# PHYTOCHEMICAL ANALYSIS AND ANTIBACTERIAL PROPERTIES OF AQUEOUS AND ETHANOL EXTRACTS OF *BRACHYLAENA ELLIPTICA* (Thurb.) DC. AND *BRACHYLAENA ILICIFOLIA* (Lam.) Phill. & Schweick.

**IDOWU JONAS SAGBO**

**Dissertation**

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**SUPERVISOR: PROF AJ AFOLAYAN**

**Co-SUPERVISOR: PROF G BRADLEY**

# DECLARATION

I, Idowu Jonas Sagbo, hereby declare that this research work was carried out by me and it has never been submitted for any other degree at this university or any other university.

I declare that I am completely aware of the university of Fort Hare policy on plagiarism and I have taken every necessary precaution to comply with the regulations of the university.

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| ABTS | 2, 2'- azinobis [3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt. |
| DPPH | 1, 1-diphenyl-2- picrylhydrazyl |
| DM | Diabetes Mellitus |
| g | Gram |
| $H_2O_2$ | Hydrogen peroxide |
| kDa | Kilo Dalton |
| L | Litre |
| m | Milli |
| M | Molar |
| NO | Nitric Oxide |
| PBS | Phosphate Buffered Saline |
| ROS | Reactive Oxygen Species |

**Abstract**

Resistance of human pathogenic bacterial strains results in selective pressure against known antibiotic. However, plant derived compounds that possess antibacterial potential are currently being investigated for treatment of wound infections in diabetic patients as they are inexpensive and non-toxic. Hence, this dissertation was designed to evaluate two medicinal plants (*Brachylaena elliptica* and *Brachylaena ilicifolia*) traditionally used in the treatment of various diseases such as diabetes, and its secondary complications in diabetic patients. The *in vitro* antioxidant activity of both plants were evaluated using DPPH (1, 1-diphenylhydrazl), ferric reducing power, ABTS (2, 2'-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid), NO (nitric oxide) and $H_2O_2$ (hydrogen peroxide) techniques. The antibacterial test and Minimum inhibitory concentration (MIC) was determined by agar dilution method against 5 bacteria strains (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pyogene*, *Proteus vulgaris* and *Proteus mirabilis*) infecting wounds in diabetic patients using amoxicillin and ciprofloxacin as positive control. The phytochemical analyses were assessed using standard published methods. Identification of bioactive components in essential oils of both plants were assessed using GC-MS. The aqueous and ethanol extracts of both plants were also evaluated to identify bioactive components using LC-MS. The results of the phytochemical analysis revealed the presence of phenols, tannins, flavanoids, flavanols, proanthocyanidins, saponins and alkaloids in both plants. Both plants indicated strong antioxidant activities which might be due to the presence of bio-active compounds.  The aqueous and ethanol leaf extracts of both plants demonstrated appreciable broad spectrum activities against these wound pathogens with MIC ranging between 5 and 0.3 mg/ml. The GC-MS analysis of the essential oils of both plants revealed the presence of monoterpenes, oxygenated sesquiterpenes, phenolics and esters. The LC-MS analysis of the

aqueous and ethanol leaf extracts of both plants showed that both plants are rich in alkaloids, terpenes, terpenoids, monoterpernoids, and flavanoids. Conclusively, this study has partially justified the ethnomedicinal use of *B. elliptica* and *B. ilicifolia* leaves for the treatment of various diseases, including diabetes and wound infections caused by bacteria in diabetic patients. These may be attributed to the presence of antioxidant compound such as phenols, flavanoids, saponins, tannins, alkaloids and other phytochemical compounds.

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# INTRODUCTION

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# INTRODUCTION

## 1.1 INTRODUCTION

Over the years, the prevalence of bacterial infection and its resistance to antibiotics drugs has brought to knowledge the importance to search for alternative treatments against infections (Sunita and Mahendra., 2008). Presently, there has been an increasing interest in the study of traditional plants and their medicinal value in different parts of the world. The medicinal properties of plants have been investigated in the light of recent scientific development throughout the world, due to their strong pharmacological activities, economic viability and low toxicity (Prashant *et al*., 2010). This tremendous interest in plants-derived drugs are mainly due to the current widespread belief that herbal medicine is safer and more reliable than the costly orthodox medicine, many of which may have adverse side effects (Jigna *et al*., 2006). Owing to this fact, the majority of the South African population depends heavily on the use of plants for their well being due to a wide array of phytochemicals with therapeutic properties (Siveen and Kutton 2010). Naturally, it has been reported that plants possess free radical scavenging molecule such tannins, alkaloids, terpernoids, phenolic and other metabolites which are rich in antimicrobial and antioxidant properties (Omoruyi *et al*., 2012). The ingestion of these natural antioxidants has been investigated to enhance the immune defense, reduce risks of cardiovascular disease, diabetes and infections (Omoruyi *et al*., 2012). Therefore, responding to the compelling need for evidence regarding herbal medicine, the phytochemical, antioxidant and antibacterial effects of selected medicinal plants on a panel of five opportunistic bacterial in diabetic infection were evaluated in this study.

## 1.2 Diabetes mellitus

Diabetes mellitus is a group of metabolic disorders that affects the metabolism of carbohydrates, fats, protein and electrolytes in the body. This is as a result of defects in insulin secretion, insensitivity of target organs to insulin or both. It is also characterized by abnormal high blood glucose level that causes glycation of the body protein which lead to serious complications (Rang *et al*., 1991). To understand diabetes, it is important to know the normal physiological process that occurs during and after a meal.

Food passes through the digestive system, where nutrients, including carbohydrates, fat and protein are taken up into the bloodstream. The presence of sugar (glucose) signals to the endocrine pancreas to release the hormone insulin. Insulin facilitates the entry and storage of glucose by almost all tissue types in the body, particularly the liver, fat and musculature tissues (Roussel, 1998). However, If the amount of insulin available is deficient, if the body cells respond poorly to the effects of insulin (insulin resistance), or if the insulin itself is defective, then glucose will not be absorbed properly by the cells that need it, and it will not be appropriately stored in the liver, muscles and fat cells. The net effect is persistently high levels of glucose in the blood, poor protein synthesis, and other long term complications such as glaucoma, retinopathy, neuropathy and acidosis. Unfortunately, there is currently no cure for diabetes. However, by controlling blood glucose level through various hypoglycemic drugs, insulin injection and life style modifications such as healthy diet, exercise and medications, the risk of diabetes complications can be lessened. Recently, attention has been focused on a number of medicinal plants used in the treatment of diabetes disease by virtue of their antioxidant and antidiabetic effects.

### 1.3 Opportunistic bacterial infections associated with diabetes mellitus.

People with diabetes are more vulnerable to developing infections, since high concentration of glucose in blood can weaken the patient's immune system defenses. In addition, diabetic patients are at higher risk for bacterial infection such as foot infection, wound infection, urinary tract infections, surgical site infections, peritonistis, osteomyelitis, sepsis and lower respiratory tract infections (Koh *et al*., 2012). Diabetic foot infections (DFIs) are one of the most serious and costly diabetes mellitus complications. Diabetic patients with DFIs are characterized by vasculopathy, increased frequency of infections, peripheral neuropathy with loss of sensation and unnoticed injuries (Roberts and Simon 2012). A prior diabetic foot ulcer (DFU) is an almost obligatory prerequisite for diabetic foot infections. Diabetic foot ulcers result from a complex interaction of a number of risk factors. Once the protective layer of skin is broken, deep tissues are prone to bacterial infection that progresses rapidly. Patients with diabetic foot ulcers often require amputations of the lower limbs and, in more than half the cases, infection is the predominant factor (Mendes and Neves, 2012). The most common causative pathogens in diabetic foot ulcers are *Staphylococcus aureus*, *Streptococcus pyogenes*, *Proteus vulgaris*and *Pseudomonas* spp (Sharma *et al*., 2006).

*Staphyloccoccus aureus* are the first microorganisms to colonize and acutely infect breaks in the skin while *Pseudomonas auroginosa* are specifically associated with wounds treated with wet dressing (Lipsky 1999).

Urinary tract infections (UTIs) are another most common infection in diabetic patients. These are infections of any part of urinary system. They are classified into disease categories according to the site of infections such as bacteriuria (urine), cystitis (bladder) and pyelonephritis (kidney) (Foxman 2003). The primary causative agents responsible for more than 80% of all UTIs are strains of *Escherichia coli* (Sadler *et al*., 1989). Infections in diabetic patients can lead to hyperglycemia and diabetic ketoacidosis (Kitabchi *et al*., 2009). Infections can also worsen the glycemic control. In summary, poor glycemic control or other factors associated with diabetes mellitus can exacerbate the development of infections in diabetes patients.

**1.4 Phytochemical in plants**

Plants produce a wide variety of secondary metabolites, many of which have been reported to be of therapeutic value. In many cases, the prospect of obtaining drugs from plants has been demonstrated by some notable examples of important pharmaceuticals derived from plant precursors. For instance, the antimalarial drug Quinine was derived from the quinoline alkaloid of *Cinchona spp*.; the topical analgesic Capsaicin was derived from a phenylalkyl-amine alkaloid of *Capsicum spp* (Raskin *et al*., 2002). The rich chemical diversity in plants has also been reported to be a promising source of antibacterial compounds (Bylka *et al*., 2004; Smith et al., 2007; Machado *et al*., 2002), raising hopes of obtaining novel antibiotics that can aid the fight against drug resistant infections. However, different phytochemicals show various mechanisms of action, such as, inhibiting specific pathogens, increasing colonic water and electrolyte re absorption (Ahmed *et al*., 2006). Lui (2003) reported that phytochemicals such as tannins, steroids, alkaloid and saponins have anti-inflammatory effect. Triterpenoids and steroids reported to possess analgesic properties (Malairagan *et al*., 2006).

**1.5 Free radical scavenging and antioxidant property of medicinal plants.**

Free radicals can be defined as an atom or group of atom containing one or more unpaired electrons in its outermost atomic or molecular orbital and is therefore unstable and highly reactive (Miller and Rice Evans, 1997). A free radical is another term applied to reactive oxygen species that can be classified into five types, four of which are reactive oxygen species such as superoxide ion (O), hydroxyl radical (OH), singlet oxygen, Hydrogen peroxide ($H_2O_2$) while the fifth one is the nitrogen reactive species. Antioxidants are molecules which can easily interact with free radicals and terminate the chain reaction by donating one of their own electrons, before vital molecules are damaged (Sa´nchez-Moreno *et al*., 1998).

Choi *et al* (2002) reported that free radicals induce cellular oxidative damage to biomolecule such lipids, protein and nucleic acids which may result in many diseases, including diabetes mellitus, inflammation, atherosclerosis, anemia, carcinogenesis and AIDS. Hence, medicinal plants with antioxidant and free radical property could have great importance as therapeutic agents in treatment of several diseases related to oxidative stress (Ramchoun *et al*., 2009). However, several synthetic antioxidant agents are reported to be toxic to animal and human being such as butylated hydoxyltoluene (BHT) and butylated hydroxylanisole (BHA) (Madhavi and salunkhe 2005). This observation prompts many researchers to search for more natural antioxidants (Nagulendran *et al*., 2007). The reliable laboratory method used to scavenge free radical includes spectrophotometric method using 2, 2-azino-bis (3-ethylbenzenthiazolidine-6-sulphonic) acid radical ($ABTS^+$), hydrogen peroxide radical ($H_20_2$), nitric oxide (NO) and 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH) with spectrophotometer (Choi *et al*., 2002).

### 1.6 Uses of medicinal plant in South Africa

In South Africa, medicinal plants are still an important cultural heritage as a large number of people now depend on herbal medicines for their health care needs, mostly because of their easier accessibility and affordability (Manders, 1988). Due to diverse cultural heritage, South

Africa has over 30,000 plant species of which about 3,000 species are currently used as herbal medicine for treatment of various diseases (Van Wyk *et al*., 1997; Van Vuuren, 2008). The literature sources detailing uses of plant medicine in South Africa include: Watt and Breyer-Brandwijk (1962), Hutching (1996), Van Wyk (1997), Olajuyigbe and Afolayan (2012), Lawal *et al*., (2014). This reliance on plant as a source of medicine for treatment of various diseases justify scientific validation of their safety effectiveness, and proper dosage of the plant material used (Masika and Afolayan, 2002).

### 1.7 Safety evaluation of medicinal plants

Medicinal plants have been used for centuries by traditional healers for treatment of various diseases, however care needs to be taken regarding dosage as these treatments at higher doses can be toxic. For example, several studied have shown that intake of plant extracts in high dosage could lead to various complications such as injury to the kidney, induce acute renal failure and interfere with renal tubular function (Bwititi *et al*., 2000; Ijeh and Agbo, 2006). Therefore, there is a need to further investigate safer concentrations of ethnomedicinal preparations accompanied with toxicology screening (Taylor *et al*., 2001; Fennell *et al*., 2004; Cos *et al*., 2006).

### 1.8 The choice of *Brachylaena elliptica* and *Brachylaena ilicifolia* for this study

The choice of *Brachylaena elliptica* and *Brachylaena ilicifolia* (Asteraceae) was based on four criteria: Firstly, these plants have ethnopharmacological data indicating their traditional usage for the treatment of diabetes mellitus. (Deutschlander *et al.*, 2009). Secondly, in the history of diabetes mellitus, there is no study in the Eastern Cape of South Africa that deals with these plants for the management of diabetes. Third and fourth criteria were based on the antidiabetes and antibacterial activities and the availability of the plant materials respectively. Based on these criteria *Brachylaena elliptica* and *Brachylaena ilicifolia* were then selected for this study.

*Brachylaena elliptica* *Brachylaena ilicifolia*

Figure 1: Medicinal plants used in this study

*Brachylaena elliptica* (Thunb.) DC. belongs to the family Asteraceae. It is a shrub or small tree up to 4m tall with a light grey to brown bark that becomes rough with age. It is distributed from Port Elizabeth, Eastern Cape Province to Durban, KwaZulu-Natal. The leaves are lanceolate, elliptic to ovate, dark green above and white felted below (Figure 1). The species occurs in bushveld on rocky outcrops and along the edge of evergreen forest. Poles from this species are used as fence posts; the sticks have been used to start a fire by friction. The leaves, which are extremely bitter tasting, are used medicinally (Van Wyk and Van Wyk, 1997) and valued by the Zulu and Xhosa for the management of diabetes. The infusion of the leaves is used as a gargle and mouthwash (Coates Palgrave, 1984).

*Brachylaena ilicifolia* (Lam.) Phill. & Schweick. belongs to the family Asteraceae. It is a shrub or small tree between 3 and 4 m in height with grey to brown bark. The leaves are frequently on short lateral branches, small, narrowly oblong, lanceolate to ovate, green above and covered with whitish-green hairs below. The plant is distributed from Port Elizabeth Eastern Cape Province to Durban, KwaZulu-Natal, in bush and scrub forest (Coastes Palgrave, 1984).The leaves, which are intensely bitter, are used by traditional healers and herbalists to treat various disease including diabetes (Coastes Palgrave, 1984). The infusion and decoction of the leaves are used to treat diarrhea (Olajuyigbe and Afolayan, 2012).

Despite the acclaimed folkloric use of *B. elliptica* and *B. ilicifolia* as an antidiabetic and antibacterial agent, there is a lack of scientific study to authenticate the claim. Hence, there is need to validate these acclaimed use by traditional healers for the treatment of various diseases.

## 1.9 Statement of research problem

The last two decades have witnessed a dramatic rise in the incidence of life threatening bacterial infections. It is a major health problem with its frequency increasing every day in most countries. Nowadays, a number of clinically efficacious antibiotic are becoming less effective due to the development of resistance. However, current medication is not readily accessible to many rural populations and many traditional plants have not been scientifically validated.

## 1.10 Aim and objectives

### 1.10.1 Aim

- The ultimate aim is to contribute to our knowledge of these two plants in their medicinal properties.

### 1.10.2 Specific objectives

- To investigate the antioxidant, antibacterial and phytochemical properties of these plants against wound infecting bacterial in diabetic patients.
- To identify volatile bioactive compounds from the essential oil of these plants using GC/MS
- To identify non volatile bioactive compounds from these plants extracts using LC/MS

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# CHAPTER 2

## ANTIOXIDANT, ANTIBACTERIAL AND PHYTOCHEMICAL PROPERTIES OF TWO MEDICINAL PLANTS AGAINST THE WOUND INFECTING BACTERIA IN DIABETIC PATIENTS

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# CHAPTER 2

## 2.1 INTRODUCTION

Numerous human diseases are caused by oxidative stress that results from imbalance between the production of reactive oxygen species and antioxidant defenses (Hazra *et al*., 2008). Free radicals such as hydrogen peroxide, hydroxyl, nitric oxide, superoxide anions, play a crucial role in damaging different cellular macromolecules, including proteins along with lipid peroxidation and DNA molecules. This damage may result in many diseases including atherosclerosis, neurodegenerative diseases, carcinogenesis and diabetes mellitus (Polterat, 1997).

Diabetes mellitus is a group of chronic metabolic diseases in which there are high blood glucose levels over a prolonged period. If left untreated, diabetes can cause serious complications including stroke, kidney failure and cardiovascular disease (WHO, 2013). Apart from organ complications, diabetic patients also suffer from various infections, which include skin and mucous membrane infection, respiratory tract infection, urinary tract infection and diabetic foot infections (Hoepelman *et al*., 2003). Hence, patients with diabetes are at high risk for infection-related mortality (Bertoni *et al*., 2001). It has been reported that poor management of diabetes contributes to the pathogenesis of microbial infection in diabetic patients. When diabetic patients develop ulcers, they become at greater risk of major complication like infection and amputation. Wound infection is caused by physical injuries that result in an opening or breaking of the skin. Wounds break the continuity of the skin and allow bacteria such as *Pseudomonas aeruginosa*, *Streptococcus pyogenes* and *Staphylococcus spp* to gain access to the tissue and cause infection (Houghton *et al*., 2001). There are several bacteria which are responsible for wound infection. One of such is *Pseudomonas aeruginosa*, characterized by the formation of a green pigment which later a black lesion (Al-Akayeh, 1999). Todar (2007) reported the isolation of *Echerichia* *coli* from surgical wounds. *Staphylococcus aureus* has also been isolated from diabetic patients with diabetic wounds and foot ulcers (Hirsh *et al*., 2008). Bacteria generally, possess the genetic ability to acquire resistance towards many antibiotics. Following the massive use of antibiotics in human therapy, some antibiotics while effective, have been associated with adverse side effect such as allergic reaction and immune-suppression (Ahmad *et al*., 1998). Due to these reasons plant derived compounds or phytomedicine having antimicrobial action are needed to be introduced and evaluated through biological trials.

Many plants naturally contain a wide variety of free radical scavenging molecules such as tannin, anthocyanins, saponin, alkaloids, steroids, terpernoids. In most cases, the pharmacological properties of medicinal plants are attributed to these phytochemicals. Some of these compounds are radical scavenging compounds or antioxidants. Muthur *et al.* (2011) reported that plants are very good source of antioxidants and also help in the treatment of various radical related diseases including wound infections. Studies and identification of bioactive compounds from plants have become a major interest to scientists (Shai *et al*., 2008). Previous work (Dekker *et al*., 2001) reported that compounds such as diterpenoid found in *Jatropha zeyheri*, showed antibacterial activity against *Streptococcus pyogenes*. In addition, some compounds such as 2-alpha hydroxyurosolic acid,ursolic acid and butulinic acid isolated in *Curtisia dentate* also possessed antimicrobial properties (Shai *etal*., 2008). This study aimed at evaluating antioxidant and phytochemical properties of *Brachylaena elliptica* and *Brachylaena ilicifolia* leaves and their effect on bacteria which infect wounds of diabetic patients.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Microorganisms used

*Pseudomonas aeruginosa* (ATCC 27853), *Staphylococcus aureus* (ATCC 2593), *Streptococcus pyogene* (Laboratory strain), *Proteus vulgaris* (KZN) and *Proteus mirabilis* (ATCC 7002) were obtained from the Medicinal Plants and Economic Development (MPED) Research Centre, Department of Botany, University of Fort Hare, South Africa. These bacteria strains were chosen for their pathological effects on wounds in diabetic patients. The following antibiotic drugs were used as control: amoxicillin and ciprofloxacin.

### 2.2.2 Collection of plant materials and preparation of extracts

The leaves of *B. elliptica* were collected from a thick forest in Amathole District in Eastern Cape, while *B. ilicifolia* leaves were collected from Grahamstown also in the Eastern Cape. The plants were identified by their vernacular names and later authenticated at the Albany Museum Grahamstown. The leaves of each plant were oven dried to constant weight at 40$^{o}$C and pulverized to a homogeneous powder using an electric blender (Waring Products Division, Torrngton, USA). Approximately 60 g of the powdered plants were extracted separately in distilled water and ethanol on a shaker for 24 h. Each extract was filtered using a Buchner funnel and Whatman No. 1 filter paper. The water extract was frozen at -40$^{o}$C and dried for 48 h using a freeze dryer to give a yield of 9 g and 8.1 g for *B. elliptica* and *B. ilicifolia* respectively. The ethanol extract was concentrated to dryness under reduced pressure at 40$^{o}$C using a rotary evaporator to give a yield of 11.6 g and 12.7 g for *B. elliptica* and *B. ilicifolia* respectively. The resulting extracts were then reconstituted in their respective solvents to give the desired concentrations used in the study.

**Phytochemical analysis**

### 2.2.3 Determination of tannin content

The tannin content was determined according to the method described by Wintola and Afolayan (2011) with some modifications. Twenty milliliter (20 ml) of 50% methanol prepared in distilled water was added to 0.2 g of plant sample and covered. The mixture was shaken vigorously on the shaker placed in a water bath at 77$^{o}$C for 1 h to ensure uniform mixing. Extract was filtered into 100 ml volumetric flask. Twenty milliliter (20 ml) of distilled water containing 2.5 ml of Folin-Denis reagent and 10 ml of 17% $Na_2CO_3$ were added to the filtrate and properly mixed together. The bluish-green colour developed at the end of the reaction mixture, and was unique at various concentrations ranges from 0 to 10 ppm. The absorbance of the tannic acid standard solution was measured after colour development at 760 nm using UV-VIS spectrophotometer (AJ-C03). Total tannins content was expressed as tannin standard equivalents (mg/g) using the following equation from the calibration curve: $Y = 0.0593x – 0.0485$, $R^2= 0.9826$, where Y is the absorbance and X is the tannic acid equivalent (mg/g).

### 2.2.4 Determination of phenol content

The total phenolics content was determined spectrophotometrically with Folin Ciocalteau's phenol reagent using the method described by Otang *et al.* (2012) with some modifications. About 0.5 ml of the extract solution was mixed with 0.5 ml of 10% Folin-Ciocalteu reagent (previously diluted with water 1:10 v/v) and 4 ml (75 % w/v) of sodium carbonate. The tubes were vortexed for 15 s and allow to stand for 30 min at $40^oC$ for colour development. The absorbance was then measured spectrophotometrically at 765 nm using UV-VIS spectrophotometer (AJ-C03). Total phenolic content was then expressed as mg/g tannic acid equivalent using the following equation based on the calibration curve: Y= 0.1216x, $R^2$ = 0.9365, where y was the absorbance x was the concentration

**2.2.5 Determination of flavanoids content**

Determination of flavonoid content was determined using the method described by Adedapo *et al* (2008). A volume of 0.5 ml of 2% $AlCl_3$ ethanol solution was mixed with 0.5 ml of extract solution. After 1 h of incubation at room temperature, the absorbance was measured at 420 nm using UV-VIS spectrophotometer (AJ-C03). A yellow colour indicated the presence of flavonoids. Extract samples were evaluated at a final concentration of 0.1 mg/ml. Total flavanoid contents were calculated as mg/g of quercetin equivalents using the following calibration curve: Y =0.0255, $R^2$ = 0.9812, where y was the absorbance x was the concentration.

**2.2.6 Determination of flavanols content**

Total flavonols content was determined according to the method described by Wintola and Afolayan (2011). The reacting mixture consisted of 2.0 ml of the sample, 2.0 ml of $AlCl_3$ prepared in ethanol and 3.0 ml of (50 g/L) sodium acetate solution. The absorption at 440 nm was read after 2.5 h at $20^oC$. Total flavonols content was calculated as mg/g of quercetin quercetin equivalents using the following calibration curve: Y =0.0255, $R^2$ = 0.9812, where y was the absorbance x was the concentration.

### 2.2.7 Determination of proanthocyanidins content

Total proanthocyanidins was determined by adopting the method described by Oyedemi *et al* (2010). 0.5 ml of 1 mg/ml extract solution was mixed with 3 ml of vanillin-methanol (4% v/v), and 1.5 ml of hydrochloric acid was added and vortexed. The mixture was left to stand for 15 min at room temperature. The absorbance was then measured at 500 nm. Total proanthocyanidins content was calculated as mg/g of catechin equivalents equivalents using the following calibration curve: Y = 0.5825x, $R^2$ = 0.9277, where y was the absorbance x was the concentration.

### 2.2.8 Estimation of alkaloids content

The alkaloids content in plant extracts was quantitatively determined following the method described by Otang *et al* (2012). Five grams (5g) of powdered plant extract was added into 200 ml of 10% acetic acid prepared in ethanol and allowed to stand for 4 h. The filtrate was collected and the extract was concentrated using water bath at $55^oC$ to $1/4^{th}$ of its original volume. Concentration ammonium hydroxide was added drop wise into the extract until precipitation was complete. The precipitate collected was washed with dilute ammonium hydroxide solution and then filtered. The residue which is the crude alkaloid was weighed and calculated using the following equation:

$$\% \text{ Alkalods} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \times 100\%$$

### 2.2.9 Estimation of saponins content

Estimation of saponin content in the plant extract was determined according to the method described by Omoruyi *et al.* (2012) with some modifications. Ten gram (10g) of the plant sample was added to 200 ml of 20% ethanol prepared in distilled water and kept in a shaker for 30 min. The plant sample was heated over a water bath at 55$^{o}$C for 4 h. The resulting mixture was filtered and the residue was re-extracted again with 200 ml of 20% aqueous ethanol. The mixture was reduced to 40 ml over water bath at 90$^{o}$C. The concentrate was transferred into 250 ml separatory funnel, extracted twice with 20 ml diethyl ether. The Ether layer was discarded while purification process was repeated. Sixty milliliter (60 ml) of n-butanol was added and the extract was washed twice with 10 ml of 5% aqueous sodium chloride. The remaining solution was heated over a water bath and evaporated to dryness to a constant at 40$^{o}$C. The saponins content was calculated using the following equation:

$$\% \text{ Saponins contents} = \frac{\text{Weight of residue}}{\text{Weight of sample}} \times 100\%$$

**Antioxidant assays**

### 2.2.10 DPPH radical scavenging assay

DPPH radical scavenging activity of the plant extracts was determined according to the method described by Wintola and Afolayan (2012) with some modifications. One milliliter (1 ml) of 0.135 mM DPPH in methanol was prepared and mixed with 1.0 ml of various concentrations (0.2 – 1.0 mg/ml) of the plant extracts, vitamin C or BHT. Both vitamin C and BHT were used as the standards. The reaction mixture was vortexed thoroughly and left in the dark at room temperature for 30 min. The absorbance of the mixture was measured spectrophotometrically at

517 nm. The ability of the plant extract to scavenge DPPH radical was calculated by the equation: DPPH radical scavenging activity = {(Abs control – Abs sample)}/ (Abs control} × 100 where Abs control was the absorbance of DPPH radical + methanol; Abs sample was the absorbance of DPPH radical + sample extract or standards (Vitamin C and BHT)

### 2.2.11 Reducing power assay

The reducing power of the plant extracts was determined according to the method described by Aiyegoro and Okoh (2010). A volume of 1.0 ml of the extract prepared in distilled water or BHT, Vitamin C (0.2 - 1.0 mg/ml) were mixed individually with 2.5 ml of phosphate buffer (0.2M, pH 6.6) and 2.5 ml of potassium ferricyanide (1%w/v). The resulting mixture was shaken well and incubated at $50^oC$ for 20 min. After incubation, 2.5 ml of trichloroacetic acid (TCA) (10 % w/v) was added to stop the reaction and then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml),  2.5 ml of distilled water and 0.5 ml of ferrous chloride ($FeCl_3$)(0.1%, w/v) were mixed and incubated for 10 min and the absorbance was measured at 700 nm on spectrophotometer.

### 2.2.12 ABTS radical scavenging assay

The method described by Adedapo *et al*. (2008) was adopted for the determination of ABTS activity of the plant extract. The radical was prepared by mixing two stock solutions of 7 mM ABTS and 2.4 mM potassium persulphate in the same ratio and allowing the solution to react for 12 h at room temperature in the dark. The resulting solution was further diluted by mixing 1 ml of $ABTS^+$ solution with 60 ml of methanol to obtain an absorbance of 0.706 ± 0.001 units. Various concentrations (0.2- 1.0 mg/ml) of the plant extracts and the standards (BHT and vitamin C) was allowed to react with the ABTS radical in the dark for 7 min and the absorbance were then measured at 734 nm. The percentage inhibition of $ABTS^+$ by the extract was calculated and compared with that of BHT and vitamin C using the following equation: $ABTS^+$ scavenging activity = {(Abs control – Abs sample)}/ (Abs control} × 100 where Abs control was the absorbance of ABTS radical + methanol; Abs sample was the absorbance of ABTS radical + sample extract or standards (Vitamin C and BHT)

**2.2.13 Nitric oxide scavenging activity**

Nitric oxide scavenging activity was determined according to the method described by Oyedemi *et al* (2010). Sodium nitroprusside (2 ml, 10 Mm) prepared in 0.5 ml phosphate buffer saline (pH 7.4) was mixed with 0.5 ml of plant extracts, vitamin C and BHT at various concentrations (0.2-1.0 mg/ml). The mixture was incubated at $25^oC$ for 150 min. After incubation, 0.5 ml of incubation solution was withdrawn and mixed with 0.5 ml of Griess reagent containing 1.0 ml of 0.33% sulfanilic acid reagent prepared in 20% glacial acetic acid at room temperature for 5 min with 1 ml of naphthylethylenediamine dichloride (0.1% w/v). The mixture was then further incubated for 30 min at room temperature. The absorbance was then measured at 540 nm. The amount of nitric oxide radical inhibited by the extract was calculated using the following equation: NO radical scavenging activity = {(Abs control – Abs sample)}/ (Abs control } × 100 where Abs control was the absorbance of NO radical + methanol; Abs sample was the absorbance of NO radical + sample extract or standards (Vitamin C and BHT)

**2.2.14 Hydrogen peroxide radical scavenging assay**

Hydrogen peroxide scavenging activity of the plants extract was determined using the method described by Oyedemi *et al* (2010). Plant extract (4 ml) prepared in distilled water at different concentrations (0.2-1.0 mg/ml) was mixed with 0.6 ml of 4 mM Hydrogen peroxide ($H_2O_2$) solution prepared in phosphate buffer (0.1 M, pH 7.4) and incubated for 10 min. After incubation, the absorbance of the solution was then measured at 230 nm against a blank solution containing the plant extract without Hydrogen peroxide. The amount of hydrogen peroxide radical inhibited by the extract was calculated using the following equation: $H_2O_2$ radical scavenging activity = {(Abs control – Abs sample)}/ (Abs control} × 100 where Abs control was the absorbance of $H_2O_2$ radical + methanol; Abs sample was the absorbance of $H_2O_2$ radical + sample extract or standard (Vitamin C and BHT).

**Antibacterial assay**

**2.2.15 Preparation of inocula**

The inoculums of the test micro-organism were carried out using the colony suspension method described by EUCAST (2003). The bacteria strains and isolates were cultured in nutrient agar overnight at $37^{o}$C. Identical colonies from the culture were suspended in sterile saline. The suspension was adjusted following the McFarland turbidy of 0.1 at 600nm to achieve 5 $x10^5$ colony forming units per/ml (Olajuyigbe and Afolayan, 2012).

**2.2.16 Bioassay**

Five bacteria strains (two Gram positive and three Gram negative) each of which was maintained in nutrient agar plate and recovered for testing by growth in nutrient broth for 24 hours. Before streaking, each culture was diluted 1:100 with fresh sterile nutrient broth.

### 2.2.17 Minimum Inhibitory Concentration (MIC)

The antibacterial activity of the plant extract was determined according to the method described by Olajuyigbe and Afolayan, (2012) with some modifications. Agar (Biolab) was prepared according to manufacturer's instructions and placed in water bath at 50$^{o}$C. The extract stock solution (100 mg/ml) was filtered and incorporated in the molten agar. Different concentrations of the extracts were prepared to final concentrations in the range of 5- 0.3 mg/ml. A volume of one milliliter (1 ml) from each dilution of the extract was mixed with 19 ml of molten sensitivity test agar at 50$^{o}$C and poured into sterile petri dishes allowing the agar to cool. Plates containing only nutrient agar and another set containing nutrient agar and the solvent of extraction were served as negative control while plates containing amoxicillin and ciprofloxacin were used as standard drugs (positive control). The surface of the agar was left to dry before streaking with standardized overnight broth cultures of the test bacteria. Plates were incubated at 37$^{o}$C for 24 hours under aerobic conditions. Each test was done in triplicate. The minimum inhibitory concentration was defined as the lowest concentration of the extract or standard that completely inhibited the visible growth of the organism.

### 2.2.18 Statistical analysis

All experiments were done in triplicates and, where applicable, the data were statistically analyzed using one way analysis of variance (ANOVA) and the difference between samples were determined by Duncan's Multiple Range test using the Minitab program (version 12 for Windows). Values were considered significant at $P < 0.05$.

## 2.3 RESULTS AND DISCUSSION

### 2.3.1 Phytochemical analysis

Phytochemical analysis of the *B. elliptica* and *B. ilicifolia* extract revealed the presence of tannins, phenols, flavanols, flavanoids, proanthocyanidins, alkaloids and saponins in aqueous and ethanol extracts (Figure 2 and 3). Most plants are used for the treatment of diseases because of the presence of these phytochemicals which have been reported to possess high medicinal value. In this study high content of tannins was observed in aqueous (105 mg/g tannic acid equivalent) and ethanol extracts (211 mg/g tannic acid equivalent) of *B. ilicifolia* leaf when compared with that of *B. elliptica*. Tannin has been reported for antibacterial activity, treatment of cancer and inhibition of lipid oxidation (Yokozawa *et al*., 1993; Dharmananda, 2003). The presence of this compound in the aqueous and ethanol extract of both plants could play a vital role in antioxidant and antibacterial properties observed in this study. Nevertheless, these results show similar trends found by Omoruyi *et al.* (2012) for the total tannin content using aqueous and ethanol extract of *C. edulis*, though the results obtained in this study was higher.

The total phenol content of aqueous (99 mg/g tannic acid equivalent) and ethanol leaf extracts (98.6 mg/g tannic acid equivalent) of *B. ilicifolia* was significantly higher ($P < 0.05$) than that of *B. elliptica* (Figure 2 and 3). Phenolic compounds are important plant components reported to possess antioxidant, antibacterial and numerous biological activities (Omale *et al*., 2010). These compounds have been indicated in several studies to be effective in scavenging free radicals due to their redox properties that allow them to act as a reducing agent (Zheng *et al*., 2001). The high content of phenol in the aqueous and ethanol leaf extracts of *B. ilicifolia*, may be a contributing factor toward its antioxidant activity.

The flavanoid content showed no significant differences ($P > 0.05$) in the aqueous and ethanol extracts of *B. elliptica* and *B. ilcifolia* leaf (Figure 2 and 3). However, flavanoids are very important bioactive polyphenols that are widely distributed in the plant kingdom and play a crucial role in photosynthesizing cells (Fernandezet *et al*., 2006). Flavanoids have been reported to possess a wide range of biological activities including anti-inflammatory, antibacterial and analgesic (Fergusion, 2001). Research undertaken by Otang *et al*. (2012) showed similar results with no significant difference for the flavanoid content between the leaf and stem bark acetone extracts of *G. bicolor* and *P. viridiflorum* respectively. Their findings were in agreement with current study.

Flavanols were present in the aqueous and ethanol extract of *B. ilicifolia* and *B. elliptica* (Figure 2 and 3). The highest content of flavanols was observed in the aqueous (20 mg/g quercetin equivalent) and ethanol (48.8 mg/g quercetin equivalent) extracts of *B. ilicifolia* leaf when compared with that of *B .elliptica*. Flavanols are phytochemical compounds found in high concentrations in variety of plants. They have been reported to have the ability to scavenge free radicals (Williamson and Manach, 2005). The results for ethanol extract of *B. ilicifolia* show similar trends found by Wintola and Afolayan, (2012) for the total flavanol contents using ethanol extracts, though the results obtained in this study was higher.

Proanthocyanidins, were one of the groups of phytochemicals that were found to be in high quantity compared to other phytochemicals investigated in this study. The maximum proanthocyanidins content was noted in both aqueous and ethanol extracts of *B. elliptica* and *B.*

*ilicifolia* (Figure 2 and 3). However, levels of proanthocyanidins one found to be significantly higher in the aqueous (219 mg/g catechin equivalent) and ethanol (417 mg/g catechin equivalent) extracts of *B. ilicifolia* when compared with that of *B. elliptica*. Proanthocyanidins are group of polyphenolics bioflavanoids which play a vital role in eliminating hydroxyl radicals (Pataki *et al*., 2002). Beninger and Hosfield (2003) reported that proanthocyanidins have high antioxidant activity usually greater than ascorbic acid and tocopherol. These results concurred with the finding of Omoruyi *et al.* (2012) who reported high content of proanthocyanidins in the aqueous and acetone extract of *C. edulis,* but lesser than what was reported in this study.

The highest content of alkaloid was observed in the aqueous extract of *B. elliptica* when compared with that of *B. ilicifolia* (Figure 2 and 3). However, the ethanol extract of *B. ilicifolia* was also showed significantly higher than that of *B. elliptica*. These results corroborated with the findings of Omoruyi *et al*. (2012) who reported the aqueous extract of *C. edulis* exhibited the highest concentration of alkaloids compared to other solvents. Alkaloid is one of the bio-active components in plants with potential to protect cells against foreign invading agents due to its toxic nature. This nature is accountable for the medicinal values of various plants used for the management of anti-malaria, analgesic and bactericidal activities (Neumann *et al*., 2004). The presence of alkaloids in the aqueous and ethanol leaf extracts of *B. elliptica* and *B. ilicifolia* may partially justify their ethnomedicinal use for treatment of various diseases.

The estimation of saponin content in the aqueous and ethanol extract of *B. elliptica* was significantly higher ($P < 0.05$) than that of *B. ilicifolia* leaf (Figure 2 and 3). The presence of saponin in *B. elliptica* and *B. ilicifolia* aqueous and ethanol leaf extracts in this study contradicts the observations of Rajamurugan *et al.* (2013) who reported that saponin were absent in *Eclipta alba* and *Alternanthera sessilis*. Their observation may be due to the methods of extraction and many other factors such as geographical distribution of the plants and environmental factors. Saponins are one of the largest secondary metabolites. They serve as a potential source of antibacterial and anticancer agents. The presence of saponin in the extracts of both plants could support antibacterial activity observed in this study.

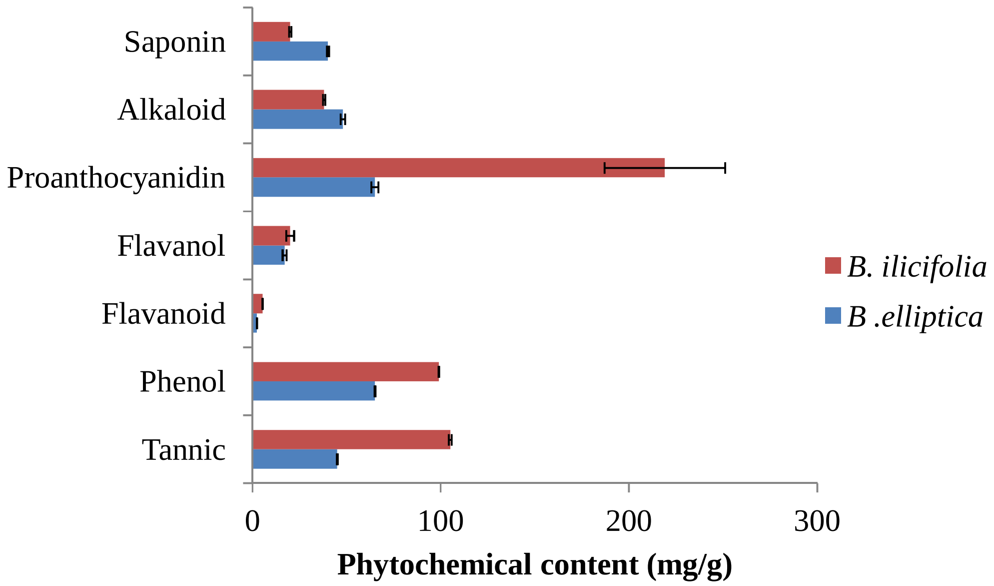


Figure 2: Phytochemical constituents identified in the aqueous extract of *B. elliptica* and *B. ilicifolia*. Each value represents mean ± S.D (n=3)

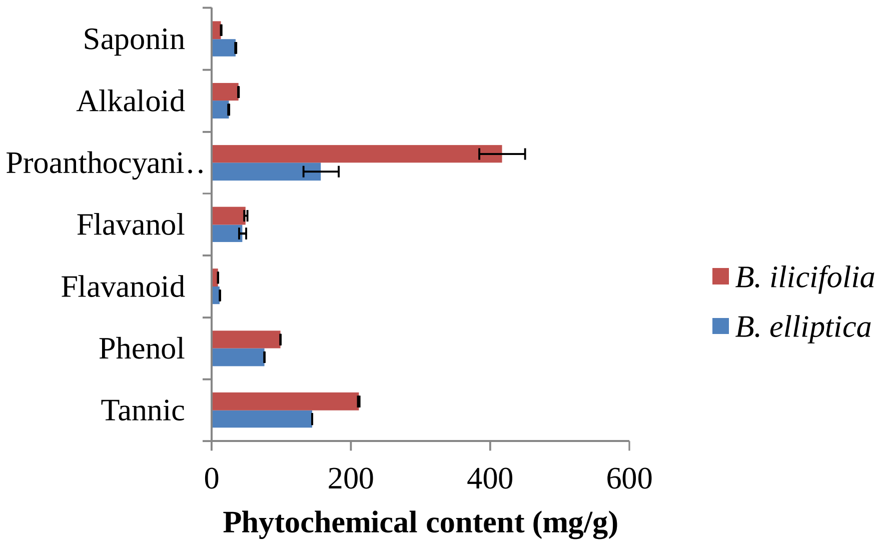


Figure 3: Phytochemical constituents identified in the ethanol extract of *B. elliptica* and *B. ilicifolia*. Each value represents mean ± S.D (n=3).

### 2.3.2 Antioxidant activity

### 2.3.3 DPPH radical scavenging activity

DPPH radical is commonly used as a model to investigate the scavenging ability of various natural compounds such as tannins, phenolics or crude extract of plants (Veerapur *et al*., 2009). This method is based on the reduction of the methanol DPPH solution in the presence of hydrogen donating antioxidant due to formation of the non radical form DPPH-H. The results of DPPH radical scavenging activity of the aqueous extract of both plants and reference compounds (Vitamin C and BHT) are presented in figure 4. The results of the aqueous extracts showed that *B. elliptica* have a higher scavenger activity than that of *B. ilicifolia* at all concentrations. At 0.2 mg/ml *B. elliptica* (87.16% ± 0.97) showed stronger inhibitory activity in removing the DPPH radical from the reaction system than *B. ilicifolia* (81.02% ± 1.94). Nevertheless, none of the extract evaluated here showed an activity that was as potent as that of vitamin C and BHT used as reference compounds in this study. It was also observed that *B. elliptica, B. ilicifolia,* vitamin C and BHT had DPPH scavenging activity with $IC_{50}$ values of 0.61, 0.61, 0.52 and 0.56 mg/ml respectively. Scavenging of DPPH radical in this study showed the effectiveness of both plants in donating a hydrogen proton to the lone pair electron of the radical. This could be suggested that both plants contain compounds capable of donating protons to the free radicals. This method has confirmed the efficacy of both plant extracts in a concentration dependent manner (Mondal *et al*., 2005).

Figure 5 illustrates the DPPH radical scavenging activity of the ethanol extract of *B. elliptica* and *B. ilicifolia* compared with vitamin C and BHT. The DPPH radical scavenging activity of *B. elliptica* was significantly higher ($P<0.05$) than that of *B. ilicifolia* within the concentration range of 0.4 to 1.0 mg/ml (Figure 5). The result also showed that both plant extracts and the standards (Vitamin C and BHT) acted in a concentration dependent manner. However, comparable scavenging activities of the ethanol extract of both plants were observed with those of reference compounds (Vitamin C and BHT).The $IC_{50}$ values of *B. elliptica, B. ilicifolia* and that of vitamin C and BHT were 0.57, 0.59, 0.53 and 0.60 mg/ml respectively. This result has proven the antioxidant effectiveness of both plants extracts in a dose dependent manner. Therefore, the results obtained from aqueous and ethanol extracts of *B. elliptica* and *B. ilicifolia* may partially justify the folkloric use of these plants as a potential source of natural antioxidant agent against radical related diseases.

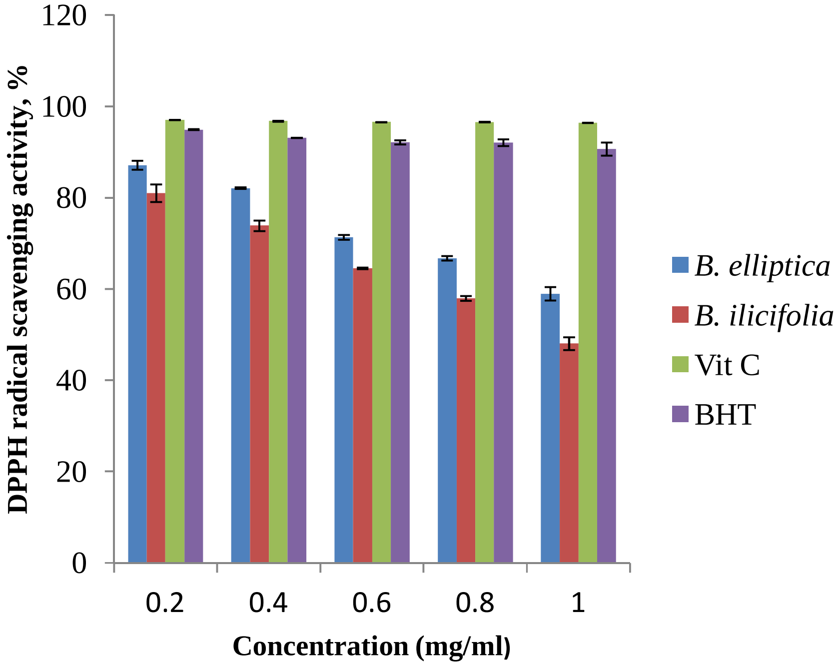


Figure 4: DPPH radical Scavenging activity of the aqueous extract of *B. elliptica* and *B. ilicifolia*. Each value represents mean ± S.D (n =3)

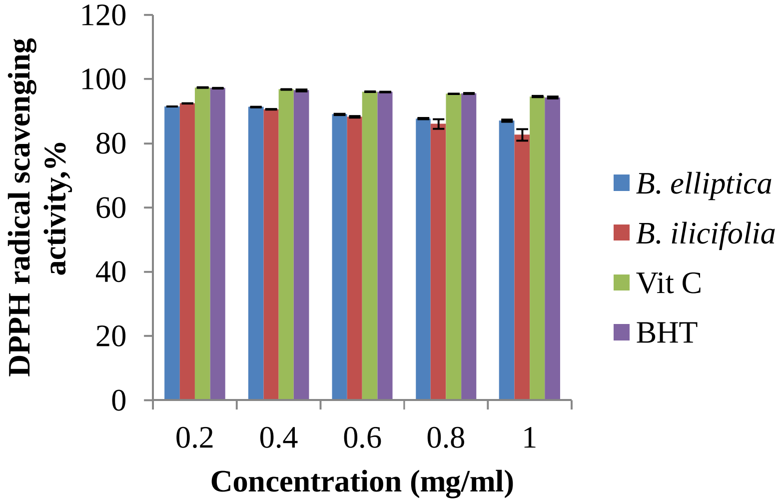


Figure 5: DPPH radical Scavenging activity of the ethanol extract of *B. elliptica* and *B. ilicifolia*. Each value represents mean ± S.D (n =3)

### 2.3.4 Reducing power

The reducing ability of the aqueous extracts of the leaf of *B. elliptica* and *B. ilicifolia* were estimated from their ability to reduce $Fe^{3+}$ to $Fe^{2+}$. This was detected by a change from the yellowish color of the test solution to different shades of green and blue depending on the concentration of plant extracts. The dose-response curves for the reducing powers of aqueous extract compared with standards are shown in figure 6. The results showed that *B. elliptica* has a stronger reducing potential at all tested concentrations compared to *B. ilicifolia*. The result also showed that both plant extracts and the reference compounds (vitamin C and BHT) had dose dependent activity. In addition, it was also observed that reducing ability of *B. elliptica* and *B. ilicifolia* aqueous leaf extracts was significantly greater than that of vitamin C but the scavenging effect was lesser than that of BHT (97.80% ± 0.02). The $IC_{50}$ of *B.elliptica*, *B. ilicifolia*, vitamin C and BHT were 0.67, 0.60, 0.75 and 0.62 mg/ml respectively. The reducing ability of a compound usually relies on the presence of reductants which show antioxidative potential by breaking the free radical chain and donating a hydrogen atom. The observed reducing capacity of both plant extracts might be due to the presence of polyphenolic compounds such as tannin, flavanoid, phenol and flavanol (Ebrahimzadeh *et al*., 2010; Yang *et al*.,2001). These results corroborated previous work which reported that reducing power of plant extracts correlated with the phenolic content (Park and Jhon, 2010; Hashasa, *et al.*,2010). Therefore, This result may partially support the folkloric use of these plants as a natural antioxidant for the treatment of oxidative stress caused diseases.

In this study, it was observed that ethanol extract of *B. elliptica* showed a stronger reducing ability than that of *B. ilicifolia* at all tested concentrations (Figure 7). The result also showed that that the activity both plant extracts and standard or reference compounds decreased with increasing concentration (Figure 7). The reducing ability of both plant extract was significantly higher than that of the reference compounds (vitamin C and BHT) used in this study. The $IC_{50}$ values of *B. elliptica*, *B. ilicifolia*, vitamin C and BHT were 0.57, 0.59, 0.53 and 0.60 mg/ml respectively. These results showed that extracts of both plants had a good reducing potential when compared with the standards. Similar observation were made in the finding of Otang *et al.* (2012) who reported the reducing power of *P. viridiflorum* (bark) and *G. bicolor* (leaf) were greater than vitamin C. Therefore, it could be inferred from this study that *B. elliptica* and *B. ilicifolia* aqueous and ethanol leaf extracts could serve as a source of natural antioxidant against pathological disease related to free radical.

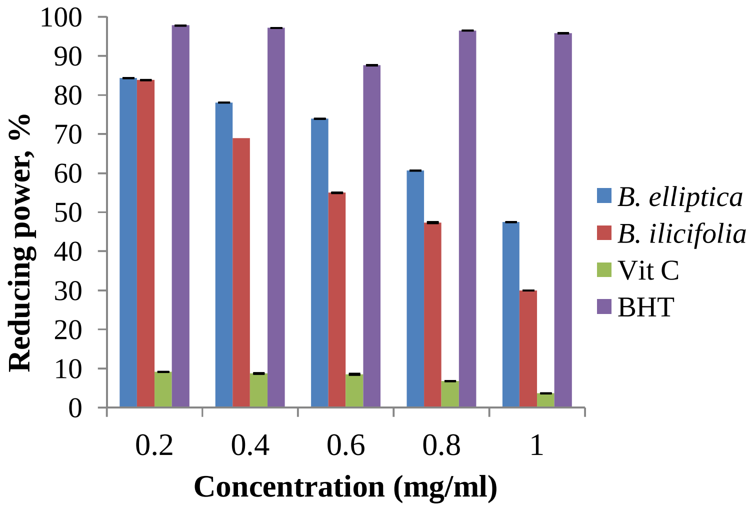


Figure 6: Reducing power activity of the aqueous extract of *B. elliptica* and *B. ilicifolia*. Each value represents mean ± S.D (n=3).

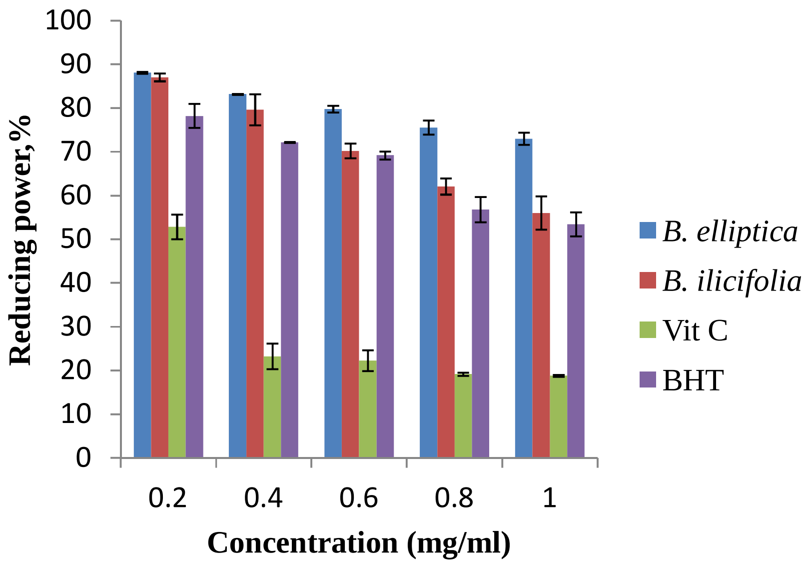


Figure 7: Reducing power activity of the ethanol extract of *B. elliptica* and *B. ilicifolia***.** Each value represents mean ± S.D (n=3).

### 2.3.5 ABTS radical scavenging activity

ABTS is a compound used to measure antioxidant potential of medicinal plants based on the decolorization of blue chromophore of ABTS radical formed by the reaction of ABTS and sodium persulphate. The scavenging activity of ABTS of *B. elliptica* and *B. ilicifolia* aqueous leaf extracts was found to be extremely high (Figure 8). At a concentration of 0.6 mg/ml, *B. elliptica* (97.27% ± 0.09) and *B. ilicifolia* (97.05 ± 0.02) showed a similar strong activity, but it was significantly different ($P < 0.05$) when compared with that of vitamin C (99.87% ± 0.01) and BHT (99.95 ± 0.01). The results also show that the ABTS radical scavenging activity of both plants and that of standards act in a concentration dependent manner. The $IC_{50}$ values of *B. elliptica*, *B. ilicifolia*, vitamin C and BHT were 0.61, 0.58, 0.59, 0.57 mg/ml respectively. However, the capability of the plants extract to scavenge DPPH radical also reflects its ability to scavenge $ABTS^{+}$. These results corroborated with the finding of Wintola and Afolayan, (2011) who reported that compounds with higher amounts of polar solvents have the ability to scavenge both ABTS and DPPH radicals as compared with non polar solvent (Bushra *et al*., 2009) Therefore, it could be deduce from this study that aqueous leaf extracts of both plants may be useful therapeutic agents for treating radical-related diseases.

The results from this assay showed that ethanol extracts of both plants are very efficient scavenger of ABTS radicals. There was no significant differences between the scavenging ability of extract of *B. elliptica* and *B. ilicifolia* but the scavenging effect was lesser than the reference compounds used in this study (Figure 9). The results also showed concentration dependent decrease in ABTS radical scavenging activity. The $IC_{50}$ values of *B. elliptica* (0.55 mg/ml), *B. ilicifolia* (0.34 mg/ml) showed a comparative activity with vitamin C (0.54 mg/ml) and BHT (0.61 mg/ml) which is a derivative of phenolic compounds. Some factors like solubility of the extract or stereoselectivity of the radicals in different testing system have been reported to affect the capability of the extracts to quench or react with different radical system (Yul *et al*., 2002). Wang *et al.* (1998) found that some compound which inhibited ABTS radical did not show DPPH scavenging property. In this study the aqueous and ethanol leaf extracts of both plants depicted strong scavenging activities against both radicals (ABTS and DPPH) in different system indicating that they may be useful therapeutic agent for treating pathological disease emanating from oxidative stress.

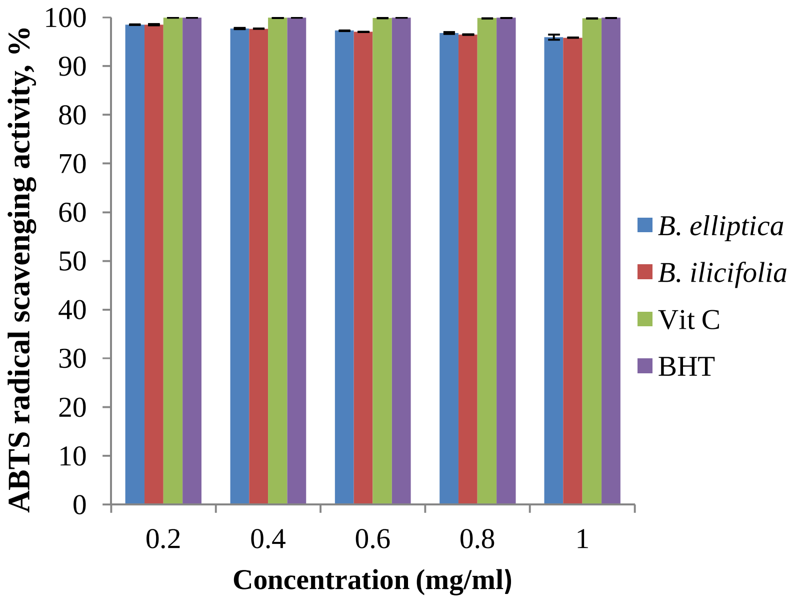


Figure 8: ABTS radical scavenging activity of the aqueous extract of *B. elliptica* and *B. ilicifolia*. Each value represents mean ± S.D (n =3)

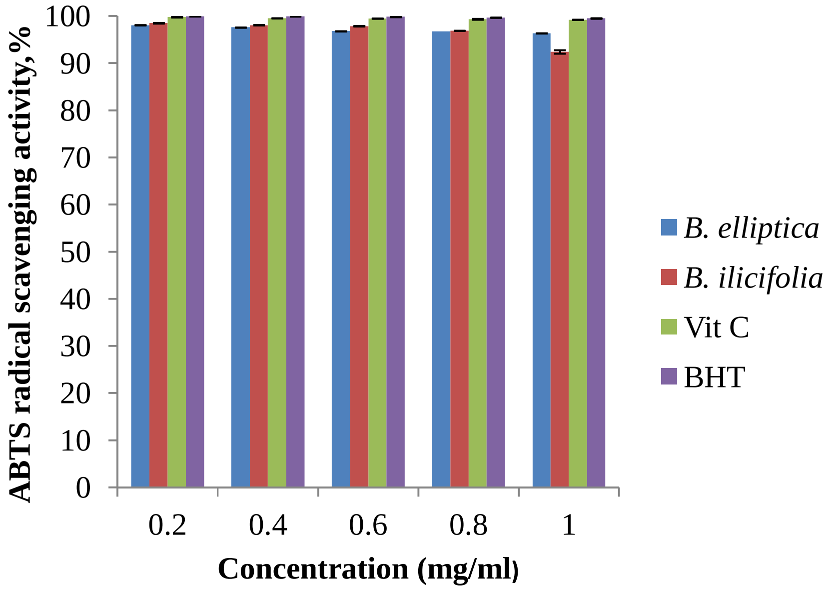


Figure 9: ABTS radical scavenging activity of the ethanol extract of *B. elliptica* and *B. ilicifolia*. Each value represents mean ± S.D (n =3).

### 2.3.6 Nitric oxide radical scavenging activity

Nitric oxide has been involved in inflammation and pathogenesis of different human ailments, for example cardiovascular disease and cancer (Li and Forstemann, 2000). In this study, the aqueous extract of *B. ilicifolia* significantly scavenged NO production from sodium nitroprusside when compared of *B. elliptica* at all tested concentrations (Figure 10). The extracts of both plants and standards (vitamin C and BHT) showed a concentration dependent decrease in NO radical scavenging activity. At 0.6 mg/ml vitamin C (85.08% ± 0.05) and *B. ilicifolia* (78.60 ± 0.03) show strong inhibitory activity when compared with that of *B. elliptica* (66.81% ± 0.21) and BHT (75.39% ± 0.04). The $IC_{50}$ values for *B. elliptica* (0.58 mg/ml), *B. ilicifolia* (0.34 mg/ml) also show comparative activity with vitamin C (0.59 mg/ml) and BHT (0.55 mg/ml). In this study, the results showed that both plants extract inhibit formation of nitric oxide by directly competing with oxygen during its reaction with nitric oxide.

In this study, the ethanol extract of *B. elliptica* showed a concentration dependent decrease in nitric oxide scavenging activity that reached a minimum at a concentration of 0.2 mg/ml and increased thereafter (Figure 11). At a concentration of 0.4 mg/ml, *B. ilicifolia* (84.81% ± 0.07) showed a strong inhibitory NO radical scavenging activity when compared with *B. elliptica* (80.45% ± 0.03), vitamin C (83.66% ± 0.02) and BHT (82.06% ± 0.07). In addition, it was also observed that the $IC_{50}$ values of *B. elliptica*, *B. ilicifolia* and the reference compounds (vitamin C and BHT) were 0.50, 0.66, 0.59 and 0.70 mg/ml respectively. These results corroborated with the findings of Otang *et al.* (2012) but contradict the report of Omoruyi *et al* (2012). Therefore, NO (nitric oxide) radical scavenging ability of aqueous and ethanol extracts of these plants may possibly assist to stop the chain reactions instigated by excessive production of nitric oxide and play a role in preventing pathological diseases emerge from oxidative stress.

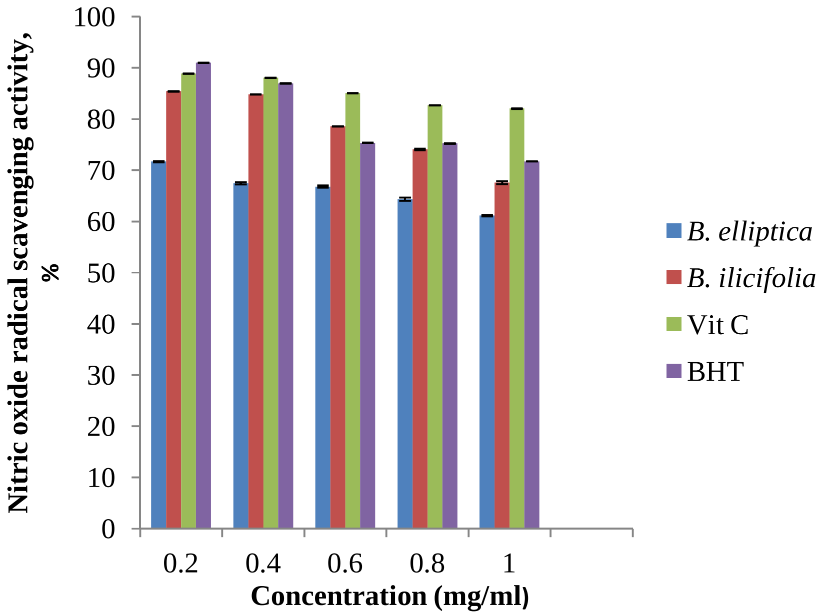


Figure 10: Nitric oxide radical scavenging activity of the aqueous extract of *B. elliptica* and *B. ilicifolia*. Each value represents mean ± S.D (n=3).

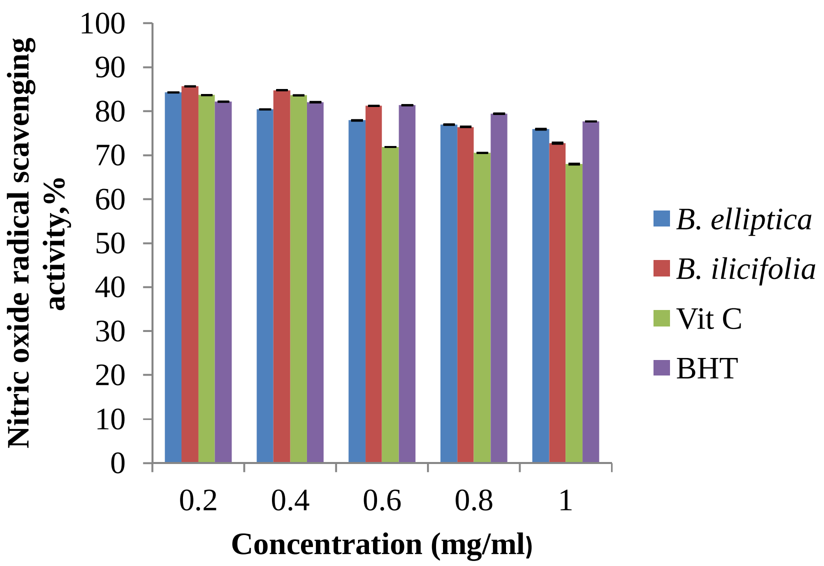


Figure 11: Nitric oxide radical scavenging activity of the ethanol extract of *B. elliptica* and *B. ilicifolia*. Each value represents mean ± S.D (n=3).

### 2.3.7 Hydrogen peroxide radical scavenging activity

Hydrogen peroxide is an important reactive oxygen species due to its strong ability to penetrate biological molecules. It is formed as a result of superoxide radical production by the action of superoxide dismutase and which is later converted to hydroxyl radical (OH) by the action of glutathione and catalase in the presence of copper or iron. Among the oxygen radicals, the hydroxyl radical (OH) is the most reactive radical capable of damaging macromolecules such as carbohydrates, proteins and lipids in the biological system (Sakanaka *et al.*, 2005). Therefore, eradication of this radical in order to defend the body system from invading agent is very important. In this study, it was also observed that the aqueous extract of *B. ilicifolia* demonstrated a significant hydrogen peroxide scavenger activity when compared with that of the *B. elliptica* aqueous leaf extracts at all the tested concentrations (Figure 12). The results also showed a concentration dependent decrease in hydrogen peroxide scavenging activity that reached a minimum at a concentration of 0.2 mg/ml. The aqueous extract of both plants and reference compounds recorded high inhibitory activities over a range of concentrations tested. The $IC_{50}$ values of 0.61, 0.57, 0.64 and 0.63 mg/ml were recorded for *B. elliptica*, *B. ilicifolia*, vitamin C and BHT respectively.

The scavenging potential of ethanol leaf extracts of *B. elliptica* and *B. ilicifolia* against hydrogen peroxide are presented in figure 13 using vitamin C and BHT as reference compounds. The result showed that the extract of *B. elliptica* had a strong potential to eradicate hydrogen peroxide scavenging activity as compared with *B. ilicifolia* at all tested concentration, but *B. elliptica* and *B. ilicifolia* showed a dose reduction in hydrogen peroxide scavenging activity which was lower than the reference compounds (vitamin C and BHT). The $IC_{50}$ values of *B. ilicifolia* (0.57

mg/ml) significantly high ($P < 0.05$) when compared with *B. elliptica* (0.60 mg/ml), BHT (0.63 mg/ml) and vitamin C (0.64 mg/ml). The ability of ethanol extracts to scavenge hydrogen peroxide radical has been reported by Wintola and Afolayan (2011). These results showed that aqueous and ethanol leaf extracts of *B. elliptica* and *B. ilicifolia* have the ability to scavenge harmful hydrogen peroxide radical.

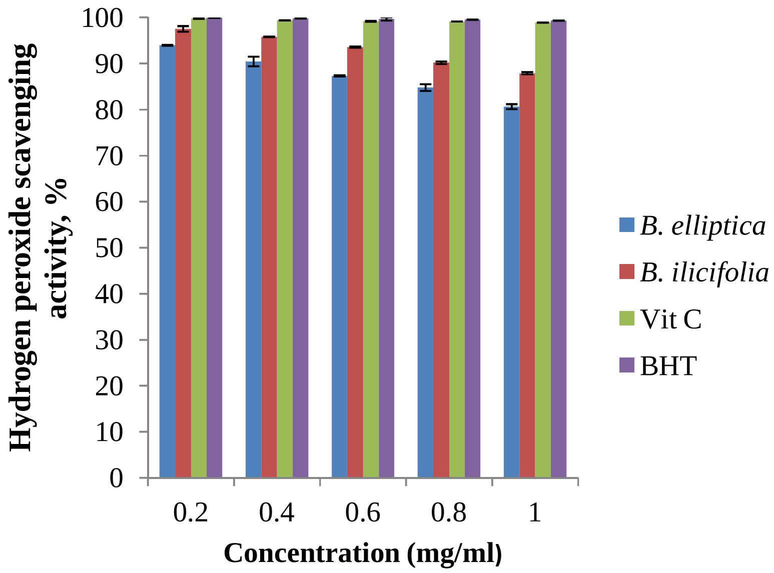


Figure 12: $H_2O_2$ radical scavenging activity of the aqueous extract of *B. elliptica* and *B. ilicifolia*. Each value represent mean ± S.D (n=3).

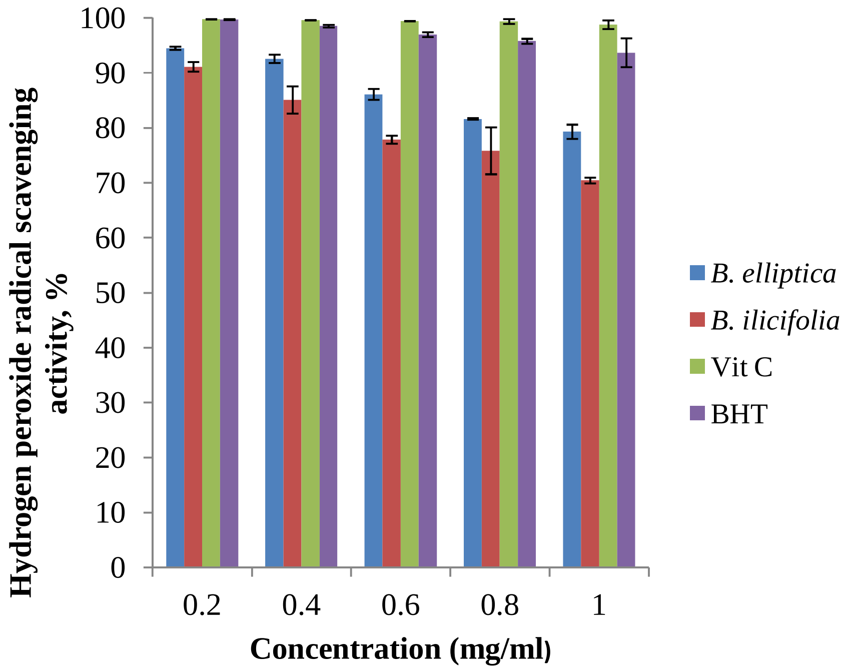


Figure 13: $H_2O_2$ radical scavenging activity of the ethanol extract of *B. elliptica* and *B. ilicifolia*. Each value represent mean ± S.D (n=3).

**2.3.8 Antibacterial assay**

The results of the effect of aqueous and ethanol extracts of *B. elliptica* and *B. ilicifolia* tested on 5 bacterial strains associated with diabetic infection using serial dilution of 5 to 0.3 mg/ml are shown in Table 1. The aqueous extracts of *B. ilicifolia* was found to be inactive in all tested 5 bacterial strains but *B. elliptica* showed moderate antibacterial activity against *Pseudomonas aeruginosa* with MIC value of 5 mg/ml but not effective against *Staphylococcus aureus*, *Streptococcus pyrogenes*, *Proteus vulgaris* and *Proteus mirabilis.* Similar observations were made in the finding of Kuduru *et al.* (2006) who reported no inhibitory effect of a water extract of the fruit and leaf of *S. aculeastrum* on *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* using agar dilution methods.

The ethanol extract of *B. elliptica* and *B. ilicifolia* showed strong antibacterial activity against *Pseudomonas aeruginosa* and *Streptococcus pyrogenes* with MIC values of 2.5 and 2.5 mg/ml respectively. The results also show that both plants exhibited effective antibacterial activity against *Proteus mirabilis* with MIC values of 5 mg/ml. The ethanol extracts of *B. elliptica* was found to be inactive in *Staphylococcus aureus* but activity was found when tested with *B. ilicifolia* with MIC value of 5 mg/ml. There was no significant antibacterial activity against *Proteus vulgaris* when tested with the ethanol extract of *B. ilicifolia* but activity was present in *B. elliptica* with MIC value of 5 mg/ml. It has been reported that Gram-negative bacteria are more resistant to anti-microbial agents than Gram-positive bacteria due to the possession of multilayered structure of Gram-negative which are not found in Gram-positive bacteria. These results show that growth of two out of three gram negative bacteria used in this study were inhibited by the ethanol extracts of both plants. The findings on *P. aeruginosa* and *Proteus vulgaris* corroborated with the report of Uma and Parvathavarthini, (2010) on hexane extract of sea urchin, *Temnopleurus alexandri*. This result on *Staphylococcus aureus* concurred with the finding of Adedapo *et al.* (2008) who reported the antibacterial properties of methanol extracts of the leaves and stem of *Calpurina aurea* but contradicts the MIC value obtained on *Streptococcus pyogenes*. The observed antibacterial activities of *B. elliptica* and *B. ilicifolia* against some bacteria associated with wounds in diabetic patient could be due to the presence of bio-active compounds. Flavonoids, tannin, alkaloid and polyphenols compounds have been reported to possess antibacterial properties and this could be responsible for this observation (Oyedemi *et al*., 2010). These results suggest that the ethanol extracts of *B. elliptica* and *B. ilicifolia* leaves can be an effective herbal remedy for treatment of wound infection caused by these bacteria in diabetic patients

Table 1: Minimum inhibitory concentration (mg/ml) of *B. elliptica* and *B. ilicifolia* aqueous and ethanol leaf extracts

| | | Aqueous | | Ethanol | | | |
|---|---|---|---|---|---|---|---|
| Bacteria | Gram (+/-) | *B. elliptica* | *B. ilicifolia* | *B. elliptica* | *B. ilicifolia* | Amoxi[a] | Cipro[b] |
| *Staphylococcus aureus* | + | na | na | 5 | 5 | 0.6 | 0.3 |
| *Streptococcus pyrogenes* | + | na | na | 2.5 | 2.5 | 0.6 | 0.3 |
| *Proteus vulgaris* | - | na | na | 5 | na | 0.6 | 0.3 |
| *Pseudomonas aeruginosa* | - | na | 5 | 2.5 | 2.5 | 0.6 | 0.3 |
| *Proteus mirabilis* | - | na | na | 5 | 5 | 0.6 | 0.3 |

na = not active; [a]Amoxicilin; [b]Ciprofloxacin

## 2.4 CONCLUSION

From the results of this study it could be concluded that aqueous and ethanol leaf extracts of *B. elliptica* and *B. ilicifolia* contain bioactive components and found to have strong antioxidant activities. The antibacterial properties of aqueous of both plants are not as effective as the ethanol leaf extracts.

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# CHAPTER 3

## GAS CHROMATOGRAPHY-MASS SPECTROMETRY (GC-MS) ANALYSIS OF BIOACTIVE COMPOUNDS FROM THE LEAVES OF *BRACHYLAENA ELLIPTICA* AND *BRACHYLAENA ILICIFOLIA*

CHAPTER 3

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# CHAPTER 3

## 3.1 INTRODUCTION

Extracts and essential oils obtained from many plants have recently gained popularity and scientific interest. Essential oils have been used for different purposes, such as food, drugs and perfumery from ancient times (Ates and Erdogrul, 2003). They are natural product obtained from plants through various extraction methods such as microwave assisted distillation, hydrodistillation, steam distillation and organic solvent extraction. Nevertheless, the properties of the essential oils extracted through these methods have been found to vary depending on the method. It has been reported that essential oils of plant origin exhibit a broad spectrum of biological activities. Jeong *et al.* (2009) reported that *Cymbopogon citrates* (DC) Stapf is a source of essential oil widely used as a component of ethnopharmaceuticals in tropical and subtropical countries. The Majority of individuals who use essential oils from plants are less prone to contract infectious disease such as wound, inflammation, skin sores and bleeding (Nicolas *et al*., 2013). However, oil users who at some point contract an infectious disease such as fungal infection, wound, syphilis and leprosy trend to improve faster than those using antibiotics (Panahi *et al*., 2012).

In South Africa, essential oils are commonly used to protect food against the growth of microorganisms. In this manner numerous of these essential oils from plants are cheaply circulated and sold in the local market center because of expanded requests (Otang *et al*., 2011).

*Brachylaena elliptica* and *Brachylaena ilicifolia* belongs to the family of Asteraceae. This family is an important source of essential oil with biological activities such as antibacterial, antidiabetic. The leaves of both plants which are intensely bitter are used traditionally to treat diabetes

(Deutshlander, 2009). At the beginning of this study ethanol leaf extracts of both plants have proven effective against wound infecting bacterial in diabetic patients. Taking into consideration of the medicinal value of these plants, the essential oil of the leaves of *B. elliptica* and *B.ilicifolia* were analysed for the first time using GC-MS. This study will help to identify the compounds of theurapeutic value. GC-MS is the best technique used to identify various bioactive constituents of alcohols, esters, alkaloids, amino acid, long chain hydrocarbons and phenolic compounds (Muthulashmi *et al*., 2012).

## 3.2 MATERIALS AND METHODS

### 3.2.1 Collection of plant material

The leaves of *B. elliptica* and *B. ilicifolia* were collected as described in section 2.2.2

### 3.2.2 Extraction of essential oil

Volatile oil from the fresh leaves (30 g) of both plants were extracted separately for 3 h using a hydro-distiller in a Clevenger's-type apparatus in accordance with the British Pharmacopeia specifications (1980). The oils were collected and analyzed immediately.

### 3.2.3 GC-MS analyses

The GC-MS analysis of the oils were performed using Agilent 6890 GC coupled to Agilent 5975 MSD with a Zebron-5MS column (ZB-5MS 30 m x 0.25 mm x 0.25 um) (5%-phenylmethylpolysiloxane). GC grade helium was used as a carrier gas at a flow rate of 2 mL/min; splitless 1 μL injections were used. Injector temp 280$^{o}$C, source temp 280$^{o}$C, Oven temperature was 70$^{o}$C, the ramp was 15$^{o}$C/min. to 120$^{o}$C, then 10$^{o}$C/min to 180$^{o}$C, then 20$^{o}$C/min. to 270$^{o}$C and held for 3 minutes.

### 3.2.4 Identification of components

Identification of the components of each essential oil was accomplished by matching their mass spectra and retention indices with those of Wiley 275 library (Wiley, New York) in the computer library (Joulain *et al*., 2001). The yield of each component was calculated per g of the plant material, while the composition was calculated from the summation of the peak areas of the total oil composition. The whole experiment was done in triplicate.

### 3.2.5 Calculation of oil yield

Prior to the final extraction and obtaining of the oil, a clean bottle of known mass was made available. At the end of extraction process, the oil obtained was carefully transferred into the bottle and final mass noted. The yield was obtained using the following equation:

Percentage (%) yield = [(B- A) ÷ X] 100 where Mass of plant material distilled (g) = X; Mass of empty bottle (g) = A; Mass of bottle + oil extracted = B; Mass of oil (g) = (B-A)

## 3.3 RESULTS AND DISCUSSION

The essential oil yields were 1.7% and 4.7% for *B. elliptica* and *B. ilicifolia*, respectively. A light pale yellowish liquid with pungent garlic like odour was produced. Fourteen compounds were identified in the essential oil of *B. elliptica* (Figure 14). The prominent compounds were oxalic acid, cyclohexylmethyltridecylester with peak area of 11.39% followed by butylated hydroxytoluene (3.97%), τ –muurolol (3.47%), caryophyllene oxide (2.73%), δ-cadinene (1.45%), eucalyptol (1.16%), 1R-.alpha.-pinene (1.05%), damascenone (1.02%), pyrazine, methoxy (0.79%), squalane (0.75%), α-calacorene (0.70%), hexadecanoic acid,15-methyl-,methylester (0.30%), benzoic acid,2.5-bis (trimethylsiloxy)-,trimethylsilyl ester (0.55%), and phenol,2,5-dichloro-4-methoxy (0.27%) (Table 2).

In the case of *B. ilicifolia*, seven compounds were identified (Figure 15). The major compounds of the oil were oxalic acid,cyclohexylmethyltetradecylester (7.87%), butylated hydroxytoluene (2.22%), caryophyllene oxide (0.54%), santolina triene (0.29%) carvone oxide, cis (0.27%), 9-borabicyclo [3.3.1] nonane,9- hydroxyl- (0.12%) and benzoic acid,2-[(trimethylsilyl)oxy]-,trmethylsilyester (0.08%) (Table 3). The GC analysis also indicated the presence of a higher percentage of ester (12.24%), followed by oxygenated sesquiterpenes (6.2%), phenol compounds (4.24%) and monoterpenes (3.23%), in the essential oil of *B. elliptica* than those in *B. ilicifolia* (Table 4). However, sesquiterpenes and the heterocyclic aromatic class of compounds were not found in the essential oil of *B. ilicifolia* but detected at a level lower than 3% in the oil of *B. elliptica.* These differences could be attributed to several factors such as species differences, climate, soil or season. However, there were no reports on the variation of the volatile oil composition with the phonologic stage. Among the identified phytochemicals, butylated hydroxytoluene and phenol, 2, 5-dichloro-4-methoxy may be employed as an antioxidant, antidiabetes and antibacterial activity (Duke, 2012) (Table 5). Compounds such as hexadecanoic acid, 15-methyl-,methyl ester(0.30%), benzoic acid,2,5-bis(trimethylsiloxy)-,trimethylsilyl ester (0.55%) oxalic acid, cyclohexylmethyltridecyl ester (11.39%) and benzoic acid,2-[(trmethylsilyyl)oxy]-, trimethylsilylester (0.08%) were observed as ester, such essential oil containing fatty acid may be an active antimicrobial (Duke, 2012).

Most volatile components analysed from plant essential oils are largely composed of terpenes (Omoruyi *et al*., 2014). Terpenes are well-known to have strong biological activities and they are involved in plant protection. (Micheal *et al.,* 2013). Martins *et al*. (2010) reported that intake of terpenes can reduce toxins from kidney and liver in the body. In this study *B. elliptica* oil was largely composed of monoterpenes dominated by 3 compounds, 1R-.apha.-pinene, eucalyptol and damascenone. Such essential oils, containing monoterpenes as their major constituents are known for pharmacological activities (Harney *et al.,* 1978; Gherlardini *et al.,* 2001). Santolina triene, one of the major monoterpenes observed in the essential oil of *B. ilicifolia* was found to possess antibacterial activity (Duke 2012). Plant essential oil containing sesquiterpenes have been used for treatment of allergies and inflammation (Enzo, 2011; Akram *et al*., 2011). It has been reported that people who use oil containing sesquiterpenes consistently, have a higher level of resistance to illness than the average person. (Martins *et al*., 2010). Oxygen-containing sesquiterpenes have apparent antimicrobial, tranquilizing action and beneficial to various systems and metabolic processes in human organism (Gurib, 2006). Compounds such as caryophyllene oxide, was detected in high amounts in the essential oil of both plants. Phenols, monoterpenes, threeterpenes, sesquiterpenes and fattyacid ester have been reported to possess antioxidant and antimicrobial activites (Chokoe *et al.,* 2008; Zsuzsanna *et al.,* 2010). These results support the finding of previous studies (Euphorbiaceae *et al.,* 2013; Mamza *et al.,* 2012; Omoruyi *et al.,* 2014). The GC/MS analysis revealed that the essential oil of *B. elliptica* and *B. ilicifolia* leaves are composed of phenolic, terpenes and fatty acid ester. These phytochemicals are responsible for various pharmacological actions like antioxidant, antimicrobial and inflammation activities. However, isolation of individual phytochemical constituents may proceed to find a novel drug or a leading compound.

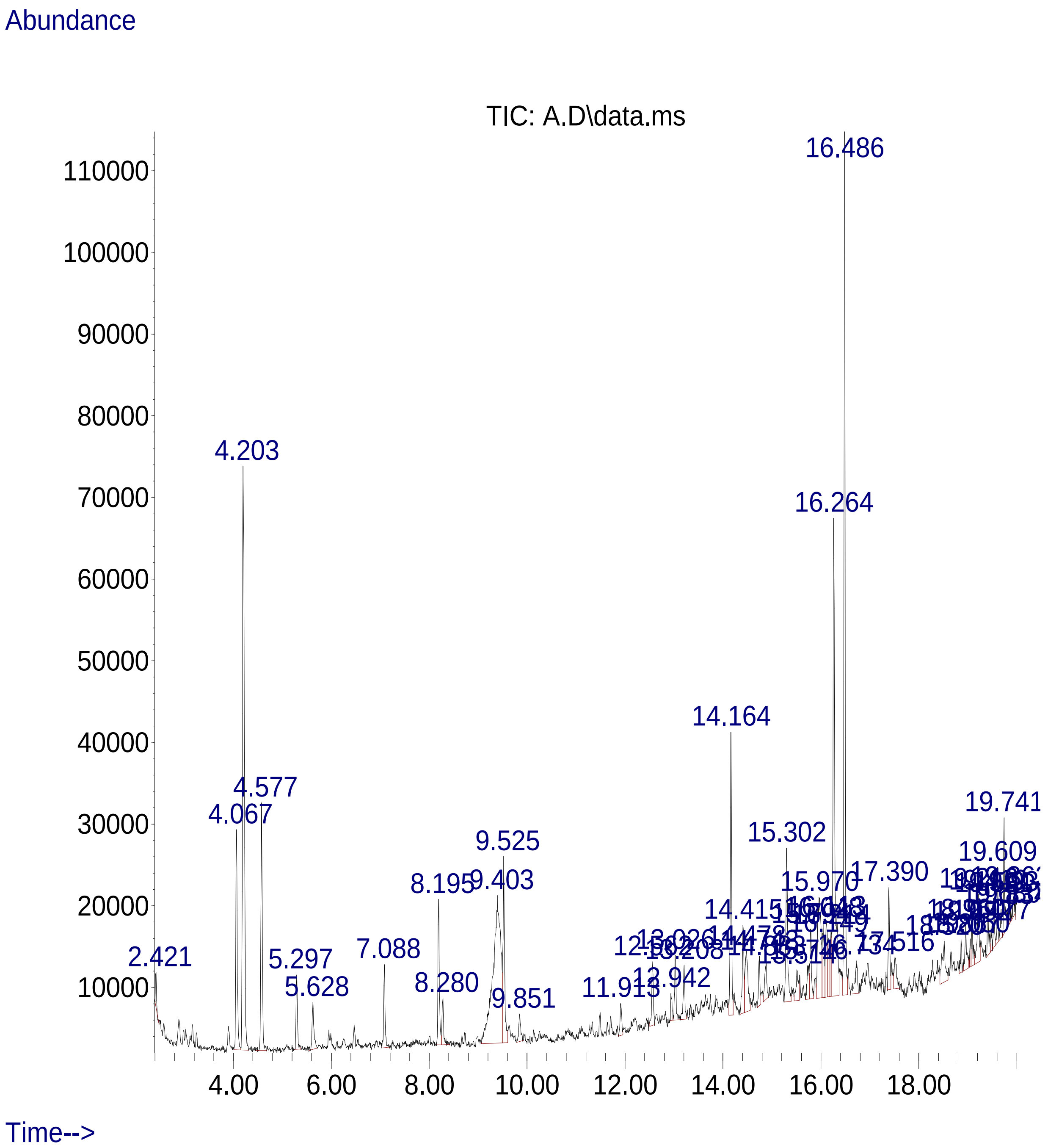


Figure 14: GC-MS chromatogram of essential oil of *B. elliptica*

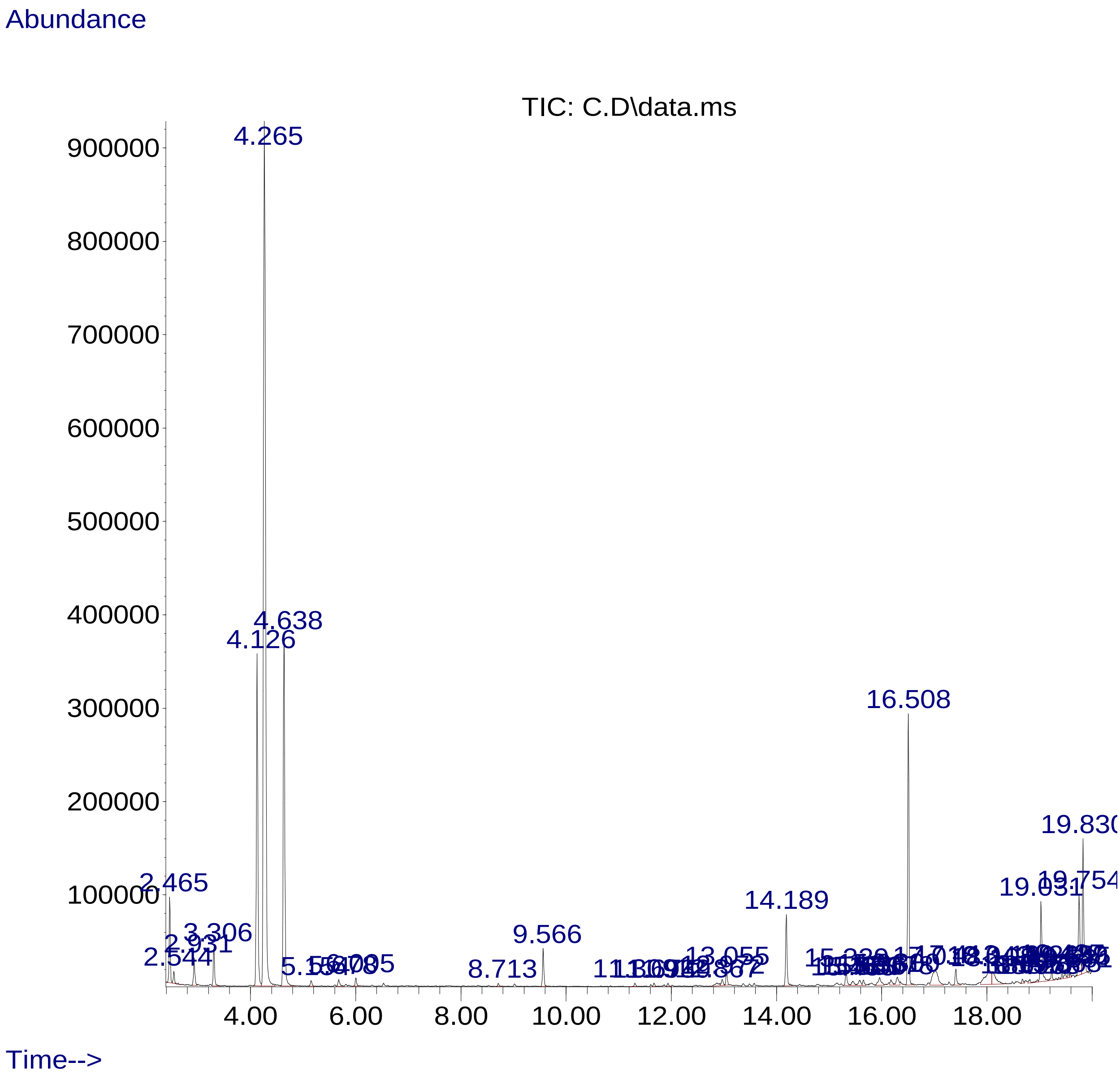


Figure 15: GC-MS chromatogram of essential oil of *B. ilicifolia*

Table 2: Chemical composition of *B. elliptica* essential oil determined by GC-MS

| S/N | RT (min) | Compounds | Peak (%) | Molecular formular | MW | Hit quality |
|---|---|---|---|---|---|---|
| 1 | 5.299 | 1R-.alpha.-pinene | 1.05 | $C_{10}H_{16}$ | 136 | 87 |
| 2 | 7.084 | Eucalyptol | 1.16 | $C_{10}H_{18}O$ | 154 | 70 |
| 3 | 12.56 | Damascenone | 1.02 | $C_{13}H_{18}O$ | 190 | 74 |
| 4 | 14.42 | δ-Cadinene | 1.45 | $C_{15}H_{24}$ | 204 | 93 |
| 5 | 14.74 | α-Calacorene | 0.7 | $C_{15}H_{20}$ | 200 | 92 |
| 6 | 15.3 | Caryophyllene oxide | 2.73 | $C_{15}H_{24}O$ | 220 | 64 |
| 7 | 15.97 | τ -Muurolol | 3.47 | $C_{15}H_{26}O$ | 222 | 62 |
| 8 | 19.61 | Squalane | 0.75 | $C_{30}H_{50}$ | 410 | 70 |
| 9 | 14.86 | Pyrazine,methoxy | 0.79 | $C_9H_{14}N_{20}$ | 166 | 72 |
| 10 | 14.17 | Butylated hydroxytoluene | 3.97 | $C_{15}H_{24}O$ | 220 | 64 |
| 11 | 19.47 | Phenol,2,5-dichloro-4-methoxy- | 0.27 | $C_7H_6Cl_2O_2$ | 193 | 60 |
| 12 | 19.86 | Hexadecanoic acid, 15-methyl-,methyl ester | 0.3 | $C_{18}H_{36}O_2$ | 284 | 68 |
| 13 | 19.53 | Benzoic acid, 2,5-bis(trimethylsiloxy)-,trimethylsilyl ester | 0.55 | $C_{16}H_{30}O_4Si_3$ | 370 | 74 |
| 14 | 16.49 | Oxalic acid,cyclohexylmethyltridecyl ester | 11.39 | $C_{22}H_{40}O_4$ | 368 | 68 |
| | | Total compounds (%) | 29.6 | | | |

Table 3: Chemical composition *of B. ilicifolia* essential oil determined by GC/MS

| S/N | RT (min) | Compounds | Peak (%) | Molecular formular | MW | Hit quality |
|---|---|---|---|---|---|---|
| 1 | 5.679 | Santolina triene | 0.29 | $C_{10}H_{16}$ | 136 | 91 |
| 2 | 15.326 | Caryophyllene oxide | 0.54 | $C_{15}H_{24}O$ | 220 | 64 |
| 3 | 14.192 | Butylated hydroxytoluene | 2.22 | $C_{15}H_{24}O$ | 220 | 96 |
| 4 | 16.507 | Oxalic acid, cyclohexylmethyltetradecyl ester | 7.87 | $C_{23}H_{42}O_4$ | 382 | 67 |
| 5 | 8.713 | Benzoic acid,2-[(trimethylsilyl)oxy]-,trimethylsilylester | 0.08 | $C_{13}H_{22}O_3SI_2$ | 282 | 70 |
| 6 | 19.487 | Carvone oxide, cis- | 0.27 | $C_{10}H_{14}O$ | 150 | 87 |
| 7 | 18.964 | 9-Borabicyclo[3.3.1]nonane,9-hydroxy- | 0.12 | $C_8H_{15}BO$ | 138 | 60 |
| | | Total compounds (%) | 11.4 | | | |

Table 4: Class composition of *B. elliptica* and *B.ilicifolia* essential oil

| Class of compound | *B. elliptica* (%) | *B. ilicifolia* (%) |
|---|---|---|
| Monoterpene | 3.23 | 0.29 |
| Triterpene | 0.75 | * |
| Sesquiterpene | 2.15 | * |
| Oxygenated sesquiterpenes | 6.2 | 0.54 |
| Phenolic | 4.24 | 2.22 |
| Ester | 12.24 | 7.95 |
| Heterocyclic Aromatic | 0.79 | * |
| Others | * | 0.39 |
| Total | 29.6 | 11.4 |

***** = Not detected

Table 5: Activity of phytocomponents identified in the essential oil from leaves of *B. elliptica* and *B. ilicifolia.*

| S/N | Name of the compound | Activity |
|---|---|---|
| 1 | 1R-.alpha.-Pinene | Antimicrobial |
| 2 | Eucalyptol | Antimicrobial |
| 3 | Damascenone | Antimicrobial |
| 4 | Butylated hydroxytoluene | Antioxidant,Antidiabetes, |
| 5 | δ-Cadinene | Antibacterial |
| 6 | α-Calacorene | Antidiabetes |
| 7 | Carvone oxide, cis | Antibacterial, Antineoplastic |
| 8 | Pyrazine,methoxy | Antibacterial |
| 9 | Caryophyllene oxide | Antimicrobial, Anticancer |
| 10 | τ -Muurolol | Antimicrobial, Anticancer |
| 11 | Oxalic acid,cyclohexylmethyltridecyl ester | Antimicrobial |
| 12 | Phenol,2,5-dichloro-4-methoxy- | Antioxidant, Antidiabetes, |
| 13 | Santolina triene | Antibacterial |
| 14 | Squalane | Antibacterial |
| 15 | Hexadecanoic acid, 15-methyl-,methyl ester | Antioxidant,Hypocholesterolemic, |
| 16 | Benzoic acid, 2,5-bis(trimethylsiloxy)-,trimethylsilyl ester | Antimicrobial |
| 17 | 9-Borabicyclo[3.3.1]nonane,9-hydroxy- | Antimicrobial |
| 18 | Benzoic,2-[(trimethylsilyl)]oxy-,trimethylsilyester | Antimicrobial |

Activity source: Dr Duke phytochemical and Ethnobotanical databases.

## 3.4 CONCLUSION

The present study is the first report of the GC-MS analysis of bioactive compound in the essential oil from the leaves of *B. elliptica* and *B. ilicifolia.* The presence of various bioactive compounds in the essential oil of both plants may partially justify their use for treatment of various ailments by traditional healers.

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# CHAPTER 4

# LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS) ANALYSIS OF BIOACTIVE COMPOUNDS EXTRACTED FROM THE LEAVES OF *BRACHYLAENA ELLIPTICA* AND *BRACHYLAENA ILICIFOLIA*

# CHAPTER 4

## LIQUID CHROMATOGRAPHY-MASS SPECTROMETRY (LC-MS) ANALYSIS OF BIOACTIVE COMPOUNDS EXTRACTED FROM THE LEAVES OF *BRACHYLAENA ELLIPTICA* AND *BRACHYLAENA ILICIFOLIA*

# CHAPTER 4

## 4.1 INTRODUCTION

At the beginning of this study, several biologically active compounds have been identified from *B. elliptica* and *B. ilicifolia*, although many questions still remain about their mode of action. Recent studies have focused on their antimicrobial and antioxidant property. Therefore the present study aimed at identifying bioactive components of these plants using LC-MS.

Liquid chromatography (LC-MS) is one of the best techniques used to increase sensitivity and accelerate analysis (Ackermann *et al*., 1996). Frequent improvements in liquid chromatography (LC-MS) interface technologies combined with powerful features for structure analysis, qualitative and quantitative, have resulted in a widened scope of application (Pavan *et al*., 2011).

## 4.2 MATERIAL AND METHODS

### 4.2.1 Collection of plant materials and preparation of extracts.

Collection of plant materials and preparation of extracts was as described in section 2.2.2

### 4.2.2 LC-MS analyses and identification of components

LC-MS analyses were performed using the Agilent LC-MS system (ABSCIEX Triple TOF, 5600) with the analyser and electron spray ionization source (ESI), source parameters were optimized to provide high sensitivity. The source parameters were: negative mode, gas temperature 600$^{o}$C, drying gas flow rate 0.5 mi/min, nebulizer pressure 50 psi, capillary voltage 5500v, separation was carried out by Ultra High Pressure Liquid Chromatography (UHPLC) using Shimadzu UFLCXR with a phenomenex kinetex 2.6 µm (100x2.1), column mobile phase used was A: water (5 Mm ammonium formate + 0.5% formic acid) and B (acetonitrile). The gradient program was : 5% B for starting condition, increased up to 95% B in 20 min, hold 25 min, how and decrease % B to 5% at the final step. The total run time was 30 min. Injection volume was 50 µl. The standardized collision energy was 35 ±15V. Identification of compounds was done with the aid of computer assisted evaluation of the resulting data by searching against the spectral library. The whole experiment was done in triplicate.

## 4.3 RESULTS AND DISCUSSION

Liquid Chromatography-Mass Spectrometry (LC-MS) has proved to be one of the best techniques provide complete information on target and non target compound in natural products (Wolfender *et al*., 2003; Singh *et al*., 2013). The LC-MS method allows the quantification of a large variety of common plant metabolites in a single chromatogram. The Components present in the aqueous and ethanol extracts of *B. elliptica and B. ilicifolia* leaf were identified by LC/MS coupled with their retention time (RT), molecular formular, molecular weight (MW) and nature of the compound (Table 6 and 7). Three compounds were identified in the aqueous leaf extracts of *B. elliptica* while four compounds were identified in *B. ilicifolia* leaf extracts. The prominent compounds were: Nerol (monoterpenoid), (-)- carvone (monotepenoid), fenchol (terpene), geraniol (monoterpenid), ibogaine (alkaloid) and enoxacine (quinolones) (Table 6). All of these identified compounds have been reported to have various biological activities especially antidiabetes and antimicrobial activity. Aggarwal *et al*., (2002) and Friedman *et al*. (2002) reported that carvone exhibited antimicrobial activity against a number of bacteria including *Listeria monocytogenes*. Enoxacine found in aqueous leaf extract of *B. ilicifolia* is reported to be active against many Gram negative bacteria and Gram positive bacteria (Chin and Neu, 1983).

Kotan, (2007) also reported that fenchol, nerol and geraniol inhibited the growth of many Gram positive and Gram negative bacteria.

The LC-MS analysis of the ethanol leaf extracts of *B. elliptica* and *B. ilicifolia* revealed the presence of eight major compounds in *B. ilicifolia* and six compounds in *B. elliptica* (Table 7). The prominent compound were: Fenchol (terpene), pelargonidin-3, 5-diglucoside (anthocyanidin), (-)- carvone (monoterpenoid), wighteone (flavanoid), vanillylanine (alkaloid), geraniol (monoterpenoid), cinchoninone (alkaloid), liquiritiginin (flavanone), geraniol (monoterpenoid), hederagenin (triterpenoid) and nerol (monoterpenoid). These compounds are of pharmacological importance as they possess analgesic, anti-diabetic and antibacterial properties. It has been reported that plants naturally possess radical scavenging molecules such as terpenoids, flavanoid, alkaloid, tannins which are rich in antioxidant and antimicrobial properties (Amini *at al*., 2012; Omoruyi *et al*., 2012). Compound such as geraniol have been reported to inhibits growth and polyamine biosynthesis in human colon cancer cell (Carnesecchi *et al* 2001). The results presented in this study showed similar observation with previous report on presence of some alkaloids, flavanone, terpernoid isolated from plants which are potent against some disease and health conditions such as oxidative stress, diabetes and infections (Srivastava *et al*., 2012; Singh *et al*., 2013). Therefore, the folkloric pharmacological activities of these plants (*B. elliptica* and *B. ilicifolia*) may be due to the presence of identified compounds. However, isolation of individual bioactive components and subjecting them to biological testing will absolutely confirm these results.

Table 6: Identified compounds in aqueous leaf extracts of *B. elliptica and B. ilicifolia* using LC/MS

| S/n | Compounds | Formular | *B. elliptica* RT(min) | *B. ilicifolia* RT(min) | MW | Nature of compound |
|---|---|---|---|---|---|---|
| 1 | Ibogaine | $C_{20}H_{26}N_2O$ | 3.1 | * | 310 | Alkaloids |
| 2 | Nerol | $C_{19}H_{24}N_2O_2$ | 13.67 | * | 312 | Monoterpenes |
| 3 | Carvone | $C_{10}H_{14}O$ | 13.57 | 11.82 | 150 | Terpenoids |
| 4 | Fenchol | $C_{10}H_{18}O$ | * | 6.63 | 154 | Terpenes |
| 5 | Enoxacine | $C_{15}H_{17}FN_4O_3$ | * | 14.36 | 320 | Quinolones |
| 6 | Geraniol | $C_{10}H_{18}O$ | * | 14.74 | 154 | Monoterpernoids |

*=Not detected

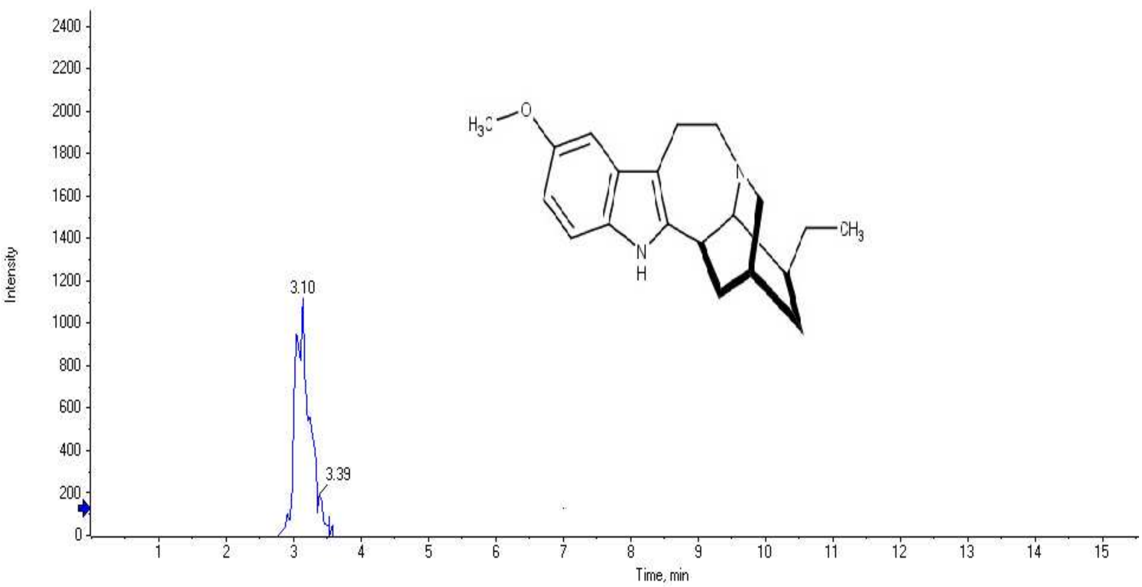


Figure 16: LC-MS spectrum of ibogaine

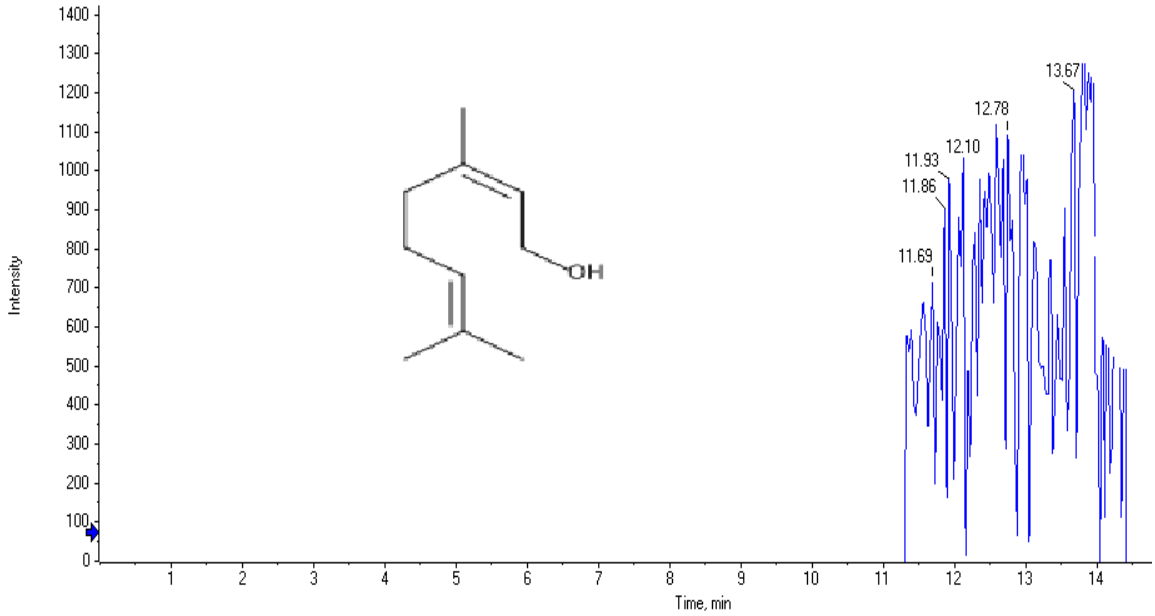


Figure 17: LC-MS spectrum of nerol

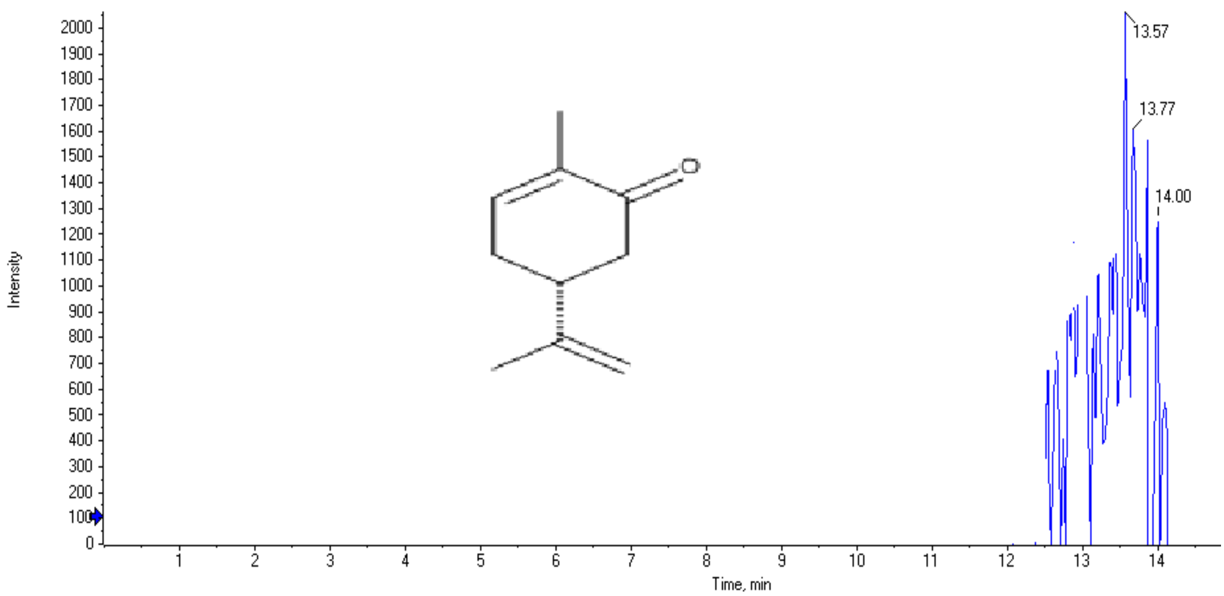


Figure 18: LC-MS spectrum of carvone

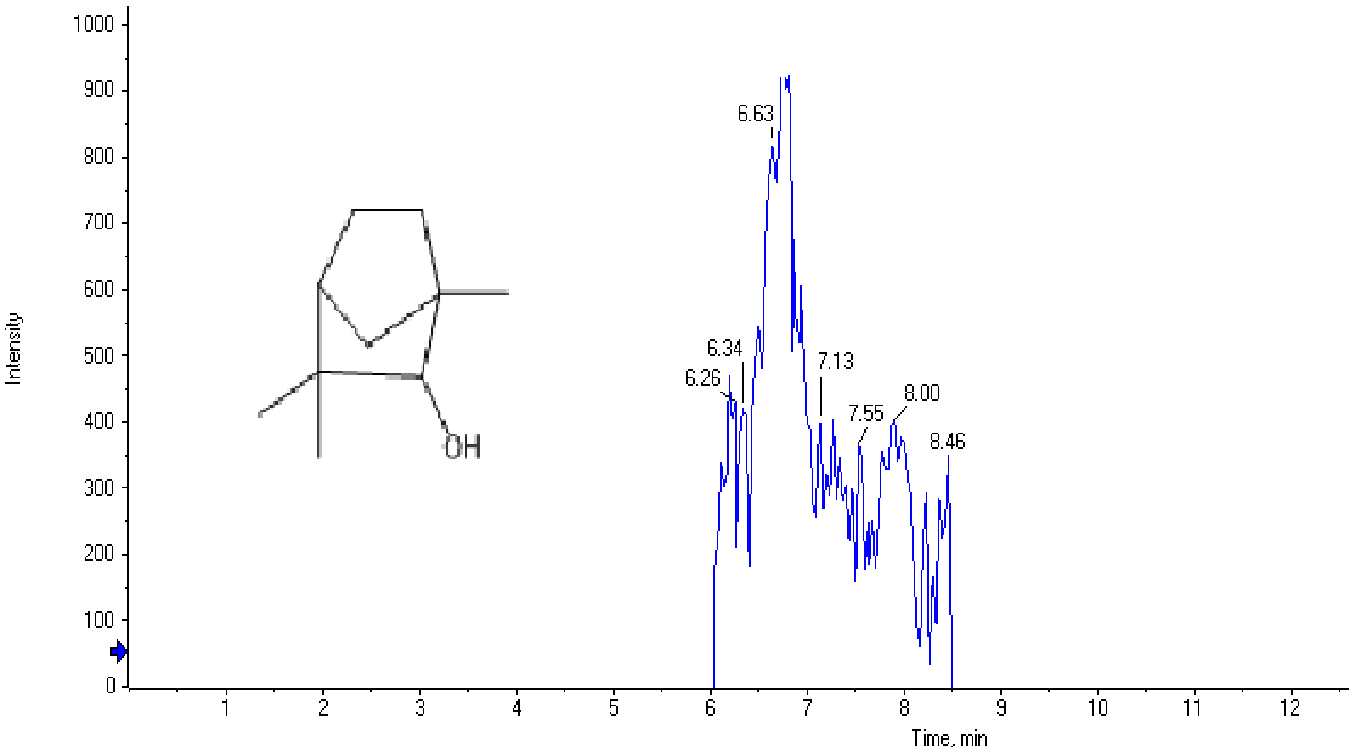

Figure 19: LC-MS spectrum of fenchol

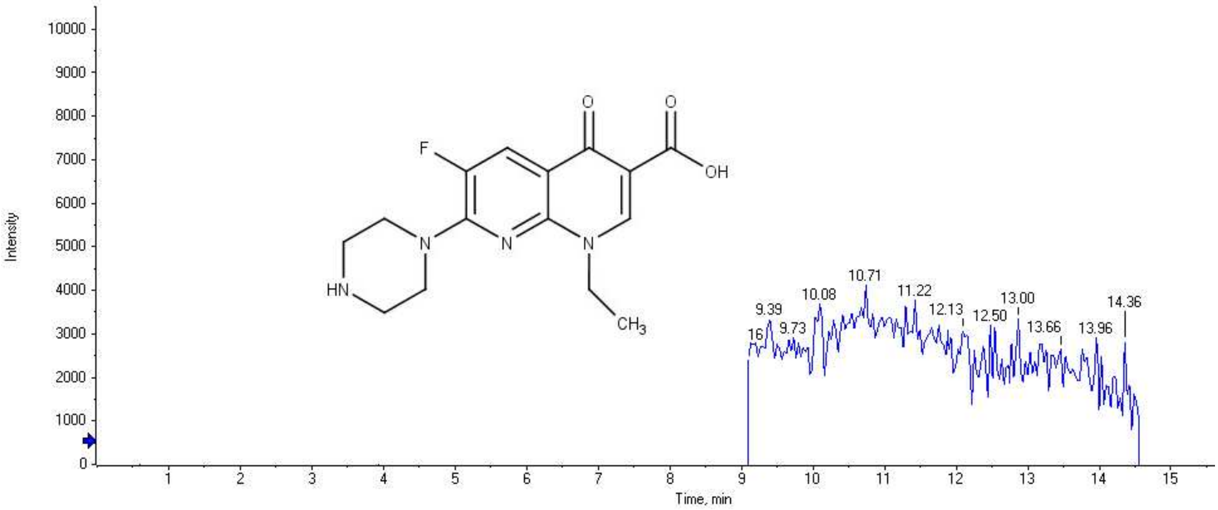

Figure 20: LC-MS spectrum of enoxacine

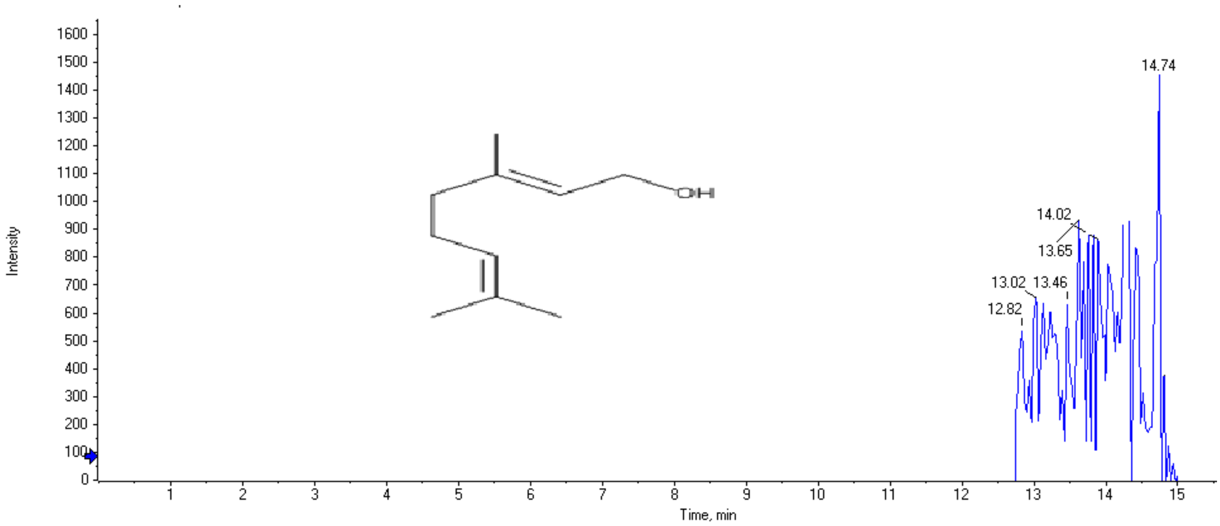


Figure 21: LC-MS spectrum of geraniol

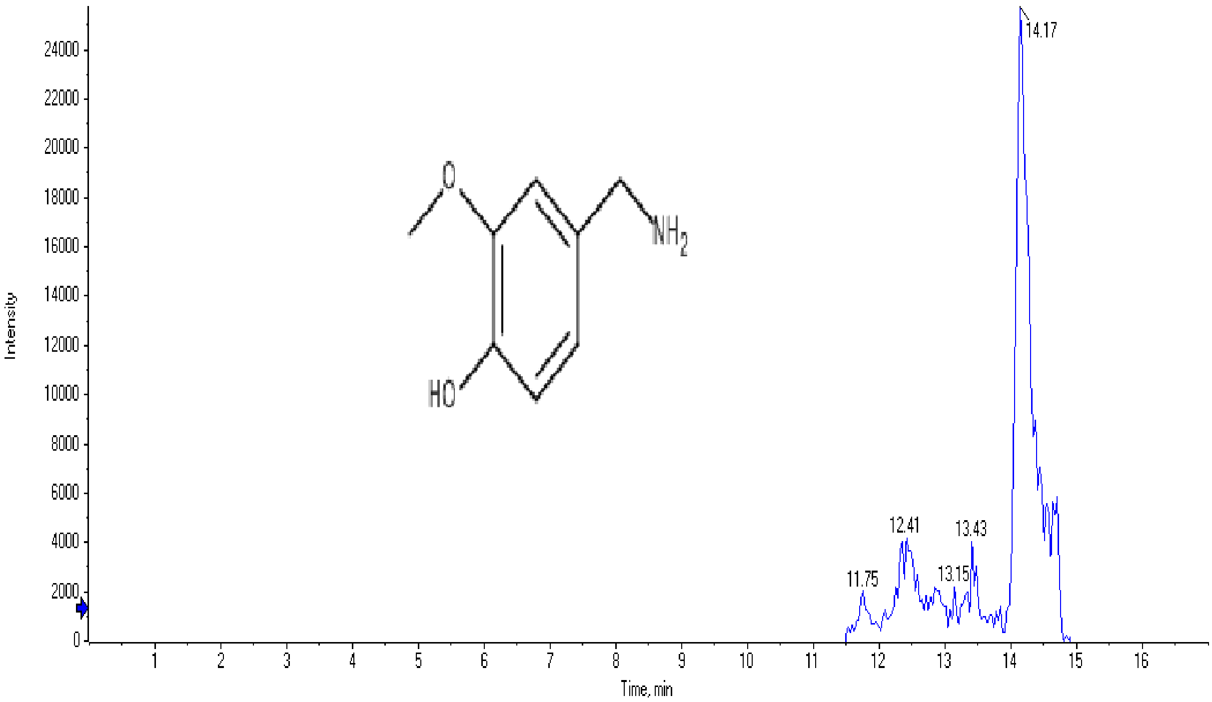


Figure 22: LC-MS spectrum of vanillylanine

Table 7: Identified compounds in ethanol leaf extracts of *B. elliptica and B. ilicifolia* using LC/MS

| S/n | Compounds | Formular | *B. elliptica* RT(min) | *B. ilicifolia* RT(min) | MW | Nature of compound |
|---|---|---|---|---|---|---|
| 1 | Fenchol | $C_{10}H_{18}O$ | * | 6.71 | 154 | Terpenes |
| 2 | Catharanthine | $C_7H_2O_6$ | 7.18 | * | 181 | Alkaloid |
| 3 | Hederagenin | $C_{30}H_{48}O_{15}$ | 10.7 | * | 180 | Triterpenoids |
| 4 | (-)- Carvone | $C_{10}H_{14}O$ | 11.66 | 11.73 | 150 | Monoterpenoids |
| 5 | Pelargonidin-3,5-diglucoside | $C_{15}H_{11}O_5$ | * | 11.01 | 271 | Anthocyanidin |
| 6 | Nerol | $C_{19}H_{24}N_2O_2$ | 13.91 | * | 312 | Monoterpenes |
| 7 | Wighteone | $C_{20}H_{18}O_5$ | * | 14.7 | 338 | Flavanoid |
| 8 | Vanillylanine | $C_8H_{11}NO_2$ | 14.17 | 14.19 | 250 | Alkaloid |
| 9 | Geraniol | $C_{10}H_{18}O$ | * | 14.84 | 154 | Monoterpernoid |
| 10 | Cinchoninone | $C_{16}H_{23}N_5O$ | * | 14.56 | 381 | Alkaloid |
| 11 | Liquiritigenin | $C_{15}H_{12}O_4$ | 14.78 | 14.81 | 132 | flavanone |

***=** Not detected

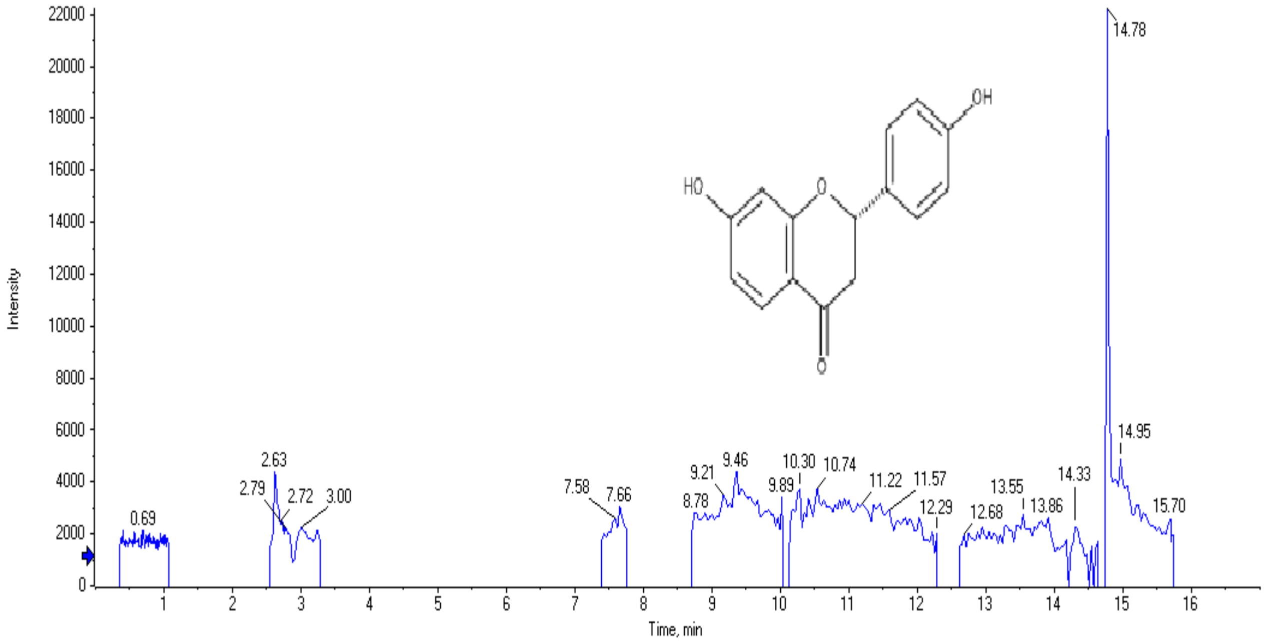


Figure 23: LC-MS spectrum of liquiritigenin

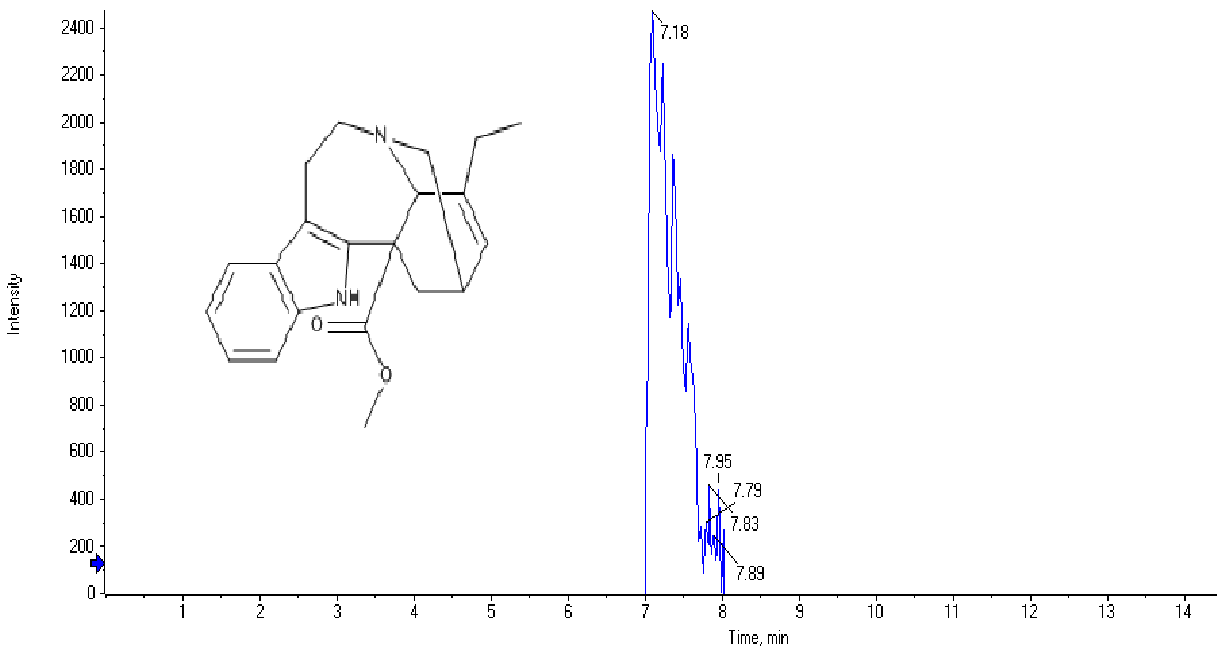


Figure 24: LC-MS spectrum of catharathine

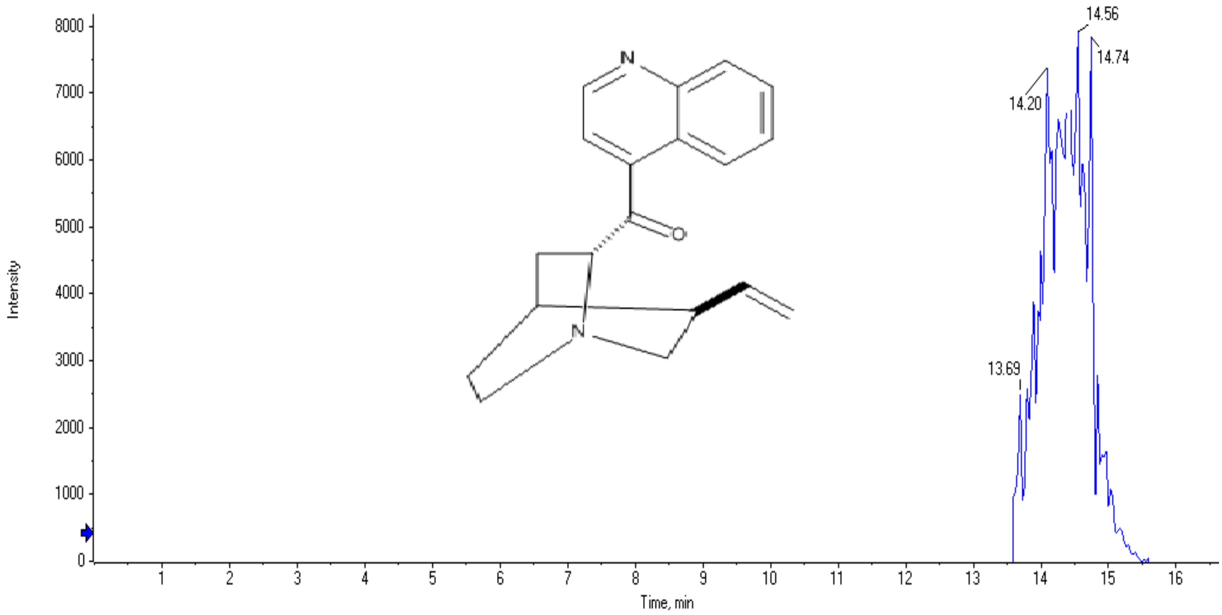


Figure 25: LC-MS spectrum of cinchoninone

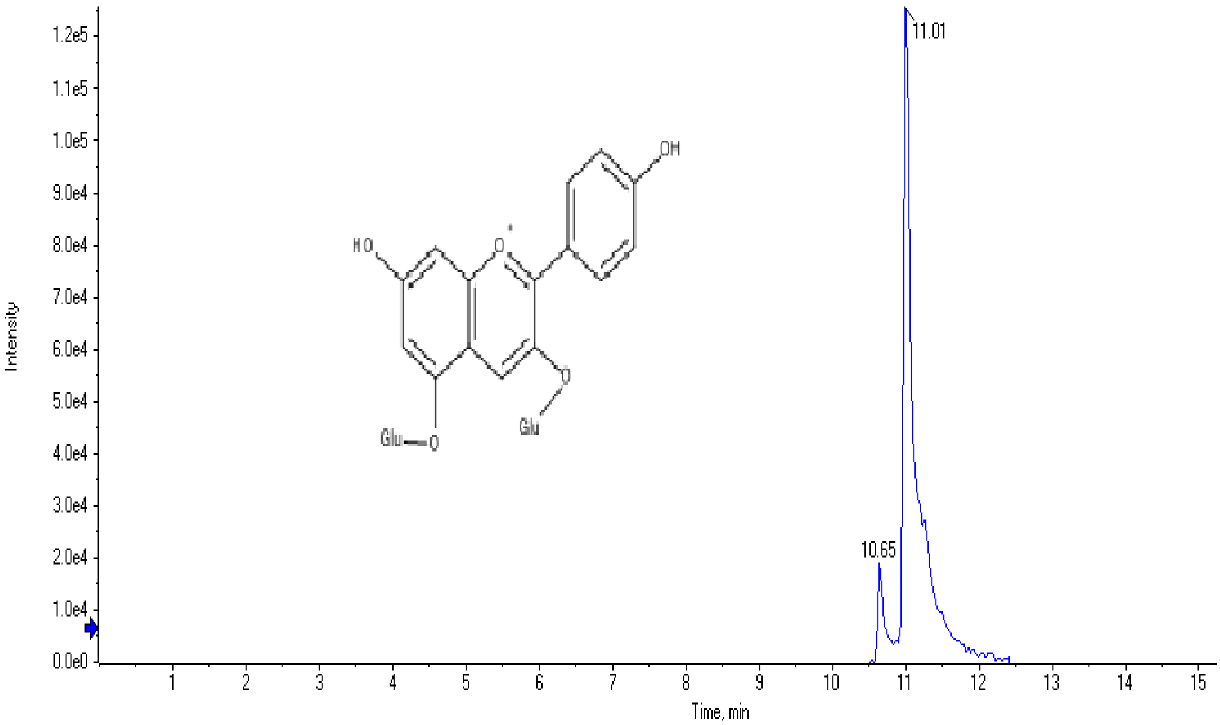


Figure 26: LC-MS spectrum of pelargonidin-3, 5-diglucoside

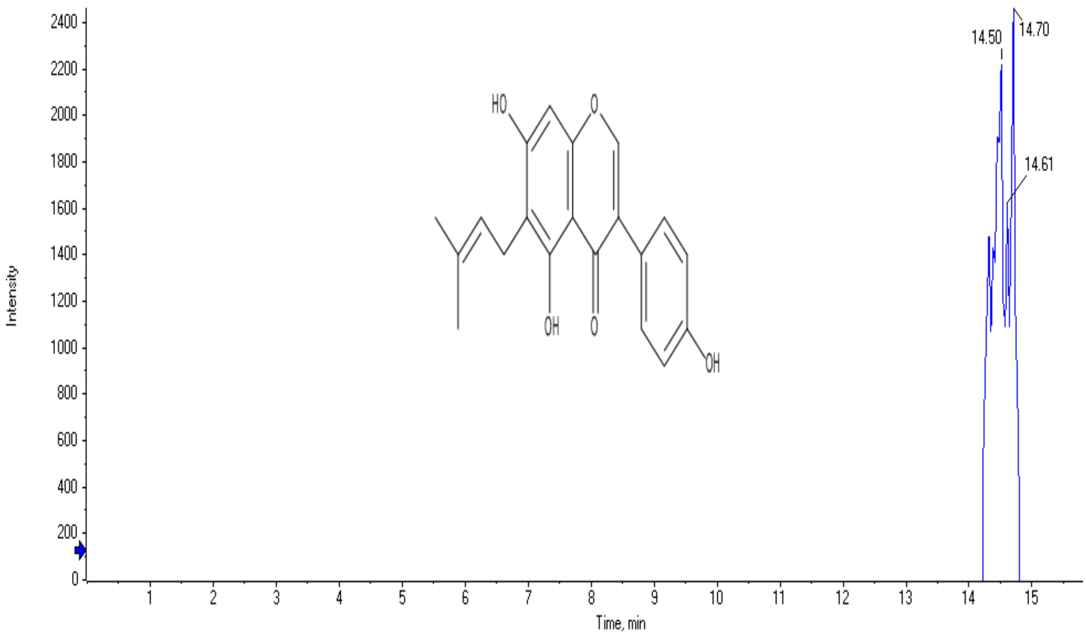


Figure 27: LC-MS spectrum of wighteone

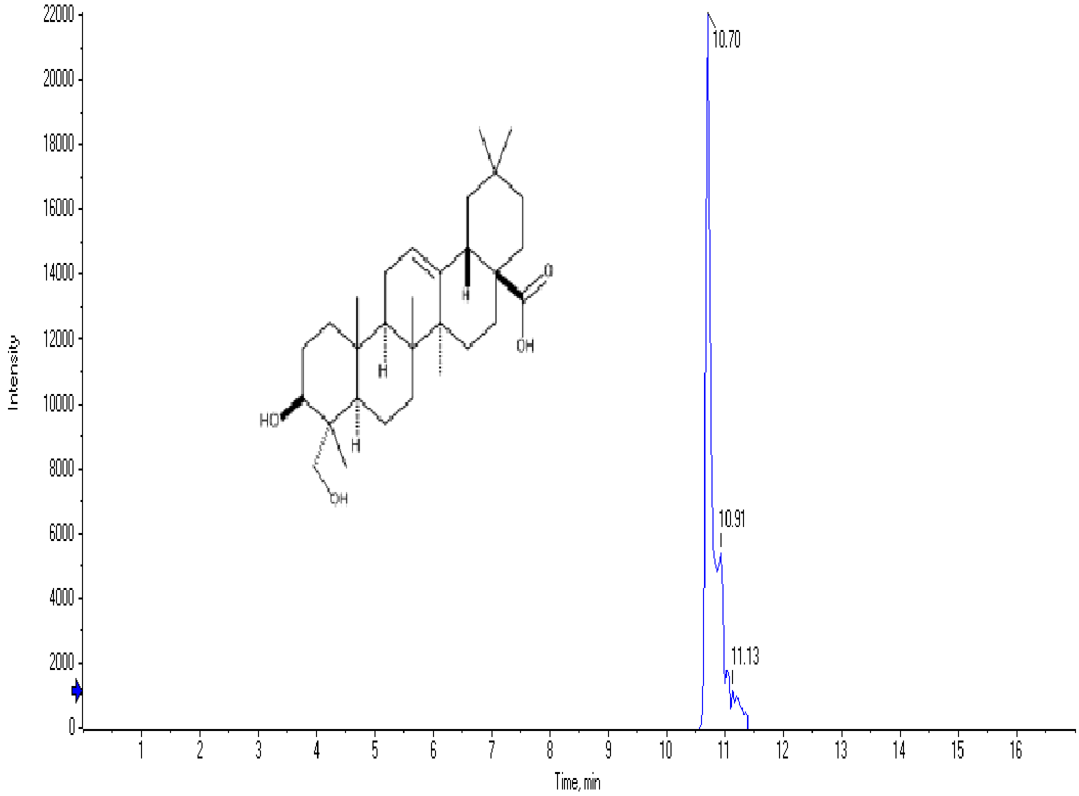


Figure 28: LC-MS spectrum of hederagenin

## 4.4 CONCLUSION

In conclusion, aqueous and ethanol extracts of *B. elliptica* and *B. ilicifolia* contain various identified bioactive compounds. Both are recommended as plants of pharmaceutical importance. However, further studies are needed to isolate and explore individual bioactive compounds.

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# CHAPTER 5

# GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

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## 5.1 GENERAL DISCUSSION

Diabetes mellitus is one of the common endocrine disorders that affect more than 387 million people around the world (IDF, 2014). It has been reported that poor management of diabetes contributes to the pathogenesis of microbial infection in diabetic patients and many pathogens are however becoming resistant to antibiotic drugs. Herbal medicine is an alternative medicine widely used for the treatment of various diseases, including diabetes and wound infections.

As a multidisciplinary science, research in the phytoscience is almost unlimited; thus, in this dissertation phytochemical, antimicrobial and antioxidant investigation of *B. elliptica* and *B. ilicifolia* have been reported. The effect of aqueous and ethanol extracts of both plants against some opportunist bacteria associated with wound infection in diabetic patients and the bioactive components present in these plants were also discussed.

**Phytochemical**

This study has revealed the presence of phytochemicals such as flavanoids, phenolics, tannins, alkaloids, proanthocyanidins, flavanols and saponins. These have been reported for their wide range of biological activity, including antidiabetes, antimicrobial, anti-inflammatory and antiviral effects (Wong *et al*., 2006). These properties have been attributed to several mechanisms of action such as stimulating defence enzyme activities and quenchers of the formation of singlet oxygen (Zhou and Yu, 2004). Previous studies (Surbhi and Leelavathi, 2010) have shown that flavanoids exhibit their action through effects on membrane permeability and by inhibition of membrane-bound enzymes. This property may explain the antioxidative action of the two medicinal plants extracts used in this study.

**Antioxidant activity**

Oxidative stress plays a role in the pathogenesis of various diseases including diabetes mellitus. It occurs as a result of increased production of oxygen free radicals and a sharp reduction of antioxidant defense systems (Fridlyand *et al.*, 2005). Antioxidant agents of natural origin have attracted special interest because they can protect the human body from oxidative damages caused by free radicals (John, 1991). In the present study, these results show that aqueous and ethanol extracts of *B. elliptica* and *B. ilicifolia* scavenge DPPH, reducing power, ABTS, NO and hydrogen peroxide radicals. The observed strong *in vitro* antioxidant activities of both plants extracts may be due to the presence of bioactive compounds. Therefore, it could be inferred from this study that aqueous and ethanol leaf extracts could serve as a source of natural antioxidants against pathological disease related to free radical.

**Antibacterial activity**

Antimicrobial compounds in plants have been reported for their enormous therapeutic potential. The active ingredients of the plant parts are better extracted with alcohol than other solvents. Okemo, (1996) reported an alcohol extract of selected medicinal plants used by Kenyan herbal doctors found to contain alkaloid, and coumarins. Coumarins have been reported for antibacterial and antihelminthic properties (Hedbeg *et al*., 1983). In the present work, the antibacterial activity of aqueous and ethanol extract of both plants was studied on five bacterial strains. The results of the study revealed that ethanol extract of both plants exhibited strong antibacterial activity against five selected pathogens used in this study when compared with that of aqueous extracts of *B. elliptica* and *B. ilicifolia* leaf. This observed antibacterial activity of ethanol extracts of both plants against these bacteria associated with wound in diabetic patients could be attributed to the presence of bio-active compounds. Tanins were reported to be outstanding antimicrobial compounds (Cowan, 1999). These compounds have diverse effects on biological system due to their capability of metal ion chelators and biological antioxidants (Hagerman, 2002). Various mechanisms of action have been proposed to explain antimicrobial activity of tannins. They include extracellular microbial enzymes and proteins, deprivations of iron as substances for microbial growth or direct action towards its membranes (Scalbert, 1991). From this investigation, it is therefore postulated that the phytochemicals compounds presents in the ethanol leaf extracts were responsible for the antibacterial activity.

**GC-MS analysis**

The GC-MS analysis of the essential oil of both plants revealed the presence of monoterpenes, oxygenated sesquiterpenes, phenolics and esters. All of these identified compounds were reported to have various biological activities and some of them were found to have antimicrobial activity. Monoterpenes compounds possess strong antimicrobial and antibacterial activities (Sokmen *et al*., 2003). Previous studies (Denyer and Hugo, 1991; Sikkema *et al*., 1994) have also reported that essential oils break through lipid components of bacterial cell membrane and mitochondria, disrupting the cell structure and making them more permeable resulting in leakage of critical molecules from within the cell and eventual death of the bacteria cells.

**LC-MS analysis**

This type of analysis provides high sensitivity and specificity, based on the fact that only ions specific to target analytes are monitored. The results of analysis showed that aqueous and ethanol extract of *B. elliptica* and *B. ilicifolia* are rich in alkaloids, terpenes, terpenoids, monoterpernoids and flavanoids. Some of the identified compounds have been reported to have great pharmacological importance such as antioxidant, antidiabetes and antimicrobial (Amini *et al*., 2012; Omoruyi *et al*., 2012). Hence both plants can be recommended for pharmacological importance due to this presence of bioactive components.

## 5.2 CONCLUSION

This study has partially justified the ethnomedicinal use of *B. elliptica* and *B. ilicifolia* leaves for treatment and wound infection caused by bacteria in diabetic patients. These may be attributed to the presence of antioxidant compound such as phenols, flavanoids, saponin, tannic, alkaloids and other phytochemical compounds. These compounds have contributed to the scavenging activity of free radicals of these plants as a means of preventing pathogenesis of radical related diseases.

## 5.3 RECOMMENDATION

It is however, recommended that further assays be performed on the two plants species to evaluate their hypoglycaemic activity and toxicity. This should be done by using aqueous extract as used by traditional healers and herbalist. It is also recommended that extracts of *B. elliptica* and *B. ilicifolia* should be tested *in vivo* in a rat or mouse model for their hypoglycaemic activity as plants extracts differ in their activity. Isolation of individual bioactive components should be tested for biological activity.

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