

**Qualitative And Quantitative Analysis of some Phytochemicals in
Diodia sarmentosa Leaf**

BY

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CERTIFICATION

This is to certify that this undergraduate project work, **Qualitative and Quantitative Analysis of Some Phytochemicals in *Diodia sarmentosa* Leaf**, was carried out by **NWADINIGWE, EBERE UZOAMAKA** with the registration number, **20101730425**, of the department of Biochemistry, Federal University of Technology, Owerri, Nigeria.

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DEDICATION

This work is dedicated to those who continually contribute to the development of Biochemistry.

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ABSTRACT

Research and reviews are continually being carried out in order to discover newer, cheaper and abundant plant sources of bioactive components which exhibit strong pharmacognostic and biochemical effects. The whole plant of *Diodia sarmentosa* has a folkloric reputation as an anti-ulcer agent. It is claimed to be useful in herbal medicine for the treatment of bacterial and fungal infections, and some other ailments. The qualitative and quantitative analysis of some important phytochemicals in *Diodia sarmentosa* (Tropical Buttonweed) leaf were determined using two forms of the plant — the aqueous extract and pulverized samples of the leaf— so as to determine and/or confirm the presence of some phytochemicals with significant biological activity. The aqueous leaf extract were prepared using maceration process. The percentage yield of the aqueous D.S leaf extracts obtained was 24%. Qualitative phytochemical analysis of the leaf sample showed the presence of tannins, saponins, alkaloids, flavonoids, steroid and cardiac glycosides, phytic acid, oxalic acid, phenols, carbohydrates, amino acids and terpenoids. Results of qualitative analysis confirmed the presence of the following expressed as percentage: Tannins (4.82 ± 0.36); saponins (4.81 ± 0.29); flavonoids (12.74 ± 0.44); alkaloids (4.12 ± 0.44); cardiac glycosides (0.62 ± 0.44); and phenolic acids (3.36625 ± 0.41). The phytic acid content was determined as 0.49%, and the oxalic acid content as 34020mg/100g of the plant sample. The phytochemical and nutrient compositions of this tropical plant are expository and depict the role of the plant in nutrition and medicine. The significance of the plant in traditional medicine and the importance of its chemical constituents were discussed with respect to the role of the plant in ethnomedicine in Nigeria.

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ABBREVIATIONS

AcOH:	Acetic acid
CNS:	Central Nervous System
D.S.:	<i>Diodia sarmentosa</i>
e.g.:	Example
FeCl ₃ :	Ferric chloride
HCl:	Hydrochloric acid
HCN:	Hydrogen cyanide
HDL:	High density lipoproteins
H ₂ O:	Water
i.e.:	That is
IUPAC:	International Union of Pure and Applied Chemistry
LDL:	Low density lipoproteins
NAD(P)H:	Nicotinamide adenine dinucleotide phosphate
NaOH:	Sodium hydroxide
NH ₄ OH:	Ammonium hydroxide
PA:	Phytic acid
PAL:	Phenylalanine lyase
PPE:	Personal protective equipment
s.d.:	Standard deviation
USDA:	United States Department of Agriculture

CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.0 BACKGROUND

Through the years, since the inception of life on earth, earnest search has been made continually for the discovery of chemical substances with curative efficacy for different human ailments. The formulation of new medicines through the evaluation of crop varieties often involves the chemical analysis of plant materials in order to assess their nutritive value, inhibitory characteristic and potency (Harborne, 1973). For novel plants, development of new potencies begin with chemical analysis of various phytochemicals present in the plant. Two broad methods are used; qualitative and quantitative phytochemical analysis. Qualitative analysis refers to analysis in which substances are identified or classified on the basis of their chemical or physical properties, such as chemical reactivity, colour formation, solubility, molecular weight, melting point, radioactive properties (emission, absorption), mass spectra, nuclear half-life, etc. Quantitative analysis is the determination of the absolute or relative abundance (often expressed as concentration in mg/g or µg/ml) of one, several or all substance(s) present in a sample. It involves the qualification of analyte(s) for which numerical estimates are given (IUPAC, 1995). Gravimetric and volumetric

analytical procedures are commonly employed for qualitative analysis of plant samples, whereas mass spectrophotometric and chromatographic procedures are more reliable for quantitative analysis as these give accurate results. The methods of analysis employed were elaborated in chapter two of this report. In this study both the quantitative and qualitative phytochemical analysis of *D. sarmentosa* leaf were conducted in order to determine specific properties of the plant sample contributed by some of its chemical constituents. Identification of the chemical constituents of plants is desirable because such information will be of value for the synthesis of new bioactive compound(s) for treating certain diseases. Quantitative analysis was done in inclusion because data obtained could easily be compared with those of other plant species that are of high repute in pharmacognosy due to their degree of action, and hence determine the potential for utilization of *D. sarmentosa* in the production of pharmaceuticals or its use as a therapeutic agent. It also is useful in dosage estimations, in case of oral intake/administration, because phytochemicals, like other nutrients and drugs have recommended maximum daily dosage beyond which they become toxic to the body system. Leaf samples of the plant, in addition to being the most abundant and substantial source of phytochemicals, would be most appropriate for preliminary phytochemical analysis due to the fact that they are sites of synthesis of phytocompounds in plants. Although, greater amounts of phytochemicals usually occur in other plant storage tissues such as shoots and roots.

Phytochemicals (from the Greek word *phyto*, which means plant) are biologically active, naturally-occurring chemical compounds that play key roles in defence system against numerous diseases and stress conditions in plants (and as has been reviewed, in humans as well.) (Hashmi *et al.*, 2013; Rahim *et al.*, 2013; Tupe *et al.*, 2013). They accumulate in different parts of the plants, such as roots, stems, leaves, flowers, fruits or seeds (Costa *et al.*, 1999). These phytochemicals are grouped into two main categories (Krishnaiah *et al.*, 2009) namely primary constituents; which include amino acids, common sugars, proteins and chlorophyll; and secondary constituents consisting of flavonoids, alkaloids, saponins, tannins, terpenoids and phenolic compounds (Edeoga *et al.*, 2005; Krishnaiah *et al.*, 2007; Jign and Sumitra, 2007). Detailed information on the various phytochemicals studied were provided in this chapter. Majority of phytochemicals have been known to bear valuable therapeutic activities such as insecticidal, antibacterial, antifungal, purgative effects and antioxidant activities (Abdeltawab *et al.*, 2012). Plants thus find their medicinal value due to respective phytochemical constituents they contain. It is important to know the structure of phytochemical constituents, thus knowing the type of biological activity which might be exhibited by the plant (Agbafor and Nwachukwu, 2011). To decipher this, phytochemicals are extracted and isolated through different chemical techniques. Extraction of bioactive compounds from medicinal plants allows the demonstration of their physiological activity. It also facilitates pharmacological

studies leading to synthesis of a more potent drug with reduced toxicity (Manna and Abalaka, 2000).

Diodia sarmentosa is a scrambling herb with yellowish-green leaves, long square-shaped stems, and a tap root system widespread across the tropics, especially; Africa and Latin America (Waterhouse and Mitchell, 1998). Diodia is a genus of flowering plants in the Rubiaceae family as described by Carl Linnaeus in 1753. There are over 112 known *Diodia* species in this family. Its ovate leaf and hairy cuboid shoot are major features which distinguish *D. sarmentosa* from other species in the same family. The Latin name for *Diodia sarmentosa* is *Diodella sarmentosa* (Bacigalupo and Cabral, 2006), and due to its relative abundance in the tropics, it is commonly referred to as Tropical Buttonweed. The aqueous leaf extract of *Diodia sarmentosa* has a folkloric reputation as antiulcer (Akah *et al.*, 1998), insecticidal (Mbata and Ekpenu, 2014), and antimicrobial agents. The plant has been used for several years in African traditional medical practices and as a condiment in soups. It is a plant indigenous to the tropical and pan-tropical regions. In addition to its antiulcer and antimicrobial properties, the plant exhibits anti-inflammatory effects, and is also used for the treatment of convulsive cough and tuberculosis. These biological effects of *D. sarmentosa* could be due to the presence of alkaloids, steroids, cardiac glycosides, saponins and tannins. Chapter three of this report contains results obtained from phytochemical screening carried out on the plant whereas

chapter four contains details of the discussions made, recommendations given and conclusions drawn from this experimental research.

Many modern pharmaceuticals have been modelled on or derived from chemicals found in plants. Presently, in the developing countries, synthetic drugs are not only expensive and inadequate for the treatment of diseases but also are often with adulterations and side effects (Shariff, 2001). There is therefore continuous and urgent need to discover new drugs with diverse chemical structures and novel mechanisms of actions because of the drastic rise in the incidence of new and re-emerging [infectious] diseases (Nair and Chanda, 2008) to replace those that have lost their efficacy. Kaufman *et al.*, (1999), had reported that more than 25% of prescribed drugs contained at least one active ingredient of plant origin.

1.1 STATEMENT OF PROBLEM

Powdered and aqueous extracts of *Diodia sarmentosa* leaf samples will be analysed to determine the various phytochemicals present in the sample.

1.2 MEDICINAL PLANTS

Medicinal plants are plants containing inherent active ingredients used to cure diseases or relieve pain (Okigbo *et al.*, 2008). Medicinal plants are the natural sources of many biologically active ingredients. The pharmacological importance

of indigenous plants herbs and fibres over the years have been overlooked by the scientific world (Adjeroh *et al.*, 2015). However, the use of medicinal plants through practices and knowledge has augmented modern medicine. This is because these plants represent a constituent part of the natural biodiversity of many countries in Africa (Okigbo *et al.*, 2008).

Furthermore, an increasing reliance on the use of medicinal plants in industrialized societies have been traced to the extraction and development of several drugs and chemotherapeutics from these plants (UNESCO, 1998). The medicinal properties of plants could be based on the antioxidant, antimicrobial, antipyretic effects of the phytochemicals in them (Adesokan *et al.*, 2008). The methanol extract of curry leaves showed high antibacterial activity against *E. coli*. This effect was as a result of its high saponin content (Bansode *et al.*, 2014). Siam weed (*C. odorata*) has been reported to possess nematicidal activity against plant-parasitic nematodes (Adekunle and Fawole, 2003; Adegbite and Adesiyan, 2005). Hot water percolates of lemon grass exhibited high antioxidant activity, a property contributed by the macrominerals in the plant such as sodium, magnesium and potassium (Godwin, *et al.*, 2014). The ethanol and aqueous leaf extracts of *Euphorbia hirta* exhibit antibacterial and antifungal properties, and the constituents of the plant extract can be useful in chemotherapy of some microbial infections (Adjeroh *et al.*, 2015).

Undoubtedly, the plant kingdom still holds many species of plants containing substances of medicinal value, which are yet to be discovered. Large numbers of

plants are constantly been screened to determine their toxicity level. Traditional use of any plant for medicinal purposes warrant the safety of such plant, particularly with regards to mutagenicity, nephrotoxicity, carcinogenicity and hepatotoxicity (Odesanmi *et al.*, 2009).

1.3 *Diodia sarmentosa* (Tropical Buttonweed)

Diodia sarmentosa (Fig.1) is a scrambling herb with a tap root system, stems of about 1-4 m long, often with numerous lateral branches, square in cross-section and with long hairs on the angles. Its leaves are yellowish-green, lanceolate, and about 1.8-6.3 - 0.7-2.8 cm in size. Its flowers are about 1-8 in axillary clusters at most nodes; each flower is 1.5-3 mm long with mauve or white petals. The seeds are dark red, 2-4 mm long, 1.5 mm wide, and 0.8 mm thick. (Waterhouse and Mitchell, 1998). From the descriptions above, it can be deduced that *D. sarmentosa* is a dicotyledonous, perennial plant. A climbing plant, often near high water mark; grassland and bushland at low altitudes, and as a weed of cultivation. Waterhouse & Mitchell, 1998). It can be multiplied through seed propagation.



Fig. 1: Shoot of *Diodia sarmentosa* (Tropical Buttonweed).

Taxonomical classification of *Diodia Sarmentosa* (Britton and Wilson, 2014)

Kingdom	Plantae – Plants
Subkingdom	Tracheobionta – Vascular plants
Superdivision	Spermatophyta – Seed plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Subclass	Asteridae
Order	Rubiales
Family	Rubiaceae – Madder family
Genus	<i>Diodia</i> – buttonweed
Species	<i>sarmentosa</i>

Ethno-botanical Distribution of *Diodia Sarmentosa*

Native:

• AFRICA

East Tropical Africa: Kenya; Tanzania; Uganda

West-Central Tropical Africa: Cameroon; Central African Republic; Zaire

West Tropical Africa: Ghana; Nigeria

South Tropical Africa: Mozambique; Zambia; Zimbabwe

Western Indian Ocean: Madagascar

- NORTHERN AMERICA
Southern Mexico: Mexico - Chiapas, Jalisco, Nayarit, Oaxaca, Veracruz
- SOUTHERN AMERICA
Caribbean: Dominican Republic; Guadeloupe; Jamaica; Martinique; Montserrat; Puerto Rico
Mesoamerica: Belize; Costa Rica; Guatemala; Honduras; Nicaragua; Panama
Northern South America: French Guiana; Guyana; Suriname; Venezuela
Brazil: Brazil
Western South America: Colombia; Ecuador

1.4 Biological Effects of *Diodia sarmentosa*

1.4.1 Therapeutic effects

Preliminary studies have shown that *Diodia sarmentosa* leaf extracts exhibit several therapeutic effects on certain human ailments, viz:

1. Anti-ulcer Activity

Diodia sarmentosa has therapeutic effects for ulcer and decreases heartburn (Brzozowski, 2012). *Diodia sarmentosa* is a major constituent of anti-ulcer recipes in Nigeria. *In vivo* studies in mice and rats revealed their anti-ulcer activities by the decreased ulcer index in aspirin-induced ulcerogenesis, delayed

intestinal transit, increased pH, and decreased volume and acidity of gastric secretion all caused by the administration of the aqueous leaf extracts (Akah *et al.*, 1998).

2. Anti-Fungal and Anti-Bacterial Effects

The anti-fungal and anti-bacterial effects of *Diodia sarmentosa* leaf extracts have been evaluated. Multi-solvent (hexane, chloroform, ethyl acetate, ethanol, methanol and water) extracts of *D. sarmentosa* were evaluated in triplicates against twelve (12) medically- important microorganisms for microbiosatic and microbiocidal activities using colorimetric broth microdilution methods. Hexane extracts of *D. sarmentosa* exhibited high potency (low minimum inhibitory concentration and minimum fungicidal concentration) against *Cryptococcus neoformans* (Khoo *et al.*, 2014); a fungus which causes cryptococcosis, an inflammatory disease which affects vital organs such as the lungs. The study corroborates the efficacy of *D. sarmentosa* in traditional medicine for treatment of cough, sores and skin diseases caused by microorganisms. *Diodia sarmentosa* (locally known as “dasa” in Ago-Iwoye, Ogun state.) leafs, usually taken with pepper and salt, is used as a curative agent for dysentery (Soladoye *et al.*, 2010).

1.4.2 Insecticidal Effects

Powder from low temperature dried shoots and leaves of *Diodia sarmentosa* was investigated for insecticidal activity against three storage insect pests:

Callosobruchus maculatus, *Callosobruchus subinnotatus* and *Sitophilus zeamais*.

D. sarmientosa was not effective in causing mortalities of the adult beetles at concentrations up to 0.75g powder per 20g of seeds. However, *C. maculatus* and *S. zeamais* females were prevented from ovipositing on the seeds. Aqueous and ethanol extracts of *D. sarmientosa* did not show any insecticidal activity against the beetles used in the study (Mbata and Ekpenu, 2014).

1.5 Phytochemicals

Phytochemicals are chemicals that are derived from plants. They may be primary plant products (such as amino acids, common sugars, proteins and chlorophyll) or secondary plant products (example; flavonoids, alkaloids, saponins, tannins, terpenoids and phenolic compounds) (Edeoga *et al.*, 2005; Krishnaiah *et al.*, 2007; Jign and Sumitra, 2007). Primary metabolites are directly involved in growth and development while secondary metabolites are not involved directly in activities that initiate or promote plant growth (although, some have been implicated as biocatalysts). Secondary plant products evolved through defunct metabolic pathways in the plants. Most of the secondary plant products could serve defensive purposes against predators; they also help in pollination, impact on taste or are simply waste products. Man has found out through either customary or ancestral practice that some of them have medicinal values. A plant may exhibit certain biological effects. This has always been attributed to the presences of phytochemicals like tannins, flavonoids and

saponins. Some of them may have stimulatory effects like alkaloids (caffeine) and some cardiac glycosides. Some may have antitumor/antioxidant/anticancer effects like tannins and flavonoids. Others may be poisonous like cyanogenic glycosides present in cassava. Many others have found good pharmaceutical uses, while others give plants and fruits their distinctive colour, taste and scent. Phytochemicals such as phytic acid, oxalic acid and glycosides are often referred to as anti-nutrients, because they reduce the bioavailability of certain nutrients, and are hepatotoxic. Qualitative and quantitative phytochemical screening is a major technique used for the identification of new sources of therapeutically and industrially important compounds.

Table 1: Major Classes of Phytochemicals

Class of Phytochemical	Sub-class and specific examples	Sources
A. Plant phenols	1. Flavonoids i. flavonones, e.g., apigenin, luteolin ii. flavonols, e.g., quercetin iii. flavanons taxifolin iv. flan-3ols, e.g., epigallocatechin, catechin v. anthocyanidins, e.g., cyanidin vi. chalcones, e.g., pedicinin 2. Stilbenes i. polyhydroxystilbenes, e.g., resveratrol 3. Bioflavonoids i. anthocyanocide 4. Lignans 5. Tannins 6. Phenols i. caffeic acids ii. ferulic acids 7. Phenolic acids i. ellagic acid	Berries, grapes, green tea, grapefruits, apples and other fruits, beans and peas
B. Terpens and Terpenoids	1. Carotenoids, e.g., β -carotene, lutein and zeaxanthine. 2. Saponin, e.g. diosgenin	Carrots, orange, lime and other citrus fruits, tomato and vegetables.
C. Plant Sterols	1. Phytosterols, e.g., sitosterol and stigmastosterol 2. Phytosterol esters and 3. Stanol esters	Soybean, wheat, green and yellow vegetables.
D. Thiols	1. Allyl and diallyl sulphides, e.g., allicin	Garlic, onions and cruciferous vegetables.
E. Indoles/glucosinolates	1. Auxin and auxin derivatives, e.g., indole-3-carbinols	Cruciferous vegetables

1.5.1 Major Classes of Phytochemicals

1. Alkaloids

These are the largest group of secondary metabolites which contain nitrogen bases, and usually are synthesized from amino acid building blocks with various radicals replacing one or more of the hydrogen atoms in the peptide ring; most containing oxygen. The compounds are alkaline in solutions. In fact, one or more nitrogen atoms present in an alkaloid, typically as 1°, 2° or 3° amines, contribute to the basicity of the alkaloid. The degree of basicity varies considerably, depending on the structure of the molecule, presence and location of the functional groups (Sarker and Nahar, 2007). They react with acids to form crystalline salts without the production of water (Firm, 2010). Majority of alkaloids exist as solids such as atropine, some as liquids containing carbon, hydrogen, and nitrogen. Most alkaloids are readily soluble in alcohol, whereas, they are sparingly soluble in water. The solutions of alkaloids are intensely bitter. These nitrogenous compounds function in the defence of plants against herbivores and pathogens, and are widely exploited as pharmaceuticals, stimulants, narcotics, and poisons due to their potent biological activities. In nature, the alkaloids exist in large proportions in the seeds and roots of plants and often in combination with organic acids. Alkaloids have pharmacological applications as anaesthetics and CNS stimulants (Madziga *et al.*, 2010). More than 12,000-alkaloids are known to exist in about 20% of plant species and only few have been exploited for medicinal purposes. The alkaloid names generally

end in the suffix *-ine*; a reference to their chemical classification as amines. Plant-derived alkaloids in clinical use include the analgesics; morphine and codeine, the muscle relaxant; tubocurarine, the antibiotics; sanguinifine and berberine, the anticancer agent; vinblastine, the antiarrhythmic; ajmaline, the pupil dilator; atropine, and the sedative; scopolamine. Other important alkaloids of plant origin include stimulants such as caffeine, nicotine, codeine, atropine, morphine, ergotamine, cocaine, nicotine and ephedrine

Amino acids act as precursors for biosynthesis of alkaloids with ornithine and lysine commonly used as starting materials.

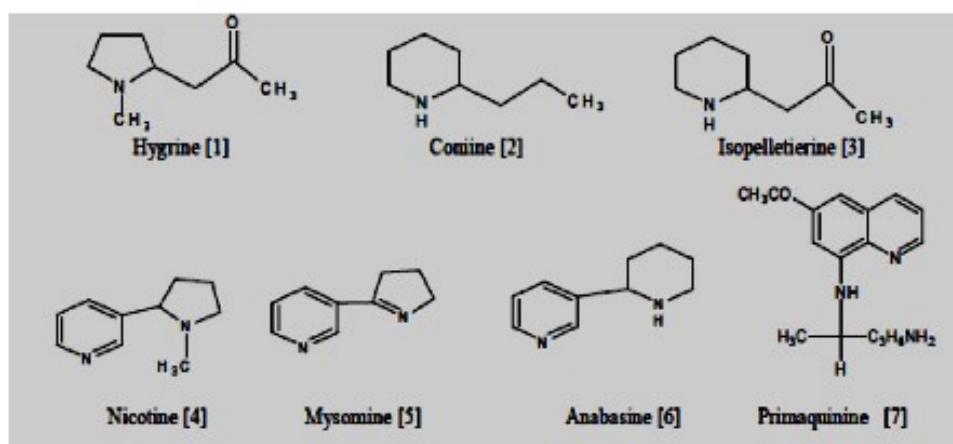


Fig. 2: Structure of Some Important Alkaloids

Source: Saxena *et al.*, 2013

2. Phenolics

Phenolics, phenols or polyphenolics (or polyphenol extracts) are chemical components that occur ubiquitously as natural pigments responsible for the colour

of fruits. Phenolics in plants are mostly synthesized from phenylalanine via the action of phenylalanine ammonia lyase (PAL). They are very important to plants and have multiple functions. The most important role may be in plant defence against pathogens and predators, and thus are applied in the control of human pathogenic infections (Puupponen-Pimiä *et al.*, 2008). They are classified into (i) phenolic acids (ii) flavonoid polyphenolics (flavonones, flavones, xanthones and catechins) and (iii) non-flavonoid polyphenolics.

Caffeic acid is regarded as the most common of phenolic compounds distributed in the plant flora followed by chlorogenic acid; known to cause allergic dermatitis among humans (Kar, 2007). Phenolics essentially represent a host of natural antioxidants, used as nutraceuticals. Apples, green-tea, and red-wine (edible materials of plant origin which contain substantial quantities of phenolics) are known for their enormous ability to combat cancer and are also thought to prevent heart ailments to an appreciable degree and sometimes are anti-inflammatory agents. Rutin and naringin are also prominent plant phenolics.

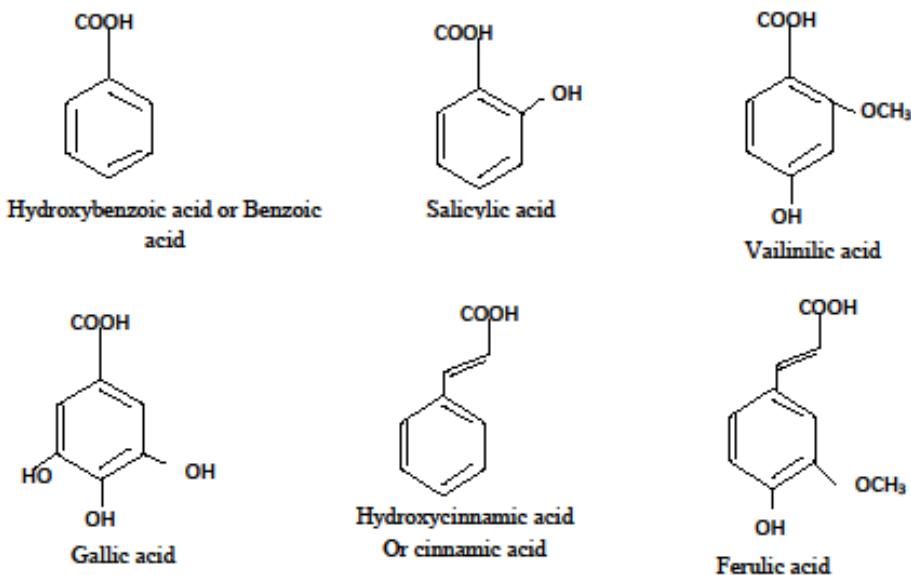


Fig. 3: Structure of some important phenolic acids

Source: Saxena *et al.*, 2013

3. Glycosides

Glycosides in general, are defined as the condensation products of sugars (including polysaccharides) with a host of different organic hydroxyl (and occasionally thiol) group-containing compounds. Glycosides are colourless, crystalline, carbon-, hydrogen- and oxygen-containing (some contain nitrogen and sulphur), water-soluble phyto-constituents found in plant cell sap. Chemically, glycosides contain a carbohydrate (glucose) portion and a non-carbohydrate part (aglycone or genin) (Kar, 2007; Firn, 2010). Alcohol, glycerol or phenols are mostly the aglycones. Glycosides are neutral in solutions and can be readily hydrolysed into their components with mineral acids. Glycosides are classified on the basis of type of sugar component, chemical nature of aglycone

or pharmacological action. The rather older or trivial names of glycosides usually have a suffix ‘in’ and the names essentially included the source of the glycoside, for instance: strophanthidin from Strophanthus, digitoxin from Digitalis, barbaloin from Aloes, salicin from Salix, cantharidin from Cantharides, and prunasin from Prunus. However, the systematic names are invariably coined by replacing the “ose” suffix of the parent sugar with “oside”. This group of drugs are usually administered in order to promote appetite and aid digestion. Glycosides are purely bitter principles that are commonly found in plants of Genitiaceae family and though they are chemically unrelated but possess the common property of an intensely bitter taste. The bitterness acts on gustatory nerves, which results in increased flow of saliva and gastric juices. Chemically, the bitter principles contain the lactone group that may be diterpene lactones (e.g. andrographolide) or triterpenoids (e.g. amarogentin). Some of the bitter principles are either used as astringents due to the presence of tannic acid, as antiprotozoan agents, or to reduce thyroxine activity and metabolism. Examples include cardiac glycosides (acts on the heart), anthracene glycosides (used as a purgative, and for treatment of skin diseases), chalcone glycoside (anticancer agents), amarogentin, gentiopicrin, andrographolide, ailanthone and polygalin. Sarker and Nahar, (2007), reported that extracts of plants that contain cyanogenic glycosides are used as flavouring agents in many pharmaceutical preparations. Amygdalin has been used in the treatment of cancer (HCN liberated in stomach kills malignant cells), and also as a cough suppressant in various preparations. Excessive

ingestion of cyanogenic glycosides can be fatal. Some foodstuffs containing cyanogenic glycosides can cause poisoning (severe gastric irritations and damage) if not properly handled (Sarker and Nahar, 2007). To test for O-glycosides, the plant samples are boiled with HCl/H₂O to hydrolyse the anthraquinone glycosides to respective aglycones, and an alkaline, e.g. NaOH or NH₄OH solution, is added to it. For C-glycosides, the plant samples are hydrolysed using FeCl₃/HCl, and an alkaline solution is added to it. In both cases a pink or violet colour in the base layer after addition of the aqueous base indicates the presence of glycosides in the plant sample.

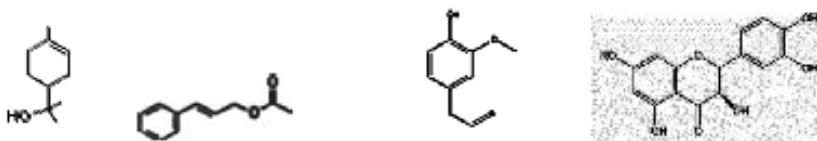


Fig.4: Basic structures of some important plant derived glycosides

Source: Saxena *et al.*, 2013

4. Flavonoids

Flavonoids are important polyphenols widely distributed among the plant flora. Structurally, they are made of more than one benzene ring in their structure (a range of C15 aromatic compounds) and numerous reports support their use as antioxidants or free radical scavengers (Kar, 2007). The compounds are derived from parent compounds known as **flavans**. Over four thousand flavonoids are

known to exist and some of them are pigments in higher plants. Quercetin, kaempferol and quercitrin are common flavonoids present in nearly 70% of plants. Other groups of flavonoids include flavones, dihydroflavons, flavonols, anthocyanidins, proanthocyanidins, calchones, catechins and leucoanthocyanidins.

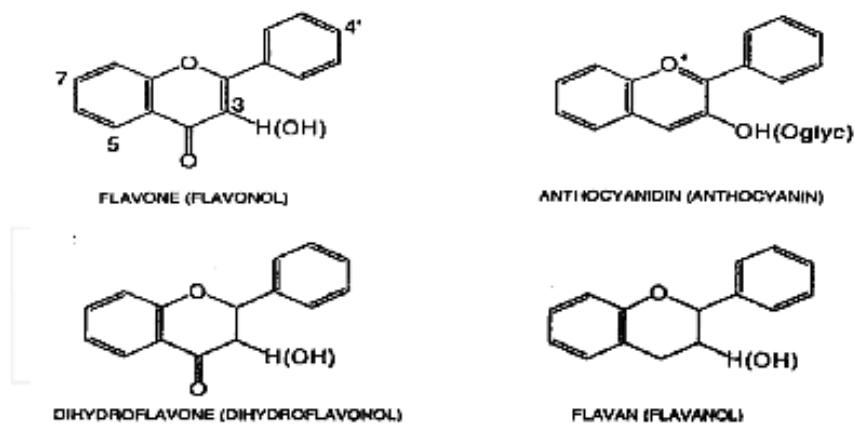


Fig.5: Basic structures of some important plant derived flavonoids

Source: Saxena *et al.*, 2013

5. Steroids

Plant steroids (or steroid glycosides) are one of the most naturally occurring plant secondary metabolites that have found therapeutic applications as arrow poisons or cardiac drugs (Firm, 2010). The cardiac glycosides are basically steroids with an inherent ability to afford a very specific and powerful action mainly on the cardiac muscle when administered through injection into man or animal. Steroids

(anabolic steroids) have been observed to promote nitrogen retention in osteoporosis and in animals with wasting illness (Maurya *et al.*, 2008; Madziga *et al.*, 2010). Caution should be taken when using steroidal glycosides as small amounts would exhibit the much needed stimulation on a diseased heart, whereas excessive dose may cause even death. Diosgenin and cevadine (from *Veratrum veride*) are examples of plant steroids.

6. Tannins

These are widely distributed in plant flora. They are phenolic compounds of high molecular weight. Tannins are soluble in water and alcohol and are found in the root, bark, stem and outer layers of plant tissue. Tannins have a characteristic feature to tan, which is to convert things into leather. They are acidic in solutions, and the acidity is attributed to the presence of phenolics or carboxylic groups (Kar, 2007). They form complexes with proteins, carbohydrates, gelatin and alkaloids. Tannins are divided into hydrolysable tannins and condensed tannins. Hydrolysable tannins, upon hydrolysis, produce gallic acid and ellagic acid and depending on the type of acid produced, the hydrolysable tannins are called gallotannins or egallitannins. On heating, they form pyrogallic acid. Tannins are used as antiseptic and this activity is due to presence of the phenolic group. Common examples of hydrolysable tannins include the a flavins (from tea), daidzein, genistein and glycinein. Tannin-rich medicinal plants are used as healing agents in a number of diseases.

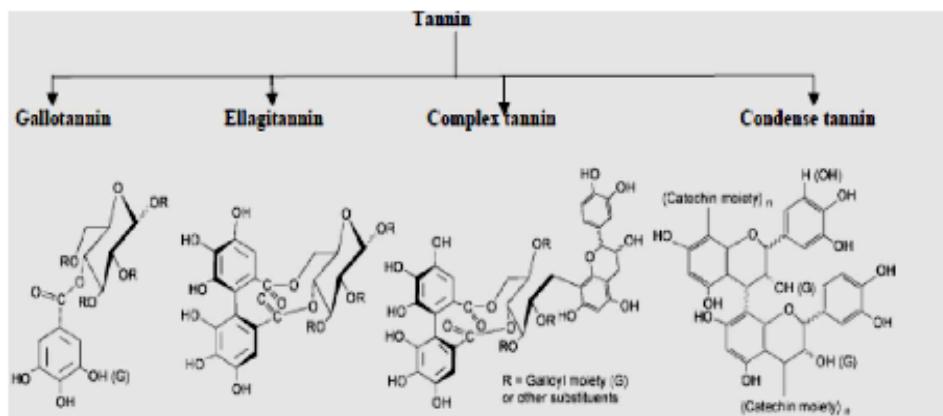


Fig.6: Structures of some pharmacologically important plant derived tannins

Source: Saxena *et al.*, 2013

8. Saponins

The term saponin was derived from *Saponaria vaccaria* (*Quillaja saponaria*), a plant, which has abundant saponins, and was once used as soap. Saponins therefore possess soap-like behaviour in water, which means they produce foam. On hydrolysis, an aglycone is produced, which is called sapogenin. There are two types of sapogenins: steroidal and triterpenoidal. Usually, the sugar is attached at C-3 in saponins, because in most sapogenins there is a hydroxyl group at C-3. *Quillaja saponaria* is known to contain toxic glycosides; quillajic acid and the sapogenin, senegin. Quillajic acid is strenutatory (causes sneezing) and senegin is toxic. Senegin is also present in *Polygala senega*. Saponins are soluble in water and insoluble in ether, and like glycosides on hydrolysis, they give aglycones. Saponins are extremely poisonous, as they cause haemolysis of blood and are

known to cause cattle poisoning (Kar, 2007). They possess a bitter and acrid taste, besides causing irritation to mucous membranes. They are mostly amorphous in nature, soluble in alcohol and water, but insoluble in non-polar organic solvents like benzene and n-hexane.

9. Terpenoids

The terpenoids are a class of natural products which have been derived from five-carbon isoprene units. Most of the terpenoids have multi-cyclic structures that differ from one another by their functional groups and basic carbon skeletons. These types of natural lipids can be found in every class of living things, and therefore considered as the largest group of natural products. Many of the terpenoids are commercially interesting because of their use as flavours and fragrances in foods and cosmetics— examples menthol and sclareol— or because they are important for the quality of agricultural products, such as the flavour of fruits and the fragrance of flowers. Terpenes are widespread in nature, mainly in plants as constituents of essential oils. Their building block is the hydrocarbon isoprene, $\text{CH}_2=\text{C}(\text{CH}_3)-\text{CH}=\text{CH}_2$. Terpenes therefore have the molecular formula $(\text{C}_5\text{H}_8)_n$ and they are classified according to the number of isoprene units:

Hemiterpenoids: consist of a single isoprene unit. The only hemiterpene is the isoprene itself, but oxygen-containing derivatives of isoprene such as isovaleric acid and prenol are classified as hemiterpenoids.

Monoterpenoids: have two isoprene units. Monoterpenes may be of two types; linear (acyclic) or cyclic e.g. geranyl pyrophosphate, limonene, camphor and pinene.

Sesquiterpenes: have three isoprene units e.g. artemisinin, bisabolol and farnesol.

Diterpenes: are composed of four isoprene units. They are derived from geranylgeranyl pyrophosphate. Examples; cembrene, and cafestol. Retinol, retinal, and phytol are the biologically important compounds made up of diterpenes.

Triterpenes: consist of six isoprene units e.g. Lanosterol and squalene found in wheat germ, and olives.

Tetraterpenoids: contain eight isoprene units which may be acyclic like lycopene, monocyclic like gamma-carotene, and bicyclic like alpha- and beta-carotenes.

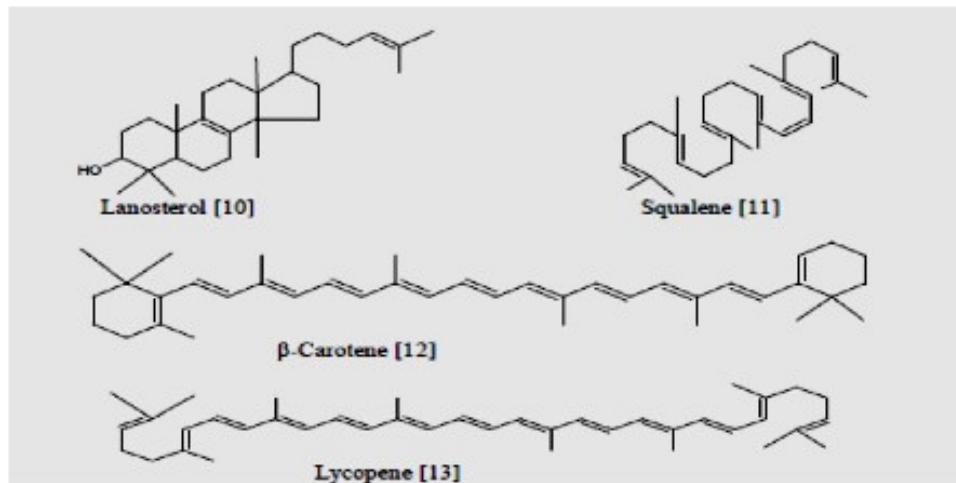


Fig.7: Structure of some important terpenoids

Source: Saxena *et al.*, 2013

10. Organic acids

a. Phytic acid

Phytic acid (PA), also known as myo-inositol hexakisphosphate, is an organic acid formed during maturation of plant seeds and grains. It is a common constituent of plant-derived foods. Phytic acid is not metabolised by plants or humans, and is therefore not a dietary source of inositol or phosphate. It is a good metal chelator which has negative nutritional impact on metal ions necessary for good health thereby preventing their absorption by the intestine; a basis for its use as an antioxidant. Ions of concern in this regard include Zn^{2+} , $Fe^{2+/3+}$, Ca^{2+} , Mg^{2+} , Mn^{2+} , and Cu^{2+} .

Since the 1990s, though, PA has been scientifically emphasized on for its beneficial effects on human health, particularly in the prevention of diabetes (Lee *et al.*, 2006), renal calculi (Saw *et al.*, 2007), Parkinson's disease (Xu *et al.*, 2008) and cancer (Vucenik and Shamsuddin, 2006).

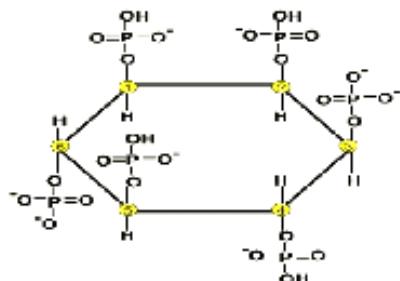


Fig. 8: Structure of Phytic acid

b. **Oxalic Acid**

Oxalic acid is a naturally-occurring component of plants, and is found in relatively high levels in dark-green leafy foods. It is also a metabolic product of ethylene glycol; a major component of petroleum products, trioses and ascorbic acid. The chemical formula for oxalic acid is $C_2O_2(OH)_2$. Oxalic acid is inherently a strong acid: it is about 3,000 times stronger than acetic acid. Oxalic acid is so strong that it is widely used industrially for bleaching and heavy metal smelting; notably for rust removal. If oxalic acid is not heavily diluted—as it is in plants—it is quite dangerous to humans, being both toxic and corrosive. The effects of oxalic acid in the human body, when ingested in foods, flow from its ability to combine chemically with certain metals commonly found in—and

important to—the human body, such as magnesium and calcium. When oxalic acid combines with such metals, the result is, in chemical terms, a "salt"; oxalic-acid+metal salts are called **oxalates**. The potential problems with oxalates in the human body are two. First, oxalates create a condition of decreased availability of macrominerals, such as calcium and magnesium. The second effect is caused by the irritation of vital organs by sharp crystals of oxalates, preeminent in diseases like kidney stones and gouty arthritis.

The only foodstuff that contains oxalic acid at concentrations high enough to cause intoxication is the leaves of the rhubarb plant.

1.5.2 Biological Effects of phytochemicals

The phytochemicals present in plants, responsible for preventing disease and promoting health, have been studied extensively to establish their efficacy and to understand the underlying mechanism of their actions. Such studies have included identification and isolation of the chemical components, establishment of their biological potency both by *in vitro* and *in vivo* studies in experimental animals, through epidemiological and clinical-case control studies in man (Saxena *et al.*, 2013). Phytochemicals may reduce the risk of coronary heart disease by preventing the oxidation of low-density lipoprotein (LDL) cholesterol, reducing the synthesis or absorption of cholesterol, normalizing blood pressure and clotting, and improving arterial elasticity (Mathai, 2000). Phytochemicals may

detoxify substances that cause cancer. They appear to neutralize free radicals, inhibit enzymes that activate carcinogens, and activate enzymes that detoxify carcinogens. Genistein prevents the formation of new capillaries that are needed for tumour growth and metastasis (Meagher and Thomson, 1999). Phytochemicals have also been promoted for the prevention and treatment of diabetes, high blood pressure, and muscular degeneration. While phytochemicals are classified by function, an individual compound may have more than one biological function serving as both an antioxidant and antibacterial agent.

Table 2: Bioactive Phytochemicals in Medicinal Plants.

Classification	Main groups of compounds	Biological function
NSP (Non-starch polysaccharides.)	Cellulose, hemicellulose, gums, mucilages, pectins, lignins	Water holding capacity, delay in nutrient absorption, binding toxins and bile acids.
Antibacterial & Antifungal	Terpenoids, alkaloids, and phenolics	Inhibitors of micro-organisms, reduce the risk of fungal infection
Antioxidants	Polyphenolic compounds, flavonoids, carotenoids, tocopherols and ascorbic acid.	Oxygen free radical scavenging and inhibition of lipid peroxidation.
Anticancer	Carotenoids, polyphenols, curcumine, Flavonoids	Inhibitors of tumour, anti-metastatic agents
Detoxifying Agents	Reductive acids, tocopherols, phenols, indoles, isothiocyanates, coumarins, flavones, carotenoids, retinoids, cyanates, phytosterols	Inhibitors of procarcinogen activation, inducers of drug binding of carcinogens, inhibitors of tumourogenesis
Other	Alkaloids, terpenoids, volatile flavour compounds, biogenic amines	Neuropharmacological agents, anti- oxidants, etc.

Source: (Saxena *et al.*, 2013)

Anti-Psychotic Activity

Chung *et al.*, (1995), developed assays to screen the phytochemical and pharmacological profiles of natural products used in Korean traditional medicine to treat psychotic illnesses. Among the 31 plants screened were *Gardenia jasminoides*, *Citrus unshiu*, *Citrus aurantium*, *Chrysanthemum indicum*, *Ginseng radix* and *Liriope koreana*. In screening assays, some of the plant products exhibited potent selectivity to receptors such as monoamine receptors that are assumed to be involved in mental disorders.

Prevention of Neurodegeneration

Phytochemicals may provide protection against neurodegenerative diseases such as Alzheimer's and Parkinson's (Lee *et al.*, 2012). Research has suggested that phytochemicals such as capsaicin (found in red pepper), curcumin (found in the spice turmeric), epigallocatechin gallate (a catechin in tea known as EGCG), and resveratrol (found in grapes, wine, and peanuts) may have neuroprotective effects (Mythri *et al.*, 2012; Davinelli *et al.*, 2012). The consumption of flavonoid-rich foods such as berries and cocoa throughout life may hold the potential to limit, prevent, or reverse normal or abnormal deterioration in cognitive function in the aging brain (Spencer, 2009).

Several studies have found an association between tea consumption and a lower risk of developing Parkinson's disease or delaying its onset by several years

(Kandinov *et al.*, 2009; Hu *et al.*, 2007; Tanaka *et al.*, 2011). It has been suggested that the association is due to its caffeine content, which also is a naturally occurring phytochemical, but flavonoid intake in general, and berries in particular have been linked to a reduced risk of Parkinson's disease (Gao, *et al.*, 2012).

Phytoestrogens, which have oestrogen-like activity and are found in soy and whole grains, may help prevent cognitive decline that can occur following menopause (Zhao *et al.*, 2009).

Prevention of Cardiovascular Disease

There is evidence to suggest that consuming foods rich in phytochemicals may reduce the risk of cardiovascular disease. One meta-analysis found that increasing fruit and vegetable consumption from fewer than three to more than five servings per day was associated with a 17% reduction in risk. Another meta-analysis suggested that the risk of coronary heart diseases would decrease by 4% for each portion per day of fruits and vegetables added to the diet (Dauchet *et al.*, 2006).

Soy, cocoa, and black and green teas have been studied extensively, and consumption of each one is associated with a reduced risk of cardiovascular disease. There's a wide range of benefits associated with the phytochemical content of these foods and beverages, including lowering blood pressure, reducing inflammation, increasing HDL cholesterol while decreasing LDL oxidation, dilating blood vessels, and decreasing the tendency of the blood to

form clots (Andujar *et al.*, 2012; Suzuki *et al.*, 2012; Bahorun *et al.*, 2012). The range of phytochemicals, including anthocyanins, phytosterols, phenolic acids, lignans, and carotenoids, that are present in wheat, rye, oats, rice, and other grains is believed to contribute to these cardioprotective effects (Fardet, 2010).

Analgesic and Anti-Inflammatory Effects

The compounds glutinol and moretenone exhibited marked analgesic action in mice, being 16- to 26-fold higher in efficacy than aspirin or paracetamol (Gaertner *et al.*, 1999). Terpenoid compounds in a hexane extract of *Eryngium foetidum* Lin. (Apiaceae), a Caribbean endemic plant used in folk medicine for treatment of several anti-inflammatory disorders, reduced oedema induced in mice (Garcia *et al.*, 1999). Phytochemicals in *Lobelia laxiflora* exhibited anti-inflammatory activity in mice through the inhibition of complement activation (Philipov *et al.*, 1998). Phytochemicals in *Teucrium buxifolium* displayed significant anti-ulcer and cytoprotective activities (Fernandez *et al.*, 1997).

Antibacterial, Anti-parasitic and Antiviral Effects

Aqueous extract of the Bulgarian medicinal plant, *Geranium sanguineum* Lin. (Geraniaceae), significantly inhibited the replication of herpes simplex virus Type 1 and Type 2 as shown by the reduction of virus-induced cytopathogenic effect and protection of cells (Serkedjieva and Ivancheva, 1999). The inhibitory effect on virus replication was reported to be related to the phytochemicals in the

plant; flavonoids, catechins, polyphenolic acid and condensed tannins. Among 13 flavanones tested in one study, tetrahydroxyflavanones from *Sophora exigua* and *Echinosopohora koreensis* actively inhibited the growth of methicillin-resistant *Staphylococcus aureus* (Tsuchiya *et al.*, 1996). Natural products – recently discovered from plants- which exhibit antibacterial properties include; salinosporamide, curacin A, dolastatin 10, turbomycin A, cryptophycin, vancomycin and theopalauamide (Doughari *et al.*, 2009). These exert their effect by disrupting membrane transport mechanisms in bacterial cells, especially those involving chemiosmosis.

Antioxidant Activity

Natural antioxidants play a key role in health maintenance and prevention of the chronic and degenerative diseases, such as atherosclerosis, cardiac and cerebral ischemia, carcinogenesis, neurodegenerative disorders, diabetic pregnancy, rheumatic disorder, DNA damage and ageing (Uddin *et al.*, 2008; Jayasri *et al.*, 2009). Plants contain a wide variety of free radical-scavenging molecules including phenols, flavonoids, vitamins, terpenoids that are rich in antioxidant activity (Cai and Sun, 2003). Many plants, citrus fruits and leafy vegetables are sources of ascorbic acid, vitamin E, caratenoids, flavanols and phenolics which possess the ability to scavenge the free radicals in human body. Many dietary polyphenolic constituents derived from plants are more effective antioxidants *in vitro* than synthetic vitamins E and C, and thus might contribute significantly to

protective effects *in vivo* (Jayasri *et al.*, 2009). Due to safety concerns of synthetic compounds, food industries have focused on finding natural antioxidants to replace synthetic compounds.

Anti-carcinogenesis

Polyphenols particularly are among the diverse phytochemicals that have the potential in the inhibition of carcinogenesis (Liu, 2004). Phenolic acids usually significantly minimize the formation of the specific cancer-promoting nitrosamines from the dietary nitrites and nitrates. Glucosinolates, isothiocyanates and indole-3-carbinols do interfere categorically in the metabolism of carcinogens thus causing inhibition of procarcinogen activation, and thereby inducing the ‘phase-II’ enzymes, namely: NAD(P)H quinone reductase or glutathione S-transferase, that specifically detoxify the selected electrophilic metabolites which are capable of changing the structure of nucleic acids (Doughari, 2012). Sulforaphane (rich in broccoli) has been proved to be an extremely potent phase-2 enzyme inducer. It predominantly causes specific cell-cycle arrest and also the apoptosis of the neoplasm (cancer) cells. Sulforaphane categorically produces D-gluconolactone which has been established to be a significant inhibitor of breast cancer. Indole-3-carbinol specifically inhibits the Human Papilloma Virus (HPV) that may cause uterine cancer. It blocks the oestrogen receptors specifically present in the breast cancer cells as well as down regulates cyclin-dependent kinase-6 (CDK6) in prostate cancer cells.

Phytosterols block the development of tumours (neoplasms) in colon, breast, and prostate glands by preventing cell-membrane transfer during mitosis (Doughari, 2012).

Anti-diabetic Activity

Research suggests that phytochemical-rich foods may directly decrease the risk of type 2 diabetes, most likely by reducing inflammation and improving insulin sensitivity, and indirectly by preventing weight gain, the most important risk factor of the disease (Bodinham *et al.*, 2011). Cinnamaldehyde have been reported to exhibit significant anti-hyperglycaemic effect resulting in the lowering of both total cholesterol and triglyceride levels and, at the same time, increasing HDL-cholesterol in streptozotocin (STZ)-induced diabetic rats (Doughari, 2012). Recent reports indicate that polyphenols with procyanidin type-A polymers exhibit the potential to increase the amount of thrombi in Thrombotic Thrombocytopenic Purpura (TTP), Insulin Resistance (IR), and Glucose Transporter-4 (GLUT4) in 3T3-L1 Adipocytes (Jakhelia *et al.*, 2010).

1.6 Treatment of Plant Materials

1.6.1 Drying of plant material

Drying of plant material is an important operation carried out during plant analysis. It can be defined as the loss of moisture from a material into an unsaturated vapour phase which leads to a subsequent reduction in weight of the

material. To examine if the samples are properly dried, weight and moisture content of the samples are evaluated. Dried samples are easily detachable, have much lighter weight and are crispy when felt. The act of drying during plant analysis is aimed at;

- The preservation of sample by minimizing mould and bacterial growth.
- Increasing the stability of certain chemical constituents of the plant material.
- Reducing weight, transportation cost and storage space.

Drying methods are classified into two based on the state of motion of the drying material. They are;

- Static bed method: this includes hot air oven, vacuum oven, freeze drying and exposure to the sun or air-drying.
- Non static bed method: involves movement of materials during drying and includes agitated drying, fluidized bed frying and spray drying.

1.6.2 Extraction

Extraction is the crucial first step in the analysis of medicinal plants, because it is necessary to separate out the desired chemical components from the plant materials. It is defined as the separation of medicinally active portions of plant or animal tissues from inactive or inert components by using selective solvents in

standard extraction procedures. The selection of solvent system largely depends on the specific nature of the bioactive compound being targeted (Sasidharan *et al.*, 2011). Prior to the onset of an extraction process, there is need to produce a **homogenate** (that is a slurry of tissues and cells which results when structures have been mechanically distributed) usually by agitation of a mixture of the material and extraction solvent. During extraction care must be taken to minimize changes in chemical composition, and to ensure that the percentage recovery is satisfactory. After extraction, the products obtained are relatively impure liquids, semi-solids or powders intended for oral or external use (Handa *et al.*, 2008).

Extracts are pharmaceutical preparations obtained from plant/animal tissues with the aid of solvents using standardized extraction procedures. Integrity of the raw material, choice of solvent, process utilization and equipment performance are determining factors for obtaining high-quality plant extracts.

Methods of extraction

The principle methods of extraction that can be employed include any of the following:

- Maceration
- Percolation
- Digestion
- Infusion

- Decoction
- (a) **Maceration** consists simply of extraction by soaking the properly comminuted plant in a solvent until the cellular structure is thoroughly penetrated and the soluble portions are softened and dissolved. This method is claimed to reduce enzyme action to a minimum.
- (b) **Percolation** requires the use of a percolator, and it involves the descent of a suitable solvent through the powdered plant.
- (c) **Digestion, decoction and infusion** are similar in that warm solvent is added to the plant. In digestion, gentle heat is applied without boiling while in decoction menstrum (extraction solvent) actually boils. In the case of infusion, boiling water is poured on the plant for the purpose of extraction. Decoction is suitable for the extraction of water-soluble, heat-stable constituents.

Other modern extraction techniques include soxhlet extraction, solid-phase micro-extraction, supercritical-fluid extraction, pressurized-liquid extraction, microwave-assisted extraction, solid-phase extraction, and surfactant-mediated techniques, which possess certain advantages.

Extraction is desirable for the following reasons;

- Potency is more readily controlled
- Denaturation caused by enzymes action is diminished
- Formulated drugs are more stable, more palatable and more elegant.

- Size reduction
- Elimination of need for bulk facilities required for storage and transport

In the extraction process, various solvents could be used but choice of solvent depends on;

- The cost
- The non-toxicity
- Rate at which the solvent dissolves the active or desirable constituents
- The ability to remove the active constituents.
- The stability of the solvent. Extensive use of wrong solvent may lead to ineffective extraction. Therefore, the type of solvent chosen must be of similar nature to active principles to be extracted.

Water is a universal solvent suitable for the extraction of proteins, glycosides, tannins and alkaloids. Appropriate mixture of alcohol and water may increase extraction efficacy. The extraction of hydrophobic compounds would require the use of polar solvents such as methanol, ethanol or ethyl-acetate, dichloromethane and n-hexane.

1.7 AIMS AND OBJECTIVES OF THE STUDY

In view of the fact that the use of local herbs in treatment of illness in Nigeria folk medicine is on the increase these days, this research involved reviews and experiments:

1. To identify some biological effects of *Diodia sarmentosa* such as antimicrobial and antioxidant effects.
2. To determine the presence of some phytochemicals in *Diodia sarmentosa* through qualitative analysis
3. To carry out the quantitative phytochemical analysis of the aqueous leaf extracts and pulverised leaf samples.
4. To compare the likely biological effects of various phytochemicals in *Diodia sarmentosa* leaf with those in plants of well-known medicinal value.
5. To predict the toxicity of the plant sample by the identification and quantification of anti-nutrients, such as oxalates and cyanogenic glycosides.

1.8 RESEARCH LIMITATION

Diodia sarmentosa may contain other active components which may either exhibit deleterious or curative effects on test organisms. The use of aqueous

extraction method alone limits the range of biologically active components (especially lipid-soluble compounds) to be obtained during the extraction process.

1.9 JUSTIFICATION

D. sarmientosa is claimed to have a lot of values such as medicinal, nutritional and pesticidal values. These claims have not been clearly justified. This research is centred on investigating, analysing and justifying the claims made on this plant. This study would provide knowledge of chemical constituents responsible for the medicinal value of the plant.

1.10 RESEARCH HYPOTHESIS

Null hypothesis (H_0) – in inferential statistics– refers to a general statement or default position that there is no relationship between two measured phenomena, or no difference among groups (Everitt, 1998). It is the probability that similar data from a previous experiment would be obtained in a current research. It denotes that there would be no significant difference between two independent sample means at a designated percentage probability. In view of this, the following ascertainment were made:

1. *Diodia sarmentosa* would contain appreciable quantities of phytochemicals such as flavonoids, alkaloids and phenolic acids with beneficial biological effects.
2. The concentration of bioactive chemicals in *D. sarmentosa* would be similar to those of other plants of prominent medicinal value.
3. *D. sarmentosa*, having been referred to as a herb, would exhibit some degree of toxicity.

CHAPTER TWO

MATERIALS AND METHODS

2.1 MATERIALS

2.1.0 Basic Materials

Plant samples (*Diodia sarmentosa* leaf)

Cotton wool

Cotton cloths

Crucibles

Whatman® (No. 1 and 42) filter papers

Spatula

Masking tape

One-litre sized plastic containers

Universal bottles

PPE; hand gloves, nose masks and laboratory coat

Practical record notebook

Glass wares

- 1000ml Erlenmeyer flasks
- Volumetric measuring cylinders; 25, 200 and 1000ml
- Funnel
- Beaker; 250 and 500ml
- Boiling tubes
- Conical flasks
- 500ml round-bottom flasks

2.1.1 Apparatus/Instruments

Oscillating mixer (BE-2011; 4-65°C, 1kg max.)
Bunsen-burner
Desiccator
Distillation apparatus
Electronic analytical balance and pocket analytical scale (XS 104; 0.0001g)
Electro-thermostatic water cabinet (water bath)
Manual grinder
Magnetic stirrer with hot plate/heating mantle
Muffle furnace
Oven
Refrigerator
Spectrophotometer (Spectrolab 755S; 320-1100nm)
Soxhlet apparatus
Thermometer

2.1.2 Chemicals/Reagents

(Glacial) acetic acid solution
Acetone solution
Aqueous chloroform
Butanol solution
Copper sulphate solution
Copper acetate solution
Distilled water
Disinfectant solutions
Dragendorff's reagent
Ethanol solution
Ferric chloride (FeCl_3) solution
Hydrochloric acid (HCl) solution
Lead acetate solution
Methyl-red indicator
Molisch's reagent
Ninhydrin reagent
Petroleum ether solution
Potassium ferrocyanide solution
Sodium chloride (NaCl) solution
Sodium hydroxide (NaOH)
Sulphuric acid (H_2SO_4) solution

2.2 METHODS

2.2.1 Collection of Plant Material

The study was done in the cropping season (April-September). Fresh and matured shoots of *Diodia sarmentosa* were collected from the surroundings of Federal University of Technology Owerri, Imo State (South-East), Nigeria. A large number of the plant material were collected from many positions in the surroundings. Since the aerial part of the plant was required, samples were carefully removed from mature plant by excising the shoot system just above soil level. Mr Francis Ogunari of the Department of Forestry and Wildlife Technology, Federal University of Technology, Owerri, identified and authenticated the plant.

2.2.2 Preparation of Plant Material

The materials were rinsed severally with clean tap water to remove dust particles and debris, and thereafter allowed to drain completely. Samples were spread out on a clean surface and allowed to air-dry for up to one week. Samples were continually stirred and monitored so as to ensure changes in chemical composition of the leaves do not occur. Rapid air-drying techniques were not used as these may lead to unwanted changes and the loss of low boiling point constituents due to volatilization. After drying, the leaves of DS were plucked from the shoot; damaged and dead leaves were removed, only dark green leaves were used. The leaves were then

pulverized using a properly cleaned manual grinder and then stored in an air tight dry container for further analysis.

2.2.3 Extraction of Plant Material

Aqueous Extraction

40g each of the milled *Diodia sarmentosa* leaves were accurately weighed into clean 1000ml Erlenmeyer flasks, and agitated for about 5 minutes with 500ml of water so as to obtain a homogenous mixture, after which the volume of the mixtures were constituted to 1000ml mark. The flasks were stoppered with cotton wool and lowered into a preheated electro-thermostatic water cabinet, and steamed for an hour at a temperature range of 60°C-67°C so as to accommodate the optimum temperature range of enzymes in the plant material (decoction process). After which the mixtures were poured off into clean plastic containers and allowed to stand for 48 hours at room temperature to allow for thorough extraction of the plants active components (maceration process). Upon the expiration of the 48-hour duration, the suspensions were then filtered with clean cotton cloths and later with Whatman® (No. 1) filter papers to obtain a homogenous filtrate. The filtrates were then concentrated (to one-tenth the original volume) at a temperature range of 37°C-40°C using magnetic stirrers with hot plate until a thick semi-solid fluid was obtained. The concentrates were allowed open in an electro-thermostatic water cabinet at 50°C for complete

dryness yielding crude greenish-brown concentrates of *Diodia sarmentosa* leaf. The concentrates were transferred into sterile universal bottles using spatula, and then refrigerated at 2°C-8°C until use.

2.3 Determination of Percentage Yield of the Extract

The percentage yield was obtained using the formula;

$$\frac{W_2 - W_1}{W_0} \times 100$$

Where; W₂ = total weight of the extract and the container

W₁ = total weight of the container alone, and

W₀ = total weight of the initial dried sample

2.4 Phytochemical Screening of *Diodia sarmentosa* (leaf)

Chemical tests for the screening and identification of bioactive chemical constituents in the medicinal plant under study were carried out on the leaf samples using standard procedures as described by (Sofowara, 1982; Trease and Evans, 1989; Harborne, 1973).

2.4.1 Qualitative Analysis

This was done prior to quantitative analysis in order to detect the presence of the different phytochemicals analysed in the leave sample.

2.4.1.1 Test for Alkaloids

Principle

Free alkaloids, which are of a basic nature form salts by condensing with acids without the liberation of hydrogen.

Preparation of Dragendorff's reagent

This reagent was freshly-prepared as follows:

Solution A: Bismuthnitrate salt (0.17g) in AcOH (2ml) and distilled water (8ml).

Solution B: 4g of KI in AcOH (10ml) and distilled water (20ml)

Both solutions were mixed as designated in a 250ml-beaker and diluted to 100ml using distilled water.

Procedure

5ml of the crude extract was mixed with 5ml of 1% HCl in a boiling tube (borosilicate glass), and warmed inside a steam bath. After which the mixture was filtered. 1ml of the freshly-prepared Dragendorff's was added to 1ml of the filtrate. Formation of an orange-red precipitate indicated a positive test (Trease and Evans, 1989).

2.4.1.2 Test for Tannins

Lead Acetate Test

Principle

Tannins cause precipitation of solutions of heavy metal salts of copper, lead and tin.

Phlobotannins react at room temperature with lead acetate to give dark-blue to black precipitate.

Procedure

In accordance with the method of Trease and Evans, (1989), about 3 drops of 5% lead acetate was added to 2ml of the extract. Formation of a dark blue precipitate indicated a positive result.

2.4.1.3 Test for Saponins

Froth Formation Test

In accordance with the method of Sofowora, (1982), 2g of the powdered leaf sample was accurately measured out into a boiling tube and dissolved with 20ml of distilled water. The mixture was shaken for 15 minutes at room temperature. Formation of 1cm stable foam which persisted on warming in a water bath indicated a positive test.

2.4.1.4 Test for Flavonoids

Principle

Sodium hydroxide from acid stable complexes with C-4 keto-groups and either the C-3 or C-5 hydroxyl groups of flavone and flavonols.

Procedure

In accordance with the method of Trease and Evans, (1989), 1ml of the filtered extract was introduced into a boiling tube, and dissolved with few drops of 10% NaOH solution. Formation of intense yellow colour which became colourless on addition of dilute HCl solution indicated the presence of flavonoids.

2.4.1.5 Test for Steroidal and/or Cardiac Glycosides

Salkowskii Test

Procedure

In accordance with the method of Sofowora, (1982), 1ml of the crude extract was dissolved with 10ml of chloroform in a boiling tube. An equal volume of concentrated sulphuric acid solution was carefully poured down the sides of the tube to form a layer. A reddish-brown colour at the interface indicated the presence of steroids.

Keller-Kiliani Test for Cadenonides

Procedure

In accordance with the method of Trease and Evans, (1989), 5ml of the diluted extract was dissolved with 2ml of 3.5% ferric chloride in glacial acetic acid. The mixture was overlaid with 2ml of conc. sulphuric acid solution. The appearance of a reddish-brown ring at the interface of the liquid indicated the presence of deoxy-sugars characteristic of cardenolides.

2.4.1.6 Test for Carbohydrate

Molisch's Test

1ml of the extract was dissolved in distilled water, and a few drops of Molisch's reagent was added to it. Next, 1ml of conc. Sulphuric acid was introduced into the mixture in boiling tube so that the acid forms a layer beneath the aqueous layer. The mixture was allowed to stand for 2 minutes, and was then diluted with 5ml of distilled water. Formation of a dull violet colour at the interface of the two layers indicated a positive test.

2.4.1.7 Test for Amino acids/Proteins

Ninhydrin Test

0.25% w/v ninhydrin reagent was added to 1ml of the crude extract in a boiling tube, and was warmed for few minutes. Formation of a blue colour indicated a positive test.

2.4.1.8 Test for Terpenoides

Copper Acetate Test

1ml of the crude extract was dissolved in distilled water and treated with three drops of copper acetate solution. Formation of emerald-green colour indicated a positive test

2.4.1.9 Test for Phenolics

Ferric chloride Test

1ml of the crude extract was treated with 3 drops of ferric chloride solution. Formation of bluish-black colour indicated the presence of phenols.

2.4.1.10 Test for Phytic acid

Principle

Phytic acids chelate trivalent cations. Most assays for phytic acids employ ferric chloride to precipitate ferric phytate. Sodium hydroxide may be used to convert the precipitate to sodium phytate and ferric hydroxide.

Procedure

1ml of Wade reagent (prepared by the dissolution of 1mg ferric chloride salt in 10ml of 0.375N HCl) was added into a boiling tube containing 0.5ml of the D.S extract. Formation of a dark red precipitate confirmed the presence of phytic acids.

2.4.1.11 Test for Oxalic acid

Procedure

1ml of the extract was acidified with acetic acid, warmed and mixed with an equal volume of a saturated solution of calcium sulphate, CaSO_4 . Formation of a white precipitate of calcium oxalate, CaC_2O_4 , indicated the presence of oxalic acid ($\text{H}_2\text{C}_2\text{O}_4$). The precipitate was washed, dissolved in warm dilute H_2SO_4 , and mixed with dilute solution of potassium permanganate (VII), KMnO_4 . Decolourization of the potassium permanganate solution confirmed the test for oxalic acid, $\text{H}_2\text{C}_2\text{O}_4$.

2.4.2 Quantitative Analysis

2.4.2.1 Determination of Alkaloids

In accordance with the method of Harbone, (1973), 5g of the crude extract was accurately weighed, introduced into a 250ml beaker and mixed with 200ml of 20% acetic acid in ethanol (1:10). The mixture was allowed to stand for 4hrs, after which it was filtered, and concentrated using a water bath. The concentrate obtained was evaporated to one-quarter its original volume and treated with drop-wise addition of conc. aqueous ammonium hydroxide until alkaloids were precipitated. The solution was allowed to sediment, after which the precipitate was filtered off, dried and weighed.

The percentage of alkaloids in the extract was calculated using the formula:

$$\text{Alkaloids (\%)} = \frac{W_2 - W_1}{\text{Weight of sample}} \times 100$$

Where, W1 = weight of filter paper

W2 = weight of filter paper + residue

2.4.2.2 Tannin Determination by Titration

The Folin-Denis titrating method as described by Pearson, (1974), was used. To 20g of the crushed sample in a conical flask was added 100ml of petroleum ether and covered for 24 hours. The sample was then filtered and allowed to stand for 15

minutes allowing petroleum ether to evaporate. It was then re-extracted by soaking in 100ml of 10% acetic acid in ethanol for 4hrs. The sample was then filtered and the filtrate collected. 25ml of NH₄OH were added to the filtrate to precipitate the tannic acids. The solution was heated with electric hot plate to remove some of the NH₄OH still in the solution. The remaining volume was measured to be 33ml. 5ml of this was taken and 20ml of ethanol was added to it. It was titrated with 0.1M NaOH using phenolphthalein as indicator until pink end point was reached. Tannin content was then calculated.

$$C_1 = \frac{C_2 V_1}{V_2}$$

$$\% \text{ of tannic acid content} = \frac{C_1 \times 100}{\text{Weight of sample analysed}}$$

C₁ = concentration of tannic acid

C₂ = concentration of base = 0.1

V₁ = volume of titrated precipitate = 5ml

V₂ = volume of base = titre value of sample

2.4.2.3 Determination of Flavonoids

In accordance with the method of Harbone, (1973), 5g of the sample was weighed, introduced into a 100ml plastic container, and was extracted repeatedly with 100ml of 80% aqueous ethanol at room temperature. The extracts were filtered into a 1000ml flask using Whatman® No. 42 filter paper. The filtrate was transferred into

a crucible dish and evaporated to dryness in a water bath. After which it was further dried in an oven at 60°C for 30 minutes and cooled in a desiccator. The crucible and its content were then weighed to obtain W2.

The percentage flavonoid content was calculated thus:

$$\text{Flavonoids (\%)} = \frac{W_2 - W_1}{\text{Weight of sample}} \times 100$$

Where, W1 = weight of empty crucible

W2 = weight of filtrate + crucible

2.4.2.4 Determination of Saponins

In accordance with the method of Harbone, (1973), 5g of the plant sample was dispersed in 200ml of 20% ethanol. The suspension was heated in a water bath for 4hrs at 55°C with continuous stirring. After which it was filtered and re-extracted with another 200ml of 20% ethanol. The combined liquid extracts were then evaporated to 40ml at 90°C. The concentrated extract was then transferred into a 250ml separator funnel. 20ml of diethyl ether was added to it and shaken vigorously. The aqueous layer formed was recovered. The purification process above was repeated using 50ml of n-butanol. The solution obtained was then washed using aqueous sodium chloride, after which it was poured into a pre-weighed crucible and dried in an oven at 60°C to a constant weight.

The saponin concentration was calculated thus:

$$\text{Saponins (\%)} = \frac{W_2 - W_1}{\text{Weight of sample}} \times 100$$

Where, W1 = weight of empty crucible

W2 = weight of saponin extract + crucible

2.4.2.5 Cardiac Glycosides Determination

In accordance with the method of Osagie, (1998), 1ml of extract was mixed with 1ml of 2% solution of 3,5 – DNS (Dinitrosalicylic acid) in methanol and 1ml of 5% aqueous NaOH in a boiling tube. It was boiled for two minutes (until brick-red precipitate was observed) and the boiled sample was filtered. The weight of the filter paper was obtained before filtration. The filter paper with the absorbed residue was dried in an oven at 50°C and the weight of the filter paper with residue was noted.

The cardiac glycoside was calculated thus:

$$\% \text{ cardiac glycoside} = \frac{(\text{weight of filter paper} + \text{residue}) - (\text{weight of filter paper}) \times 100}{\text{Weight of sample analysed}}$$

2.4.2.6 Oxalate Determination by Titration Method

This was determined according to Osagie, (1998).

This determination involved three major steps; digestion, oxalate precipitation and permanganate titration.

i. Digestion

- 1) 2g of sample was suspended in 190ml of distilled water in a 250ml volumetric flask
- 2) 10ml of 6M HCl was added and the suspension digested at 100°C for 1 hour
- 3) The digest was cooled, and then made up to 250ml mark before titration

ii. Oxalate precipitation

Duplicate portions of 125ml of the filtrate were measured into beakers and four drops of methyl red indicator added. This was followed by the addition of NH₄OH solution (dropwise) until the test solution changed from pink to faint yellow colour. After which each portion was heated to 90°C, cooled and filtered to remove precipitate containing ferrous ions. The filtrate was again heated to 90°C and 10ml of 5% CaCl₂ solution was added while being stirred constantly. After heating, it was cooled and left overnight at 25°C. The solution was then centrifuged at 2500rpm for 5 minutes. The supernatant was decanted and the precipitate completely dissolved in 10ml of 20% (v/v) H₂SO₄ solution.

iii. Permanganate Titration

At this point, the solution in sulphuric acid was titrated against 0.05M standardized KMnO_4 solution to a faint pink colour which persisted for 30 seconds (using phenolphthalein indicator). The calcium oxalate content is calculated using the formula:

$$\text{Oxalate Content (mg/100g)} = \frac{\text{T} \times (\text{Vme})(\text{Df}) \times 10^5}{\text{ME} \times \text{Mf}}$$

Where: T = titre value of KMnO_4 (ml);
Vme = volume – mass equivalent (i.e. 1ml of 0.05M KMnO_4 solution
 is equivalent to 0.00225g anhydrous oxalic acid);
Df = dilution factor V_t/A (2.4);
Vt = total volume of titrate (300ml);
A = volume of aliquot used (125ml);
ME = molar equivalent of KMnO_4 in oxalate (KMnO_4 redox
 reaction); and
Mf = mass of sample used.

2.4.2.7 Determination of Phytic acid Content

Phytic acid content was determined using the method of Young and Greaves, (1940). 0.2g of each of the differently processed samples were weighed into different 250ml conical flasks. Each sample was soaked in 100ml of 2% concentrated HCl for 3hrs.

The samples were then filtered. 50ml of each filtrate was placed in 250ml beaker and 100ml distilled water was added to each sample. 10ml of 0.3% ammonium thiocyanate was added to the resulting solutions to serve as indicator which were titrated to end point with standard iron (111) chloride solution which contained 0.00195g iron per ml. The percentage phytic acid content was calculated using the formula:

$$\text{Phytic acid (\%)} = \frac{\text{Titre value} \times 0.00195 \times 1.19 \times 100}{2}$$

2

2.4.2.8 Determination of Total Phenolic acids

Determination of total phenolic acids was performed according to procedure described in European Pharmacopoeia, (2004). 2g of the plant sample was boiled with 50ml ethanol for 50minutes. 5ml of the boiled sample was then pipetted into a 50ml volumetric flask and 10ml of distilled water was added. After the addition of distilled water, 2ml of NH₄OH solution and 5ml of concentrated pentanol were added to the mixture. The sample was made up to mark, left for 30minutes to react for colour development and measured at 505nm wavelength using a spectrophotometer and distilled water as blank. The percentage total phenolic acid content was calculated according to the following expression: (%) = A × 2.5/m,

where A is the absorbance of the test solution at 505 nm, and m is the mass of the sample, in grams. Analysis of each sample was performed in triplicate.

2.5 STATISTICAL ANALYSIS

Each experiment was done for three concordant readings and results were expressed as Mean \pm standard deviation. Means were tested with student t-test and significance accepted at $P \leq 0.05$ probability levels. All statistical analyses were carried out using Microsoft Excel 2013 software.

CHAPTER THREE

3.0 RESULTS

The percentage yield of the aqueous leaf extracts was obtained as 23.89% \approx 24%.

This shows the efficacy of the solvent in the recovery of desired constituents from the D.S leaf samples.

3.1 Qualitative Analysis

Table 3: Data Obtained from Qualitative Analysis of D.S (leaf)

Phytochemicals	Leaf
Tannins	+++
Saponins	+++
Alkaloids	+++
Flavonoids	+++
Cardiac Glycosides	++
Phytic acid	+
Oxalic acid	+
Phenolics	+
Steroids	+++
Carbohydrates	+++
Proteins	+++
Terpenoids	+++

Key: - (negative) absent; + (positive) present; +++ = High concentration, ++ =

Moderate concentration, + = Low concentration

Table 3 above shows data obtained from the qualitative analysis of the plant sample (aqueous leaf extracts). The notation + + + was assigned based on the intensity of colour – or any other indication of positive result(s) – observed. The aqueous D.S extracts contained significant quantities of tannins, saponins, alkaloids and flavonoids. Analytical procedures to detect their presence showed high concentrations of these phytochemicals.

3.2 Quantitative Analysis

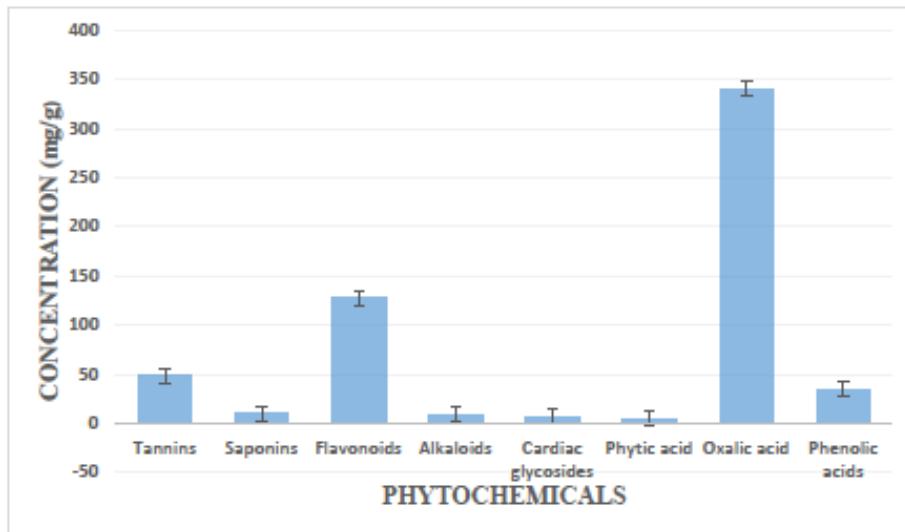


Fig. 9: Concentration (mg/g) of phytochemicals in D.S (leaf)

Figure 9 above depicts the concentration of various phytochemicals assayed for in the aqueous extracts and the pulverized samples of D.S (leaf). Results show the leaf samples contained appreciable quantities of flavonoids, tannins phenolic and oxalic acids. Comparison of the concentrations of the phytochemicals showed significant differences ($p \leq 0.05$) amongst values. Oxalic acid was the highest in concentration (340.2mg/g of sample) followed by flavonoids (127.4mg/g of sample).

CHAPTER FOUR

DISCUSSION, CONCLUSION AND RECOMMENDATION

4.1 DISCUSSION

The medicinal value of plants lies in some chemical substances that have definite physiological actions on the (human) body (Edeoga *et al.*, 2005). The study carried out on *Diodia sarmentosa* plant revealed the presence of bioactive constituents. Qualitative analyses were carried out on the aqueous leaf extracts of the plant whereas quantitative analyses were done using the pulverized leaf samples. From Table 3, tannins, saponins, alkaloids, flavonoids, cardiac and steroid glycosides, phytic acid, oxalic acid, phenols and terpenoids were present in the sample. These phytochemicals have been found to possess a wide range of activities which may help in protection against chronic diseases.

Alkaloids are natural products that contain heterocyclic nitrogen atoms, are basic in character, and easily react with acids to form salts. They have a bitter taste. Their concentration in the samples were not high enough to induce a bitter taste on the extracts. Alkaloids have many pharmacological activities including antihypertensive effects (many indole alkaloids), antiarrhythmic effect (quinidine, sparteine), and anticancer actions (dimeric indoles, vincristine, and vinblastine). Alkaloids are significant for the protection of plants; especially against micro-organisms

(antibacterial and antifungal activities), insects and herbivores and also against other plants by means of allelopathically active chemicals which inhibit synthesis of microbial cell wall polysaccharides and neurotransmission (Wink *et al.*, 1998). This could be the basis for the use of the plant by Igbo natives as a curative for eczema; a fungal infection. Alkaloids such as; caffeine and nicotine have stimulant effects; morphine is used as an analgesic and quinine is a major active ingredient in antimalarial drugs (Rao *et al.*, 1978). Quinine blocks hemozoin crystallization during heme degradation, thereby preventing red blood cell disintegration with subsequent accumulation of toxic metabolic products *in vitro* which has deleterious effects on malaria-causing protozoa (Bray *et al.*, 2005). The alkaloid content of the D.S leaf samples analysed ($8.24\pm0.44\text{mg/g}$) were significantly higher ($p\leq0.05$) than that obtained for *Vernonia amygdalina*; 6.65 ± 0.06 (Yusuf and Obiegbara, 2015), a plant with prominent antimalarial activity. D.S would therefore possess significant antimalarial and stimulating activities. Extraction and quantification of alkaloids in D.S can be done; using high performance liquid chromatography or microwave-assisted techniques, in order to support this hypothesis.

Tannins are a heterogeneous group of high molecular weight polyphenolic compounds with the capacity to form reversible and irreversible complexes with proteins (mainly), polysaccharides (cellulose, hemicellulose, pectin, etc.), alkaloids,

nucleic acids and minerals (Schofield *et al.*, 2001). The tannin-containing plant extracts are used as astringents, against diarrhoea, as diuretics, against stomach and duodenal tumours (De Bruyne *et al.*, 1992), and as anti-inflammatory, antiseptic, antioxidant and haemostatic pharmaceuticals (Dolara *et al.*, 2005). Plants with tannins are used for healing of wounds, varicose ulcers and burns (Nafiu *et al.*, 2011). The tannin content of the D.S leaf samples analysed was $48.2\pm0.36\text{mg/g}$, which is significantly greater than those in *Chromolaena odorata* leaf; 41.0mg/g (Agaba *et al.*, 2015). This justifies the folkloric use of D.S as an antiulcer agent. In the food industry, tannins are used to clarify wine, beer, and fruit juices. Other industrial uses of tannins include the manufacture of textile dyes, as antioxidants in fruit juice, beer, and wine, and as coagulants in rubber Production (Gyamfi and Aniya, 2002).

Saponins have been reported to protect against hypercholesterolemia, to kill protozoans and molluscs, to be antioxidants, to impair the digestion of protein and the uptake of vitamins and minerals in the gut, to cause hypoglycaemia, and to act as antifungal, anticancer and antiviral agents due to their ability to depolarise lipids and target carcinoma growth factors (Morrissey and Osbourn, 1999; Takechi *et al.*, 1999; Traore *et al.*, 2000). Saponin concentration in the D.S leaf samples analysed was $9.62\pm0.29\text{mg/g}$, a value significantly greater ($p\leq0.05$) than those obtained for

both *Moringa oleifera* (leaf) and *Hibiscus sabdariffa* (flower); 1.13 ± 0.03 and 1.19 ± 0.11 , respectively. Both vegetables exhibited high antioxidant properties in suppressing ferric thiocyanate and malonaldehyde formations in linoleic acid emulsions (Ijeomah *et al.*, 2012).

Flavonoids have been stated to possess many useful properties; containing anti-inflammatory activity (through the inhibition of cyclooxygenases and lipoxygenases), enzyme inhibition property, antimicrobial activity, oestrogenic activity, anti-allergic activity, vascular activity and hepatoprotective activity (Tapas *et al.*, 2008). Flavonoids constitute a wide range of substances that play important role in protecting biological systems against the harmful effects of oxidative processes on macromolecules such as carbohydrates, proteins, lipids and DNA (Atmani *et al.*, 2009). Transport and cycling of vitamin C is flavonoid-related. Flavonoids and/or their metabolites easily reduce free radicals and other oxidants in the plasma. In addition to this, flavonoids induce the expression of antioxidant enzymes such as manganese superoxide dismutase, catalase, glutathione peroxidase (Chae *et al.*, 2013). They exhibit high nucleophilicity due to the numerous unsaturated bonds in their chemical structures. Most flavonoids are aromatic, and are used to improve food quality. The D.S samples did not possess any perceivable aromatic property. The percentage flavonoid content of the plants would comprise

more of free radical scavenging flavonoids rather than aromatic flavonoids. Flavonoid concentration in the D.S leaf samples analysed was 127.4 ± 0.44 mg/g, a value significantly greater ($p \leq 0.05$) than those obtained for both *Moringa oleifera* (leaves) and *Hibiscus sabdariffa* (flowers); 29.42 ± 0.49 and 5.54 ± 0.37 , respectively (Ijeomah *et al.*, 2012). The high flavonoid concentration in the D.S samples correlates with its oxalic acid content. The D.S plants from which the samples were gotten may have grown on soil with high mineral content, hence the need for increased free radical scavenging activity.

Phenolic acids possess diverse biological activities, for instance, antiulcer, anti-inflammatory, antioxidant, cytotoxic and antitumor, antispasmodic, and antidepressant activities (Silva *et al.*, 2007; Ghasemzadeh *et al.*, 2010). Possible use of phenolic acids in the control of diabetes is being reviewed. Ingested phenolics are metabolised in the body system by glucuronosylation in the liver (Shangari *et al.*, 2005). The modified phenolics are either stored in the liver or excreted via the kidney. Release and re-entry of glucuronic acid into circulation may occur when the need arises. Utilization of glucose in this process ensures timely regulation of glucose concentration in the blood. Significant amounts of phenolic acid (33.66 ± 0.41 mg/g) were obtained in the D.S leaf samples analysed which could exhibit sufficient hypoglycaemic activity.

Qualitative analysis revealed the presence of terpenoids in the D.S leaf samples. Terpenes are important signal transducers and growth regulators (phytohormones) in plants. In addition, terpenoids have medicinal properties such as anticarcinogenic (e.g. perilla alcohol), antimalarial (e.g. artemisinin), anti-ulcer, hepaticidal, antimicrobial or diuretic (e.g. glycyrrhizin) and anticancer activities (Langenheim, 1994; Dudareva *et al.*, 2004).

Oxalic acid is a naturally-occurring component of plants found in relatively high concentrations in dark green leafy plants like D.S. Results from phytochemical analysis data showed high concentration of oxalic acids in the D.S leaf samples analysed (340.2 ± 0.43 mg/g) which were significantly higher than those obtained for rhubarb leaf; 5.2mg/g (Pucher *et al.*, 1938), known for the toxic level of oxalates. Owing to this fact aqueous D.S leaf extracts may not be suitable for ingestion by humans. High oxalic acid concentration is quite dangerous to humans, being both toxic and corrosive. These effects are due to its ability to combine with certain metals (calcium and magnesium, especially) with resultant reduction in bioavailability of these metals and the denaturation of metalloproteins. However, oxalic acid concentration of the plant can easily be reduced by dilution or by the regulation of plant growth conditions. Studies have shown that oxalic acid levels depend on the age of the plant as well as its growth rate (Albihn and Savage, 2001; Kaminishi and

Kitai, 2006). Paul *et al.*, (2012), evaluated the effect of cooking and processing methods on oxalate content of green leafy vegetables and pulses. Blanching for 10 minutes at 95°C lowered oxalic acid content by 40-115% in various green vegetables. The study also showed an average 25% reduction in oxalic and phytic acid contents in pulses that were soaked for 48 hours before being boiled. Beneficial activities of oxalic acids include physical or chemical defence of plants against herbivores, detoxification of heavy metals, calcium regulation, antioxidant activity, and anti-bacterial activity (as a result of its denaturation of bacteria proteins and/or enzymes) (EMEA, 2004). Use of D.S in phytoremediation can be reviewed as the plant may exhibit toxic metal entrapping and/or accumulating properties in intoxicated soils. Phytic and oxalic acids have strong affinity for divalent cations. Genetic engineering can be exploited to increase the plant's affinity for heavy metal ions like lead (Pb^{2+}) and chromium (Cr^{2+}) ions.

4.2 CONCLUSION

Phytochemical analysis showed presence of medicinally-important constituents in both the aqueous extracts and pulverised samples of *D. sarmentosa* leaf. Tannins, saponins, alkaloids, flavonoids, cardiac and steroidal glycosides, phytic acid, oxalic acid, phenols and terpenoids were present in the samples. The relative concentrations of these compounds varied significantly ($p \leq 0.05$). Oxalic acid was the most

abundant compound followed by flavonoids. Many evidences gathered in previous studies confirmed the identified phytochemicals to be bioactive. Therefore, *Diodia sarmentosa* leaves could be seen as a good source of useful drugs. High concentrations of antinutrients (saponins, oxalic acids and phytic acids) denote the leaf extracts would be toxic and would have a low LD₅₀ value. The presence of significant amount of flavonoids and tannins showed its potential use as an antioxidant and an insecticidal agent.

4.3 RECOMMENDATION

I recommend the analysis of the other plant parts – stem and roots in order to determine and identify which contains greater quantities of phytochemicals. Use of suitable techniques in carrying out studies on the toxicity of the aqueous extract to ascertain its safety for therapeutic use is also recommended. D.S contains significant quantities of saponins, possible analysis and/ or identification of steroidal saponins in the plant should be done for use in commercial production of steroidal anti-inflammatory agents. The metal chelating activity of the leaf extracts should be carried out. Considering the high oxalic acid content of the plant (leaf), it would exhibit sufficient activity that would inculcate its use as a toxic metal accumulator.

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APPENDICES

Appendix 1: Determination of the Percentage Yield of the Extract:

$$W_0 = 1080 \text{ (40g each} \times 27)$$

$$W_1 = 41.94\text{g}$$

$$W_2 = 299.9 \pm 5\text{g (lost concentrates/extraneous materials)}$$

$$\begin{aligned}\text{Percentage Yield of the extract} &= \frac{(299.9 - 41.94)\text{g}}{1080\text{g}} \times 100 \\ &= \frac{257.96}{1080} \times 100 \\ &= 0.23885 \times 100 \\ &= 23.89\% \approx 24\%\end{aligned}$$

Appendix 2: Preparation of Reagents

All analytical procedures were carried out at New Concepts Chemical Engineering and Environmental Services, Obinze, Owerri, Imo State.

For salts and other amorphous chemicals, solutions were prepared using the formulae below:

$$\text{Grams of Compound required} = \text{Molarity (mol/l)} \times \text{Molar mass (g/mol)} \times$$

$$\text{Volume (in litres)}$$

$$\text{Mass concentration (g/l)} = \text{Molarity (mol/l)} \times \text{Molar mass (g/mol)}$$

For aqueous reagents, solutions were prepared using the formulae below:

$$\text{Molarity} = \frac{n}{\text{proton/basicity}}$$

$$n = \frac{\text{Mass (g)}}{\text{Molar mass (g/mol)}} = \text{Concentration (mol/l)} \times \text{Volume (in litres)}$$

$$\text{Mass of aqueous solution} = \text{percentage purity} \times \text{Specific gravity (g/cm}^3) \times 1000\text{cm}^3$$

Volume of reagent required from stock solution (V_r) is given by:

$$V_r = \frac{\text{Concentration of solution required (mol/l)} \times \text{Volume required (l)}}{\text{Concentration of stock solution (mol/l)}}$$

Percentage concentrations were obtained by dilution of the stock/concentrated solutions with appropriate volume of distilled water.

All mass measurements were taken using electronic analytical balance.

Appendix 3: Statistical Analysis

Scatterthwaite's Approximate Test, a method in the Behrens-Welch family (Armitage and Berry, 1994), was used for comparison of values. Assuming unequal variances, significant differences between unpaired variables were computed using the formulae below:

$$d = \frac{|\bar{X}_1 - \bar{X}_2|}{\sqrt{\frac{S_1^2 + S_2^2}{n_1 + n_2}}} \quad d.f = \sqrt{\frac{\left[\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2} \right]^2}{\frac{(S_1^2/n_1)^2}{n_1-1} + \frac{(S_2^2/n_2)^2}{n_2-1}}} \quad S = \frac{\sum(X - \bar{X})^2}{n-1} \quad \bar{X} = \frac{\sum(X)}{n}$$

Where:

d = Behrens-Welch Test Statistics

\bar{X}_1 = Mean of group 1

\bar{X}_2 = Mean of group 2

N_1 = Number of samples in group 1

N_2 = Number of samples in group 2

σ = Population variance

S_1 = Standard deviation of group 1

S_2 = Standard deviation of group 2

$N_1 + N_2 - 2$ = degree of confidence (df)

Two means are statistically different if the calculated t value, that is, t statistics (d) is greater than or equal to the tabulated/critical t . value (t_{tab}) obtained from t-distribution table at 95% confidence level.

All percentage concentrations were converted to mg/g using the formulae:

$$\text{Concentration of Phytochemical (mg/g)} = \frac{\text{Mass of Phytochemical (g)}}{\text{Mass of sample used (g)}} \times 1000\text{mg}$$

$$\text{Mass of Phytochemical (g)} = \frac{\text{Percentage concentration}}{100} \times \text{Mass of sample used (g)}$$