

PHYTOCHEMICAL ANALYSIS, RADICAL SCAVENGING AND ANTI-MICROBIAL
ACTIVITY OF THE LEAVES OF *GOMPHOCARPUS FRUTICOSUS*

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Abstract

Gomphocarpus fruticosus is a wild plant that belongs to the *Asclepiadaceae* family and has many traditional uses. The plant has demonstrated by this study a significant level of microbial and phytochemical activity; the drug-resistant strains of microbes which have emerged in the last decade will be eradicated by the compounds this indigenous plant carries. This particular study aimed to scientifically validate the traditional uses of the plant *G. fruticosus*. The objectives of this study were (a) to extract and screen the phytochemicals in the leaves of *G. fruticosus*, (b) to quantify the detected phytochemicals, (c) to evaluate the anti-microbial activity of the leaf extracts and (d) to determine the antioxidant activity. The plant samples that were used in this research study were collected in Windhoek, Khomas Region of Namibia and in particular the areas surrounding the University of Namibia. Phytochemical screening was done followed by the evaluation of total flavonoid, phenol and tannin content using aluminium chloride method, folin-ciocalteu method and the potassium ferrocyanide methods respectively. Antimicrobial screening as well as MIC determination was done using the disk diffusion method. The antioxidant activity was determined using the DPPH Free Radical Scavenging Method. The quantitative phytochemical determinations were performed in triplicate and expressed as means (\pm standard deviation) and of the phytochemicals that were quantified, tannins were present in large amounts with 6.937 ± 0.115 mg TA/g in the methanolic extract and 5.356 ± 0.185 mg TA/g in the ethanolic extract. The MIC values for antimicrobial analysis were recorded as the least concentration of plant extracts that completely inhibited the growth of the microorganism. The MIC value for the ethanolic extract on *Staphylococcus aureus* was 1 mg/ml and for *Escherichia coli* it was 6 mg/ml. For DPPH, the extract with the lowest IC_{50} was the one with the highest scavenging activity. The standard used was Vitamin C and it had an IC_{50} value of 0.37 mg/ml. The IC_{50} value of the methanolic extract and ethanolic extract were 0.25 mg/ml and 0.34 mg/ml

respectively. At an overall point of perspective, it can be concluded that the leaf extract of *G. fruticosus* is of great medicinal significance due to the phytochemicals present, antimicrobial activity and antioxidant activity that was exhibited.

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List of Abbreviations

A1	Ethanollic Extract
A2	Methanollic Extract
Ca	<i>Candida albicans</i>
CPGS	Centre of Post Graduate Studies
DPPH	2, 2- diphenyl-1-picrylhydazyl
Ec	<i>Escherichia coli</i>
Et 1	Ethanollic extract Trial 1
Et 2	Ethanollic extract Trial 2
IC	Inhibition concentration
Kp	<i>Klebsiella pneumoniae</i>
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
Mt 1	Methanollic extract Trial 1
Mt 2	Methanollic extract Trial 2
Sa	<i>Staphylococcus aureus</i>
TDW	Triple Distilled Water
T1	Trial 1
T2	Trial 2
T3	Trial 3
URECU	University of Namibia Research Ethic Committee

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Dedication

This work is dedicated to my late grandfather, Alfred Komboni Maguraushe and to my late father Kudakwashe Francis Maguraushe and my immediate family for their unconditional support during my studies

Declaration

I, Kudzashe Arnold Maguraushe hereby declare that this study is a true reflection of my own research and that this work or part of it has not been submitted for a degree at any other tertiary institution.

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Signature.....

Chapter 1: Introduction

1.1 Background of study

Gomphocarpus fruticosus locally known as //horapob in the Nama/Damara dialect of Namibia is an indigenous plant belonging to the Asclepiadaceae family. There are basically two common species of *Gomphocarpus* in Africa and these are *G. physocarpa* and *G. fruticosus* (1). The main distinction between the two species are that of *G. physocarpa* has larger and more rounded ornamental seed balls than *G. fruticosus* whose inflated seed balls have sharp pointed ends and covered in short stout hairs. *G. fruticosus* demonstrates an erect growth habit with multiple stems in terms of morphology and these stems are of 1 to 3 m in height. The plant leaves are dull in colour and the flowers are suspended on a pedicel and they are often brightly coloured with a characteristic five-fold symmetry. The pollen is found in pollen sacs (2) and the follicles split open when they are ripen and the seeds attached to the floss are blown away by wind (3).

Traditional medicines are used in the maintenance of health by prevention, diagnosis or treatment of physical and mental illness (4). The reliance on traditional medicines has been of great significance since pre-colonial rule specifically amongst rural populations and this has been due to the general lack of access to public healthcare facilities in such areas (5). Plants are endowed with various phytochemical compounds that have different medicinal activities that are traditionally used in the management of diseases (6). The plant *G. fruticosus* is traditionally used to treat various ailments such as coughing, diabetes, tumors, skin disease, scabies and itching. The root is used as snuff for headache, convulsions, veterinary medicine for dogs and chickens, treating stomach ailments and body pains. The root is also used to treat sexually transmitted diseases (7).

Around the globe about 3.4 billion people representing 80% world population depends primarily on plant-based traditional medicines (4). The therapeutic efficiency of these traditional medicines has mainly been attributed to the presence of various phytochemicals such as vitamins, terpenoids, phenolic acids, lignin, stilbene, tannins, flavonoids, quinones, coumarins, alkaloids, amines, betalains, and other secondary metabolites. Studies have demonstrated that many of these phytochemicals contribute as antioxidants, anti-inflammatory, anti-atherosclerotic, antitumor, anti-mutagenic, anti-carcinogenic, antibacterial, and antiviral agents (8).

Phytochemical screen testing was evaluated for the leaves of *G. fruticosus* in this study and the phytochemicals that were screened are alkaloids, tannins, flavonoids, saponins, terpenoids, cardiac glycosides, phenols and quinines. Quantification was done for the phenolic content, flavonoid content and tannin content. In terms of the antimicrobial activity study, screening was done to determine whether the ethanolic and methanolic extracts have inhibition of growth activity on the strains. Minimum Inhibitory Concentration (MIC) was then determined for the extracts on the different strains.

1.2 Statement of the problem

There is a need to scientifically validate the traditional uses of *G. fruticosus* as an effective remedy for the treatment of disease such as coughing that are caused by microbes. In the past decade there has been the development of drug-resistant strains of microbes and this has necessitated the development of new inexpensive drugs to treat related diseases. The detailed determination of the phytochemical composition of *G. fruticosus* has not previously been performed.

1.3 Objectives of study

The objectives of the study were:

- a) To extract and screen the phytochemicals in the leaves of *G. fruticosus*.
- b) To quantify the detected phytochemicals.
- c) To determine the radical scavenging activity of the leaf extracts.
- d) To evaluate the anti-microbial activity of the leaf extracts.

1.4 Significance of study

This study will contribute to the phytochemical knowledge of this indigenous medicinal plant and scientifically validate its traditional use. The fact that there has been the emergence of drug-resistant strains of microbes; it might be that this indigenous plant carries phytochemicals as well as antioxidant and antimicrobial properties that can inhibit the manifestation and growth of these disease causing microbes which will be of great significance.

1.5 Limitation of study

The limitation of this study was that of time limitation due to the sensitivity of the biological assay and the requirements that the supplier needed.

1.6 Delimitation of study

Four strains available in the department were selected based on the plant's ethno-medicinal knowledge.

Chapter 2: Literature Review

2.1 Introduction

According to the World Health Organisation (4), more than 80% of the world's population in poor and underdeveloped countries depend on traditional plant based medicines for their primary healthcare needs. In developing nations like Namibia, microbial infections are still directly responsible for merely half of all cases of death (9). The plant *G. fruticosus* is widely distributed in the Southern part of Africa stretching to the eastern parts of the continent. The plant grows naturally and it is found abundantly in old cultivated lands and along roadsides. In countries like Kenya, the plant has been categorized as an ornamental flower which actually brings revenue in terms of commercial production. Fig 1 below shows the distribution of the plant on African continent where the regions that is grey in color is the areas where the plant is found.

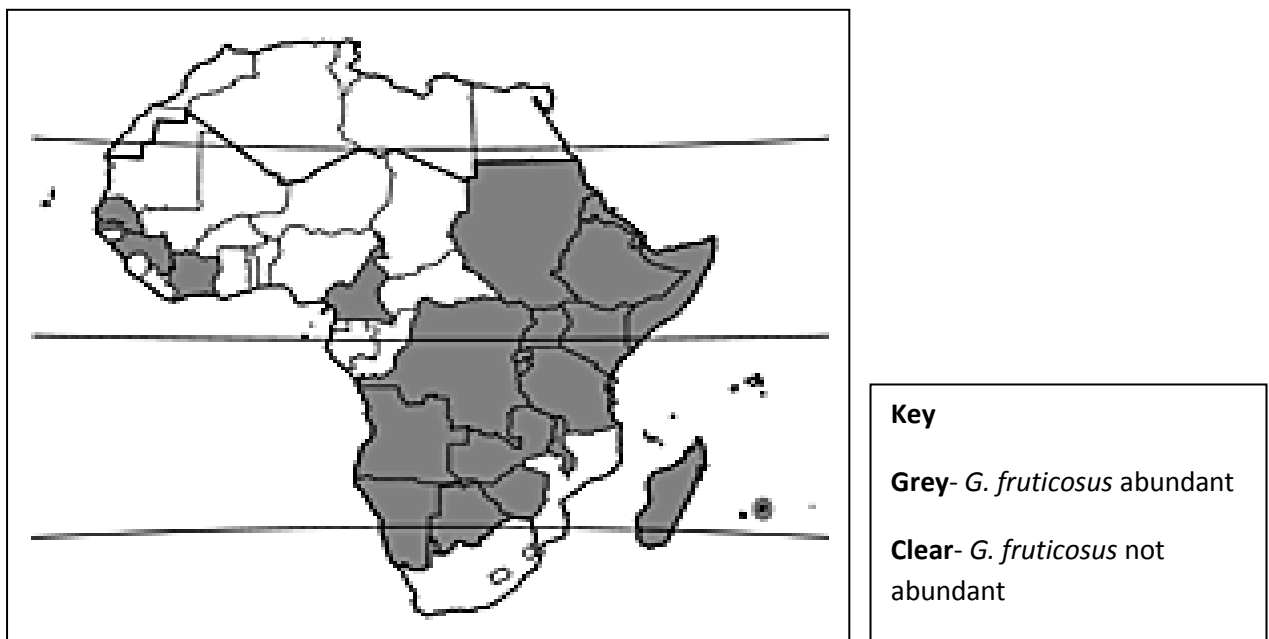


Fig 1: Distribution of the plant *G. fruticosus* on the African continent

2.2 Ethno-botanical aspects of *Gomphocarpus fruticosus*

Gomphocarpus fruticosus is a wild plant that is also known as *Asclepias fruticosus* and it is a species of milkweed native to South Africa (10). The plant is found along roadsides and old cultivated lands. Asclepiadaceae is the family to which this plant belongs to and

Gomphocarpus comprises about 22 species in tropical Africa and Peninsular Arabia (11). Five subspecies are distinguished in *Gomphocarpus fruticosus* most importantly depending on the hairiness of the plant, the colour of the flower and form of the fruit. The sub species are namely *G. cancellatus* (Namibia and South Africa), *G. glaucophyllus* (Zimbabwe), *G. purpurascens* (Ethiopia), *G. stenophyllus* (Kenya) and *G. solstitialis* (Togo) (1).



Fig 2: An Image of *Gomphocarpus fruticosus*

It is an erect, sparsely branched perennial shrub that can grow up to 2 m tall and all parts of the plant contain milky latex. This plant has quite a number of vernacular names that include narrow-leaved cotton plant, cotton bush, swan plant, bristle- fruited silkweed and mobydick (1).

2.3 Traditional Uses of *Gomphocarpus fruticosus*

The plant has quite a number of uses depending on its geographical location on the African continent. *Gomphocarpus fruticosus* is actually of toxic nature to livestock and humans, and has caused deaths in cattle, sheep and poultry (8). Severe gastroenteritis is the main

symptom of poisoning by this species and dense infestations may also reduce the productivity of pastures.

In Namibia, a tea made using the root is drunk daily to treat diabetes and the San people use the latex as an arrow poison ingredient (12). The leaves are used to rub the body in order to treat skin cancer in Southern parts of Namibia implying that the plant has a vital medicinal significance. In Uganda, an extract from the roots is taken to treat backache and swellings of the neck (13).

In Botswana, the Bayei drink the root decoction to treat gonorrhoea and for the treatment of hepatitis fresh leaves are soaked in water and the liquid is drunk to induce vomiting. The inner bark of the plant yields a white fibre that is spun into cotton that is used for sewing clothes and for snaring birds as stated by Miller (14). In Zambia, bitter latex is smeared over eggs in chicken sheds to prevent snakes and dogs from eating them. In Lesotho, the rootstock are cooked and eaten as vegetable and in Kenya the Masai people eat the fruit (14).

2.4 Antimicrobial activity of *Pergularia daemia*, *Secamone afzelii*, and *Leptadenia hastate* of the Asclepiadaceae family used in traditional medicine in South Togo

Pergularia daemia, *Secamone afzelii*, and *Leptadenia hastate* contain latex and they belong to the Asclepiadaceae family. This family consists of about 130 genera and 2000 species distributed all over the world of which some of them are tropical and subtropical shrubs or perennial herbs. According to Okusa (15) the emergence of multi-drug resistant phenotypes is a major public health problem in the treatment of bacterial infections. As a result of the alarming worldwide incidence of antibiotic resistance, an increase in the need for new compounds has resulted and medicinal plants represent a valuable source for these kinds of compounds (16). A number of herbal medicines are widely used to treat a variety of

infectious diseases but not all of them have been studied chemically and biologically in order to identify their active constituents (17).

Several plants from Togo are traditionally used against infectious diseases and among these plants is *Pergularia daemia*, *Secamone afzelii*, and *Leptadenia hastata*. Plant latex is a good source of various secondary metabolites, which shows growth inhibition effect against bacteria, fungi, viruses, tumours and cancer cell lines. These three latex plants have quite a number of traditional medicinal uses which vary from plant to plant. *P. daemia* is used as anthelmintic, laxative, antipyretic and expectorant, and is also used to treat infantile diarrhoea and malarial intermittent fevers (18). The plant *Secamone afzelii* is used to treat stomach problems, diarrhoea, gonorrhoea, malaria, cough, catarrhal conditions and diabetes (19). *Leptadenia hastata* is used in the management of onchocercosis, scabies, hypertension, catarrh, skin diseases, sexual potency, and wound-healing. The aim of this particular literature review study was to evaluate antibacterial potentials of the aqueous and ethanolic extracts of *P. daemia*, *S.afzelii*, and *L. hastata* against six selected human pathogenic bacteria. The influence of plant material harvest times on the antibacterial activities of *P. daemia* was also studied.

In previous studies that were tested in India, the ethanolic extract of *P. daemia* reveals the presence of medicinally valued bioactive components like tannins, saponins, terpenoids, alkaloids, flavonoids, phenols and steroids (20). The presence of alkaloids, tannins, cardiac glycosides and saponins in the leaves extract of *S. afzelii* was demonstrated in Nigeria and *Leptadenia hastata* was reported to contain alkaloids, saponins, phenolic glycosides, tannins, flavonoids, proanthocyanidins and triterpenes (21). So the antibacterial activities observed can be due to the presence of these components and in agreement with the results, it was

found in India that the methanolic extract of leaves of *P. daemia* was active against *E. coli*, *Pseudomonas aeruginosa*, and *S. aureus* (22). In Ghana, it was demonstrated that the methanolic extract of aerial part of *S. afzelii* inhibited the growth of *S. aureus* and *E. coli* and was inactive against *P. Aeruginosa* (22).

The lowest MICs were determined in the study for *P. daemia* and in order to elucidate whether the observed antibacterial effects were bactericide or bacteriostatic, MBC/MIC ratios were calculated. Extracts with ratios greater than 1 were considered as bacteriostatic, while the extracts with ratios equal to 1 are bactericide (23). Thus, the ethanolic extract of leaves of *P. daemia* had a bactericide effect against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *S.aureus* ATCC 29213 and *S. aureus*, and a bacteriostatic effect against *S. typhi* and *K.pneumoniae*. The extract of leaves of *S. afzelii* had a bactericide activity against *E.coli* ATCC 25922 and a bacteriostatic effect against *S. aureus* ATCC 29213 and *S.aureus*. A bacteriocidal activity was observed for the extract of *L. hastata* against the two strains of Staphylococcus tested and a bacteriostatic activity against *S. typhi* and *K.pneumoniae*.

Table 1: A summary of the Minimum Inhibitory Concentration and Minimum Bactericidal Concentration

<i>Bacteria</i>	<i>Pergularia Daemia</i>			<i>Secamone afzelii</i>			<i>Leptademia hastata</i>		
	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC	MIC	MBC	MBC/MIC
<i>E.coli</i>	0.62	0.62	1	1.25	1.25	1	NA	NA	NA
<i>P.aeruginosa</i>	1.25	1.25	1	NA	NA	NA	NA	NA	NA
<i>S. aureus</i> <i>ATCC</i>	0.62	0.62	1	1.25	2.5	2	1.25	1.25	1
<i>S. aureus</i>	1.25	1.25	1	1.25	2.5	2	1.25	1.25	1
<i>S. typhi</i>	1.25	2.5	2	NA	NA	NA	1.25	2.5	2
<i>K. pneumoniae</i>	1.25	2.5	2	NA	NA	NA	1.25	5	2

Table 2: A summary of the activity of the active ethanolic extracts of *Pergularia daemia*, *Secamone afzelii*, and *Leptadenia hastate*

	Activity		
	<i>P. Daemia</i>	<i>S. afzelii</i>	<i>L.hastata</i>
Bacteria			
<i>E.coli</i>	Bactericide	Bactericide	None
<i>P.aeruginosa</i>	Bactericide	None	None
<i>S. aureus ATCC</i>	Bactericide	Bacteriostatic	Bactericide
<i>S. aureus</i>	Bactericide	Bacteriostatic	Bactericide
<i>S. typhi</i>	Bacteriostatic	None	Bacteriostatic
<i>K. pneumoniae</i>	Bacteriostatic	None	Bacteriostatic

The biological activities of medicinal plants vary widely based on the type of plant part, geographic location and solvent used in extraction. These biological activities can also depend on the condition of the plant parts and according to Tchacando et al (21), traditional healers follow certain practices such as harvesting plant material early in the morning to prepare their herbal medicines. Based on the findings of this literature review study, it was confirmed that the leaves of *P.daemia* should be taken early in the morning to optimize the antibacterial activity. This literature review study tested not only the ethanolic extract but also the aqueous decoction of leaves of these three plants. The activities obtained for these extracts were consistent with the use of such plants in the treatment of bacterial infections. This study provided useful data concerning the antibacterial activities of leaves extracts however the toxicological investigations are yet to be done to provide the medicinal safety uses of these species.

2.5 Phytochemical Screening and Antioxidant activity of ethanolic extract of *Boucerosia truncato-coronata* gravelly muyar

The Asclepiadaceae family has quite a number of species and the members of this family show great morphological diversity. The plant *Gomphocarpus fruticosus* belongs to this

family and the phytochemistry of plants belonging in this family are characterized by many pregnane glycosides while in recent studies, megastimane glycosides have also been isolated (24). The main phytochemicals that are found in medicinal plants are saponins, flavonoids and polyphenols and they are known to be major bioactive compounds in Ayurvedic medicine (25). The antioxidants that have been isolated from most medicinal plants have depicted quite a number of activities which include antibacterial, anti-carcinogenic and antiviral. An antioxidant is defined as any substance that prevents or removes free radicals or oxidative damage to a target molecule. In this particular literature review study, the extract activity of *B. truncato-coronate* was tested for antioxidant activity by DPPH and the phytochemical studies were also done for this plant.

For the qualitative phytochemical analysis that was done on this plant, confirmation of the presence of various phytochemicals was done. It was confirmed that secondary metabolites such as alkaloids, terpenoids, saponnins, flavonoids and phenols were present in *B. truncato-coronate*. In terms of the radical scavenging activity, both the in vivo and the in vitro extracts exhibited significant scavenging activity which actually increased with an increase in concentration of the extract. There are three main techniques that can be used to measure the antioxidant capacity measurement. They are divided into three main classes which are Spectroscopic technique, Electrochemical Techniques and the Chromatographic technique. In this particular literature study, the DPPH radical scavenging activity technique was used and it is categorized as a Spectroscopic Techniques. It is a less complex technique as compared to the other techniques that could have been used to determine the antioxidant capacity measurement. DPPH technique is commonly used in the determination of antioxidant capacity in many fruit extracts and juices hence making it possible for DPPH technique to be used on medicinal plant extracts.

2.6 Phytochemistry of medicinal plants

Phytochemicals are naturally occurring chemical compounds found in plants, which provide health benefits for humans further than those attributed to macronutrients and micronutrients (26). More than 4,000 phytochemicals have been catalogued (36) and are classified by three main factors which are protective function, physical characteristics and chemical characteristics (27). Phytochemicals are available in supplementary forms as well but there is not enough evidence to prove that they provide the same health benefits as dietary phytochemicals found in plants (28). It is well-known that plants produce these chemicals to protect themselves, but many studies have demonstrated the fact that many of these phytochemicals can also protect humans against diseases (29).

Phytochemicals are classified as either primary or secondary constituents which is basically dependent on their main role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids and chlorophyll's while the secondary constituents are the remaining plant chemicals such as alkaloids, terpenes, flavonoids, lignans, plantsteroids, curcumines, saponins, phenolics, flavonoids and glucosides (30).

Phenolic phytochemicals are the largest category of phytochemicals and the most widely distributed in the plant kingdom and there are three main groups of dietary phenolics that are of vital importance and these are flavonoids, phenolic acids, and polyphenols (31). Phenolic compounds contain an –OH bonded directly to an aromatic hydrocarbon group and phenol is considered to be the simplest class of this group of natural compounds since phenolic compounds are considered to be a large and complex group of chemical constituents found in plants (32).

Flavonoids are considered to be the largest group of the plant phenols and the most studied (33). They can be defined as polyphenolic compounds that are ubiquitous in nature with more than 4,000 flavonoids having been recognised and many of which occur in vegetables, fruits and beverages (34). Flavonoids have appeared to have played a major role in successful medical treatments because of their broad biological and pharmacological activities. They have been reported to exert multiple biological properties including antimicrobial, cytotoxicity, anti-inflammatory as well as antitumor activities but the well known property of almost every group of flavonoids is their capacity to act as powerful antioxidants which can protect the human body from free radicals and reactive oxygen species (35).

Tannins are another class of phytochemicals and they are a heterogeneous group of high molecular weight polyphenolic compounds with the capacity to form reversible and irreversible complexes with proteins, polysaccharides, alkaloids, nucleic acids and minerals (36). Several health benefits have been recognized for the intake of tannins and some epidemiological associations with the decreased frequency of chronic diseases have been established (37). In Asian natural healing practices, the tannin-containing plant extracts are used as astringents against diarrhoea, as diuretics against stomach and duodenal tumours (38). They are also used as anti-inflammatory, antiseptic, antioxidant and haemostatic pharmaceuticals (39). Tannins are used in industry as caustics for cationic dyes and also in the production of inks. In the food industry tannins are used to clarify wine, beer, and fruit juices. The other industrial uses of tannins include textile dyes and as antioxidants in fruit juice, beer, and wine industries (40).

Alkaloids are natural products that contain heterocyclic nitrogen atoms and they have such a variety of molecular structures which thus makes their rational classification to be difficult.

The best approach to the problem is to however group them into families based on the type of heterocyclic ring system present in the molecule (41). Alkaloids have many pharmacological activities including antihypertensive effect, antiarrhythmic effect, antimalarial activity and anticancer actions. Some alkaloids have stimulant properties such as caffeine, nicotine, and morphine which are used as the analgesics and quinine as the anti-malarial drug (42).

Terpenoids are defined as a class of natural products which have been derived from five-carbon isoprene units and 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 are thus considered as the largest group of natural products (43).

The physiological role of saponins in plants is not yet fully understood however there are a number of publication describing their identification in plants and their multiple effects in animal cells and on fungi and bacteria. Saponins are considered to be a part of plant's defence systems and as they belong to a large group of protective molecules found in plants named phytoanticipins or phytoprotectants (44).

Chapter 3: Research Methods

3.1 Research Design

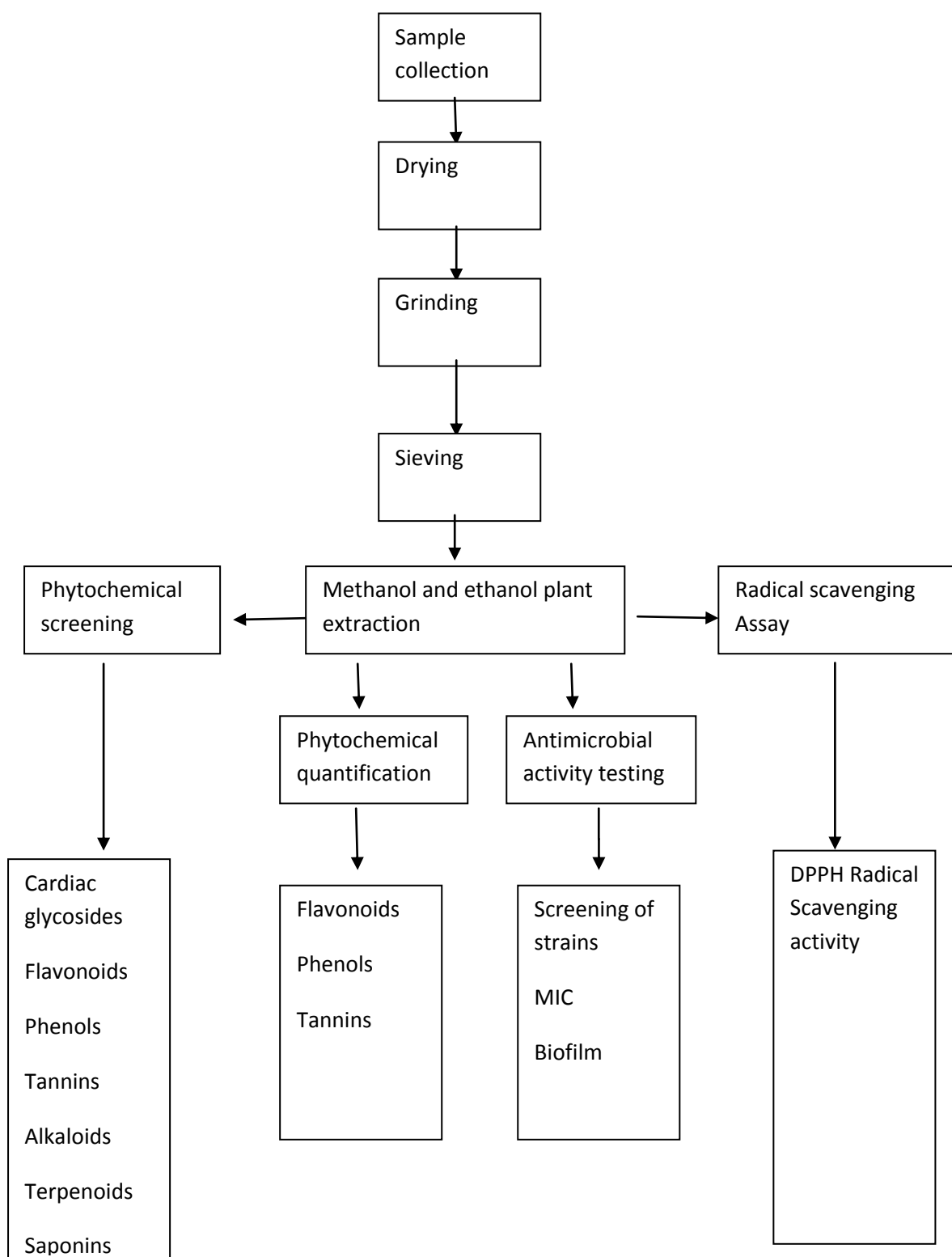


Fig 3: Flow diagram of the Research laboratory work

3.2 Ethical consideration

A Research/Plant Collection Permit was obtained from the Ministry of Environment and Tourism and plants were collected using the procedures outlined by the Guide for Collecting Plants from the National Botanical Research Institute however *G. fruticosus* is not on the endangered species list. The disposal of all waste generated in the laboratory was completed according to the UNAM Occupational Safety and Health guidelines. Ethical clearance was obtained from University of Namibia Research Ethic Committee (URECU) and research permission from Centre of Post Graduate Studies (CPGS) before commencement of the study.

3.3 Sample Collection

The collection of plant samples was done on the 12th of April 2017 in the Khomas region particularly in the areas surrounding the University of Namibia.



Fig 4: The plant *G. fruticosus* at the site of collection

3.4 Sample preparation

The plant samples were air dried for 15 days in the shade. Separation of the leaves from the stems was completed and the weight of the leaves was taken before grinding. The grinding was done using a mortar and pestle until a fine powder was obtained. Fine powders of ground samples are shown in fig 5 below.



Fig 5: Ground leaves of *G. fruticosus*

3.5 Plant Extraction

3.5.1 Procedure for Plant Extraction

The plant extraction was done according to the method described by Packirisamy et al (45). A mass 25 g of ground plant material was added into two separate 250 mL Erlenmeyer flasks and 125 mL of ethanol and methanol each was added into the two separate flasks containing the ground plant material. The mixtures were then shaken for 30 minutes using an orbital shaker. When shaking was completed the mixtures were allowed to stand in the refrigerator for 24 hrs so as to allow the mixture to settle. See fig 6 below.



Fig 6: An image of the ethanolic and methanolic plant extract

3.5 Screening of the Phytochemicals in *G. fruticosus*

The phytochemical screen testing was carried out according to the procedure previously reported by Packirisamy et al (45).

3.5.1. Test for Alkaloids

3.5.1.1. Preparation of Dragendorff's reagent

Bismuth nitrate (0.5 g) was added into the beaker and then 10ml of distilled water was added into the same solution. Concentrated HCl (10 ml) was added and then the solution was stirred. Potassium iodide (4 g) was added in another beaker and 1 ml water until the KI had completely dissolved. The two solutions were mixed and a dark orange solution was observed indicating that the reagent was ready for use. See fig 7 below.



Fig 7: Showing the preparation of Drangedorf's reagent.

3.5.1.2. Procedure for testing Alkaloids

Plant extract (0.2 g) was warmed up with 2% sulphuric acid for 2 minutes. The solution was filtered and a few drops of 8% Dragendoff's reagent were added and an orange red precipitate indicated the presence of alkaloids. See fig 8 below.

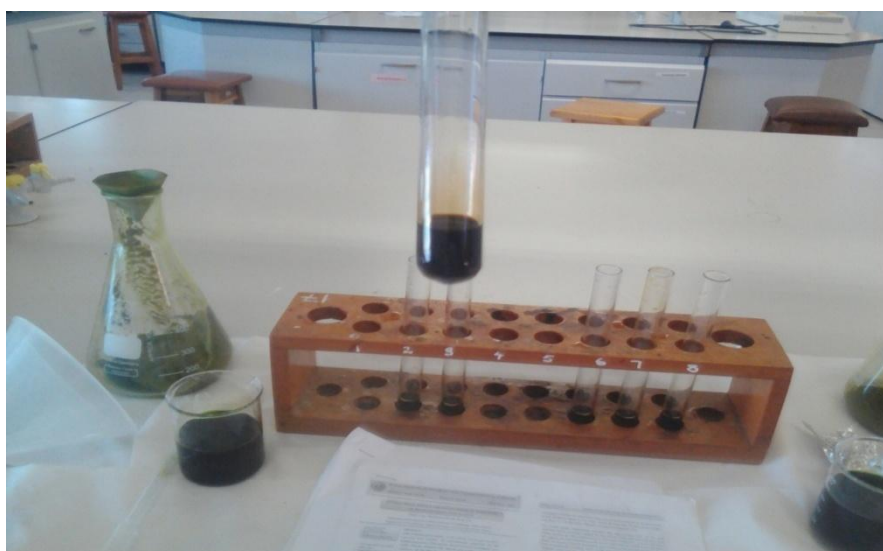


Fig 8: A positive test for the presence of alkaloids

3.5.2 Test for Saponins

Plant extract (0.5 ml) was added into two separate test tubes. One plant extract was ethanolic and the other one was methanolic. Into each test tube, 10 ml distilled water was added. The contents were thoroughly shaken. Frothing and bubbling indicated the presence of saponins as shown in fig 9 below.

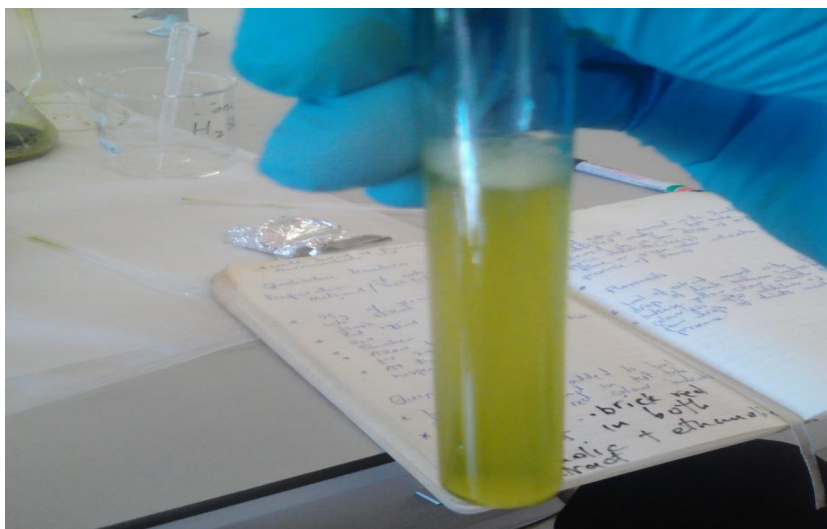


Fig 9: A positive test for the presence of saponins

3.5.3 Test for Flavonoids

The extract (0.2 g) was dissolved in diluted 10% NaOH and 1% HCl was added. A yellow solution that turns colourless indicated the presence of flavonoids.

3.5.4 Cardiac glycosides

Approximately 5 g of the plant extract was treated with 2ml of glacial acetic acid containing one drop of ferric chloride solution. The solution was added with 1 ml of concentrated sulphuric acid and a brown ring of the interface indicated a deoxy-sugar which is a characteristic of cardenolides.

3.5.5 Test for Terpenoids

About 0.2 g of extracts was mixed with 2 ml of chloroform and 3 ml of 2% sulphuric acid was carefully added to form a layer. A reddish brown coloration of the interface indicated the presence of terpenoids.

3.5.6 Test for Balsams

Two drops of alcoholic ferric chloride solution were added to 5 ml of each extract and a green colour indicated the presence of balsam as shown in fig 10 below.

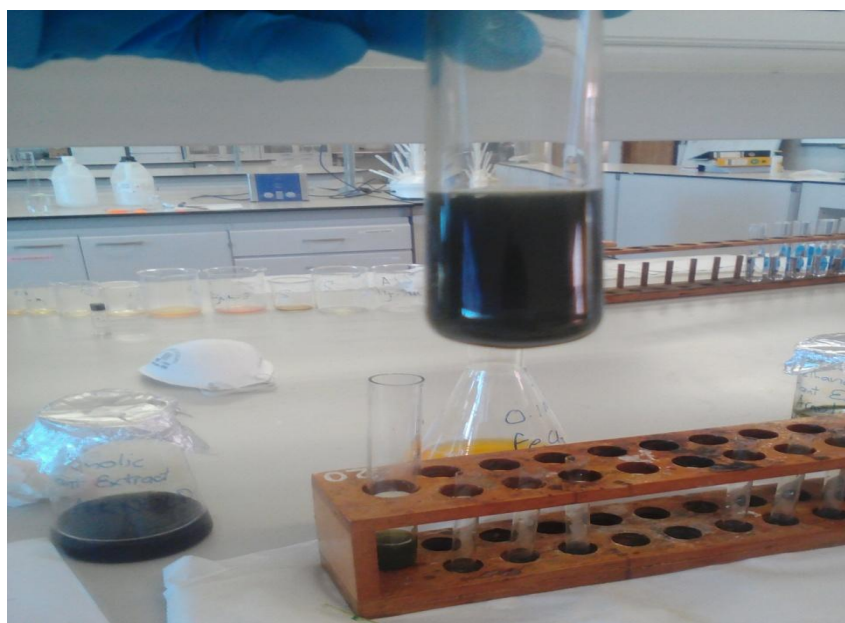


Fig 10: A positive test for the presence of balsams

3.6 Quantification of Phytochemicals

3.6.1. Determination of total flavonoids

Determination of total flavonoids was conducted as previously reported by Kumaran et al (46). Plant extract (100 μ l) in methanol (10 mg/ml) was mixed with 100 μ l of 20 % aluminum trichloride in methanol and a drop of acetic acid, and then diluted with methanol to 5ml. The absorption at 415 nm was observed after 40 minutes. Blank samples were prepared from 100 ml of plant extracts and a drop of acetic acid, and then diluted with 5ml methanol. The absorption of standard quercetin solution (0.5 mg CE/ml) in methanol was then measured under the same conditions and all the tests were repeated in triplicates.

3.6.2 Determination of total phenolic compounds

Determination of total phenolics was conducted as previously reported by Hagerman et al (47).

A mass of 50 g of the extract was dissolved in 100 ml of triple distilled water. A volume of 1 ml of this solution was then transferred to a test tube. Folin-Ciocalteu reagent (0.5ml) and 1.5 ml 20% of Na_2CO_3 solutions were added and the volume was made up to 8 ml with TDW. Vigorous shaking was done and allowed to stand for 2 hours after which the absorbance was taken at 765 nm. An estimate of the total phenolic content was then determined using a standard calibration curve obtained from various diluted concentrations of gallic acid which were 0, 0.01, 0.02, 0.03, 0.04 & 0.05 mg GAE/g.

3.6.3 Determination of total tannin content

Determination of total tannin content was completed according to the method reported by Gracelin et al (48).

A mass of 50 g of the sample was weighed into a 50 ml plastic bottle and 50 ml distilled water was added and shaken for 1 h in a mechanical shaker. It was filtered in 50 ml volumetric flask and made up to the mark and then 5 ml of the filtered was pipette out into a test tube and mixed with 2 ml of 0.1 M FeCl_3 in 0.1 N HCl and 0.008 M potassium ferrocyanide. The absorbance was measured at 120 nm within 10 min.

3.8 Antimicrobial activity

3.8.1 Preparation of Nutrient Broth

A mass of 13 g nutrient broth powder was dissolved in 1 l of distilled water. Heating was done to dissolve the medium. The mixture was distributed into the final container and then sterilized by autoclaving at 121 °C for 15 mins.

3.8.2 Preparation of Nutrient Agar

Nutrient agar powder (28 g) was suspended in 1 l of distilled water. The solution was boiled using a water bath to dissolve the powder completely. Sterilization of the solution was done by autoclaving at 121 °C for 15 mins.

3.8.3 Antimicrobial screening Procedure

The screening procedure was done as reported by Purity Kaaria et al (49).

Culture plates were prepared using the nutrient agar solution and the plates were left for 5 hours so that the nutrient agar could properly solidify. The bacterial strains that were used to do screening were *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus* and *Candida albicans*. Each microbial strain was cultured onto nutrient agar and incubated at 30°C for 18 to 24 hr. A 100µl of each strain was placed on different Nutrient agar plates; spread evenly using a glass spreader then allowed to stand for 1 hr before the extract was introduced. About 10µl of plant extracts (1 mg/ 1ml) were placed onto a disc and placed on to the plate, allowed to stand for 30 min then the plates were incubated for 24 hrs at 37°C. The plant extract that inhibited the growth of bacterial strains after 24 hr were recorded as positive and were investigated further whereas bacterial strains in plates where no inhibition took place at all were recorded as negative and were not investigated further.

3.8.4 Minimum Inhibitory Concentration

The determination of the MIC was done as reported by Purity Kaaria et al (49).

Filter paper discs approximately 6 mm in diameter were soaked with 10 µl of the plant extract with different concentrations and placed in the prepared agar plates as mentioned above. Each disc was pressed down to ensure complete contact with the agar surface and distributed evenly so that they were no closer than 24 mm from each other, centre to centre. The agar plates were then incubated at 37 degrees and after 24 hrs of incubation, each plate was examined. The least concentration that showed inhibition on the different strains was the minimum inhibitory concentration (MIC).



Fig 11: MIC trials being done

3.9 Antioxidant activity

Free radical scavenging activity testing was completed as previously reported by K.

Kalimuthi (50) however with minor modifications.

3.9.1 Procedure

A volume of 100 μ l DMSO was added into wells respective wells. A volume of 100 μ l plant sample was added into the wells which were then followed by dilutions that were done by pipetting 100 μ l from each of the wells. A volume of 100 μ l DPPH was added into the wells and the absorbance was measured at 517 nm. The formula that was used for calculating the % Scavenging activity was as follows

$$\% \text{ Scavenging} = (\text{Absorbance of Sample}) / (\text{Absorbance of DPPH}) \times 100$$

The formula used for % inhibition is as follows

$$\% \text{ Inhibition} = 100 - \% \text{ Scavenging}$$

The results that were obtained were expressed as IC₅₀.



Fig 12: An image of 96 well plates loaded with sample, DMSO and DPPH before measuring the absorbance at 512nm.

3.9 Data analysis procedure

The quantitative phytochemical determinations was performed in triplicates and expressed as means (\pm standard deviation). The MIC values for antimicrobial analysis were recorded as the least concentration of plant extracts that has completely inhibited the growth of the microorganism.

Chapter 4: Results

4.1 Phytochemical screening Results

Table 3: Phytochemical screening results

Phytochemical	Methanol Extract	Ethanol Extract
Quinones	+	++
Saponins	++	+++
Flavonoids	++	++
Alkaloids	+	++
Tanins	++	++
Glycosides	+	+
Phlobotaninis	+	+
Terpenoids	-	-
Resin	+	+
Balsams	+++	+++

Key

+++ : abundantly present

++ : moderately present

+ : less present

- : not detected

4.2 Phytochemical quantification

Quantitative analysis of Phytochemicals in the leaves of *Gomphocarpus fruticosus* in mg/g

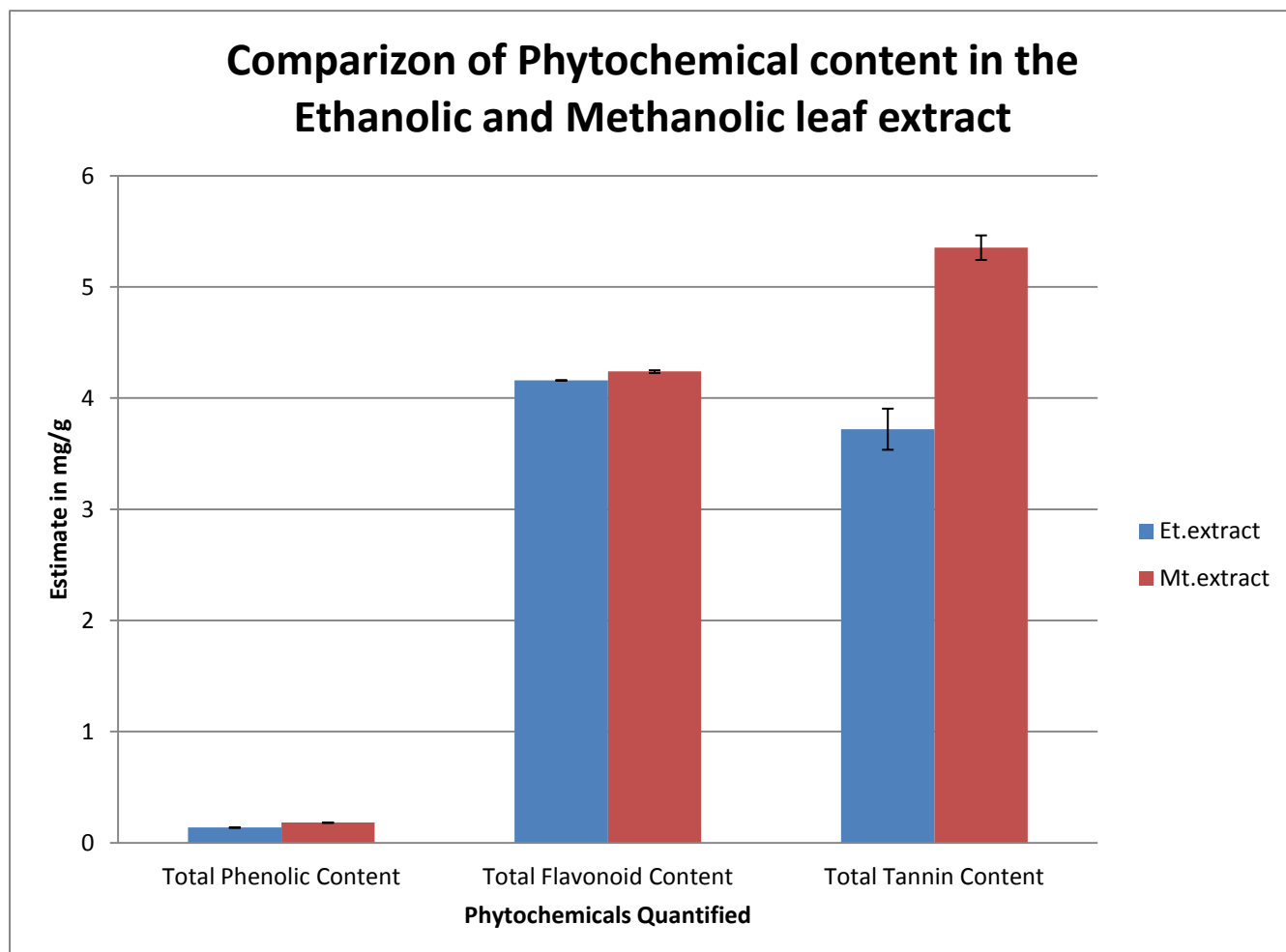


Fig 13: Comparison of phytochemical content in the ethanolic and methanolic leaf extract

The amount of the total flavonoid in leaf was 4.16 ± 0.01 CEmg/g in the ethanolic plant extract and 4.239 ± 0.012 CEmg/g in the methanolic plant extract. The considerable amount of the flavonoid present in the leaf could be the main reason for its antioxidant activity. The leaf of *G. fruticosus* has little amounts of phenolic compounds in both the ethanolic plant extract and the methanolic plant extract. The ethanolic and methanolic plant extracts have 0.732 ± 0.001 GAEmg/g and 0.961 ± 0.001 GAEmg/g of phenolic content respectively. The tannin content in

the methanolic plant extract was observed to be 6.937 ± 0.115 TAmg/g and 5.356 ± 0.185 TAmg/g in the ethanolic plant extract.

4.3 Antioxidant activity

Table 4: DPPH radical scavenging results

	Ethanol extract	Methanol extract	Vitamin C
IC ₅₀ (µg/ml)	343.2	245.4	365.9

The standard in this study was Vitamin C and it had an IC₅₀ value of 0.37mg/ml. The ethanolic extract showed a DPPH radical scavenging activity with an IC₅₀ value of 0.34mg/ml as compared with the methanolic extract which had an IC₅₀ value is 0.24mg/ml.

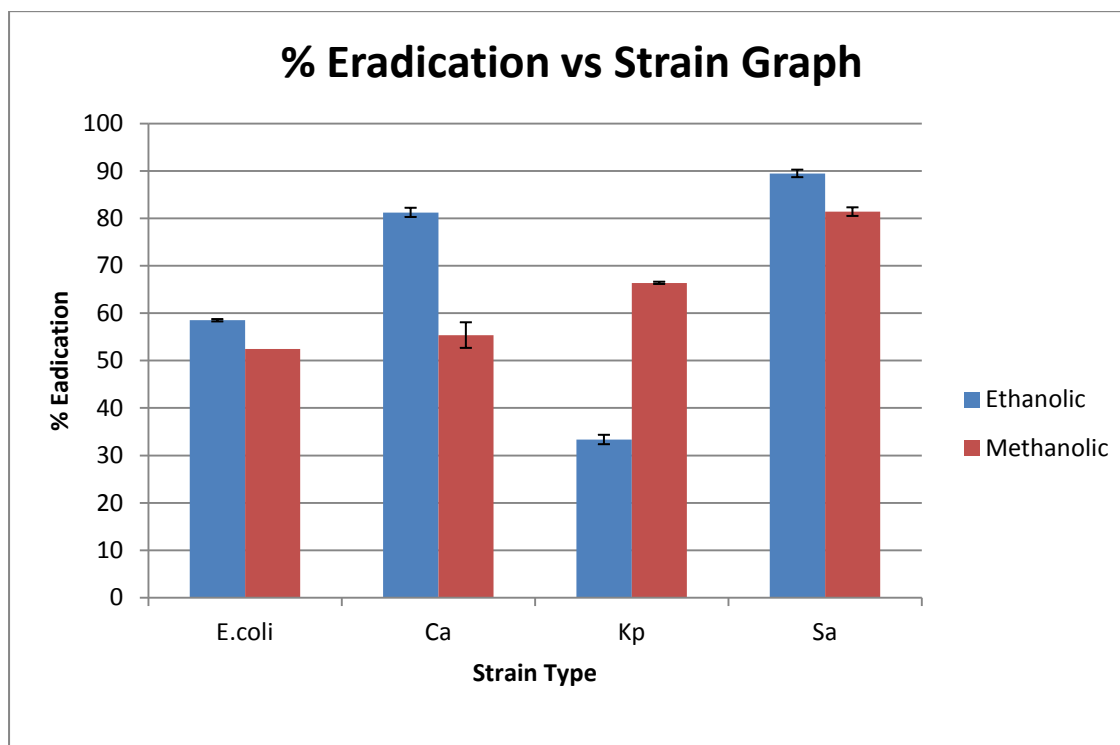


Fig 14: % Eradication versus strain graph

4.4 Antimicrobial activity

Antimicrobial activity screening

Table 5: Antimicrobial activity screening

		<u>Sa</u>	<u>Ec</u>	<u>Ca</u>	<u>Kp</u>
<u>T1</u>	<u>A1</u>	<u>10 mm</u>	<u>5 mm</u>	<u>5 mm</u>	<u>6 mm</u>
	<u>A2</u>	<u>8 mm</u>	–	–	–
<u>T2</u>	<u>A1</u>	<u>11 mm</u>	<u>6 mm</u>	<u>4 mm</u>	<u>5 mm</u>
	<u>A2</u>	<u>10 mm</u>			
<u>T3</u>	<u>A1</u>	<u>9 mm</u>	<u>4 mm</u>	<u>5 mm</u>	<u>6 mm</u>
	<u>A2</u>	<u>8 mm</u>	–	–	–

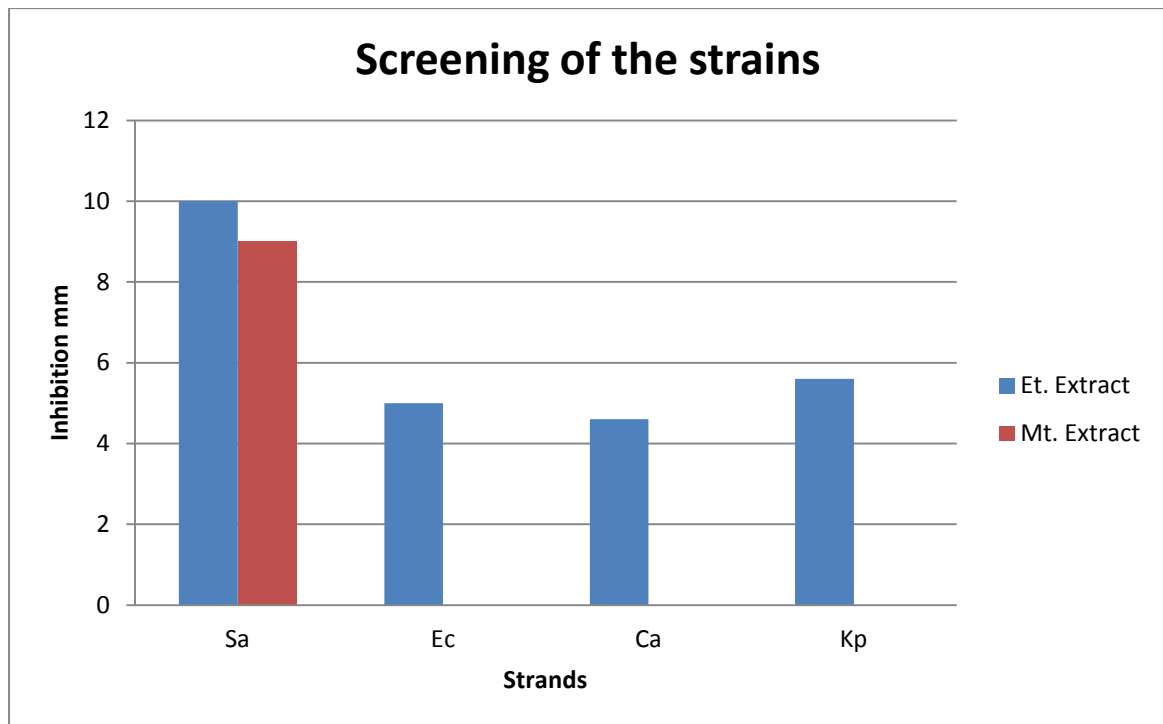


Fig 15: Graph for showing the inhibition against the strains

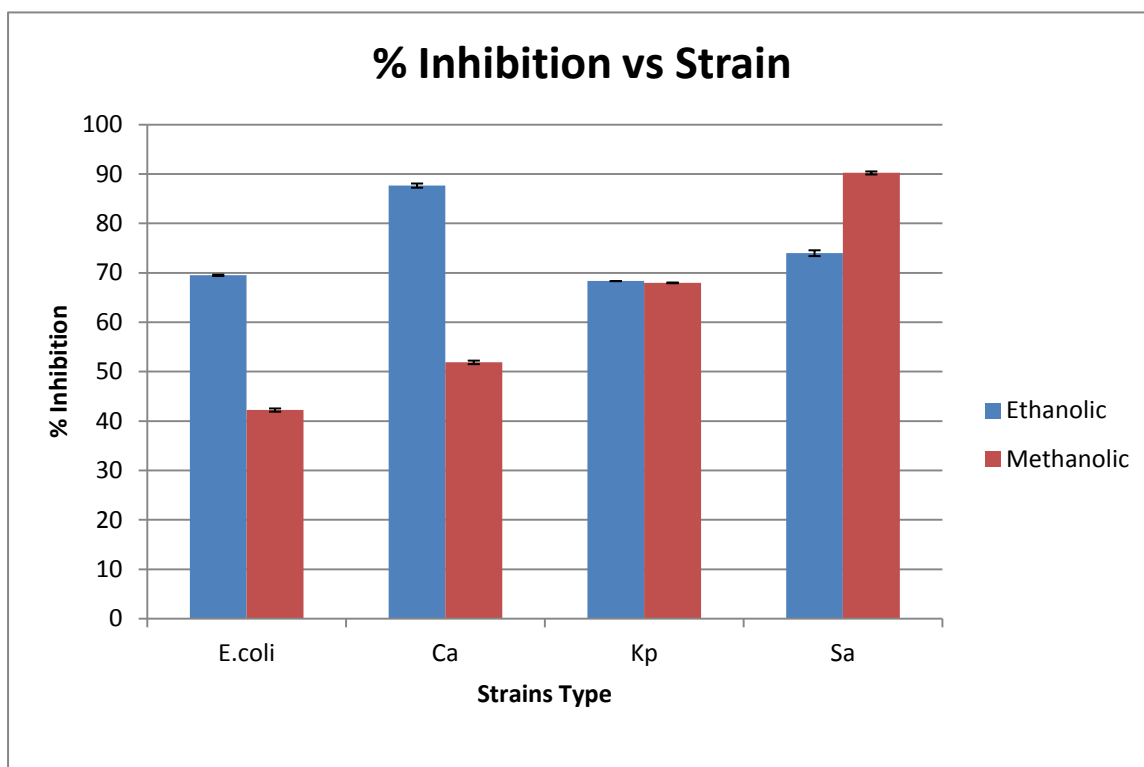


Fig 16: % Inhibition versus strain graph

Table 6: Summary of MIC of the extracts on the different strains

Strain	Ethanolic Extract	Methanolic Extract
Ca	2 mg/ml	-
Sa	1 mg/ml	1 mg/ml
Ec	6 mg/ml	-
Kp	8 mg/ml	-

Of the strains that were under study, *Staphylococcus aureus* had the least MIC value in the ethanolic extract which was 1 mg/ml and it is the only strain that had an MIC value from the methanolic extract.

Chapter 5: Discussion

In this study, dried samples were used instead of fresh samples due to the fact that fresh samples are very fragile and they tend to deteriorate much faster than dried ones. In a study that was done to make a comparison between dried and fresh *Moringa Olifera* leaves in terms of phytochemical quantification by were reported by Vongsak et al (51), no significant effect was observed in total phenolics but with flavonoids, significant effect was observed. The dried samples quantified more flavonoid content as compared to the fresh samples. This basically implies that it would be preferable to use dried samples than fresh samples in terms of phytochemical quantification since it seems that dried samples tend to yield more phytochemicals in terms of quantification as compared to the fresh samples.

Dried samples were grinded using a mortar and pestle in this study and the reason for grinding was to create smaller particles which enable a better contact of the particles with the solvents that were used and this case the solvents were ethanol and methanol (52). Methanol and Ethanol were the preferred solvents in this study due to the fact that these two solvents have the ability to dissolve both polar and non polar molecules. Methanol has a polarity index of 5.1 and ethanol is a very polar solvent due to the hydroxyl group with high electro-negativity of oxygen allowing the hydrogen bonding to place with other molecules (52).

There are quite a number of methods that can be used for extraction such as maceration, infusion, percolation and decoction according to the review paper that was reported by Azwanida (53). In this particular study, maceration was the preferred method of extraction it is widely used in medicinal plant research.

The results obtained from the screening of phytochemicals indicated the presence of saponins, flavonoids, alkaloids, tannins, cardiac glycosides, phlobotannins, resins and balsams in the plant extracts. Only terpenoids tested negative for screening and of the phytochemicals that were detected in the plant, and only three of them were subjected to quantification and these are tannins, flavonoids and the phenols. The reason why only three of these phytochemicals were quantified is because of their significance in terms of contributing to antioxidant and antimicrobial activities in several literature review papers. Cardiac glycosides are an important class of naturally occurring compounds in plants and they have both beneficial and toxic properties (54). Based on the amounts administered, they can be either deemed toxic or beneficial and in terms of their importance, they are used in the treatment of various cardiovascular conditions such as cardiac failure (55). The San people of Namibia use the latex as an arrow poison ingredient (10) and this is attributed to the presence of cardiac glycosides in the plant *G. fruticosus*. The fact that cardiac glycosides possess cytotoxicity at a certain extent; it might be that these compounds are of great significance in terms of cancer treatment.

Alkaloids are a class of nitrogen containing compounds and the bioactive compounds from plants containing alkaloids play an important role in developing anti-tumor drugs in human being. The increase of the inhibition of tumor growth with the increasing concentration of plant extract had been documented in several studies (56). One of the phytochemicals considered to be having a high molecular weight are saponins and they contain a sugar molecule that belongs to the class of glycosides and has cholesterol binding property. Therapeutically, saponins are very important as they possess hypolipidemic and anti-cancer activity (57).

Flavonoids are an important group of polyphenols that are widely distributed among the several medicinal plants which includes the plant of this particular study. They are having a

benzopyrone which is used for antioxidant activity or free radical scavenging and also have a cardio-protective roll (58). By inhibiting the estrogen producing enzyme, flavonoid suppresses the progression of cancer and a good example is the inhibition of estrogen synthetase which binds estrogen to its receptor.

Natural antioxidants essentially represented by the phenolic compounds are used as nutraceuticals found in apples, green-tea, red wine and in many medicinal plants. Phenolic compounds have the ability to combat cancer and also prevent heart ailments to an appreciable degree and sometimes act as anti-inflammatory agents (59). They are potent vasodilators and the presence of hydroxyl group that phenols contain possesses potential scavenging activity (60). The detection and quantification of total phenolic compound present in the leaf of *G. fruticosus* may contribute in the field of herbal remedy as a potent antioxidant in the future.

Tannins are phenolic compounds that are of high molecular weight and are used as antiseptic and this activity is due to the presence of the phenolic group. It is associated with antiviral activity. According to Rajan (61), in presence of very high quantity of tannin, there are many viruses like polio virus, herpes simplex viruses have been found to get inactivated. The leaf of *G. fruticosus* contains very high level of tannin content in both the ethanolic and methanolic plant extracts.

The IC_{50} is a measure of the effectiveness of a particular substance in terms of inhibiting a specific biological or biochemical function. Two extracts were used to determine the IC_{50} values of the leaf extract in this study and these were the methanolic plant extract and the ethanolic plant extract. The antioxidant activity was determined by the DPPH radical scavenging activity test. The antioxidant on interaction with DPPH both transfer electron or hydrogen atom to DPPH and thus neutralizing its free radical character and convert it to 1-

1,1-diphenyl-2-picryl hydrazine and the degree of discoloration indicates the scavenging activity of the drug. The reduction capacity of DPPH radical activity is determined by the decrease in its absorbance at 517 nm induced by antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants is because of the reaction between antioxidant molecules and radical progress which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a change in color from purple to yellow.

This implies that both the methanolic and the ethanolic extracts have a good antioxidant activity since their IC_{50} values did not deviate that much from the IC_{50} value of the standard used. However, between the two extracts, the methanolic extract had the lowest IC_{50} which means that it has the highest radical scavenging activity.

The screening that was executed for the antimicrobial activity of the ethanolic plant extract and the methanolic plant extract exhibited that the ethanolic extract had activity against all the strains in this study however the methanolic extract only had activity on *S. aureus*. The Minimum Inhibitory Concentration (MIC) of the different concentrations of the methanolic and ethanolic plant extracts was determined. The difference in the MIC values of the two extracts on the different strains is attributed by the presence of the various phytochemicals such as phenols, saponins and flavonoids in different quantities in the extracts

Chapter 6: Conclusion

In conclusion of the present study, the leaves of the plant *G. fruticosus* have quite a number of phytochemicals that are present and these are alkaloids, quinones, saponins, cardiac glycosides, flavonoids, tannins, phlobotannins, resins and balsams. Of the phytochemicals that were quantified, a considerable amount of tannin content in both the ethanolic and methanolic plant extracts was determined. In terms of the antimicrobial activity, Minimum inhibitory concentration was determined for each other extracts against the four strains. *Staphylococcus aureus* had the least MIC values for both the extracts and regardless of the fact that terpenoids that are considered to be of great significance in terms of antioxidant activity tested negative in the leaf extract of the plant, antioxidant activity was however determined. At an overall point of perspective, it can be concluded that the leaf extract of *G. fruticosus* is of great medicinal significance due to the antimicrobial and antioxidant activity that was exhibited by the experimental work done and all these activities are attributed to the presence of the numerous phytochemical constituents that are found in the leaf extracts of *G. fruticosus*.

Chapter 7: Recommendation

Further research studies have to be conducted in terms of the identification of bioactive compounds of the plant which are responsible for the pharmacological action against the disease causing microorganisms and cancer. One of the phytochemicals that tested positive in the leaf extract of *G. fruticosus* is the cardiac glycosides. The fact that cardiac glycosides possess cytotoxicity at a certain extent; it might be that they are of great significance in terms of cancer treatment.

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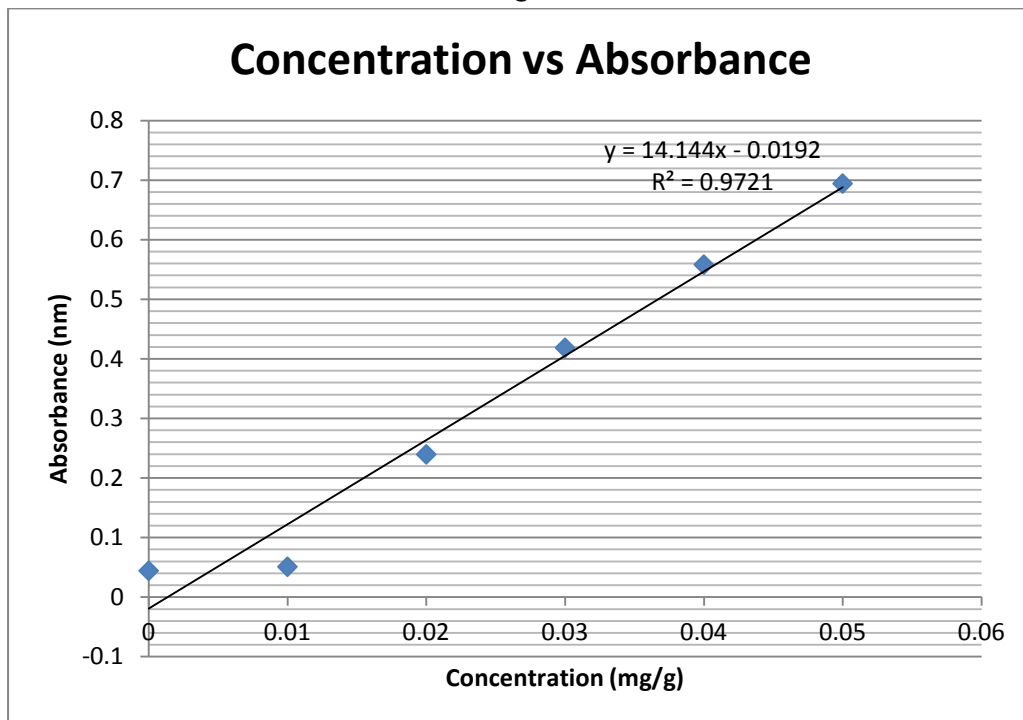
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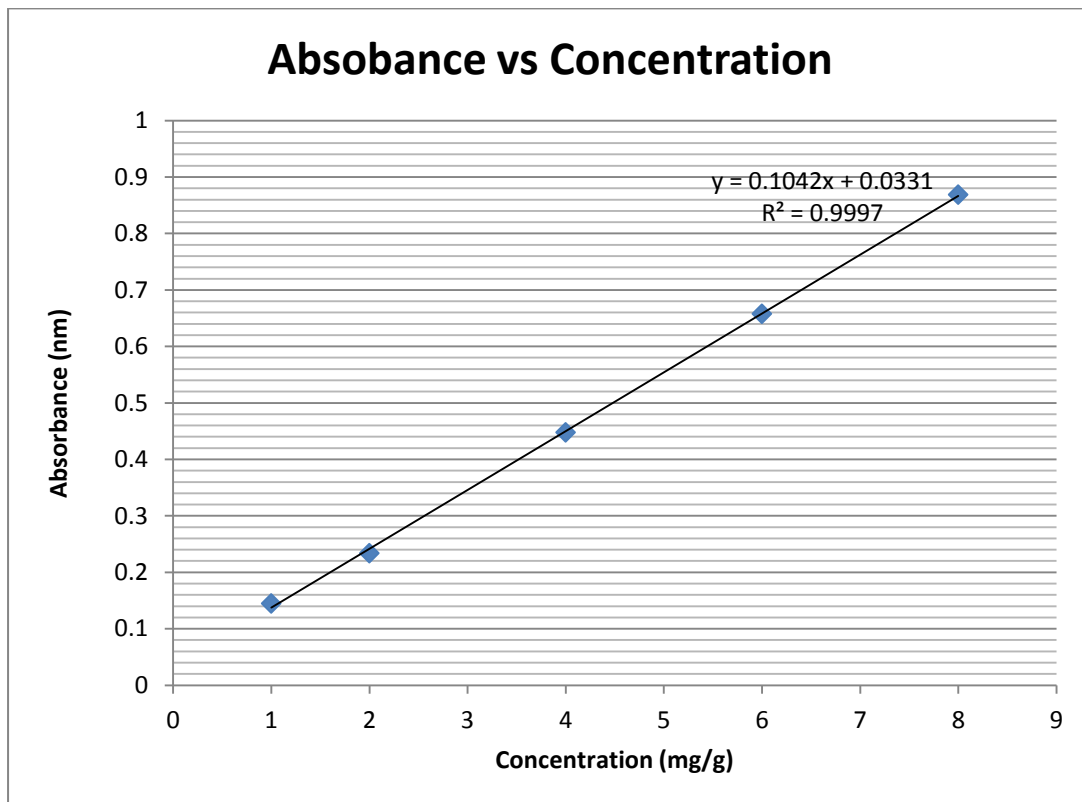
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APPENDIX A –Standard Curve for Quercetin



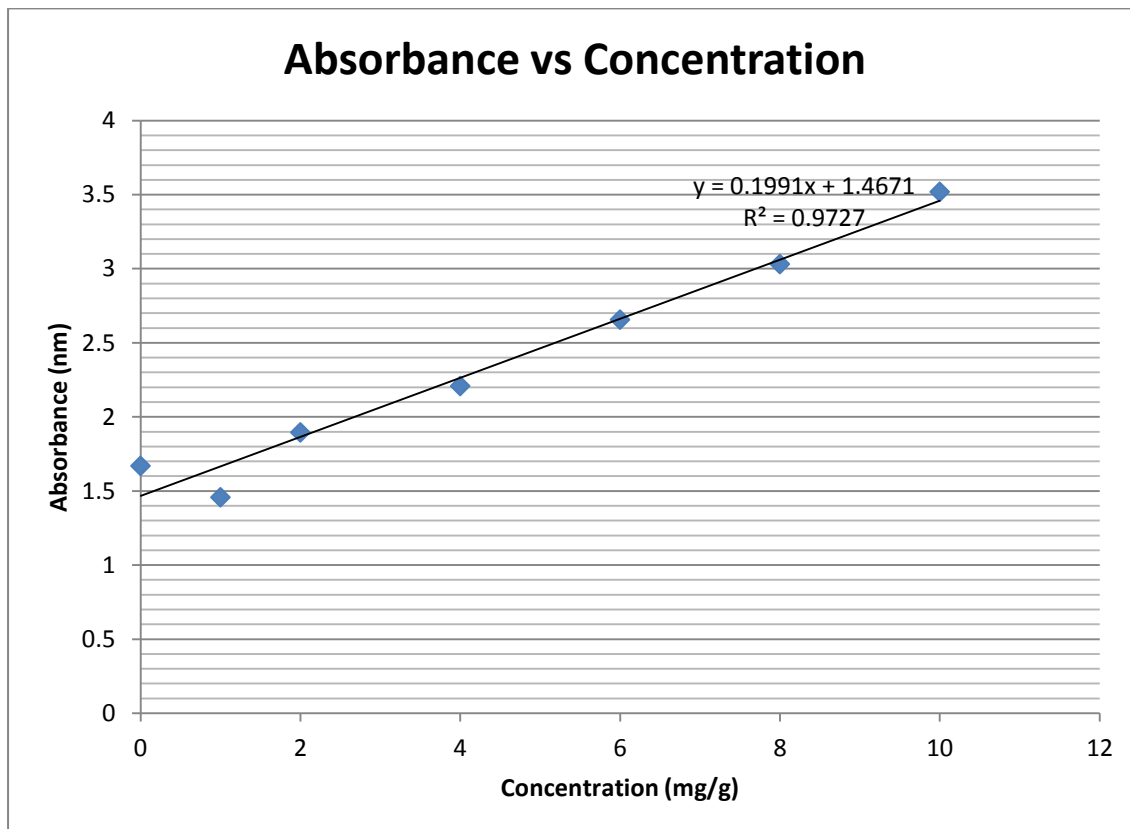
Absorbance vs Concentration (Quercetin Standard)				
Conc	T1	T2	T3	Average
2	0.24	0.238	0.224	0.234
4	0.453	0.451	0.44	0.448
6	0.652	0.659	0.658	0.658
8	0.857	0.859	0.891	0.869

APPENDIX B- Standard Curve for Tannic acid



Absorbance vs Concentration (Tannic acid standard)			
Conc.	T1	T2	Taverage
0	1.6318	1.7086	1.6702
1	1.4274	1.4886	1.458
2	1.7712	2.0208	1.896
4	2.269	2.149	2.209
6	2.463	2.851	2.657
8	3.0531	3.0107	3.0319
10	3.4325	3.6071	3.5198

APPENDIX C- Standard Curve for Gallic Acid



Absorbance vs Concentration (Gallic Standard)				
Conc.	T1	T2	T3	Average
0	0.0422	0.0413	0.0497	0.0444
0.01	0.0496	0.0518	0.0519	0.0511
0.02	0.2175	0.3012	0.2007	0.2398
0.03	0.4009	0.4396	0.4156	0.4187
0.04	0.5932	0.5194	0.5623	0.5583
0.05	0.6543	0.6845	0.7444	0.6944

APPENDIX D- Calculations for preparation of reagents

Preparation of 1% HCl

Reagents

- 32% HCl
- Distilled water

$$\text{Concentration} = V1/V2$$

$$C1V1 = C2V2$$

$$32\% \times V1 = 1\% \times 100 \text{ ml}$$

$$V1 = (1\% \times 100 \text{ ml})/32\%$$

$$V1 = 3.1 \text{ ml}$$

This therefore implies that 3.1 ml HCl has to be added to 100 ml of distilled water to make 1% HCl.

Preparation of 5% NaNO₃

$$5\% = (\text{mass of NaNO}_3) / (250 \text{ ml}) \times 100$$

$$(250 \times 5) / 100 = \text{mass of NaNO}_3$$

$$\text{Mass of NaNO}_3 = 12.5 \text{ g}$$

Therefore 12.5 g of NaNO₃ was dissolved into 250 ml of distilled water.

Preparation of 10% AlCl₃

$$10\% \text{ AlCl}_3 = 10 \text{ g} / 100 \text{ ml}$$

Dissolving 10 g of AlCl₃ in distilled water will make 10% AlCl₃

Preparation of 1M NaOH

$$\text{Molarity} = 1 \text{ mol} / 1 \text{ litre}$$

Therefore to prepare 1 l of one molar solution of Sodium hydroxide we need one mole of NaOH.

$$1 \text{ mole NaOH} = 40 \text{ g of NaOH}$$

Dissolving 40 g of NaOH in 1 l of distilled water will make 1 l of 1 molar solution

Preparation of 20% Sodium Carbonate

10 g of Sodium Carbonate was put into a volumetric flask.

Distilled water was added up to the 50 ml mark.

This produces 20% w/v solution of Na_2CO_3

Preparation of 0.1N HCl

Normality = Molarity

$N = M$

32% HCl = 10.2 M = 10.2 N

$C_1V_1 = C_2V_2$

$C_1 = 10.2 \text{ M} / 10.2 \text{ N}$

$V_1 = ?$

$C_2 = 0.1 \text{ M} / 0.1 \text{ N}$

$V_2 = 100 \text{ ml}$

$V_1 = (C_2 \times V_2) / C_1$

$V_1 = (0.1 \text{ N} \times 100 \text{ ml}) / 10.2 \text{ N}$

$V_1 = 0.98 \text{ ml HCl}$

Therefore to make 0.1 N add 1ml HCl to 99 ml of distilled water.

Preparation of Potassium ferrocyanide

Mass = $0.008 \text{ M} \times 329.26 / 1 \text{ M}$

Mass = 2.634 g

Therefore 2.634 g of potassium ferrocyanide was dissolved in 1 l distilled water to make 0.008 M potassium ferrocyanide.

APPENDIX E- Phytochemical quantification values in mg

Phytochemicals	Ethanollic extract	Methanolic extract
Phenols	0.732±0.001	0.961±0.001
Flavonoids	4.16±0.01	4.239±0.012
Tannins	5.356±0.185	6.937±0.115

APPENDIX F- MIC values for each strain*Candida albicans*

Concentration (mg/ml)	Sector #	Et 1	Et2	Me 1	Me 2
1	1	x	x		
2	2	x	x		
6	3				
8	4				
10	5				

Minimum Inhibitory Concentration for the Ethanolic and Methanolic extract on Ca.

Staphylococcus aureus

Concentration (mg/ml)	Sector #	Et 1	Et 2	Me 1	Me 2
1	1	x	x	x	x
2	2				
6	3				
8	4				
10	5				

Minimum Inhibitory Concentration for the Ethanolic and Methanolic extract on Sa.

Escherichia coli

Concentration mg/ml	Sector #	Et 1	Et2	Me 2	Me 1
1	1	x	x		
2	2	x	x		
6	3	x	x		
8	4				
10	5				

Minimum Inhibitory Concentration for the Ethanolic and Methanolic extract on Ec.

Klebsiella pneumoniae

Concentration mg/ml	Sector #	Et 1	Et 2	Me 1	Me 2
1	1	x	x		
2	2	x	x		
6	3	x	x		
8	4	x	x		
10	5				

Minimum Inhibitory Concentration for the Ethanolic and Methanolic extract on Kp.

APPENDIX G- Raw Data for IC₅₀ Graph

	Ethanol extract	Methanol extract	VitC
log(inhibitor) vs. normalized response -- Variable slope			
Best-fit values			
LogIC ₅₀	-0.4645	-0.6101	-0.4366
HillSlope	0.9622	0.8884	0.5395
IC ₅₀	0.3432	0.2454	0.3659
Std. Error			
LogIC ₅₀	0.03272	0.02651	0.09295
HillSlope	0.06779	0.04887	0.08321
95% Confidence Intervals			
LogIC ₅₀	-0.5347 to -0.3943	-0.6670 to -0.5533	-0.6359 to -0.2372
HillSlope	0.8168 to 1.108	0.7836 to 0.9932	0.3610 to 0.7179
IC ₅₀	0.2919 to 0.4033	0.2153 to 0.2797	0.2312 to 0.5791
Goodness of Fit			
Degrees of Freedom	14	14	14
R square	0.9744	0.9827	0.8182
Absolute Sum of Squares	358.3	210.5	1364
Sy.x	5.059	3.877	9.872
Number of points Analyzed			
	16	16	16
	Ethanol extract	Methanol extract	VitC
IC ₅₀	0.3432	0.2454	0.3659
	0.0044	0.0021	0.03925