83
Pru-B-C-EtOH	-0.43	0.69
Pru-R-C-Ace	0.99	-0.97
Pru-R-C-Met	-0.50	0.50
Pru-R-C-EtOH	0.95	-1.00
Pru-B-K-Ace	0.62	0.24
Pru-B-K-Met	0.00	0.17
Pru-B-K-EtOH	0.56	-0.19
Yom-B-Ace	-0.76	0.88
Yom-B-Met	-1.00	-0.19
Yom-B-EtOH	-0.96	-0.39
Orth-R-Ace	-0.11	-0.11
Orth-R-Met	0.11	-0.42
Orth-R-EtOH	-0.93	-0.88

	Top 10% positive correlations
	Bottom 10% negative correlations
	Between 0 and Top 10% positive correlations
	Between 0 and bottom 10% negative correlations

C - Cameron, K - Kenya; Pru - *Prunus africana*, Yom – *Pausinythalia yohimbe*, Orth - *Othero*; Ace - acetone, Met - methanol, EtOH - ethanol; B - bark, R - root

TFC and DPPH (Table 12). Since the radical scavenging ability of DPPH depends on the flavonoid and phenolic contents of the extracts, the TPC and TFC suggest high antioxidant power (Dajanta et al., 2011; Tabbikha, 2017). This indicates that phenolics and flavonoids are potent antioxidants (Ahmed et al., 2015), and as expected, a higher percent of DPPH scavenging is correlated to a higher antioxidant activity (H. Liu, Qiu, Ding, & Yao, 2008; Sultana et al., 2007). Several studies have shown that high antioxidant activity correlates with high total phenolic and flavonoid contents (Madivoli et al., 2012; Singh et al., 2016). The presence of flavonoids and phenolic compounds translates into effective antioxidant properties of the plant extracts (Madivoli et al., 2012). In

addition, TPC and TFC correlated strongly (0.96 and 0.93) for the methanolic extract of *P. yohimbe* and the acetonic extract of *P. africana* barks, respectively.

Table 12: Correlation between Total Phenols, Flavonoids, and Antioxidant Assays

	TPC			
Extracts	**TFC**	**FRAP**	**%DPPH**	**TEAC**
Pru-B-C-Ace	0.93	-0.99	-0.76	-0.87
Pru-B-C-Met	0.45	-0.13	0.99	-0.67
Pru-B-C-EtOH	-0.82	-0.94	0.71	-0.41
Pru-R-C-Ace	0.81	-0.99	-1.00	0.99
Pru-R-C-Met	0.53	-0.50	-0.50	-1.00
Pru-R-C-EtOH	0.16	-0.11	-0.41	0.14
Pru-B-K-Ace	-0.50	-0.19	0.65	-1.00
Pru-B-K-Met	-0.90	0.46	0.89	0.95
Pru-B-K-EtOH	-0.76	-0.50	0.43	-0.76
Yom-B-Ace	0.77	0.30	0.40	-0.19
Yom-B-Met	0.96	1.00	-0.99	-0.13
Yom-B-EtOH	-0.38	0.23	0.06	-0.99
Orth-R-Ace	-0.87	-0.50	0.92	0.92
Orth-R-Met	-0.96	0.86	0.61	-0.83
Orth-R-EtOH	0.87	0.58	-0.84	-0.13

	Top 10% positive correlations
	Bottom 10% negative correlations
	Between 0 and Top 10% positive correlations
	Between 0 and bottom 10% negative correlations

C - Cameron, K - Kenya; Pru - *P. africana*, Yom - *Yohimbe pausinythalia*, Orth - *Othero*; Ace - acetone, Met - methanol, EtOH - ethanol, B - bark, R - root

In addition, the methanolic extract of *P. africana* (C) bark exhibited a strong positive correlation (0.99) between TPC and DPPH. These results suggest that flavonoids in the methanolic and acetonic extracts of the plants might be the major constituents of the total phenols as also reported by Singh et al. (2016). Only three extracts showed strong positive correlations amidst the high negative correlations between TPC and FRAP and TPC and TEAC. These included the methanolic extract of *P. yohimbe* bark (1.00; TPC and FRAP); the acetonic extract of *P. africana*

(C) root (0.99; TPC and TEAC), and the methanolic extract of *P. africana* (K) bark (0.95; TPC and TEAC) (Table 12). This indicates that the influence of the electron donor's rich TPC of the extracts under study impacted their antioxidant capabilities, and phenolic compounds present in the extracts were a good source of electron donors, exhibiting reducing power as Singh et al. (2016) also reported.

3.5 Conclusion

The plant matrices and solvents used for extraction substantially affected the composition and quantity of measured phytochemicals. The total phenolic content of several plant parts used in this study had strong positive correlations with TFC and DPPH radical scavenging activity. There were strong negative correlations with other antioxidant assays explaining why flavonoids are often more abundant than other phenolics in medicinal plants; and demonstrating the necessity of performing more than one type of antioxidant capacity measurement. This would also ensure greater data reliability since there are various mechanisms of antioxidant action. Even when plant extracts do not contain a large quantity of phenolics they can sometimes exert effective antioxidant action (DPPH, FRAP and TEAC). Therefore, in addition to phenolics, other bioactive compounds can be present in plants, which contribute to their antioxidant potential through synergistic, additive, and potentiating effects.

CHAPTER 4: Antimicrobial Potential of Selected Plant Extracts

4.1 Introduction

The present emergence of antibiotic drug resistance by bacteria has necessitated a search for new antimicrobial substances from other sources including medicinal plants. Evolved bacterial mechanisms have developed their genetic abilities to acquire drug resistance, especially those utilized in the medical community as therapeutic agents (S.-S. Cheng et al., 2009). For example, the exhibition of active efflux as reported in many studies has been the resistance mechanism developed by most bacteria species against almost all antibiotics (J. Lin, Michel, & Zhang, 2002). Non-drug specific proteins which make up the majority of the efflux systems have recognition and exportation capabilities for a broad range of chemically and structurally unrelated bacterial compounds without drug alteration or degradation (Kumar & Schweizer, 2005).

4.2 *Escherichia coli*

Escherichia coli is a gram-negative, rod-shaped motile/non-motile, non-spore forming aerobic/facultative anaerobic intestinal pathogen or commensal of the human or animal intestine that is voided in faeces or consumed in contaminated drinking water. Although gram negative *E. coli* is part of the normal flora in the human intestine and is naturally harmless, it may cause gastro-intestinal diseases ranging from mild, self-limiting diarrhea to hemorrhagic colitis (Greenwood, Slack, Peutherer, & Duguid, 2002), lower urinary tract infections (UTIs), cholecystitis, or septicemia (Amenu, 2014). Multidrug-resistant Enterobacteriaceae, mostly *E. coli*, produce extended-spectrum β-lactamases (ESBLs) enzymes with greater activity against cefotaxime. Although there are reports about the susceptibility of *E. coli* strains to antimicrobial agents that are active against Enterobacteriaceae species, the frequent acquisition of resistant plasmids *via*

conjugation challenges this susceptibility. Consequently, therapy must be guided by susceptibility testing (Amenu, 2014). Resistance to several antimicrobial agents such as ampicillin, trimethoprim-sulfamethoxazole, ceftazidime, tetracycline and sulfonamide was exhibited and reported by a surveillance study conducted on antimicrobial resistance at tertiary healthcare facilities (Blomberg et al., 2004). The unusually virulent enterohemorrhagic strains of *E. coli* (i.e., O4:H-, O11:H-, O26:H11, O45:H2, O103:H2, O104:H21, O111:H8, and O145:H-) including the O157:H7 serotype have prompted food microbiologists to rewrite the rule book on food safety (Buchanan & Doyle, 1997). These pathogens are more dangerous than other well-recognized foodborne pathogens causing severe infections that affect all age groups, requiring a low infectious dose, tolerating significant acidity, and demonstrating a special but inexplicable association with all foods derived from ruminants. Several foods or food handling practices have been implicated in contamination with O157:H7 including undercooked ground beef, raw milk, unpasteurized apple juice/cider, dry cured salami, lettuce, produce from manure-fertilized garden, handing potatoes, radish sprouts, alfalfa sprouts, yogurt, and sandwiches (Buchanan & Doyle, 1997).

4.3 *Staphylococcus aureus*

Staphylococcus aureus bacteria are known as anaerobic, gram-negative cocci, that are part of the normal bacterial flora of the skin and mucosal surfaces of the respiratory, upper alimentary and urogenital tracts of mammals and birds (Habib et al., 2015). They are opportunistic pathogens that cause hospital acquired Staph infections (Haque et al., 2011; Klein, Smith, & Laxminarayan, 2007), ulcers, burns, pneumonia, osteomyelitis, septicemia, mastitis, meningitis, toxic skin exfoliation (Cheesbrough, 2000); post-operative wound infections, toxic shock syndrome, endocarditis, osteomyelitis and food poisoning (Benayache, Benayache, Benyahia, Chalchat, &

Garry, 2001). These infections are increasingly difficult to control because the organisms have acquired new antimicrobial resistance determinants (Ajoke, Okeke, Odeyemi, & Okwori, 2012; Jombo & Enenebeaku, 2007). Methicillin-resistant *S. aureus* (MRSA) and vancomycin-resistant *S. aureus* (VRSA) inflict very difficult-to-cure infections that resist almost all clinically available antibiotics (Amenu, 2014). Currently, almost all isolates of *S. aureus* in hospitals are resistant to penicillin (Bhatia, Rattan, & Ichhpujani, 2008; Klein et al., 2007). Akano et al. (2009) reported that 62% of *S. aureus* isolates were resistant to multiple commonly used antimicrobial agents (Akano, Daini, Ojo, Smith, & Akinsade, 2009). Additionally, resistance to nafcillin (oxacillin and methicillin) occurs in about 35% of *S. aureus* and ~75% of *S. epidermidis* isolates (Brooker, Slee, Connors, & Duffy, 2002). Some *S. aureus* strains are able to produce staphylococcal enterotoxins (SEs) that cause food poisonings, a major cause of food-borne diseases (FBD), because it can contaminate food products during preparation and processing (Le Loir, Baron, & Gautier, 2003). Foods that are not cooked after handling, such as sliced meats, puddings, pastries, and sandwiches, are more at risk for Staphylococcal food poisoning.

Plants produce hundreds to thousands of diverse chemical and antimicrobial compounds with different biological activities which have actively inhibit the growth of plant and human pathogenic microorganisms. Thus, extracts from targeted plant parts are suspected to exert antimicrobial properties against drug-resistant microbial pathogens (Amenu, 2014). Traditional herbal plants have been utilized for treatments of various diseases but need to be studies to determine their effectiveness in treating microbial infections. Spectrophotometric and chromatography studies on a variety of polar and non-polar solvent extracts of *P. africana* stem bark suggested antioxidant activities attributed to secondary metabolites (e.g., phenolic compounds such as phenolic acids and flavonoids; phytosterols, terpenoid saponins and

triterpenes) (Maiyo et al., 2016; Ngule et al., 2014; Papaioannou et al., 2010; Rider et al., 2012; Roell & Baniahmad, 2011; Shenouda et al., 2007). These secondary metabolites have different mechanisms of action against microorganisms (Cowan, 1999; Pandey, 2013). Methanol and ethanol extracts of the bark of *P. africana* from diverse geographical locations exhibited moderate to strong antimicrobial activities on a variety of clinical strains including gram (+) *S. aureus*, methicillin-resistant *S. aureus*, *B. subtilis*, gram-negative *P. aeruginosa*, *E. coli*, *C. albicans*, *C. koseri*, *E. aerogenes*, and *E. cloaceae* (Bii et al., 2010; Gashe & Zeleke, 2017; Madivoli et al., 2012; Mwitari et al., 2013). The bark extract of *P. yohimbe* contains up to 5.9% of the total indole alkaloid yohimbine, depending on the age of the plant, the height at which the bark was collected, and the season as yohimbine content increases by about 1% during the rainy season (Zanolari, 2003). For these reasons, the selected medicinal plants extract in this study were assessed for their phytochemical composition and antimicrobial properties, prior to testing them on microbes.

4.4 Materials

Cells were grown on Tryptic Soy agar (TSA) (Soybean-casein digest agar; Difco), and overnight cultures were started in Trypticase Soy broth (TSB) (Soybean-casein digest broth; BBL). Sterile blank 6 mm antimicrobial discs (Becton, Dickinson and Company; Sparks, MD) served as negative controls.

4.4.1 Plant Extraction

Ten grams of each powdered plant material were extracted with 100 mL (1:10 w/v) of aqueous acetone, methanol, and ethanol as described in section 3.2.1 above. The extracts were then stored at 4°C for antimicrobial analysis.

4.4.2 Preparation of Agar Media and Overnight Bacterial Cultures

Media used for the experiment (TSB and TSA) were prepared in accordance with manufacturer's guidelines.

4.4.3 Plant Extracts Antimicrobial Activities

The Agar disc diffusion method (Alizadeh Behbahani et al., 2016; Das, 2017; Mwitari et al., 2013) was used to test the ability of the various extracts to inhibit bacterial growth. Five (5) extracts from the phytochemical assessment study in descending order of EC_{50} (Pru-B-C-EtOH > Pru-B-C-Met > Pru-B-C-Ace > Yom-B-Ace > Pru-R-C-Ace) were selected for evaluation of their antimicrobial potential. Solubilizing 50 and 100 mg of extract respectively in 1 mL of its appropriate solvent prepared two concentrations of each plant extract (50 and 100 mg/mL). An aliquot of 150 µL (0.15 mL) of a 3 mL overnight culture of the bacteria isolates, respectively was spread on a TSA plate for the disk diffusion assay. Three sterile blank paper discs (6 mm in diameter) were dispensed on the lawn of bacteria. Each disk was either saturated with 25 µL (0.025 mL) of the solubilized plant extracts (50 mg/mL or 100 mg/mL), control solvent (extraction solvent) or neither (blank control disk), respectively. To encourage absorption of extract /solvent control into the agar prior to incubation at 37°C for 24 h incubation, plates were left on the bench undisturbed for 2 h.

The diameter of ZOI around each disc (external diameters of visible zones of growth inhibition) were measured and recorded as described in numerous studies (Alizadeh Behbahani et al., 2016; Cheruiyot, Olila, & Kateregga, 2009; Das, 2017; Madivoli et al., 2012; Mann, Yahaya, Banso, & Ajayi, 2008; Mwitari et al., 2013; Singh et al., 2016; Stefanović, Radojević, Vasić, & Čomić, 2012). The average of the triplicate ZOI tests was calculated. The degree of activity of the

extracts was classified according to inhibition zone diameter as presented by Mwitari et al., (2013) with some modification; no inhibition: <7 mm; low inhibition: 7 - 7.9 mm; active inhibition: 8 - 11 mm; highly active inhibition: 11.1 - 12 highly active; and very active inhibition: >12 mm.

4.4.4 Statistical analysis

Results were analyzed by utilizing SPSS version 25.0 (SPSS Inc., Chicago, IL). One-way analysis of variance (ANOVA) followed by Tukey's test were used for inter-group comparison of means, while considering $P \leq 0.05$ as the significant level.

4.4.5 Results and Discussion

The five selected extracts of *P. africana* (C) bark and root and *P. yohimbe* bark ranged in inhibition from low to very active against both organisms (Table 13), suggesting that the bacteria were susceptible to the plant extracts. As demonstrated by others, inhibition varied by extract and microbial organism (Cheruiyot et al., 2009; Mann et al., 2008; Mwitari et al., 2013; Singh et al., 2016; Prescott et al., 2003). This study demonstrated that the antibacterial activity of the extracts was higher in more concentrated extracts (Table 13) as previously suggested (Cheruiyot et al., 2009; Mann et al., 2008; Mwitari et al., 2013). The growth inhibition of *E. coli and S.* aureus in methanolic extracts of *P. africana* (C) bark was similar to results recorded by Ngule et al. (2014) who applied hydromethanolic extracts of *P. africana* (K) bark (9:1, v/v) on *Salmonella typhi* (17.33±0.882), *Proteus vulgaris* (16.67±0.333), *Serratia marscecens* (16.67±0.333), *E. coli* (12.33±0.333) and *Bacillus cereus* (11.67±0.333) (Ngule et al., 2014). As expected, the blank sterile disc controls exhibited no diameter of ZOI, but the three solvent controls exhibited ZOI against the microorganisms. As demonstrated in the above-mentioned study by Ngule et al. (2014),

this study also exhibited the importance of increasing polyphenolic content (such as the antimicrobial flavonoids) by utilizing aqueous solvent systems (ethanol-, methanol-, or acetone-water) to extract compounds from the plant. The presence of these important pharmacologically-active compounds in the selected plant extracts and their bacterial growth inhibition is a justification of the plants' ethnobotanical use in the treatment of various diseases in traditional medicine (Ngule et al., 2014).

Of all the selected extracts, only the acetonic extract of *P. africana* (C) bark (50 and 100 mg/mL) exhibited no activity against *E. coli*, but a very active growth inhibition against *S. aureus* (Table 13), demonstrating the specificity of an extract for inhibiting the growth of a microorganism. Furthermore, several plant extracts in this study (especially highlighting the ethanolic and methanolic extracts of *P. africana* (C) bark as listed in their decreasing order) demonstrated highly active growth inhibition against *S. aureus* but not *E. coli* (Table 13). This could be due to differences in gram (+) versus gram (-) cell wall structures. The gram-negative outer membrane acts as a diffusional barrier thus making those bacteria less susceptible to many environmental substances (antimicrobial agents) including antibiotics (Cheruiyot et al., 2009). However, the exceptions demonstrate that despite this difference in permeability, some extracts have antimicrobial potential in gram (-) organisms (Holetz et al., 2002). The antimicrobial effect could also be attributed to a possible synergistic interaction of one phenolic with another or a phenolic with a non-phenolic compound or compounds within the plant extracts (Singh et al., 2016). The ethanolic extract of *P. africana* (C) bark exhibited an active to highly active inhibitory effect (9.3 mm 50 mg/mL; 12.0 mm mg/mL for 100 mg/ml) on *E. coli*. In comparison, the methanolic extract of *P. africana* (C) bark (8.0 mm for 50 mg/mL; 9.0 mm for 100 mg/mL) inhibited growth moderately but significantly ($p \leq 0.05$; Table 13).

Table 13: Average Zone of Inhibition (mm) of Plant Extracts on Microorganisms

Extract/Control	Concentration of plant extracts (mg/mL)					
	PAB		PYOH		PAR	
	50	100	50	100	50	100
Control; Acetone						
E. coli	8.67±0.58^{b}	8.67±1.15^{b}	12.00±1.00^{c}	11.00±1.00^{b}	10.67±0.58^{b}	11.67±1.53^{a}
S. aureus	9.00±1.00^{b}	9.67±0.58^{bc}	9.33±0.58^{b}	8.33±0.58^{a}	9.67±0.58^{ab}	10.00±0.00^{a}
Control; Methanol						
E. coli	10.33±0.58^{bcd}	8.33±1.15^{b}	ND	ND	ND	ND
S. aureus	10.00±1.00^{bc}	10.00±0.00^{bcd}	ND	ND	ND	ND
Control; Ethanol						
E. coli	13.33±1.53^{de}	13.67±1.15^{ef}	ND	ND	ND	ND
S. aureus	13.00±1.73^{cde}	12.67±1.15^{cde}	ND	ND	ND	ND
Acetone; Organism						
E. coli	-	-	7.33±0.58^{a}	8.33±0.58^{a}	8.67±1.15^{a}	9.33±1.53^{a}
S. aureus	12.67±1.53^{cde}	16.00±2.65^{ef}	7.67±0.58^{ab}	8.33±1.53^{a}	8.33±0.58^{a}	10.67±0.58^{a}
Methanol; Organism						
E. coli	8.00±0.00^{b}	9.00±1.00^{bc}	ND	ND	ND	ND
S. aureus	14.00±1.00^{e}	16.67±2.31^{f}	ND	ND	ND	ND
Ethanol; Organism						
E. coli	9.33±1.15^{b}	11.67±1.53^{bcd}	ND	ND	ND	ND
S. aureus	15.00±1.00^{e}	17.33±0.58^{f}	ND	ND	ND	ND

PAB - *P. africana* bark, PYOH - *P. yohimbe* bark, PAR - *P. africana* root, ND - Not determined. Values are mean of three determinations (n = 3) ± standard deviation (SD). Values followed by the same superscript in the same column are not significantly different ($p \leq 0.05$).

Plant polyphenols such as phenolic acids have antimicrobial properties, generally exerted by disrupting the function of bacterial cell membranes which retards bacterial growth/ multiplication (Singh et al., 2016). Other compounds such as quercetin, act by inhibition of DNA gyrase (Cushnie & Lamb, 2005). However, according to previous, a higher total content in phenolic compounds including

flavonoid does not always correlate with higher antibacterial activity (Singh et al., 2016). As highlighted in this study and a similar antimicrobial study by Sing et al. (2016), the most plant extracts that were most potent against the studied microorganisms did not always contain high or the highest amount of phenolics and/or antioxidant power (EC_{50}) in comparison with other selected extracts. For example, a higher TPC (1088.33 mg GAE/g) in acetonic extract than methanolic extract (1050.32 mg GAE/g) of *P. africana* (C) bark did not correlate with their antimicrobial activity against gram (-) *E. coli* (Table 13). The more potent EC_{50} exhibited by the acetonic extract of *P. yohimbe* bark (0.194) in comparison with the acetonic extract of *P. africana* (C) root (0.214) resulted in lesser growth inhibition against *E. coli* (Table 13). Therefore, the antibacterial activity exhibited by these extracts could be attributed to the presence of specific phenolic compounds and to possible existence of synergistic effects with other non-phenolic compounds present in the extracts (Singh et al., 2016).

4.4.6 Conclusion

Antimicrobial activity in the selected extracts was dependent on their concentrations. An increase in concentration results in an increase the zone of inhibition. All solvent controls exhibited some antimicrobial activity against both *E. coli* and *S. aureus* but known concentrations of plant extracts in their antimicrobial activity. A high TPC or antioxidant power in plant part extracts did not always result in significant growth inhibition zones. Combinations of phenolic and/or non-phenolic compounds present in plant extracts can produce a synergistic or antagonistic effect on their antimicrobial properties.

CHAPTER 5: General Conclusions and Recommendations

5.1 Conclusion

The plant matrix and solvent used for extraction substantially affected the composition and quantity of phytochemicals measured. Total phenolic content present in the plant parts used in this study correlated positively with TFC and DPPH radical scavenging activity. It had a significant negative correlation with other antioxidant assays, further explaining why flavonoids are often more abundant than other phenolics in medicinal plants; and demonstrating the importance of performing more than one type of antioxidant capacity measurement. However, plant extracts without a high phenolic content can sometimes exercise efficient antioxidant activities (% DPPH and TEAC) due to the presence of other bioactive compounds, which contribute to the antioxidant potential through synergistic, potentiating, or additive effects. The presence of phenolic acids in the eight selected plant extracts in this study were identified and quantified using HPLC equipped with SPD-M20A prominence UV/Vis diode array detectors (DAD). These contributed to the overall antioxidant activity and antimicrobial properties of some of the selected plant part extracts as confirmed by other studies. Results from the spectrophotometric analysis of pigment contents found in the selected medicinal plant extracts clearly indicated that the solubility of photosynthetic pigments in different solvents depends on the chemical nature of bio-molecules (chlorophyll a, chlorophyll b, and carotenoids). The extracts of both *Orthero* root and *P. yohimbe* bark, based on their high concentration of phytopigments, appear to be promising sources of water-soluble colorants that could be marketed as natural food colorants. This study demonstrated that aqueous acetone is the best solubilizer for chlorophylls and carotenoids for most of the sampled species and of all pigment contents assessed. Variations in aqueous solvent solubility of pigmented compounds

among the experimented plants species may be attributable to inherent physiological characteristics of individual species as also suggested by Sumanta & Haque (2014).

The antimicrobial zone of inhibition in the selected extracts increased with increasing concentrations of plant extract. All positive controls inhibited microbial activity in both gram-negative *E. coli* and gram-positive *S. aureus* although known concentrations of plant extracts differed from one species and/or plant part to another. A high TPC, TFC, antioxidant activity or power in plant part extracts does not necessarily result in a significant microbial growth inhibition zone. In addition, the presence of other phenolic or non-phenolic compounds in the extracts could result in synergistic and antagonistic influences on microbial growth inhibition. Potential synergistic effects of phytochemicals and their mechanisms of actions should be further studied.

5.2 Recommendations for future work

This study suggests that the aqueous extracts of *P. africana* (C) root and *P. yohimbe* bark exert antibacterial activity. The potential synergistic, additive, or potentiating antioxidant and antimicrobial activity of the detected phenolic acids in combination with other polyphenols should be evaluated for potential application in food safety or medicine. Since potential secondary compounds have not been identified and the antibacterial mechanisms of these extracts are currently unclear; future research should involve isolation and identification of all active compounds and their mechanisms of action. Geographical (temporal and seasonal) differences and local geological conditions can contribute to variations in pigment concentrations and phenolic compositions in plants, requiring further study. Evaluation of the safety of pigments (especially anthocyanins) found in *Orthero* root and *P. yohimbe* bark for use in food processing as natural colorants or pharmaceuticals needs to be evaluated. The acidification of polar solvents (ethanol or

methanol) with citric acid should be evaluated to determine whether this increases the extraction yield of polyphenols such as anthocyanins. The use of the SFE technique as a green alternative using CO_2 as a solvent in the extraction of tetraterpenes such as carotenoids and polyphenols (e.g., anthocyanins) should be studied since this would be less hazardous and free of toxic wastes, requiring no solvent removal and eliminating the problem of thermal extract degradation.

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APPENDICES

APPENDIX A: PHYTOCHEMICAL SCREENING

A.1. Phenolic Content

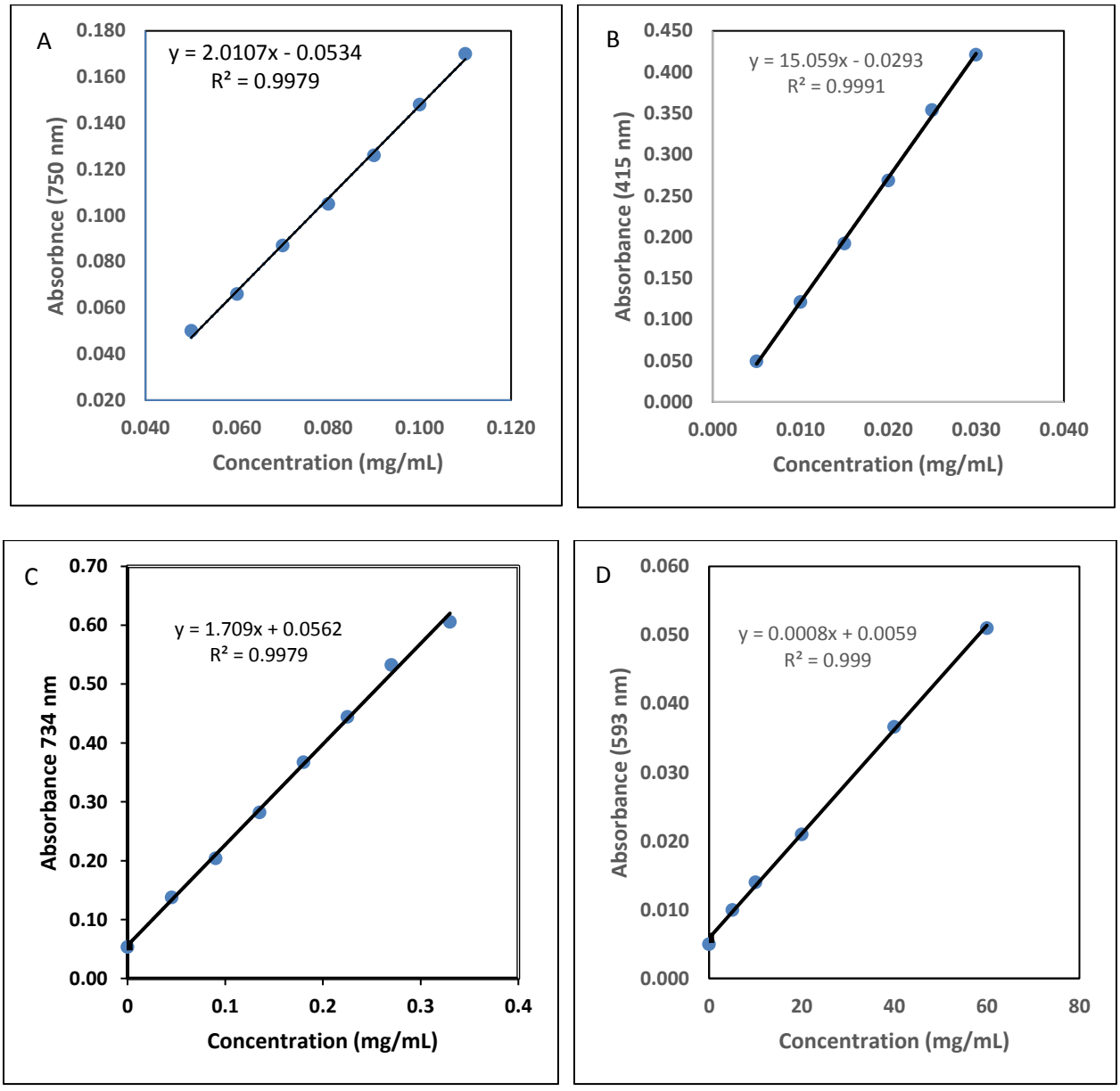

Fig. A.2.2.1. Calibration curve of **A**) Gallic acid, **B**) Rutin, **C**) Trolox and **D**) Ferrous sulphate heptahydrate

A.3. HPLC Analysis

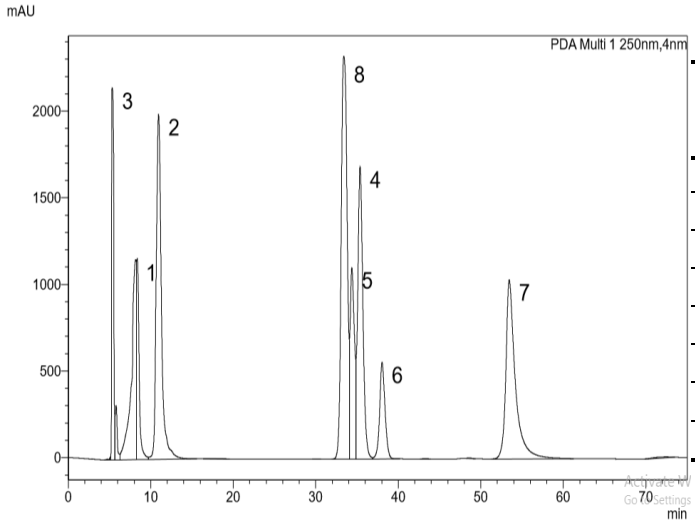

Table A.3.1.: Phenolic acids in standard mix

Peak	Phenolic acid	Retention Time (min)	HPLC-PAD (nm)
1	Methyl 4-hydroxybenzoate	8	250
2	Protocatechuic acid	10.9	
3	Trans-sinapic acid	5	
4	Syringic acid	35	
5	Trans-ferulic acid	34	
6	p-Coumaric acid	38	
7	Caffeic acid	53	
8	Vanillic acid	33	

Fig. A.3.1.: HPLC chromatogram of standard mix (internal/ reference) obtained from optimized conditions (**1**: methyl 4-hydroxybenzoate, **2**: protocatechuic acid ethyl ester, **3**: trans-sinapic acid, **4**: syringic acid (internal standard), **5**: trans-ferulic acid, **6**: p-coumaric acid, **7**: caffeic acid, **8**: vanillic acid)

Table A.3.2.: Phenolic acids in *P. africana* (C) bark and root, and *P. yohimbe* bark extracts

Peak	Phenolic acid	RT, Ace (min)	RT, MeOH (min)	RT, EtOH (min)	HPLC-PAD (nm)
Phenolic acids in *P. africana* (C) bark extracts					
1	Methyl 4-hydroxybenzoate	7.8	7.8	7.8	250, 320
2	Protocatechuic acid	10.9	10.9	10.9	250
3	Trans-sinapic acid	5	5	5	250, 320
4	Syringic acid	35	34.9	35	250, 320
Phenolic acids in *P. yohimbe* bark extracts					
1	Methyl 4-hydroxybenzoate	8	8	-	250
2	Protocatechuic acid	10.9	10.9	-	250
4	Syringic acid	34.7	34.8	-	250, 320
Phenolic acids in *P. africana* (C) root extracts					
1	Methyl 4-hydroxybenzoate	8	8	8	250, 320
2	Protocatechuic acid	10.9	10.9	10.9	250
3	Trans-sinapic acid	5	5	5	250, 320
4	Internal standard	35	35	35	250, 320
5	Trans-ferulic acid	34	34	34	250

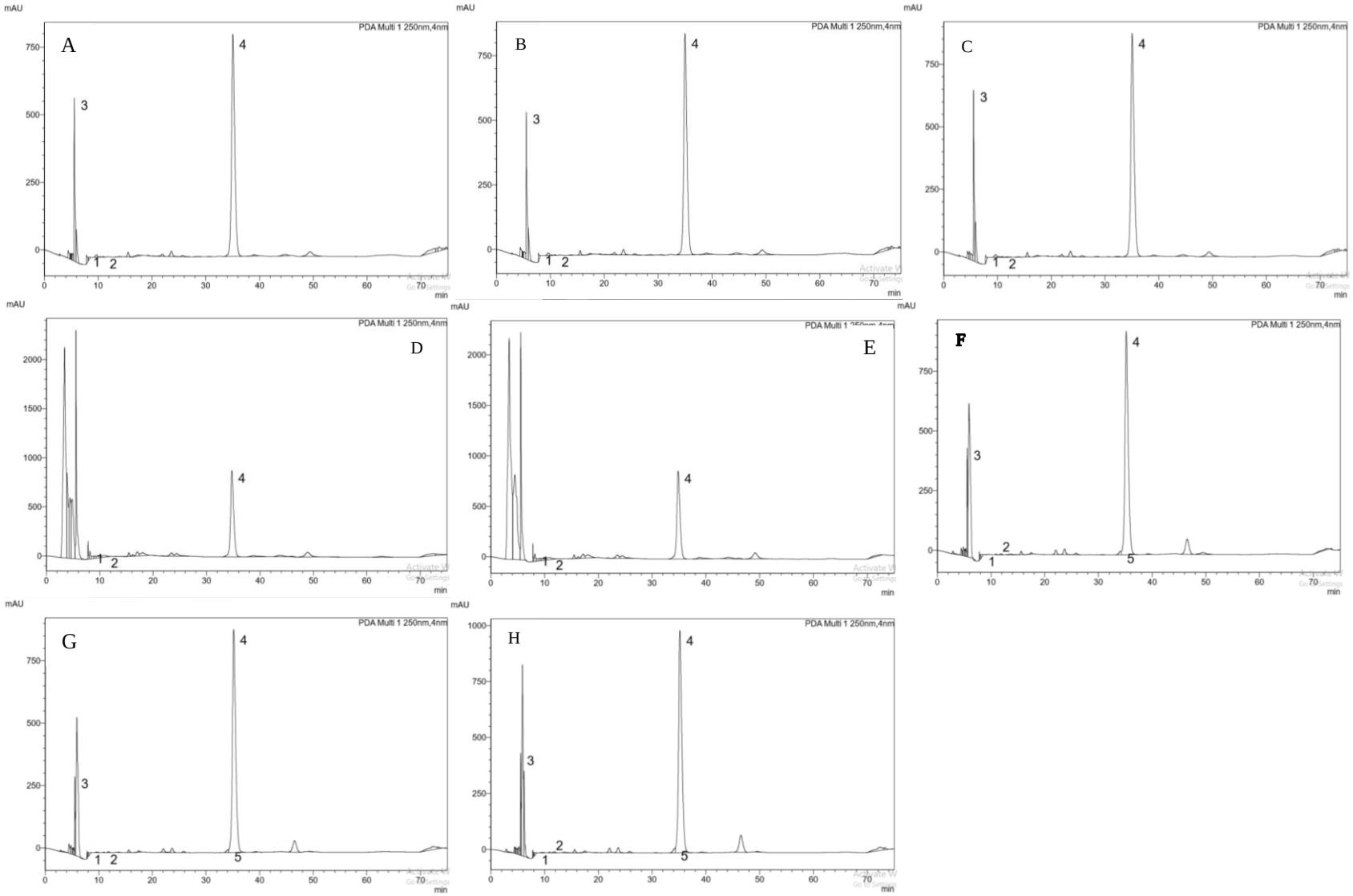

Fig. A.3.2.1. HPLC chromatograms of *P. africana* (C) bark extract (**A**: acetone; **B**: methanol; **C**: ethanol); *P. yohimbe* (C) bark (**D**: acetone; **E**: ethanol) and *P. africana* (C) root (**F**: acetone; **G**: methanol; **H**: ethanol) obtained from optimized conditions (**1**: methyl 4-hydroxybenzoate, **2**: protocatechuic acid ethyl ester, **3**: trans-sinapic acid, **4**: syringic acid (internal standard))

APPENDIX B: ANTIMICROBIAL ACTIVITY TESTS

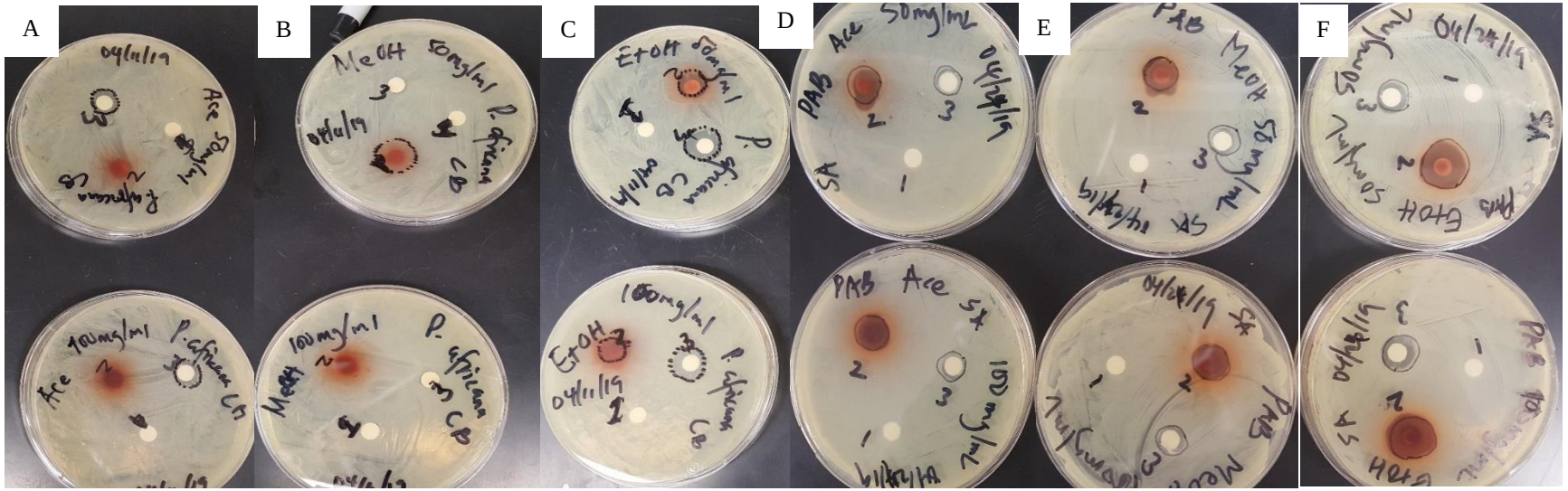

Fig. B.1.1.1.1. Antimicrobial activity of *P. africana* (C) bark extract (**PAB**) against *Escherichia coli* (**EC**); and *P. africana* (C) bark extract (**PAB**) against *Staphylococcus aureus* (**SA**); using **50** (top) and **100** mg/mL (bottom) (**A** and **D**: acetonic, **B** and **E**: methanolic, **C** and **F**: ethanolic extracts; **1**: negative control (sterile 6 mm blank disc); **2**: plant extract, **3**: solvent control (solvents: Acetone, **Ace**; Methanol, **MeOH**; Ethanol, **EtOH**)

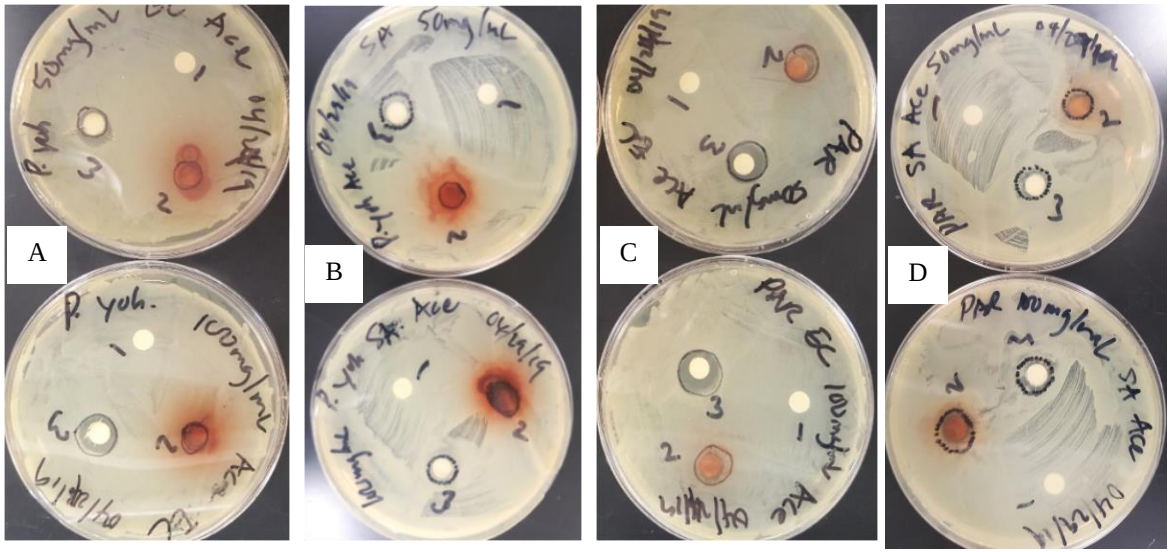

Fig. B.1.1.2.1 Antimicrobial activity of *P. yohimbe* bark extract (**PYOH**) against; **A**: *Escherichia coli* (**EC**) and **B**: *Staphylococcus aureus* (**SA**); and *P. africana* root extract (**PAR**) against **C**: *Escherichia coli* (**EC**) and **D**: *Staphylococcus aureus* (**SA**); using: **50** (top) and **100** mg/mL (bottom) **1**: negative control (sterile 6 mm blank disc); **2**: plant extract, **3**: positive control/solvent extract (solvent: Acetone, **Ace**)

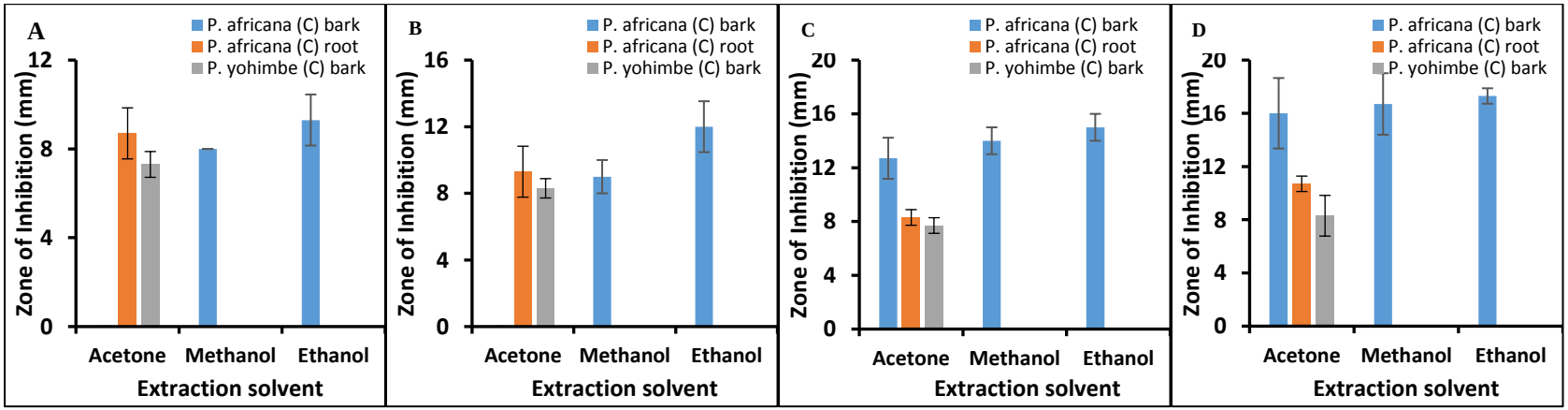

Fig. B.1.2.1. Average diameter of Zone of Inhibition (ZOI) using **A** and **C**: 50 mg/mL and **B** and **D**: 100 mg/mL of plant extracts against *E. coli* (A and B) and *S. aureus* (C and D)