TLC SEPARATION AND ANTIOXIDANT ACTIVITY OF FLAVONOIDS FROM SELECTED ZIMBABWEAN TRADITIONAL FRUITS.

BY

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Approval Form

The undersigned certify that they have supervised, read and recommend to the Bindura University of Science Education for acceptance of a research project entitled:

**TLC SEPARATION AND ANTIOXIDANT ACTIVITY OF FLAVONOIDS FROM SELECTED ZIMBABWEAN TRADITIONAL FRUITS.**

Submitted by **Mkululi Mangena**

In partial fulfilment of the requirements for the **MASTER OF SCIENCE EDUCATION IN CHEMISTRY**

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(Signature of Student)                      Date

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(Signature of Supervisor)                   Date

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(Signature of the Chairperson)             Date
Dedication

I dedicate this project to my lovely wife Cynthia, my strong pillar and source of inspiration. She encouraged me all the way and this pushed me to finish what I had started. To the rest of the family who have been affected in every way possible by this quest, thank you. My love for you is endless. Stay blessed.
Acknowledgements

My thanks and appreciation to Mr P. Dzomba and Dr. L. Gwatidzo, as my supervisors, for their valuable academic and professional input in refining the topic and the design of the study, offering expert assistance and advice throughout the time it took me to complete this research and write the dissertation. Their expert guidance nurtured my academic growth. I am also grateful to the Bindura University of Science Education Laboratory technicians and technical staff for their valuable technical assistance.
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Abstract
Thin Layer Chromatography (TLC) was used to isolate flavonoids present in three selected Zimbabwean wild fruits, namely, *C. bispinosa*, *F. sycomorus* and *G. bicolar* using methanol: chloroform: hexane (7:2:1, v/v/v) as mobile phase. The flavonoid positive fractions were separated for antioxidant and fourier transform infra-red (FT-IR) spectroscopy analyses. The 2,2-Azino-bis 3-ethylbenz-thiazoline-6-sulphonic acid, (ABTS) and 2,2-diphenyl-1-picrylhydrazyl, (DPPH) radical scavenging method was used for testing the antioxidant activity of the samples, using quercetin and catechin as reference standards. FT-IR was used to characterise the functional groups in the flavonoid fractions. Thin layer chromatographic profiling revealed six different types of flavonoids. *C. bispinosa* yielded two flavonoids with R$_f$ values of 0.113 and 0.375. *G. bicolar* also yielded two flavonoids with R$_f$ values of 0.625 and 0.812; while *F. sycomorus* yielded two types of flavonoids with R$_f$ values of 0.094 and 0.812. All the extracted flavonoid exhibited significant antioxidant activity of over 80 % at 200 mg/ml concentration. The order of radical scavenging activity for the 200 mg/ml samples is *G. Bicolar*, R$_f$ (0.812) > *C. bispinosa*, R$_f$ (0.113) > *F. sycomorus*, R$_f$ (0.094) > *F. sycomorus*, R$_f$ (0.047) > *C. bispinosa*, R$_f$ (0.375) > *G. bicolar*, R$_f$ (0.63). *G. bicolar* (R$_f$ = 0.812) exhibited antioxidant activity superior to that of Catechin. FT-IR revealed the presence of functional groups, (C=O, C-H, -OH, and –OCH$_3$) characteristic of flavanones, flavones and flavonols. The results of the present study show that *C. bispinosa*, *F. sycomorus* and *G. bicolar* contain flavonoids which are responsible for the observed antioxidant activity, hence improved intake of the fruits can lead to improved health in people, especially against oxidative stress related conditions like diabetes, cancer and so on. This new knowledge can also be used to produce nutraceuticals and can also be used in development of new organic non-toxic preservatives to be used in packed foods.
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABTS</td>
<td>2, 2-Azino-bis 3-ethylbenz-thiaz-oline-6-sulfonic acid</td>
</tr>
<tr>
<td>BHA</td>
<td>Butylated hydroxyl anisole</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated hydroxyl toluene</td>
</tr>
<tr>
<td>TBHQ</td>
<td>tert-butyl hydroquinone</td>
</tr>
<tr>
<td>PG</td>
<td>propyl gallate</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin Layer chromatography</td>
</tr>
<tr>
<td>UV</td>
<td>Ultra violet light</td>
</tr>
<tr>
<td>DPPH</td>
<td>2, 2-Diphenyl-1- picrylhydrazyl</td>
</tr>
<tr>
<td>ORAC</td>
<td>oxygen radical antioxidant capacity</td>
</tr>
<tr>
<td>FRAP</td>
<td>the ferric reducing antioxidant power</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION

1.0 Flavonoids
Flavonoids are natural plant antioxidants found in most higher plants especially in fruits, leaves, bark, roots, and seeds, (Gulcin, 2006; Roberts et al., 2013; Ioana et al., 2010). There are over 4000 known flavonoids, which can be classified as flavanols, flavones, flavanones, isoflavonones, flavonols, anthocyanins and anthocyanidins, (Gulcin, 2006; Roberts et al., 2013; Ioana et al., 2010; Lihua et al., 2009; Ashokkumar and Ramaswamy, 2014; Nazck and Shahidi. 2004; Stefanat et al., 2004; Joshi, 2011; Afroz et al., 2014). There has been notable developments in research focused on the extraction, identification and quantification of flavonoids as cosmetic, medicinal and dietary molecules, (Gulcin, 2006; Roberts et al., 2013). In several of these investigations, solvent extraction was the main method used to extract flavonoids since flavonoids are soluble in water and in organic solvents, (Gulcin, 2006). Thin layer chromatography coupled with ultra-violet (UV) spectrophotometry and sometimes high performance liquid chromatography (HPLC) have been used extensively in isolating, identifying and sometimes quantifying flavonoids, with subsequent employment of chemical spraying procedures to visualize the developed chromatograms, (Roberts et al., 2013; Ioana et al., 2010, Ashokkumar and Ramaswamy., 2014; Stefanat et al., 2004; Afroz et al., 2014; Gulcin, 2006). Flavonoids are compounds of interest because they consist of significant antioxidant activity. Antioxidant activity of flavonoids have largely been studied using the technically simple and sensitive, 2,2-diphenyl-1-picryl hydrazil (DPPH) and 2,2-Azino-bis 3-ethylbenz-thiazoline-6-sulphonic acid (ABTS) scavenging assay tests, (Gulcin, 2006; Afroz et al., 2011; Roberts et al., 2013). Spectrophotometric methods have generally been applied to characterize flavonoids that would have been separated chromatographically, (Roberts et al., 2013; Nazck and Shahidi, 2004;
Gulcin, 2006; Dzomba and Musekiwa, 2014). In this study, antioxidant activities of flavonoids isolated from selected local fruits was investigated.

1.1 Background

Antioxidants are organic molecules that scavenge free radicals and increase shelf life of packed food items by delaying, preventing and/or interrupting lipid peroxidation, (Gulcin, 2006). Recent studies has shifted focus from the use of synthetic antioxidants such as butylated hydroxyl anisole, (BHA), tert-butyl hydroquinone, (TBHQ), butylated hydroxytoluene, (BHT) and propyl gallate, (PG), which have been in wide use in food and pharmaceutical industries to elongate shelf life of packed foods and drugs, (Gulcin, 2006). The shift from synthetic to natural antioxidants like flavonoids has been necessitated by the need to find alternative safe and non-toxic antioxidants since some synthetic antioxidants, for example, BHA and BHT have been restricted by legislative rules and regulations over their possible carcinogenic effects, (Gulcin, 2006).

Flavonoids are an important class of organic molecules formed in plants by the shikimic acid and acetate pathways from aromatic amino acids (phenylalanine and tyrosine) and malonate (Gulcin, 2006). Flavonoids consist of the basic flavan nucleus, which is basically a 15 carbon molecule, with the atoms arranged in a 3-ring ($C_6-C_3-C_6$) configuration (Mohammed, 1996). The most significant benefits of flavonoids are their ability to protect against oxidative stress (Rice-Evans and van Acker, 1998), ability to modulate the activity of various enzymes, and their ability to interact with specific receptors, (Williams, et al., 2004, Mohammed, 1996, Bhat, et al., 2011) and hence have far reaching effects on the chemistry of organisms at cellular levels. Flavonoids are good natural antioxidants whose antioxidant activity depends on their metal-chelating potential, a property directly linked to the arrangement of hydroxyl and carbonyl groups around the
molecule. Their antioxidant ability is further dependent on presence of hydrogen or electron donating substituents that can reduce free radicals. Furthermore, flavonoids ability to act as antioxidants depends on their ability to delocalize the unpaired electron forming stable low energy phenoxy radical, (Gulcin, 2006; Ioana et al., 2010; Josh, 2011). Research has shown that flavonoids, as a consequence, have important biological functions such as antioxidant, anti-carcinogenic, anti-inflammation, anti-atherosclerosis, antiopoptosis, anti-aging, cardiovascular protection, improvement of the endothelial function, inhibition of angiogenesis and cell proliferation activity (Joshi, 2011; Harborne, 1998; Iona et al., 2010; Gulcin, 2006). In plants they have been shown to be important anti-feedants and attractants for pollinators. They further contribute towards color and sensory characteristics of fruits and vegetables. Due to these reasons research to characterize flavonoids in plant tissues has increased over the years.

Flavonoids help to protect the plant against UV light, fungal parasites, herbivores, pathogens and oxidative cell injury (Ioana et al., 2010; Pinheiro, et al., 2012). They are known to reduce incidence of diseases such as cancer and heart disease in humans. They are important antioxidants because they have very high redox potentials which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Ioana et al., 2010; Afroz et al., 2014). Flavonoids have aroused considerable interest recently because of their potential benefits to human health, that is, they are reported to have antiviral, anti-allergic, anti-inflammatory, anti-tumor and antioxidant activities.

Antioxidants are compounds known to protect cells against damaging effects of reactive oxygen species, like, singlet oxygen, superoxide peroxyl radicals, hydroxyl radicals and peroxy-nitrile. Imbalances between antioxidants and reactive oxygen species results in oxidative stress which causes cellular damage. Research has established a link between oxidative stress and cancer,
aging, atherosclerosis, ischemic injury inflammation and neurodegenerative diseases (Afroz et al., 2014; Gulcin, 2006). Thus flavonoids may help provide protection against these diseases by contributing antioxidant vitamins and enzymes, to the total antioxidant defense system of the human body.

Hence, it is against this background, and overwhelming emerging research evidence that fruits, vegetables, and medicinal plants are major sources of flavonoids (Gulcin, 2006), therefore, this study sought to investigate antioxidant capacity of flavonoids from C. bispinosa, F. sycomorus and G. bicolar fruits. C. bispinosa, F. sycomorus and G. bicolar are Zimbabwean traditional fruit trees that grow in the wild. Currently, information on antioxidant activity of these fruits is still limited.

1.2. Statement of the Problem
The induction of many chronic and degenerative diseases is a direct result of oxidative stress. During metabolism free radicals and other reactive oxygen species, (ROS), and reactive nitrogen species, (RNS), can cause oxidative damage of amino acids, lipids, proteins and DNA, (Ioana et al. 2010; Gulcin, 2006). This causes degenerative diseases like ischaemic heart disease, diabetes mellitus, cancer, immunosuppression, neurodegenerative diseases, and ageing. Antioxidants like vitamin C, vitamin E, butylated hydroxyanisole, (BHA), butylated hydroxyl toluene, (BHT), propyl gallate and tert butyl hydroquinone, (Li et al., 2012) have been in extensive use. However, the synthetic antioxidants like BHT and BHA are now being restricted by legislation because of possible toxic and carcinogenic effects, (Zheng and Wang. 2001; and Li et al., 2012, Roberts et al., 2013; Afroz et al., 2014; Gulcin, 2006). On the other hand, the antioxidants from plants are not only non-carcinogenic but reportedly have anti-carcinogenic effects, (Afroz et al., 2104). Hence, there is need to investigate the nature of flavonoids in the selected Zimbabwean
traditional fruits and the need to evaluate the antioxidant potential of these as well. The information obtained can then be used to professionally plan human diet that takes into account recommendations to use the traditional fruits in mainstream diet. The fruits are readily available in most Zimbabwean provinces and if found to have significant amounts of flavonoids and discovered to display significant antioxidant activity, then with awareness rural folks maybe educated to take more of traditional fruits to supplement their natural antioxidants to improve their health under oxidative stress.

1.3 Justification
Plants have been shown to be rich sources of flavonoids, which are molecules that can act as antioxidants to prevent heart disease, reduce inflammation, lower the incidence of cancers and diabetes, as well as reduce rates of mutagenesis in human cells (Roberts et al., 2013; Gulcin, 2006). It has been noted that the protection afforded by the consumption of plant products such as fruits, vegetables and legumes is mostly associated with the presence of flavonoids. Several researches have been done on separation and characterization of flavonoids in fruits, leaves, bark, roots and seeds. However, little information is available regarding C. bispinosa, F. sycomorus, and G. bicolar flavonoid content and antioxidant activity and this provides the rationale behind this study. This study may also give impetus to the sustainable use of the plants.

1.4 Aim
To evaluate the antioxidant activity of flavonoids in selected traditional Zimbabwean fruits, C. bispinosa, F. sycomorus, and G. bicolar.

1.5 Objectives
The objectives of this study are to:

- To extract flavonoids from selected traditional Zimbabwean fruits using preparative TLC
To profile flavonoids in the selected traditional fruits using analytical and preparative thin layer chromatography and FT-IR.

To evaluate the flavonoids’ antioxidant activity in the selected traditional fruits using UV spectrophotometry

1.6 Limitations

While UV-Vis and FT-IR will provide vital information of functional groups in the flavonoid bands, and TLC bio-autography or profiling will complement the former with information on retention factors, the information may not be used conclusively on structural elucidation. The use of HPLC-MS/MS and other chromatographic tandem methods like GC-MS/MS may provide more information.

1.7 Delimitation

A lot of Zimbabwean traditional fruits might contain flavonoids and may display antioxidant activity but this research has been limited to the study of antioxidant properties of three selected Zimbabwean fruits, namely: *C. bispinosa*, *F. sycomorus* and *G. bicolar*. 
CHAPTER TWO: LITERATURE REVIEW

2.0 Introduction

Flavonoids are naturally occurring antioxidants commonly found in plants. They are a major class of polyphenolic compounds found in significant amounts in fruits and vegetables, and have the ability to sequester free radicals (Bentz, 2009). The other major classes of polyphenolics include phenolic acids, tannins, stilbenes and lignins, (Ioana et al., 2010; Afroz et al., 2014; Lihua et al., 2009; Koua, et al., 2011; Bhat, et al., 2011; Maobe, et al., 2012). Polyphenolic compounds contain hydroxyl groups on aromatic rings. Polyphenols are classified on the basis of number of rings and also to the structural elements binding rings to one another. Figure 2.1 is a representative structure of flavonoids.

![Flavonoid structure](image)

**Figure 2.1: Flavonoid structure**

Generally, flavonoids are low molecular weight compounds, with a C6–C3–C6 configuration. Flavonoids structurally consist of two aromatic rings, A and B, joined by a 3-carbon bridge, which is usually in the form of a heterocyclic ring, C (Ioana, et al., 2010; Khodhami et al., 2013; Pinheiro, et al., 2012; Uivarosi, et al., 2016; Heneczkowski, et al., 2001). Bentz, (2009) explained that the aromatic ring A (Figure 2.1) is derived from the acetate/malonate pathway, while ring B is derived from phenylalanine through the shikimate pathway, (Ioana et al., 2010).
2.1 Classification of flavonoids

The major flavonoid classes include flavonols, flavones, flavanols (or catechins), isoflavones, flavanones, isoflavones flavanones and anthocyanidins (see Figure 2.2) (Hollman and Katan, 1999, in Ioana et al., 2010, Khodhami et al., 2013). Bentz (2009) explained that the different flavonoid classes arise as a result of substitutions at rings A and B, and these may include oxygenation, alkylation, glycosylation, acylation and sulphonation, (Ioana et al., 2010).

![Figure 2.2: Major flavonoid subclasses](image-url)
2.1.2 Chemistry of Flavonoids

Generally, the chemical behavior of flavonoids can be explained using quercetin as an example since a lot of documented studies have been carried out on quercetin, (Pinheiro, et al., 2012; Uivarosi, et al., 2016; Heneczkowski, et al., 2001; Bentz, 2009). Quercetin has been shown to belong to the flavonoid subclass of flavonols. Research has shown that quercetin is found in considerable amounts in fruits and foods, and is believed to have a protective effect against a lot of degenerative diseases like lipid peroxidation. Quercetin is an approved antioxidant.

2.2 Free Radicals

Free radicals are reactive molecular species that possess unpaired electrons that are capable of oxidizing other molecules by gaining electrons. The oxidative reaction will produce a new radical and this results in a domino effect of free radical stabilization and generation, (Bentz, 2009). It is believed that free radicals can oxidize very large molecules including DNA, proteins, carbohydrates, and lipids, (Udin and Ahmad, 1995; Pinheiro, et al., 2012; Uivarosi, et al., 2016; Heneczkowski, et al., 2001). The oxidative damage due to chemical effects of free radicals is called oxidative stress. Oxidative stress is linked to degenerative diseases which include cardiovascular and inflammatory diseases, cancer, aging and stroke (Bentz, 2009). There are a wide variety of free radicals. These include reactive oxygen species, (ROS), reactive nitrogen species (RNS) and non-free radical species. Gulcin, (2011) listed free radicals as summarized in Table 2.1 and Table 2.2
Table 2.1 Common reactive oxygen species

<table>
<thead>
<tr>
<th>Name of reactive oxygen species</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Super oxide radical</td>
<td>$O_2^-$</td>
</tr>
<tr>
<td>Hydroxyl radical</td>
<td>HO'</td>
</tr>
<tr>
<td>Hydroperoxyl radical</td>
<td>HOO'</td>
</tr>
<tr>
<td>Lipid radical</td>
<td>L'</td>
</tr>
<tr>
<td>Peroxyl radical</td>
<td>ROO'</td>
</tr>
<tr>
<td>Lipid alkyl radical</td>
<td>LO'</td>
</tr>
<tr>
<td>Nitrogen dioxide</td>
<td>NO$_2^-$</td>
</tr>
<tr>
<td>Nitric oxide</td>
<td>NO'</td>
</tr>
<tr>
<td>Nitrosyl cation</td>
<td>NO'</td>
</tr>
<tr>
<td>Thiyl radical</td>
<td>RS'</td>
</tr>
<tr>
<td>Protein Radical</td>
<td>P'</td>
</tr>
</tbody>
</table>
Table 2.2 Common non free radical species

<table>
<thead>
<tr>
<th>Name of non-free radical species</th>
<th>Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydrogen peroxide</td>
<td>H₂O₂</td>
</tr>
<tr>
<td>Singlet oxygen</td>
<td>^1O₂</td>
</tr>
<tr>
<td>Ozone</td>
<td>O₃</td>
</tr>
<tr>
<td>Lipid hydroperoxide</td>
<td>LOOH</td>
</tr>
<tr>
<td>Peroxy nitrile</td>
<td>ONOO⁻</td>
</tr>
<tr>
<td>Dinitrogen trioxide</td>
<td>N₂O₃</td>
</tr>
<tr>
<td>Nitrous Acid</td>
<td>HNO₂</td>
</tr>
<tr>
<td>Nitryl Anion</td>
<td>NO⁻</td>
</tr>
<tr>
<td>Nitryl chloride</td>
<td>NO₂Cl</td>
</tr>
<tr>
<td>Peroxy nitrous acid</td>
<td>ONOOH</td>
</tr>
<tr>
<td>Nitrous oxide</td>
<td>N₂O</td>
</tr>
</tbody>
</table>

Reactive oxygen species can be made in cells during respiration and other mediated cell immune functions (Gulcin, 2006). Reactive oxygen species are not always harmful. For instance they are required at certain levels in order for the cell to function normally. However, when their levels exceed the normal limit reactive oxygen species can cause destruction of crucial biomolecules like lipids, proteins, polyunsaturated fatty acids and carbohydrates (Gulcin, 2006). It is also suggested that reactive oxygen species may be produced during phagocytic actions in cells. Reactive oxygen species have been shown to cause diseases like:
• Malaria
• acquired immune deficiency syndrome
• heart disease
• stroke
• arteriosclerosis
• diabetes
• cancer

2.3 Risk of oxidation in Organisms
Physiological conditions in cells of organism can limit oxidative catalytic effects of transition elements by providing binding sites for the transition elements. Naturally, all organisms are at risk of oxidative stress since they use oxygen. Plants produce flavonoids which counter oxidative stress. Since flavonoids are poly phenols, they have numerous double bonds and hydroxyl groups that can donate electrons through resonance which leads to stabilization of free radicals, (Bentz, 2009; Pinheiro, et al., 2012; Uivarosi, et al., 2016; Heneczowski, et al., 2001). By so doing, antioxidants, like quercetin can reduce oxidative stress in plants. The ability to scavenge free radicals by flavonoids arise from their chemical structure and thus these flavonoids when consumed can help to defend against oxidative stress. This action will therefore, help to reduce heart diseases, prevent cancer and slow the aging process in cells responsible for the degenerative diseases (Bentz, 2009).

2.4 Common sources for Flavonoids
Table 2.3 summarizes natural sources of flavonoids as listed by Majewski et al, 2012 and Bentz, 2009:
Table 2.3: Flavonoid subclasses and some common sources.

<table>
<thead>
<tr>
<th>Flavonoid subclass</th>
<th>Common sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonols</td>
<td>Red grapes, red wine, green tea, beer, cider, chocolate, apricot,</td>
</tr>
<tr>
<td>Flavanones</td>
<td>Citrus foods, (oranges, lemons, grapes), tomatoes, aromatic plants, e.g. Mint</td>
</tr>
<tr>
<td>Flavones</td>
<td>Green leafy spices, parsley, celery, carrots, chicory, lettuce.</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Soy foods, that is, foods from legumes</td>
</tr>
<tr>
<td>Anthocyanidins</td>
<td>Berries, cabbage</td>
</tr>
<tr>
<td>Flavanols</td>
<td>Present in almost all foods</td>
</tr>
</tbody>
</table>

2.5 Availability of flavonoids in Foods

Availability of flavonoids in food depends on a number of factors. Bentz, (2009) singled out several researches that had revealed that boiling food reduces the levels of quercetin (Aleasn and Obrien, 2002). This happens because high temperatures needed to achieve boiling cause thermal degradation of quercetin, making it less available. Furthermore, another study (Beecher, 2003) discovered that onions lose 25-33% quercetin in first twelve days of storage but showed minimal loss after that. In contrast, quercetin levels were observed to increase in broccoli heads with storage, for example, quercetin levels were observed to increase by up to 32% when stored at -20˚C for nine months (Bentz, 2009). Flavonoid availability also depends on environmental conditions the plant grew in. Plants grown in green-houses have less quercetin as compared to
those exposed to UV light. Exposure to UV light triggers antioxidant defense mechanism against oxidative stress hence an increase in quercetin in plants exposed to UV light.

Factors affecting flavonoid availability can be summarized as:

- Type of food
- Stage of ripening of the fruit
- Preparation of food
- Storage of food
- How plants are grown
- Region where plant was grown

### 2.6 Antioxidants

Antioxidants are compounds that can either delay or inhibit the oxidation process which occur under the influence of atmospheric oxygen or reactive oxygen species, and or free radicals (Magdalena et al., 2011; Afroz et al., 2014; Gulcin, 2006). In the food industry, antioxidants are viewed as any substance that significantly delays or inhibits the oxidation of food substrate (Gulcin, 2006). Antioxidants can be used to stabilize polymeric products in petrochemicals, foodstuffs, cosmetics and pharmaceuticals. In the food industry, antioxidants may be added in small amounts to pre-packed food in order to scavenge reactive oxygen species, hence, lengthening the shelf life of packed food. They help in the defense mechanism of organism against degenerative diseases caused by free radicals.

### 2.7 Effects of Antioxidants on Health

A healthy person has good defense system which includes enzymatic antioxidants like superoxide dismutase, catalase and glutathione peroxide, inclusive of non-enzymatic antioxidants such as glutathione, ascorbic acid, vitamin E and alpha–tocopherol. The
antioxidants scavenge oxidants, hence, protecting the body against oxidative stress. Under different pathologic conditions, a person’s defense system may be disrupted, and in such situations the person’s body will depend on exogenous antioxidants to scavenge for free radicals (Koua, et al., 2011; Afroz, et al., 2014; Magdalena et al., 2011). At health centers, butyl hydroxyanisole or butyl hydroxytoluene can be prescribed as antioxidants. However, these synthetic antioxidants have been discovered to be carcinogenic, causing stomach and liver cancer (Gulcin, 2009). On the contrary, natural antioxidants, for example, vitamin E (tocopherol) and C are known to have anti-carcinogenic effects. This raises the need to look for nontoxic antioxidants, and recent scientific research point towards natural products. It is against this background that this research was undertaken to separate and evaluate the antioxidant activity of flavonoids found in selected Zimbabwean traditional fruits.

2.8 Types of Antioxidants
There are two types of antioxidants. These are endogenous and exogenous antioxidants.

2.8.1 Endogenous Antioxidants
These antioxidants are manufactured within the organism. They include enzymes, for example, superoxide dismutase, catalase glutathione peroxidase or non-enzymatic compounds like uric acid, bilirubin, albumin, metallothiones (Magdalena et al., 2011).

2.8.2 Exogenous Antioxidants
These are antioxidants that are taken in as food by animals to supplement their internal systems. Important exogenous antioxidants include vitamin E and C, β-carotene, vitamin D and K3, flavonoids, and minerals such as selenium (Magdalena et al., 2011). Most of the exogenous antioxidants are derived from natural sources.
2.9 Functions of Flavonoids in Plants
Harborne and Williams, (2000) outlined that flavonoids:

- Are important in flower and seed pigmentation.
- Act as attractants for pollinators.
- Are important in fertility and reproduction.
- Are involved in various defense mechanisms to protect plants against abiotic and biotic stresses (Ioana, et al., 2010)
- Are important for nitrogen fixing in legumes, by regulating the synthesis of nod factors in rhizobium which causes inception of nodules.
- Act as molecule signal in the transduction pathways of nod factor in legumes leading to the inhibition of auxin transport within the root cortical cells and therefore, to the formation of nodule primordial.
- Are responsible for the characteristic red and blue colors of berries, wines and certain vegetables.
- Are antioxidants, (Ioana, et al., 2010; Khodhami et al., 2013; Joshi, 2011).

2.9.1 Functions of Flavonoids in Foods
Flavonoids contribute to color, flavor, odor and oxidative stability of plant products. They have been reported to have proven efficacy, as antimycotic, antibacterial, antiviral, anti-inflammatory, antioxidant, immune modulators, and enzyme inhibitors.

2.10 Recommended dietary intake of flavonoids
A recommended dietary intake for flavonoids does not currently exist. This may be due to the lack of documented information on flavonoid composition data on fruits and generality of food available to the general population. Pokorny., (1991) reported that a number of researches have
been done on phytochemical analysis but there is still a lack of true understanding of the composition of phytochemicals in food and lack of knowledge in some instances on the absorption and metabolism of phytocompounds. Today however, many health authorities such as the American Cancer Society and the American Heart Association recommend consuming a diet high in fruits and vegetables to ensure that an individual ingests an adequate amount of beneficial phytocompounds. The phytocompounds are flavonoids present in fruits, which will help avoid oxidative stress, in the patients (Bentz, 2009).

2.11 Synthetic Antioxidants
Widely used synthetic antioxidants are butylated hydroxyanisole, (BHA), butylated hydroxytoluene, (BHT), tert-butyl hydroquinone, (TBHQ) and propylgallate, (PG) (see Fig 2.3) (Gulcin, 2006). They are used for preserving food and drugs. They have substituted alkyls to improve their ability to dissolve. BHA and BHT have been shown to be toxic and to be carcinogenic, hence, their use has been restricted by legislative rules.
2.12 Traditional Fruits

The common staple diet for Zimbabwe, as ranked by Chitsiku, (1989) consist of the following food items:

- Grains and grain products
- Milk and milk products
- Eggs
- Fats and oils
- Meat, poultry, and edible insects
- Fish
- Vegetables and vegetable products
- Fruits
• Nuts and seeds
• Sugars and syrups
• Beverages

These food stuffs are eaten according to a certain pattern that reflects one’s economic class and cultural background. The fruits are eaten by most Zimbabweans as and when they get them and not necessarily as a part of a planned diet. With more availability of information on the flavonoid composition of locally available fruits and awareness campaigns, Zimbabweans may benefit more from the intake of these fruits health-wise and not just as a food source. This research focuses on the antioxidant activity of three traditional fruits, *C. bispinosa, F. sycomorus* and *G. bicolar.*

2.13 Extraction of Flavonoids
Most flavonoid aglycones exist in glycosylated form in plant cells. It is believed that flavonoids in this form protect cells from degradation by reducing the toxic effects of metabolic products and also help in transporting them across membranes by increasing their solubility in water. The aglycones or glycosylated forms of flavonoids are polar and so are found in the soluble fraction. As a result, polar solvents like, methanol, ethanol and water or combinations of these can be used to extract flavonoids (Respail, et al., 2005; Nazck, et al., 2004) while lignins and tannins that strongly bind to proteins remain in the residue as part of the insoluble residue in polar solvents. The flavonoids extracted using polar solvents can be separated and quantified using reversed phase HPLC equipped with photo diode array detector (Ioana, et al., 2014). Flavonoids are aromatic compounds and show intense absorption in the UV region and so can be visualized in
UV light. Flavonoids can also be separated by thin layer chromatography and then characterized by FT-IR

2.14 Solid Phase Extraction, (SPE)
Solid phase extraction is a form of stepwise chromatography designed to extract, partition and/or adsorb one or more components from a liquid phase (the sample) onto a stationary phase (sorbent/resin).

2.14.1 Advantages of SPE
- Offers rapid and selective sample preparation prior to analytical chromatography.
- Extends a chromatographic system’s life time and improves qualitative and quantitative analysis.
- Simplifies matrix environment of the analyte, leaving the analyte in a form more suitable for analysis.
- Suitable for samples that contain particulate matter that may cause clogging and high back flow.
- Removes components that may cause high background interference, misleading peaks and/or poor sensitivity.
- Can be used for trace element analysis as a pre-concentration step.

2.14.2 Steps in SPE
Conditioning
This step wets or activates the bonded phases to facilitate significant and consistent interaction between analyte and functional groups. This is done by introducing two tube volumes of a water miscible solvent like methanol or acetonitrile.

Equilibration
This step involves the introduction of a solution similar to the sample load in terms of solvent strength and pH in order to maximize retention. One /two volumes of buffer or water are preferred choices for reversed phase equilibration.

**Sample Loading**

Sample must be applied at consistent and reduced rate of about 1-2 drops per second to guarantee maximum retention.

**Wash**

This step is necessary to elute interferences that may have been retained together with the analyte of interest. The wash solvent must be chosen with great care to avoid premature elution of analyte of interest. For reversed phase analysis a 5-20% methanol in water wash solvent is recommendable.

**Elution**

A solvent that can disrupt the hydrophilic interactions between the analyte and the sorbent is administered. Normally 1-2 tube volumes of methanol or acetonitrile are used as elution solvent.

### 2.15 Isolation and Identification of Flavonoids

Mishra et al, (2012) reported successful separation of flavonoids from *Launaea Procumbens Roxb* using an ethyl acetate: glacial acetic acid: formic acid: distilled water in ratios of (12.1: 1.3: 1.1: 2.8), by HP-TLC. The flavonoids were then identified using basic qualitative methods. On the other hand, (Kaya, et al., 2012,) isolated flavonoid compounds in *Alchemilla L. Species*, using TLC and HP-TLC and identified them against retention factors and they listed flavonoids and their retention factors, as given in Table 2.4. Mohammed, (1996) also tabulated various retention factors and expected colors of flavonoids as viewed under UV at 365 nm. These have been incorporated in (Table 2.4).
Table 2.4: Characteristic colors and retention factors of some flavonoids found in plants.

<table>
<thead>
<tr>
<th>Name of flavonoid</th>
<th>Color under UV/Vis</th>
<th>Retention factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Orientini (luteolin-8-C-glucoside)</td>
<td>yellow</td>
<td>0.70</td>
</tr>
<tr>
<td>Vitexin (apigenin-8-C-glucoside)</td>
<td>green</td>
<td>0.77</td>
</tr>
<tr>
<td>Rutin (quercetin-3-O-rutinoside)</td>
<td>orange</td>
<td>0.44</td>
</tr>
<tr>
<td>Hyperoside (quercetin-3-O-galactoside)</td>
<td>orange</td>
<td>0.65</td>
</tr>
<tr>
<td>Isoquercetin (quercetin-3-O-glucopyranoside)</td>
<td>orange</td>
<td>0.72</td>
</tr>
<tr>
<td>Quercetin (quercetin-3-O-rhamnoside)</td>
<td>yellow</td>
<td>0.64 -0.84</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>Bright yellow</td>
<td>0.83</td>
</tr>
<tr>
<td>Flavonoid</td>
<td>Color</td>
<td>Retention Factor</td>
</tr>
<tr>
<td>-----------------------------------</td>
<td>----------------------------</td>
<td>------------------</td>
</tr>
<tr>
<td>Chrysoeriol</td>
<td>Yellow</td>
<td>0.82</td>
</tr>
<tr>
<td>Tricin</td>
<td>Yellow-green</td>
<td>0.73</td>
</tr>
<tr>
<td>Iso-orientin</td>
<td>Yellow-green</td>
<td>0.41</td>
</tr>
<tr>
<td>Kayaflavone</td>
<td>Dull brown</td>
<td>0.98</td>
</tr>
<tr>
<td>Chalcone (Isoliquirigenin)</td>
<td>Dark brown</td>
<td>0.06</td>
</tr>
<tr>
<td>Chalcone (Isosalipurposide)</td>
<td>Deep red</td>
<td>0.04</td>
</tr>
<tr>
<td>Flavanones (Naringenin)</td>
<td>Faint Purple</td>
<td>0.66</td>
</tr>
<tr>
<td> (Hesperetin)</td>
<td>Faint Purple</td>
<td>0.67</td>
</tr>
<tr>
<td> (Hesperidin)</td>
<td>Light yellow-green</td>
<td>0.85</td>
</tr>
<tr>
<td> (Naringin)</td>
<td>Light yellow-green</td>
<td>0.87</td>
</tr>
<tr>
<td>Dihydroxyquercetin</td>
<td>Faint purple</td>
<td>0.67</td>
</tr>
<tr>
<td>Isoflavones</td>
<td>Faint yellow</td>
<td>0.73</td>
</tr>
<tr>
<td>Mangiferin</td>
<td>Yellow-green</td>
<td></td>
</tr>
</tbody>
</table>

The first two flavonoids are flavone-C-glycosides, while the last four are flavanol-O-glycosides (Kaya, et al., 2012). The same authors also reported three new flavonoids with retention factors 0.36; 0.54 and 0.68.

**2.16 Flavonoid identification on TLC plates**

Flavonoids may appear as yellow-brown spots against a white background when they react with iodine vapor. They may as well appear as dark spots against a green background when observed under UV-light at 254 nm and may be viewed as yellow, green or blue, fluorescent spots, when viewed under UV-light at 365 nm. The colors depend on the structure of the flavonoid that will be present. Table 2.5 below gives the general colors observed for different flavonoids when viewed under UV-light at 365 nm:
Table 2.5: Characteristic colors of flavonoids when observed under UV at 365 nm.

<table>
<thead>
<tr>
<th>Name or type of Flavonoid</th>
<th>Color observed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin, myricetin and 3&amp;7-o-glycosides</td>
<td>Orange-yellow</td>
</tr>
<tr>
<td>Kaempferol, isorhamnetic and 3&amp;7-o-glycosides</td>
<td>Yellow- green</td>
</tr>
<tr>
<td>Luteolin and 7-o-glycosides</td>
<td>Orange</td>
</tr>
<tr>
<td>Apigenin and 7-o-glycosides</td>
<td>Yellow-green</td>
</tr>
<tr>
<td>Anthocyanidin-3-glycosides</td>
<td>Blue</td>
</tr>
<tr>
<td>Anthocyanidin-3,5-diglycososides</td>
<td>Blue</td>
</tr>
<tr>
<td>Compound Details</td>
<td>Color Range</td>
</tr>
<tr>
<td>---------------------------------------------------------------------------------</td>
<td>------------------------------</td>
</tr>
<tr>
<td>6-hydroxylated flavonoids and flavones, (including some chalcone glycosides)</td>
<td>Dark brown or black</td>
</tr>
<tr>
<td>Most chalcones</td>
<td>Dark red or bright orange</td>
</tr>
<tr>
<td>Auorones</td>
<td>Bright orange or red</td>
</tr>
<tr>
<td>Most flavonol glycosides</td>
<td>Bright yellow or yellow brown</td>
</tr>
<tr>
<td>Most flavone glycosides bisflavonyls and unusually substituted flavones</td>
<td>Vivid yellow-green, or dark brown</td>
</tr>
<tr>
<td>Most isoflavones and flavonels</td>
<td>Faint brown</td>
</tr>
<tr>
<td>5-deoxyisoflavones and 7,8-dihydroxy-flavanones</td>
<td>Intense blue</td>
</tr>
<tr>
<td>Flavanones and flavanonols 7-glycosides</td>
<td>Pale yellow or yellow-green</td>
</tr>
</tbody>
</table>

**NB:** Table was compiled using information from Mohammed, 1996.

### 2.17 Methods of measuring antioxidant activity in fruits

Antioxidant activity can be measured by monitoring the effects of the antioxidant in controlling the extent of oxidation (Doughari, 2012). Several methods for measuring the antioxidant activity have been reported (Gupta et al., 2012). Some methods involve a mechanism that has a distinct oxidation step, before measurement of the outcome. For example, the oxidation of linoleic acid is followed by determination of diene conjugation. However, other methods have no clear distinction between the various steps in the procedure (Marinova & Batchvarov, 2011). The main features of an oxidation are the substrate, the oxidant, the initiator, intermediates and final products. Measurement of any one of these can be used to assess antioxidant activity (Prior et al., 2005). For instance, in monitoring antioxidant activity in samples, reaction could be followed by measuring free fatty acid content, polymer content, viscosity, color, and ratio of unsaturated to saturated fatty acids (Prior, et al., 2005). Thus in antioxidant activity research work, it is important to consider the source of reactive oxygen species and the target substrate. This is very
important because an antioxidant may protect lipids against oxidative damage while worsening or accelerating damage to other biological molecules. Therefore in the evaluation of antioxidant efficacy, the use of a number of different measures of activity was a common feature in the reviewed literature (Prior, et al., 2005; Marinova & Batchvarov, 2011; Doughari, 2012).

2.18 Colorimetric Methods of measuring Antioxidant Activity
Marinova and Batchvarov, (2011) singled out four commonly used colorimetric based methods for antioxidant activity testing. These are 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay, the oxygen radical antioxidant capacity (ORAC) assay, the ferric reducing antioxidant power (FRAP) and the 2,2-Azino-bis 3-ethylbenz-thiaz-oline-6-sulfonic acid (ABTS) assays. The choice of method depends on number of factors. The factors include:

- Whether the method chosen effectively measures and/or follows the chemistry actually taking place in the system.
- Whether or not the method utilizes a biologically relevant radical source
- Simplicity of the method.
- Whether or not the method has a well-defined end-point and chemical mechanism.
- The availability of instrumentation for the method.
- Routine quality control measures.

The DPPH assay has been used in determining the antioxidant activity of flavonoids in food samples (Prior, et al., 2005). The DPPH assay is preferred by most researchers because of the following advantages, (Marinova & Batchvaroy, 2011); the method is:

- Rapid
- Simple
- Accurate
• Convenient
• In-expensive.
• Not affected by the sample polarity.
• Suitable for screening many different samples.

The DPPH assay is used to measure the ability of compounds to act as free radical scavengers or hydrogen donors and may be applied in the evaluation of antioxidant activity of foods and beverages (Marinova & Batchvarov, 2011).

2.19 The DPPH Method.

Principle of DPPH antioxidant activity determination

The DPPH radical reacts with an antioxidant from a fruit sample, resulting in the unpaired electron on the DPPH radical pairing up with hydrogen from the antioxidant to form the reduced DPPH-H species. The DPPH radical solution is purple and it turns to yellow on reduction, and this is accompanied by a change in molar absorptivity (Prakash, et al., 2002; Prior, et al., 2005).

The extent of the decolorization is proportional to the number of electrons captured and thus can be used to quantify the amount of antioxidant present (Marinova & Batchvarov, 2011), the decrease in absorbance can be used to calculate percentage antioxidant capacity using the formula:

\[
Percentage \text{ antioxidant activity} = \frac{Ac - As}{Ac} \times 100
\]

Where Ac is absorbance of control and As is the absorbance of the sample.

2.19.1 Free radical scavenging assays

There are two generally accepted mechanisms for free radical scavenging assays.

Hydrogen atom transfer mechanism, (HAT)
ArOH action (hydrogen atom transfer)

Ar-OH → Ar-O’ + H’

Then there is single electron transfer, followed by proton transfer, (SET-PT).

ArOH → ArOH++ + e−
ArOH++ → ArO’ + H+

Sequential proton loss electron transfer, (SPLPT)

ArOH → ArO’ + H+
ArOH + ROO’ → ArO’ + ROOH

The 7-OH group is the site of ionization and electron transfer in SPLPT.

Mechanism of DPPH assay

ArOH + DPPH’ → ArO’ + DPPH-H (HAT)
ArOH → ArO’ + H+ (SPLPT)
ArO’ + DPPH’ → DPPH’ + ArO’
DPPH’ + H+ → DPPH-H

2.20.0 The ABTS antioxidant activity assay

The ABTS assay measures antioxidant radical scavenging by monitoring the depletion of the ABTS cation which reflects as a decrease in absorbance at 734 nm. It is also a decolorization assay. The method is based on the ability of antioxidants to quench the long lived ABTS radical cation which is blue-green to the colorless reduced form (Pellegrini, et al., 1999). The ABTS++ scavenging assay is applicable to both lipophilic and hydrophilic flavonoids (Gulcin, 2006).

2.20.1 Generation of ABTS++

The ABTS++ radical can be generated by reaction of 7 mM ABTS and 2.45 mM potassium persulfate solutions in distilled water (1:1, v/v). The mixture is left to stand in a dark cupboard for
16 hours to allow for the generation of a stable mono cationic ABTS radical that absorbs at 734 nm. After 16 hours, the absorbance of the ABTS radical is measured, (Fig 4.3). Generation of the ABTS$^{+}$ radical in the ABTS/K$_{2}$S$_{2}$O$_{8}$ system require 16 hours to produce a stable mono cation radical. The presence of peroxy-disulphate has been shown to increase the rate of ABTS$^{+}$ cation generation (Gulcin, 2006). The following reactions have been shown to occur in the system.

\[ \text{S}_2\text{O}_8^{2-} + \text{ABTS} \rightarrow \text{SO}_4^{2-} + \text{SO}_4^{2-} + \text{ABTS}^{+} \]

It is thought that the scission of the peroxy-disulphate happens after transfer of the electron. In excess ABTS the predominant reaction will be:

\[ \text{SO}_4^{2-} + 2\text{ABTS} \rightarrow \text{SO}_4^{2-} + 2\text{ABTS}^{+} \]

The final equation will be:

\[ \text{S}_2\text{O}_8^{2-} + 3\text{ABTS} \rightarrow 2\text{SO}_4^{2-} + 3\text{ABTS}^{+} \]

Then antioxidant radical scavenging is calculated using the equation:

\[ \text{Percentage antioxidant capacity} = \frac{A_c - A_s}{A_s} \times 100 \]

The decrease in absorbance is related to the amount of ABTS radical that will have been quenched. This means a sample with high antioxidant activity will produce a very low absorbance that will translate to a very high antioxidant percentage.

2.20.2 Advantages of using ABTS assay

- ABTS is soluble in both water and organic solvents so can be used to study antioxidant activity of both hydrophilic and lipophilic samples (Gulcin, 2006).

- It is a relatively technically simple and sensitive method.

2.20.3 Criticism of ABTS

The major shortfall of ABTS assay is that the ABTS$^{+}$ radical is not representative of any biomolecule and it has not been found in any biological or food system (Gulcin, 2006).
Generally, it is important to assess and evaluate flavonoid antioxidant activity by using conditions that mimic the real life scenarios.

2.20.4 Analytical methods for quantifying flavonoids in fruits
Recently there have been remarkable developments in research focused on the extraction, identification and quantitation of flavonoids from traditional medicinal herbs and/or dietary molecules (Roberts et al., 2013). Largely, organic solvents are used in the extraction processes. For identification, chemical spray reagents like 1% aluminium chloride in ethanol or ferric chloride solution are used. Spectrophotometry and chromatography can be used in identifying and quantifying extracted flavonoid samples. The most commonly applied methods/techniques to quantify flavonoids are the high performance liquid chromatography (HPLC) and gas chromatography (GC), or their combinations with mass spectrometry. In this research chromatography and spectrophotometry shall be used.

2.21.0 The UV/Vis spectrophotometric assays
This is a qualitative method which can be turned quantitative by measuring color intensities using a spectrophotometer. The principle behind the method is that a prepared colored mixture is self-decolorized due to a self-oxidative process after a period of time, usually 25 minutes. (Doughari, 2012; Gupta et al., 2012). However, when an antioxidant-containing substance such as a fruit extracts is added, the antioxidant prevents the oxidative decolorization of the mixture so that the extent of decolouration of the dye is proportional to the amount of the antioxidant present in the fruit sample. A fruit extract with very high antioxidant activity will prevent or inhibit oxidative decolouration of the dye so that it remains blue-green in the case of ABTS and the opposite holds true for a fruit extract that contains insignificant amount or has no antioxidant activity.
2.22.0 Spectral characteristics of flavonoids

Flavones and flavonols have been shown to exhibit two major absorption bands, termed Band I (320-385 nm) which is a result of ring B chromophore absorption, and Band II (250-285 nm) corresponding to absorption due to ring C (see Fig 2.1) (Kumar and Pandey, 2013). On the other hand, Pinheiro, et al., (2012) quotes band I of B-ring as stretching between 300 – 550 nm, and band II falling in the range, 240 – 285 nm for flavones. They further pointed out that flavanones which have no unsaturation on ring C absorb between 270 – 295 nm. Pinheiro, et al., (2012) further explained that Band II of flavanones without a 3-OH group lies around 303 – 304 nm. And that of the 3-OH flavonols will stretch around 352 nm. Chemical shifts from these observed can occur due to the functional groups attached to the flavonoid skeleton. Kumar & Pandey, (2013) reported that the 371 nm absorption noticed in quercetin (3, 5, 7, 3’, 4’ - hydroxyl groups) and the 374 nm absorption in myricetin (3, 5, 7, 3’, 4’, 5’ -hydroxyl groups) are characteristic chemical shifts due to functional groups on the flavonoid skeleton. The difference between flavones and flavonols is the absence of 3-hydroxyl group in flavones.

Flavanones do not have conjugation between A and B rings because they have a saturated heterocyclic C-ring. Due to this, flavanones spectra display a very strong Band II absorption between 270 nm and 295 nm, specifically, at 288 nm for naringenin and 285 nm for taxifolin and just a shoulder for Band I at 326 and 327 nm (Kumar & Pandey, 2013). Usually, Band II may appear as a single peak at 270 nm in flavonoids with a B-ring that has no substitution, but may appear as double peaks or a single peak at 258 nm with a shoulder at 272 nm if there is a di-, tri- or –o- substituted B ring in the compound (Kumar & Pandey, 2013).

On the other hand, anthocyanins may show distinctive Band I peak in the 450-560 nm region as a result of the presence of the hydroxyl cinnamoyl system of the B ring, with Band II peaks in the 240-280 nm region, a characteristic influenced by the benzooyl system of the A ring, and
subsequently, the color of the anthocyanins varies with the number and position of the hydroxyl groups (Kumar & Pandey, 2013).

CHAPTER THREE: MATERIALS AND METHODS

3.0 Introduction
This chapter explores the methodologies used in this research. Both qualitative and quantitative methodologies were employed.
3.1 Equipment

A Mettler Toledo digital analytical balance AB204-S (4d.p) was used to measure the masses of samples during sample preparation. The same balance was used to measure masses of reagents during the preparation of solutions. A Labotec horizontal shaker was used to agitate the ethanol-fruit sample mixtures during liquid-solid extraction to optimize the extraction. A KnF Neuberger vacuum suction pump was used to enhance filtration to separate the liquid sample from the solid residue during sample preparation. A CE Serial N° 15 102295 Vilber Lourmat UV-Visualization detector set at 365 nm was used to visualize developed TLC chromatograms. The Genesys 10s-UV-Vis spectrophotometer was used to measure the absorbance of flavonoid containing fractions obtained from preparative TLC and the standards used. A Thermo scientific iD1 FT-IR spectrophotometer was used for generating spectra of sample extracts.

3.2 Chemicals

Analytical grade solvents were used in all liquid-solid and TLC procedures.

3.3 Sample collection and pre-treatment

Ripened and un-ripened *C. bispinosa, F. sycomorus* and *G. bicolar* fruits were gathered from Mberengwa district in February-March of 2016. The fruit samples were shade dried. *C. bispinosa* was still not dry by April. The dried *F. sycomorus and G. bicolar* fruits were ground to a powder using a grinder. The *C. bispinosa* was ground to a thin paste using a pestle and mortar. The dry powders were stored in polythene bags and kept in cup board prior to liquid-solid extraction. The *C. bispinosa* paste was put in polythene bag and stored in fridge at 4°C prior to liquid-solid extraction.
3.4.0 Sample preparation

3.4.1 Solvent extraction
10.0 g of powdered samples of each of *F. sycomorus*, and *G. bicolar* and 10 g paste of *C. bispinosa*, was weighed and mixed with 20 mL of analytical grade absolute ethanol in a 50 mL volumetric flask. The samples were shaken for 30 minutes on a Labotec horizontal shaker. The samples were then filtered using Whitman’s grade 1 filter paper and placed in reagent bottles. The solvent maceration protocols were repeated three times for each set and the collected filtrates were combined and concentrated under reduced pressure on a rotor vapor set at 40 °C.

3.4.2 Solid phase extraction
The solid phase extraction method as reported by Mumin et al., 2006 with minor modifications was used. Each set was conditioned by passing 5 mL of absolute ethanol through 0.4 g of the Hydrophobic-Lipophilic Balanced sorbent by gravity in an SPE cartridge. Equilibration was achieved by passing 5 mL of distilled water through the sorbent. This was followed by loading the extract through the cartridge again under gravity. To maximize sample clean-up, 1ml of acetone was used to wash the sorbent. Finally, 5 mL of absolute ethanol were added to elute the samples. The collected samples were stored in glass vials.

3.4.3 Analytical thin layer chromatography
3.4.3.1 Mobile phase optimization
The TLC method as used by Lihua, et al., 2009 with modifications was used. 10.0 cm x 5.0 cm silica gel TLC plates were cut to 10.0 cm x 1.5 cm and activated by heating at 100 °C for 10 minutes, and were allowed to cool to room temperature. Pencil lines were drawn 1.5 cm from one edge of the plates. Samples were spotted using thin capillary pipettes onto the pencil line. The plates were placed in a development chamber with a trial solvent. The development chamber
was a 250 ml beaker closed with tapped plastic sheet to make it air-tight. The following solvent systems were tried out during mobile phase optimization:

- Methanol: acetic acid: water, (2:2:6, v/v/v),
- Methanol: chloroform: hexane, (7:2:1, v/v/v)
- Hexane: ethyl acetate: acetone, (8:2:0.5, v/v/v)

3.4.3.2 Visualization of chromatograms
The solvent front was allowed to travel until 1 cm from the top end. The TLC plates were removed and solvent front was marked using a soft pencil. They were air dried and then sprayed with a fine spray of 1% ethanolic aluminium chloride solution, left to dry and then visualized under UV/Vis at 365 nm. The chromatograms were marked and retention factors were calculated and recorded. The resultant chromatograms were captured on camera. The methanol: chloroform: hexane, (7:2:1, v/v/v), produced the best separation of the spot.

3.4.4 Preparative thin layer chromatography
Pre-coated thick silica gel on glass TLC plates measuring 20 cm x 20 cm were used. The methanol: chloroform: hexane, (7:2:1, v/v/v) solvent, which was determined as the optimal mobile phase was used as the mobile phase. Each of the ethanol extracts from the three fruit samples was deposited as a concentrated band 1.5 cm from the edge of its respective TLC plate and allowed to dry. The plates, with dried samples were gently placed in development tank and left to develop. The plates were removed when the solvent front had travelled three quarters of the plates’ length. The position of the solvent front was immediately marked with a soft pencil. The retention factor, (RF), values of the different bands were then calculated using the equation:
Using the method reported by Mittal, 2013 with modifications the bands that tested positive for the flavonoids, in the analytical TLC, were scratched off, mixed with 5 ml of absolute ethanol allowed to stand for 10 minutes and then filtered with Whatman’s grade 1 filter paper and collected in glass vials.

### 3.5 Fourier Transform Infra-Red Spectrophotometry

The FT-IR method as described by Ashokkumar & Ramaswamy, 2014 with minor modifications was used. Dried powder of ethanol extracts of *F. sycomorus*, *G. bicolar* and the *C. bispinosa* from the flavonoid positive bands were taken for FT-IR analysis. About 10 mg of the dried extract powder were subjected to FT-IR analysis using the Therm Scientific iD1 FTIR spectrophotometer with diode array detector with a scan range from 400 to 4000 cm\(^{-1}\) with a resolution of 4 cm\(^{-1}\).

### 3.6.0 Total antioxidant capacity analysis

The total antioxidant capacity was determined using ABTS assay as described by (Miller, et al., 1993) with modifications. Briefly, the ABTS assay depends on decolorization of the blue-green ABTS\(^{•+}\) radical when it is reduced to the colorless ABTS (2, 2-azinobis 3-ethyl benzthioazoline-6-sulphonic acid).

### 3.6.1 Preparation of standard solutions

A mass of 0.01 g catechin was dissolved in 50 cm\(^3\) of methanol to make 200 mg/ml solution. The 200 mg/ml solution was serially diluted to give solutions of 100 mg/ml, 50 mg/ml and 10 mg/ml. Similarly, 0.01 g of quercetin was also dissolved in 50 cm\(^3\) of methanol making a 200 mg/ml stock solution. The stock solution was then serially diluted to give solutions of concentration 100 mg/ml, 50 mg/ml and 10 mg/ml.
3.6.2 Preparation of sample solutions for ABTS assay
The recovered solutions from flavonoid positive bands were measured. Serial solutions of concentration 200 mg/ml, 100 mg/ml, 50 mg/ml, 10 mg/ml and 5 mg/ml of extract were prepared and tested for antioxidant activity.

3.6.3 ABTS$^{*+}$ radical generation
The ABTS$^{*+}$ radical was generated by reaction of 0.00205 M (1.057 g) ABTS and 0.000426 M (0.1153 g) potassium per-sulfate solutions in 250 cm$^3$ of distilled water. The mixture was left to stand in a dark cupboard for 16 hours to allow for the generation of a stable mono cationic ABTS radical that absorbs at 734 nm. After 16 hours, the absorbance of the ABTS radical was measured.

3.6.4 ABTS assay of the samples and standards
The sample (0.5 cm$^3$) or standard was mixed with 1.0 cm$^3$ of ABTS$^{*+}$ solution and left to stand for six minutes. Absorbance of the samples or standards was read after 6 minutes at 734 nm. Catechin and quercetin were used as standards. Then percentage antioxidant capacity was calculated using the following equation:

\[ \% \text{Antioxidant scavenging capacity} = \frac{A_c - A_s}{A_c} \times 100 \]  

Where $A_c$ is absorbance of control and $A_s$ is absorbance of sample.

The percentage total antioxidant scavenging activity for the standards and samples were plotted against the concentration in mg/ml of standard or sample used.
3.7 The DPPH radical scavenging assay
The free radical scavenging activity of the flavonoid extracts, that is, *C. bispinosa*, *F. sycomorus*, and *G. bicolar* was also estimated using the 1,1-diphenyl-2-picryl-hydrazyl, (DPPH) standard method as described by (Afroz, et al., 2014), with some minor modifications. DPPH is a stable free radical that strongly absorbs at 517 nm because of the presence of its odd electron. In the presence of a free radical scavenging antioxidant (an electron donor), the odd electron of DPPH will be paired up, thus decreasing the intensity of the absorption at 517 nm. The extract solution (1 mL) was mixed with 1.5 mL of 0.003 % DPPH in a methanolic solution at different concentrations (62.50, 125, 250, 500, 1000 and 2000 μg/mL) and the percentage of DPPH inhibition was calculated using the following equation:

\[
\% \text{ DPPH Inhibition} = \frac{A_{dpplh} - A_S}{A_{dpplh}} \times 100
\]

3.3

Where \( A_{dpplh} \) = absorbance of DPPH in the absence of the extract; and \( A_S \) = absorbance of DPPH in the presence of either the extract or the standard. The DPPH scavenging activity was expressed as the concentration of the extract required to decrease the DPPH absorbance by 50% (IC\(_{50}\)) and was graphically determined by plotting the % of inhibition of DPPH radical against concentration of extract using linear regression and the IC\(_{50}\) value, was calculated using the linear regression equation:

\[
y = mx + c
\]

Where \( y = 50 \); \( m \) = gradient and \( c \) = intercept.

3.8 Statistical analysis
The analysis of variance (ANOVA) was conducted through the use of a General Treatment Structure (in randomized blocks) for both ABTS and DPPH analyses. The data was analyzed in GenStat 7 (Version 7.2.0.220). Each experiment was replicated three times giving a total of 96
experimental units for ABTS. For DPPH, each experiment was replicated three times giving a total of 126 experimental units. The results of the experiments were presented in graphical form. Student’s t-test, linear regression analyses (performed, in SPSS Version 16.0.2007) and Excel graphing was employed in order to explore the relationships between the variables.
4.1 Analytical TLC Results Analysis

Table 4.1 shows the flavonoid test results performed on all the separated fractions. The revealing agent used was 1% ethanolic AlCl₃. The spots that confirmed the presence of flavonoids were designated positive while those which did not show the presence of flavonoid were designated negative. Four spots indicating four different compounds were noted from each fruit extract. The retention factors were calculated using equation 3.1. Each fruit extract contained at least two flavonoids.

**Table: 4.1 Flavonoid analysis using 1% ethanolic aluminium chloride revealing reagent**

<table>
<thead>
<tr>
<th>Fruit name</th>
<th>Rᵢ value</th>
<th>Test for flavonoids</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>G. bicolar</em></td>
<td>0.25</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>0.47</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>0.63</td>
<td>positive</td>
</tr>
<tr>
<td></td>
<td>0.81</td>
<td>positive</td>
</tr>
<tr>
<td><em>C bispinosa</em></td>
<td>0.11</td>
<td>positive</td>
</tr>
<tr>
<td></td>
<td>0.38</td>
<td>positive</td>
</tr>
<tr>
<td></td>
<td>0.69</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>0.84</td>
<td>negative</td>
</tr>
<tr>
<td><em>F. sycomorus</em></td>
<td>0.094</td>
<td>positive</td>
</tr>
<tr>
<td></td>
<td>0.47</td>
<td>positive</td>
</tr>
<tr>
<td></td>
<td>0.56</td>
<td>negative</td>
</tr>
<tr>
<td></td>
<td>0.88</td>
<td>negative</td>
</tr>
</tbody>
</table>
Figure 4.1 shows the thin layer chromatograms for *G. bicolar, C. bispinosa* and *F. sycomorus* developed by spraying with 1% ethanolic AlCl$_3$, and heating at 100 °C for 5 minutes, and then visualizing under UV at 365 nm. *G. bicolar* revealed two spots which contained flavonoids, (R$_f$ = 0.63, color - blue) and (R$_f$ = 0.812, color – dark brown). *C. bispinosa* revealed two spots which consists of flavonoids at R$_f$ values 0.1125 (color – dark blue) and 0.375 (color – green). *F. sycomorus* also revealed two spots with flavonoids at retention factors of 0.094 (color – grey) and 0.047 (color orange/yellow).

![Chromatograms for G. bicolar, C. bispinosa and F. sycomorus viewed under UV at 365 nm. F = positive for flavonoid](image)

**Fig: 4.1:** Chromatograms for *G. bicolar, C. bispinosa and F. sycomorus* viewed under UV at 365 nm. **F** = positive for flavonoid
4.2 Preparative TLC

Fig: 4.2 is a chromatogram of *G. bicolar* fruit extract, showing the two bands consisting of flavonoid. Bands labelled *b* and *c* tested negative for flavonoids. Band labelled *a* shows a positive test for flavonoids and there are two thin bands close together which may suggest that these bands may be actually two different flavonoids which did not separate out completely. Their retention factor corresponds to that of band of retention factor 0.094 in *F. sycomorus*. This may imply that the compound responsible for band *a* is similar or identical to fraction 0.094 in *F. sycomorus*. 
4.3 FTIR Spectrophotometric Analysis

Fig 4.3 is an FT-IR spectrum of *C. bispinosa* ethanolic extract. The peaks as observed reveal the presence of various functional groups at key peak values as listed in Table: 4.2.

![FTIR spectrum of C. bispinosa ethanolic extract](image)

**Fig. 4.3:** FT-IR analysis of *C. bispinosa* ethanolic extract.

**Table 4.2 Key peak values and chemical constituents assigned to each for *C. bispinosa***

<table>
<thead>
<tr>
<th>Peak Values/cm⁻¹</th>
<th>Functional group assigned and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3382.94</td>
<td>OH⁻</td>
</tr>
<tr>
<td>2916.37</td>
<td>O-CH₃</td>
</tr>
<tr>
<td>2849.11</td>
<td>Probably methylamine, N-CH₂</td>
</tr>
<tr>
<td>1731.12</td>
<td>Aldehyde or ester</td>
</tr>
<tr>
<td>1640.88</td>
<td>C=C, aryl substituted or C=O, like in a conjugated ketone</td>
</tr>
<tr>
<td>1464.19</td>
<td>Carbonate ion</td>
</tr>
<tr>
<td>1380.00</td>
<td>Aliphatic nitro compound</td>
</tr>
</tbody>
</table>
Fig 4.4 shows the FT-IR spectrum for *G. bicolar* ethanolic extract. The peaks reveal the presence of the chemical constituents as listed in Table 4.3.

**Fig 4.4: FTIR Spectrum of *G. bicolar* ethanolic extract.**

**Table 4.3 Key peak values and assigned functional groups for *G. bicolar***

<table>
<thead>
<tr>
<th>Peak values/cm(^{-1})</th>
<th>Functional group assigned</th>
</tr>
</thead>
<tbody>
<tr>
<td>3679.89</td>
<td>OH (broad band due to hydrogen bonding).</td>
</tr>
<tr>
<td>2921.48</td>
<td>O-CH(_3)</td>
</tr>
<tr>
<td>2896.96</td>
<td>O-CH(_2)</td>
</tr>
<tr>
<td>2074.63</td>
<td>-NCS</td>
</tr>
<tr>
<td>1712.86</td>
<td>-COOH</td>
</tr>
<tr>
<td>1652.38</td>
<td>C=C or C=O, from conjugated ketones. Band may also be due to NH from aromatic amine</td>
</tr>
</tbody>
</table>
1275.90  OH from aromatic ethers arising from aryl-O stretch
1054.51  CN from amine

Figure 4.5 is an FT-IR spectrum \textit{F. sycomorus} ethanolic extract. Table 4.4 lists the functional groups assigned to the prominent peaks.

\textbf{4.5 FTIR Spectrum of \textit{F. sycomorus} ethanolic extract}

\textbf{Table 4.4 Key peak values and the assigned functional groups for \textit{F. sycomorus}}

<table>
<thead>
<tr>
<th>Peak values/cm$^{-1}$</th>
<th>Functional group assigned and comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>3679.95</td>
<td>OH$^-$ (narrow band, most likely from phenols non bonded hydroxyl group).</td>
</tr>
<tr>
<td>3384.34</td>
<td>OH$^-$ (broad band due to hydrogen bonding)</td>
</tr>
<tr>
<td>2922.15</td>
<td>O-CH$_3$ (due to non-symmetric stretch of CH$_3$).</td>
</tr>
<tr>
<td>2855.86</td>
<td>O-CH$_2$ (a direct result of symmetric C-H stretch)</td>
</tr>
<tr>
<td>1710.91</td>
<td>COOH</td>
</tr>
<tr>
<td>1557.80</td>
<td>Aromatic nitro compound$^a$</td>
</tr>
<tr>
<td>1455.40</td>
<td>Carbonate ion$^b$</td>
</tr>
<tr>
<td>1243.94</td>
<td>OH$^-$ (due to aryl-O stretch)$^b$</td>
</tr>
</tbody>
</table>
The functional groups were assigned to peak values by comparing with documented literature
peaks labelled $a$ were also reported by Sing & Mendhulkar, 2015, while those labelled $b$ were also identified by Dzomba & Musekiwa, 2015.

4.4.0 Antioxidant Activity

4.4.1 The ABTS Assay Results

Fig 4.6 shows plots of antioxidant activity of samples + ABTS against concentration of the samples. Generally, antioxidant activity was increasing with increase in concentration.

Quercetin, a proven antioxidant, produced the best antioxidant activity.
Fig. 4.6 Plots of percentage antiradical scavenging against concentration of flavonoid extracted.
4.4.2 DPPH Antiradical Scavenging Activity Results

Fig 4.7 shows plots of antioxidant activity of samples against concentration using DPPH as the antioxidant. There is also a general increase in the antioxidant activity as observed in the ABTS assay. The range of linearity was up to 250 mg/ml. Thereafter, any increase in concentration did not produce any notable changes in antioxidant activity.
Figure 4.7 DPPH free radical scavenging percentages against concentrations of samples and standards.
4.5 Statistical Analysis
The one way ANOVA (in Randomised Blocks) revealed that there were significant differences in percentage scavenging activity of the eight samples tested as a result of increasing the level of concentration of the flavonoid extract (P-value < 0.001).

4.6 ABTS Linear regression analysis

Figure 4.8 is a plot of the linear regression analysis of the antioxidant activity of sample + ABTS. The analysis reveals that the variance in the dependent variable can be explained to varying strengths against the independent variable as shown by the different $R^2$ values.

Table 4.5 summarizes the $R^2$ values from the linear regression analysis of samples using ABTS as the antioxidant.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Retention factor</th>
<th>$R^2$ value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>-</td>
<td>0.9946</td>
</tr>
<tr>
<td>Catechin</td>
<td>-</td>
<td>0.9783</td>
</tr>
<tr>
<td><em>G. bicolar</em> (extract)</td>
<td>0.812</td>
<td>0.6758</td>
</tr>
<tr>
<td><em>G. bicolar</em> (extract)</td>
<td>0.63</td>
<td>0.897</td>
</tr>
<tr>
<td><em>F. sycomorus</em> (extract)</td>
<td>0.094</td>
<td>0.9968</td>
</tr>
<tr>
<td><em>F. sycomorus</em> (extract)</td>
<td>0.47</td>
<td>0.9482</td>
</tr>
<tr>
<td><em>C. bispinosa</em> (extract)</td>
<td>0.375</td>
<td>0.9168</td>
</tr>
<tr>
<td><em>C. bispinosa</em> (extract)</td>
<td>0.1125</td>
<td>0.9926</td>
</tr>
</tbody>
</table>
Fig 4.8: Linear regression analysis plots for samples + ABTS

**KEY**

- Antioxidant Activity of G. bicolar extract with Rf=0.63
- Antioxidant activity of F. sycomorus extract with Rf=0.47/
- Antioxidant activity of C. bispinosa extract with Rf=0.1125/
- Antioxidant activity of extract of C. bispinosawith Rf=0.375/
- Antioxidant activity of extract of G. bicolar with Rf=0.812/
- Antioxidant activity of extract of F. sycomorus with Rf=0.094/
- Antioxidant Activity of quercetin standard/(%)
- Antioxidant activity of catechin standard/(

---

\[
y = 0.1078x + 60.75 \\
R^2 = 0.9857
\]

\[
y = 0.2029x + 52.513 \\
R^2 = 0.9482
\]

\[
y = 0.1814x + 58.912 \\
R^2 = 0.9926
\]

\[
y = 0.1206x + 66.997 \\
R^2 = 0.6758
\]

\[
y = 0.1931x + 64.97 \\
R^2 = 0.9968
\]

\[
y = 0.1394x + 58.912 \\
R^2 = 0.9926
\]

\[
y = 0.1678x + 58.184 \\
R^2 = 0.9968
\]

\[
y = 0.0119x + 96.818 \\
R^2 = 0.9946
\]
4.7 Significance of $R^2$ values
When $R^2$ value is 1 it means that 100% variance is shown, that is, an exact prediction of the value of one would be possible by knowing the value of the other. Similarly, intermediate values for $R^2$ provide a good measure of the degree of the relationship between the independent and dependent variables, (Pdhzur, 1997). From Table 4.5 compounds or extracts with $R^2 > 0.9$ show strong positive relationship between concentration of samples and the antioxidant activity. This implies that we can predict the values of antioxidant activity of each sample by over 90% certainty. On the other hand, change in concentration explains 67.58% of the variance of the antioxidant activity for the *G. bicolar* extract with Rf value of 0.812. This means that there is a moderate positive relation between concentration and antioxidant activity for the *G. bicolar* extract with Rf value of 0.812.

4.8 DPPH statistical analysis results
Figure 4.9 shows plots of the linear regression analysis of antioxidant activity of samples using the DPPH as the antioxidant. The analysis reveals that the variance in the dependent variable can be explained to varying strengths against the independent variable as shown by the different $R^2$ values.

Table 4.6 summarizes the $R^2$ values from the linear regression analysis of samples using DPPH as the antioxidant.
Table 4.6: $R^2$ values of DPPH linear regression analysis and IC50 values.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Retention factor</th>
<th>$R^2$ value</th>
<th>IC50 value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. bispinosa (extract)</td>
<td>0.375</td>
<td>0.9952</td>
<td>183.38</td>
</tr>
<tr>
<td>G. bicolar (extract)</td>
<td>0.812</td>
<td>0.7003</td>
<td>142.94</td>
</tr>
<tr>
<td>C. bispinosa (extract)</td>
<td>0.1125</td>
<td>0.8873</td>
<td>127.5</td>
</tr>
<tr>
<td>F. sycomorus (extract)</td>
<td>0.47</td>
<td>0.9976</td>
<td>155.83</td>
</tr>
<tr>
<td>G. bicolar (extract)</td>
<td>0.63</td>
<td>0.6484</td>
<td>188.54</td>
</tr>
<tr>
<td>F. sycomorus (extract)</td>
<td>0.094</td>
<td>0.9966</td>
<td>98.13</td>
</tr>
<tr>
<td>Quercetin</td>
<td>-</td>
<td>0.7136</td>
<td>115.69</td>
</tr>
</tbody>
</table>

The $R^2$ values reflect a very strong positive relationship between concentration of sample and antioxidant activity for C. bispinosa compound of Rf = 0.375; F. sycomorus compound of Rf = 0.47; and F. sycomorus compound of Rf = 0.094; where the linear model can explain 99.52 %, 99.76 % and 99.66 % of variance in antioxidant activity respectively. On the other hand, G. bicolar compound of Rf = 0.812, C. bispinosa compound of Rf = 0.1125, G. bicolar compound of Rf = 0.63 and quercetin showed moderate positive relationships between concentration and antioxidant activity; where the linear model could be used to explain for 70.03 %, 88.73 %, 64.84 % and 71.36 % variation in antioxidant activity respectively.
Figure 4.9 Linear regression analysis plots for DPPH antioxidant Results
4.9 Determination of IC\textsubscript{50} values

The IC\textsubscript{50} values were determined from the linearly regressed graphs by calculation using the statistically significant regression analysis equations. (Table 4.6). For example the calculation of IC50 for \textit{F. sycomorus} extract of Rf of 0.094 was calculated as follows:

\[ y = 0.248x + 25.663 \]

To obtain IC50, we substitute \( y = 50 \) and then solve for \( x \). This will reduce to:

\[ 50 = 0.248x + 25.663 \]

Hence,

\[ x = \frac{50 - 25.663}{0.248} \]

\[ x = 98.1331 \]

\[ x = 98.13 \]

NB: The other IC50 values were calculated in a similar manner, and then tabulated in Table 4.6 above.

The IC\textsubscript{50} values were shown to be concentration dependent and also varied according to the sample. Figure 4.10 is a graphical representation of how IC\textsubscript{50} values varied with sample.
Figure 4.10 shows that *F. sycomorus* extract of $R_f$ 0.094, has the least IC$_{50}$ followed by quercetin, *C. bispinosa* extract of $R_f$ 01125, *G. bicolar* extract of $R_f$ 0.812, *F sycomorus* extract of $R_f$ 0.47, *C. bispinosa* extract of $R_f$ 0.375, and *G. bicolar* extract of $R_f$ 0.63, in ascending order.
CHAPTER FIVE: DISCUSSION OF RESULTS

5.1 Preliminary Studies

A variety of mobile phases were tried out to determine the most applicable separating mobile phase for the flavonoids in the fruit extracts. The selected solvents gave good separations and the methanol: chloroform: hexane (7:2:1, v/v/v) gave the best results and was singled out for the final TLC analysis of the three fruit extracts studied.

5.2 Analytical TLC for Fruit extracts

TLC was performed on all three samples using methanol: chloroform: hexane (7:2:1, v/v/v). The developed plates were air dried and observed under UV/VIS at 365 nm. All three fruit samples showed four spots. This indicates the presence of four different compounds and the $R_f$ values have been calculated and recorded in Table 4.1. The $R_f$ values ranged from 0.094 to 0.88 for the three fruit extracts analyzed. This shows that the compounds have a wide range of different polarities.

*F. sycomorus* gave four spots at $R_f$ values, 0.094, 0.47, 0.56 and 0.88. *C. bispinosa* had spots at $R_f$ values 0.11, 0.38, 0.69 and 0.84 while *G. bicolar* had spots at $R_f$ values 0.25, 0.47, 0.63 and 0.81. 1% aluminium chloride in ethanol was used as the revealing reagent and was viewed under UV/Vis at 365 nm. Orange-yellow, yellow-green and dirty blue indicated the presence of flavonoids in the fruit extract. The orange-yellow is indicative of presence of flavonol glycosides. The vivid yellow-green (brown) color maybe due to presence of flavone glycoside biflavonols and unusually substituted flavones, (Mohammed, 1996). The blue bands could be due to the presence of 5-deoxyisoflavones and 7, 8-dihydroxy-flavanones, (Mohammed, 1996; Koua, et al., 2011). Furthermore, the blue bands could be due to presence of anthocyanidins 3-
Mohammed (1996) explained that the blue band is mostly due to anthocyanidins 3, 5-diglycosides.

Koua et al., (2011) screened *Striga hermothica* (Del.), *Benth callus* and intact plants for phytochemicals and discovered that they contained phenolic compounds like flavonoids which they analyzed further using TLC and obtained orange and blue spots. They suspected that spots with similar retention factors could be similar compounds. Their results also indicated that there was no variation of flavonoid constituent with age of a plant. They discovered a total of five different flavonoids. In a different study (Dzomba and Musekiwa, 2014) investigated anti-obesity and antioxidant activity of flavonoids from *Dioscorea Steriscus* tubers using TLC for profiling and preparative TLC for the extraction of flavonoid active fractions. They reported the presence of eight different flavonoids, (two from ethyl acetate extract, two from the chloroform extract and four from the ethanol extract). Antioxidant activity was evaluated using DPPH method and their ethanol extract had higher antioxidant activity. In the current study, three ethanolic extracts of selected Zimbabwean plants, namely *C. bispinosa, F. sycomorus* and *G. bicolar* (Fig 4.1) were analyzed using TLC profiling. A total of two flavonoids were discovered per fruit extract and none of these spots had similar retention values suggesting that the flavonoids are different. The results show that *G. bicolar* contained flavonoids in spots at R_f values of 0.625 and 0.812; *C. bispinosa* contained flavonoids in two spots at R_f values of 0.11 and 0.375 while the third fruit, *F. sycomorus* had flavonoids in also two spots at R_f values 0.094 and 0.47. *G. bicolar* (R_f = 0.812), maybe related in structure to flavonols especially, quercetin 3-O-rutinoside, whose mean R_f value is quoted as 0.9 (Mohamed, 1996; Koua, et al., 2012).
5.3 FT-IR functional group identification of fruit extracts.

FT-IR analysis carried out on ethanol extracts of *C. bispinosa* (Figure 4.3), *F. sycomorus* (Figure 4.5) and *G. bicolar* (Figure 4.4) revealed the presence of various functional groups in the fruit samples. The functional groups observed suggest the presence of flavonoids like flavanones, flavonols and flavones. For example, flavones have been shown to have a 2,3 – double bond and a carbonyl group in the 4 position of the C-ring and the B-ring attached to the 2-position of the C-ring. On the other hand, flavanols, sometimes referred to as catechins, also exist in monomer form and as polymers called pro-anthocyanidins. They are similar to flavones in that they contain a 2,3-double bond and/or the carbonyl group in the 4-position of the C-ring. Both *C. bispinosa* and *G. bicolar* ethanolic extracts gave peaks at 1640.88 cm⁻¹ and 1652.38 cm⁻¹ respectively and these were assigned to be due to the presence of either the C=O or C=C in conjugated ketones. This may suggest that both *C. bispinosa* and *G. bicolar* ethanolic extracts contain flavones or flavanols.

Flavanones are have been shown to be similar in structure to flavones but do not possess the 2,3-double bond in the C-ring. The *F. sycomorus* extract does not have either the C=O or C=C characteristic peak at around 1640 cm⁻¹, hence, this could point out the *F. sycomorus* extract could be containing flavanones. All the three fruit extracts produced key peaks at 2916.37 cm⁻¹ (*C. bispinosa*), 2921.89 cm⁻¹ (*G. bicolar*) and 2922.15 cm⁻¹ (*F. sycomorus*) which were assigned to be due to the methoxyl group –OCH₃. The –OCH₃ group is usually present in flavonols, flavanones and flavones.

The three ethanolic extracts also gave peaks that were assigned to be arising from the –OH stretch. Notably *G. bicolar* and *F. sycomorus* extracts produced peaks at 3679.89 cm⁻¹ and 3679.95 cm⁻¹ respectively. These were assigned to have been due to –OH stretch especially those
from phenols which are non-bonded. In addition, broad peaks were observed in the 3300 cm$^{-1}$ region for *C. bispinosa* and *F. sycomorus* extracts, and these were assigned to be due to –OH functional group. The broadness is due to hydrogen bonding. The presence of hydroxyl groups in the extracts explains their observed antioxidant activity. Other characteristic peaks were also observed and assigned to various other functional groups.

The non-appearance of any peak at 2260 cm$^{-1}$ region is quite significant. This signifies that all the three fruit extracts studied are thus nontoxic, (Sigh & Mendhulkar, 2015). All the three fruit extracts contain the hydroxyl functional groups but these occur at different stretching frequencies showing that the extracts have different flavonoids.

### 5.4 ABTS and DPPH scavenging Tests

Fig 4.6 shows plots of percentage antioxidant scavenging activity of flavonoid extracts and selected standards against their concentration. The results show that none of the identified flavonoid extracts revealed an antioxidant activity which surpassed that of quercetin, a known and proven antioxidant (Dzomba & Musekiwa, 2014). *G. bicolar*, extract with $R_f$ of 0.812 revealed the greatest antioxidant activity surpassing that of the catechin standard from concentrations around 25 mg/mL up to the 200 mg/mL range. This implies that the *G. bicolar* extract of Rf of 0.812 contain flavonoid of better antioxidant activity than catechin. Other flavonoid fractions such as *F. sycomorus*, ($R_f = 0.094$ and 0.47), *C. bispinosa*, ($R_f = 0.375$), and *G. bicolar*, ($R_f = 0.63$) revealed antioxidant scavenging activity inferior to that of catechin standard at all concentration levels. On the other hand, the *C. bispinosa*, flavonoid fraction ($R_f = 0.1125$) demonstrated antioxidant activity inferior to that of catechin standard at concentrations lower than 95 mg/ml, but then equaled and surpassed that of catechin standards at concentrations
in excess of 95 mg/ml. Furthermore, the catechin standard demonstrated superior antioxidant scavenging activity over all flavonoid extracts at 10 mg/mL. *C. Bispinosa*, (R<sub>f</sub> = 0.1125) and *G. bicolar* (R<sub>f</sub> = 0.63) have comparable antioxidant scavenging activity at concentrations between 25 mg/mL and 50 mg/mL, but *C. bispinosa* (R<sub>f</sub> = 0.1125) then surpasses *G. bicolar* (R<sub>f</sub> = 0.63) from concentration greater than 50 mg/mL up to 200 mg/mL. This observed trend could imply that these two flavonoid fractions contain similar functional groups and the cross over at 50 mg/ml could be due to increased glycosylation in the *G. bicolar* fraction at higher concentrations which could suppress antioxidant activity. *C. bispinosa* (R<sub>f</sub> = 0.375) and *G. bicolar*, (R<sub>f</sub> = 0.63), also demonstrate comparable antioxidant activity at concentrations of (45-55) mg/mL, but *C. bispinosa* revealed better antioxidant activity in concentrations in excess of 55 mg/mL up to 200 mg/mL.

All the three fruit extracts demonstrated some considerable antioxidant activity as demonstrated by a reduction in absorbance of the ABTS radical at 734 nm. The reduction in absorbance is a strong indicator that the three fruit extracts have antioxidant characteristics. Furthermore, the ABTS scavenging results are comparable to those obtained in the DPPH scavenging test, where lower absorbance of the mixture indicates higher DPPH scavenging. A decrease in the absorbance of the remaining ABTS•+ in sample, implies a higher antioxidant activity of the samples.

Fig 4.6 shows plots of percentage antioxidant scavenging activity of flavonoid extracts against concentration. Quercetin was used as a positive reference. The results show that percentage inhibition varies linearly with concentration from 0 – 250 µg/L. The results in the linear range were statistically analyzed in order to determine the IC<sub>50</sub> values for the sample and standard, (Figures 4.8 and 4.9). The IC<sub>50</sub> values (Table 4.6) were used to reflect antiradical scavenging
ability. Basically, IC₅₀ is the concentration of a sample necessary for antioxidant activity to drop to 50% of scavenging DPPH free radical scavenging capability (Afroz, et al., 2012). This means that a small IC₅₀ value means higher free radical scavenging capability while large IC₅₀ means relatively low scavenging capability (Figure 4.10).

5.5 Antioxidant Scavenging Capacity

From the ABTS radical assay, quercetin standard has antioxidant capacity that was greater than catechin and all samples at 200 mg/ml concentration. On the other hand, the flavonoid at R_f value 0.812 in G. bicolar has an ABTS•⁺ scavenging activity greater than catechin at a concentration of 200 mg/ml. The order of radical scavenging activity for the 200 mg/ml flavonoid fractions is G. bicolar extract of R_f 0.812 > C. bispinosa extract of R_f of 0.1125 > F. sycomorus, extract of R_f 0.094) > F. sycomorus extract R_f of 0.047 > C. bispinosa extract of R_f of 0.375 > G. bicolar extract of R_f of 0.63. All the spots gave antioxidant scavenging activity greater than 80%. Fig 4.6 shows plots of percentage radical scavenging capacity against concentration of the samples. The linear regressions analyses (Figures 4.8 and 4.9) show moderate to strong correlation between antioxidant activity and concentration, in the range 0 - 200 mg/ml.

On the other hand, the DPPH scavenging assay revealed that the antiradical scavenging order was: F. sycomorus extract with R_f of 0.094 (IC₅₀ = 98.13) > C. bispinosa extract with R_f of 0.1125 (IC₅₀ = 127.5) > G. bicolar extract with R_f of 0.812 (IC₅₀ = 142.94) > F. sycomorus extract with R_f of 0.47 (IC₅₀ = 155.83) > C. bispinosa extract with R_f of 0.375 (IC₅₀ = 183.38) > G. bicolar extract with R_f of 0.63 (IC₅₀ = 188.5). The seeming discord in the antiradical scavenging ordering can be explained by observing that at very low concentration, F. sycomorus extract with R_f of 0.094 has better antioxidant activity than both G. bicolar extract with R_f of
0.812 and *C. bispinosa* extract with R<sub>f</sub> of 0.1125, in the ABTS assay at concentrations lower than 10 mg/mL.

In related research studies, Re, et al., 1999 carried out studies on antioxidant activity of ABTS radical and reported that percentage inhibition against concentration revealed positive correlation with R<sup>2</sup> values for analyzed samples being greater than 0.993 in all cases. They found out that the inhibition percentages of their samples were comparable to those of glutathione, uric acid, ascorbic acid, and α-tocopherol and flavonoid aglycones antioxidants kaempferol and cyaniding, with trolox as the positive standard. The differences in antioxidant activity of samples suggest that the flavonoids in the studied samples were of a different nature. Suppressed antioxidant activity may be due to glycosylation of the 3-OH, (figure 5.1). Notable reduced suppression of antioxidant activity may also arise due to glycosylation at positions 4<sup>′</sup> and 7-OH. Furthermore, Afroz, et al., 2012 also concluded that using at least two antioxidant scavenging methods which complement each other reduces bias, and helps to make quality deductions.

![Figure 5.1: Basic flavonoid structure showing the numbering system](image)

5.6 Conclusion
This research has shown that *C. bispinosa*, *F. sycomorus* and *G. bicolar* contain different flavonoids with significant antioxidant activity of over 80% each at a concentration of 200 mg/ml. The fruits contain flavonoids that produce antioxidant activity comparable to that of quercetin and catechin. Hence, *F. sycomorus* extract with R<sub>f</sub> of 0.094, *C. bispinosa* extract with R<sub>f</sub> of 0.1125 and *G. bicolar* extract with R<sub>f</sub> of 0.812 can be used as substitutes for quercetin or
catechin. TLC confirmed that each fruit extract has at least two flavonoid components and FT-IR confirmed the presence of functional groups characteristic to flavonoids. *G. bicolar F. sycomorus*, and *C. bispinosa*, demonstrated significant antioxidant activity.

5.7 **Recommendations**

The study has revealed that the selected Zimbabwean fruits, (*C. bispinosa, F. sycomorus* and *G. bicolar*) contain flavonoids with significant antioxidant properties. The next step will be to conduct investigations to determine the identity of flavanones, flavonols and flavanones present in the studied fruits. This characterization can lead to creation of a data base on Zimbabwean fruits and their flavonoid content and hence, make dietary recommendations to Zimbabweans on the important health benefits of local fruits. The extraction of the flavonoids can be commercialized and used for making nutraceuticals, or be used as food preservatives, and flavoring of packed foods. It may also be imperative to evaluate the antioxidant properties of the fruits using other antioxidant determining methods.

5.8 **Areas for further studies**

Further studies may be carried out to elucidate the structure of the flavonoids and to identify the flavonoids in the fruit extracts, using mass spectrometry and other tandem methods and also proton nuclear magnetic resonance.
References
[http://opendodcs.ids.ac.uk/opendocs/handle/123456789/6602](http://opendodcs.ids.ac.uk/opendocs/handle/123456789/6602) [Accessed 26th June, 2016]


**Appendix**

Table A4.1 Quercetin Antioxidant Scavenging UV-Vis Results

<table>
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<td>Replicate 3</td>
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Absorbance of Control = 1.199

Table A4.2 Absorbance of Catechin + ABTS radical

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Table A4.3 Absorbance of F. sycomorus (Rₜ = 0.094) + ABTS radical

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### Table A4.4 Absorbance of F. sycomorus ($R_f = 0.47$) + ABTS radical

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### Table A4.5 Absorbance of C. bispinosa ($R_f = 0.1125$) + ABTS radical

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### Table A4.6 Absorbance of C. bispinosa ($R_f = 0.375$) + ABTS radical

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### Table A4.7 Absorbance of G. bicolar ($R_f = 0.63$) + ABTS radical

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### Table A4.8 Absorbance of G. bicolar ($R_f = 0.812$) + ABTS radical

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Table A4.9 Percentage Inhibition of Quercetin standards

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Absorbance of Control = 1.199

Table A4.10. Percentage inhibition of Catechin Standards

Absorbance of the control=0.130

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4.4.0 ABTS Scavenging Activity of the three ethanolic plant extracts

Table A4.11 Antioxidant Activity of the flavonoid containing spots for *F. sycomorus*.

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<th>% Antioxidant Activity</th>
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### Table A4.12 Antioxidant Activity of flavonoid containing spots for *C. bispinosa*

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### Table A4.13 Antioxidant Activity of flavonoid containing spots for *G. bicolar*

<table>
<thead>
<tr>
<th>Retention Factor</th>
<th>Concentration/mgL(^{-1})</th>
<th>Absorbance</th>
<th>% Antioxidant Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0,625</td>
<td>5</td>
<td>0,053</td>
<td>59,2</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0,050</td>
<td>61,5</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0,043</td>
<td>66,9</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0,038</td>
<td>70,8</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0,025</td>
<td>80,8</td>
</tr>
<tr>
<td>0,812</td>
<td>5</td>
<td>0,059</td>
<td>54,6</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>0,056</td>
<td>56,9</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>0,023</td>
<td>82,3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0,010</td>
<td>92,3</td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>0,006</td>
<td>95,4</td>
</tr>
</tbody>
</table>
Statistical analysis

891...........................................................................................................

***** ABTS Analysis of variance *****

Variate: Inhibition_%

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample stratum</td>
<td>7</td>
<td>7641.74</td>
<td>1091.68</td>
<td>41.35</td>
<td></td>
</tr>
<tr>
<td>Sample.<em>Units</em> stratum</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Concentration</td>
<td>3</td>
<td>9525.94</td>
<td>3175.31</td>
<td>120.26</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Residual</td>
<td>85</td>
<td>2244.33</td>
<td>26.40</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>95</td>
<td>19412.00</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* MESSAGE: the following units have large residuals.

Sample Quercetin              21.16  s.e. 8.92

Sample Quercetin  *units* 1    12.32  s.e. 4.84
Sample Quercetin  *units* 2    12.32  s.e. 4.84
Sample Quercetin  *units* 3    12.40  s.e. 4.84
Sample Quercetin  *units* 10   -12.74 s.e. 4.84
Sample Quercetin  *units* 11   -12.74 s.e. 4.84
Sample Quercetin  *units* 12   -12.74 s.e. 4.84

***** Tables of contrasts *****

Variate: Inhibition_%

***** Tables of means *****
Variate: Inhibition_%

Grand mean  76.91

Concentration  10.00  50.00  100.00  200.00  
                  63.44  73.28  80.18  90.75

*** Standard errors of means ***

Table          Concentration
rep.                    24
d.f.                    85
e.s.e.               1.049

*** Standard errors of differences of means ***

Table          Concentration
rep.                    24
d.f.                    85
s.e.d.               1.483

*** Least significant differences of means (5% level) ***

Table          Concentration
rep.                    24
d.f.                    85
l.s.d.               2.949

***** Stratum standard errors and coefficients of variation *****

Variate: Inhibition_%

Stratum          d.f.      s.e.        cv%
Sample              7  9.538        12.4
Sample.*Units*    85  5.138        6.7

Summary statistics for Inhibition_%: Sample C. bispinosa Rr 0,1125

Number of values = 12
               Mean = 75.20
Summary statistics for Inhibition_%: Sample *C. bispinosa* R$_f$ 0.375

Number of values = 12
Mean = 68.97

Summary statistics for Inhibition_%: Sample Catechin

Number of values = 12
Mean = 77.29

Summary statistics for Inhibition_%: Sample *F. sycomorus* R$_f$ 0.047

Number of values = 12
Mean = 70.58

Summary statistics for Inhibition_%: Sample *F. sycomorus* R$_f$ 0.094

Number of values = 12
Mean = 73.20

Summary statistics for Inhibition_%: Sample *G. bicolar* R$_f$ 0.625

Number of values = 12
Mean = 70.19

Summary statistics for Inhibition_%: Sample *G. bicolar* R$_f$ 0.812

Number of values = 12
Mean = 81.79

Summary statistics for Inhibition_%: Sample Quercetin

Number of values = 12
Mean = 98.07

895 PRINT [CHANNEL=_tmptext; SQUASH=yes; IP=*]'Boxplot for',!p(Inhibition_%); FIELD=1;
896 JUSTIFICATION=left
897 BOXPLOT [WINDOW=1; TITLE=_tmptext] Inhibition_%; GROUPS=Sample
898 UNITS [NVALUES=*]
899 DELETE [redefine=yes] _rest_
900 READ [print=*,setnvalues=y] _rest_
RESTRICT Concentration,Sample,Inhibition_%; _rest_

describe [selection=nval,mean; groups=Sample] Inhibition_

No data for Inhibition_%: Sample C. bispinosa Rf 0.1125

No data for Inhibition_%: Sample C. bispinosa Rf 0.375

No data for Inhibition_%: Sample Catechin

No data for Inhibition_%: Sample F. sycomorus Rf 0.047

Summary statistics for Inhibition_%: Sample F. sycomorus Rf 0.094

  Number of values = 12
  Mean = 73.20

Summary statistics for Inhibition_%: Sample G. bicolar Rf 0,625

  Number of values = 12
  Mean = 70.19

Summary statistics for Inhibition_%: Sample G. bicolar Rf 0,812

  Number of values = 12
  Mean = 81.79

Summary statistics for Inhibition_%: Sample Quercetin

  Number of values = 12
  Mean = 98.07

PRINT [channel=_tmpextent; squash=yes; ip=*] 'Boxplot for', !p(Inhibition_%); field=1;
justification=left
boxplot [window=1; title=_tmpextent] Inhibition_%; groups=Sample
units [nvalues=*]
delete [redefine=yes] _rest_
read [print=*,setnvalues=y] _rest_
restrict Concentration,Sample,Inhibition_%; _rest_
"General Treatment Structure (in Randomized Blocks)."
919  BLOCK Sample
920  TREATMENTS Concentration
921  COVARIATE "No Covariate"
922  ANOVA [PRINT=aovtable,information,means,%cv,contrast; FACT=32;
FPROB=yes; PSE=diff,\n923  lsd,means; LSDLEVEL=5] Inhibition_%
***** Analysis of variance *****

Variate: Inhibition_%

Source of variation   d.f.   s.s.  m.s.  v.r.  F pr.
Sample stratum        7  4333.4077  619.0582  729.96
Sample.*Units* stratum 16  13.5691   0.8481
Total                 23  4346.9768

***** Information summary *****

Aliased model terms
Concentration

* MESSAGE: the following units have large residuals.

Sample Quercetin                33.51  s.e. 13.44
Sample Catechin                *units* 2            2.73  s.e. 0.75

***** Tables of contrasts *****

Variate: Inhibition_%

***** Tables of means *****

Variate: Inhibition_%
Grand mean  63.44

***** Stratum standard errors and coefficients of variation *****

Variate: Inhibition_%

Stratum   d.f.   s.e.  cv%
Sample    7    14.365  22.6
Sample.*Units*        16      0.921      1.5

924  A PLOT [RMETHOD=standardized] fitted,normal
925  AGRAPH [METHOD=means]
926  "Completely Randomized Design."
927  BLOCK "No Blocking"
928  TREATMENTS Sample
929  COVARIATE "No Covariate"
930  ANOVA [PRINT=aovtable,information,means,%cv,contrast; FACT=32;
931             FPROB=yes; PSE=diff,\                                    
932             lsd,means; LSDLEVEL=5] Inhibition_%
***** Analysis of variance *****

Variate: Inhibition%

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>7</td>
<td>4333.4077</td>
<td>619.0582</td>
<td>729.96</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Residual</td>
<td>16</td>
<td>13.5691</td>
<td>0.8481</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>4346.9768</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* MESSAGE: the following units have large residuals.

*units* 5 2.73 s.e. 0.75

***** Tables of contrasts *****

Variate: Inhibition%

***** Tables of means *****

Variate: Inhibition%

Grand mean 63.44

Sample \( C. bispinosa \) \( R_\ell 0,1125 \) \( C. bispinosa \) \( R_\ell 0,375 \) Catechin

\( 59.74 \) 54.61

67.27

Sample \( F. sycomorus \) \( R_\ell 0,047 \) \( F. sycomorus \) \( R_\ell 0,094 \) \( G. bicolar \) \( R_\ell 0,625 \)

\( 51.05 \) 59.47

61.50

Sample \( G. bicolar \) \( R_\ell 0,812 \) Quercetin

\( 56.91 \) 96.94

*** Standard errors of means ***

Table Sample rep. 3
d.f. 16
e.s.e. 0.532
*** Standard errors of differences of means ***

Table | Sample
--- | ---
rep. | 3
d.f. | 16
s.e.d. | 0.752

*** Least significant differences of means (5% level) ***

Table | Sample
--- | ---
rep. | 3
d.f. | 16
l.s.d. | 1.594

***** Stratum standard errors and coefficients of variation *****

Variate: Inhibition_

<table>
<thead>
<tr>
<th>d.f.</th>
<th>s.e.</th>
<th>cv%</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>0.921</td>
<td>1.5</td>
</tr>
</tbody>
</table>

932 A PLOT [RMETHOD=standardized] fitted, normal
933 AGRAPH [METHOD=means]
938 DESCRIBE [SELECTION=mean, sd; GROUPS=Sample] Inhibition_

Summary statistics for Inhibition_%: Sample C. bispinosa Rf 0,1125

Mean = 59.74
Standard deviation = 0.44

Summary statistics for Inhibition_%: Sample C. bispinosa Rf 0,375

Mean = 54.61
Standard deviation = 0.77

Summary statistics for Inhibition_%: Sample Catechin

Mean = 67.27
Standard deviation = 2.37

Summary statistics for Inhibition_%: Sample F. sycomorus Rf 0.047

Mean = 51.05
Standard deviation = 0.43
Summary statistics for Inhibition_%: Sample *F. sycomorus* $R_f$ 0.094

Mean = 59.47  
Standard deviation = 0.46

Summary statistics for Inhibition_%: Sample *G. bicolar* $R_f$ 0.625

Mean = 61.50  
Standard deviation = 0.00

Summary statistics for Inhibition_%: Sample *G. bicolar* $R_f$ 0.812

Mean = 56.91  
Standard deviation = 0.01

Summary statistics for Inhibition_%: Sample Quercetin

Mean = 96.94  
Standard deviation = 0.05

PRINT [CHANNEL=_tmptext; SQUASH=yes; IP=*]'Boxplot for',!p(Inhibition_%); FIELD=1;
JUSTIFICATION=left
BOXPLOT [WINDOW=1; TITLE=_tmptext] Inhibition_%; GROUPS=Sample
UNITS [NVALUES=*]
DELETE [redefine=yes] _rest_
READ [print=*,setnvalues=y] _rest_
RESTRICT Concentration,Sample,Inhibition_%; _rest_
"Completely Randomized Design."
BLOCK "No Blocking"
TREATMENTS Sample
COVARIATE "No Covariate"
ANOVA [PRINT=aovtable,information,means,%cv,contrast; FACT=32; FPROB=yes; PSE=diff,\l
lsd,means; LSDLEVEL=5] Inhibition_%
***** Analysis of variance *****

Variate: Inhibition %

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>7</td>
<td>2648.59</td>
<td>378.37</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>16</td>
<td>5.17</td>
<td>0.32</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>2653.76</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* MESSAGE: the following units have large residuals.

*units* 16  -1.28  s.e. 0.46
*units* 18  1.03  s.e. 0.46

***** Tables of contrasts *****

Variate: Inhibition %

***** Tables of means *****

Variate: Inhibition %

Grand mean  73.28

Sample  C. bispinosa Rf 0,1125  C. bispinosa Rf 0.375  Catechin
        68.47      66.66

72.30

Sample  F. sycomorus Rf 0.047  F. sycomorus Rf 0.094  G. bicolar Rf 0,625
        64.86      66.90

67.16

Sample  G. bicolar Rf 0,812  Quercetin
        82.56      97.36

*** Standard errors of means ***

Table  Sample  rep.  d.f.  e.s.e.
      rep.  3    16    0.328
*** Standard errors of differences of means ***

<table>
<thead>
<tr>
<th>Table</th>
<th>Sample rep.</th>
<th>d.f.</th>
<th>s.e.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>16</td>
<td>0.464</td>
</tr>
</tbody>
</table>

*** Least significant differences of means (5% level) ***

<table>
<thead>
<tr>
<th>Table</th>
<th>Sample rep.</th>
<th>d.f.</th>
<th>l.s.d.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3</td>
<td>16</td>
<td>0.984</td>
</tr>
</tbody>
</table>

***** Stratum standard errors and coefficients of variation *****

Variate: Inhibition_

<table>
<thead>
<tr>
<th>d.f.</th>
<th>s.e.</th>
<th>cv%</th>
</tr>
</thead>
<tbody>
<tr>
<td>16</td>
<td>0.569</td>
<td>0.8</td>
</tr>
</tbody>
</table>

956 APLOT [RMETHOD=standardized] fitted, normal
957 AGRAPH [METHOD=means]
958 DESCRIBE [SELECTION=mean, sd; GROUPS=Sample] Inhibition_

Summary statistics for Inhibition_%: Sample C. bispinosa Rf 0.1125

Mean = 68.47
Standard deviation = 0.77

Summary statistics for Inhibition_%: Sample C. bispinosa Rf 0.375

Mean = 66.66
Standard deviation = 1.17

Summary statistics for Inhibition_%: Sample Catechin

Mean = 72.30
Standard deviation = 0.00

Summary statistics for Inhibition_%: Sample F. sycomorus Rf 0.047

Mean = 64.86
Standard deviation = 0.45

Summary statistics for Inhibition_%: Sample F. sycomorus R_f 0.094

   Mean = 66.90
   Standard deviation = 0.00

Summary statistics for Inhibition_%: Sample G. bicolar R_f 0.625

   Mean = 67.16
   Standard deviation = 0.46

Summary statistics for Inhibition_%: Sample G. bicolar R_f 0.812

   Mean = 82.56
   Standard deviation = 0.45

Summary statistics for Inhibition_%: Sample Quercetin

   Mean = 97.36
   Standard deviation = 0.05

959  UNITS [NVALUES=*]
960  DELETE [redefine=yes] _rest_
961  READ [print=*;setnvalues=y] _rest_
965  RESTRICT Concentration,Sample,Inhibition_%; _rest_
966  "Completely Randomized Design."
968  BLOCK "No Blocking"
969  TREATMENTS Sample
970  COVARIATE "No Covariate"
971  ANOVA [PRINT=aovtable,information,means,%cv,contrast; FACT=32;
PPROB=yes; PSE=diff,\n972  lsd,means; LSDLEVEL=5] Inhibition_%
**** Analysis of variance ****

Variate: Inhibition_%

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>7</td>
<td>2100.8492</td>
<td>300.1213</td>
<td>1707.90</td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Residual</td>
<td>16</td>
<td>2.8116</td>
<td>0.1757</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>2103.6608</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* MESSAGE: the following units have large residuals.

*units* 10 -0.787 s.e. 0.342
*units* 12 0.753 s.e. 0.342

**** Tables of contrasts ****

Variate: Inhibition_%

**** Tables of means *****

Variate: Inhibition_%

Grand mean 80.181

<table>
<thead>
<tr>
<th>Sample</th>
<th>C. bispinosa Rf 0,1125</th>
<th>C. bispinosa Rf 0,375</th>
<th>Catechin</th>
<th>Catechin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>78.230</td>
<td>72.047</td>
<td>72.047</td>
<td>72.047</td>
</tr>
<tr>
<td>77.700</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>F. sycomorus Rf 0,047</td>
<td>F. sycomorus Rf 0.094</td>
<td>G. bicolar Rf 0,625</td>
<td>G. bicolar Rf 0,625</td>
</tr>
<tr>
<td></td>
<td>76.167</td>
<td>75.650</td>
<td></td>
<td></td>
</tr>
<tr>
<td>70.533</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sample</td>
<td>G. bicolar Rf 0,812</td>
<td>G. bicolar Rf 0,812</td>
<td>Quercetin</td>
<td>Quercetin</td>
</tr>
<tr>
<td></td>
<td>92.300</td>
<td>98.824</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*** Standard errors of means ***

<table>
<thead>
<tr>
<th>Table</th>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>rep.</td>
<td>3</td>
</tr>
<tr>
<td>d.f.</td>
<td>16</td>
</tr>
<tr>
<td>e.s.e.</td>
<td>0.2420</td>
</tr>
</tbody>
</table>
*** Standard errors of differences of means ***

Table  
rep.  3  
\(d.f.\)  16  
\(s.e.d.\)  0.3423

*** Least significant differences of means (5% level) ***

Table  
rep.  3  
\(d.f.\)  16  
\(l.s.d.\)  0.7256

***** Stratum standard errors and coefficients of variation *****

Variate: Inhibition_\%

\[
\begin{array}{ccc}
\text{d.f.} & \text{s.e.} & \text{cv\%} \\
16 & 0.4192 & 0.5 \\
\end{array}
\]

973 APLOT [RMETHOD=standardized] fitted, normal  
974 AGGRAPH [METHOD=means]  
975 DESCRIBE [SELECTION=mean,sd; GROUPS=Sample] Inhibition_\%

Summary statistics for Inhibition_\%: Sample C. bispinosa \(R_t 0.1125\)

Mean = 78.23  
Standard deviation = 0.47

Summary statistics for Inhibition_\%: Sample C. bispinosa \(R_t 0.375\)

Mean = 72.05  
Standard deviation = 0.44

Summary statistics for Inhibition_\%: Sample Catechin

Mean = 77.70  
Standard deviation = 0.00

Summary statistics for Inhibition_\%: Sample F. sycomorus \(R_t 0.047\)

Mean = 76.17
Standard deviation = 0.77

Summary statistics for Inhibition_%: Sample F. sycomorus Rf 0.094

Mean = 75.65
Standard deviation = 0.43

Summary statistics for Inhibition_%: Sample G. bicolar Rf 0.625

Mean = 70.53
Standard deviation = 0.46

Summary statistics for Inhibition_%: Sample G. bicolar Rf 0.812

Mean = 92.30
Standard deviation = 0.00

Summary statistics for Inhibition_%: Sample Quercetin

Mean = 98.82
Standard deviation = 0.00

"Completely Randomized Design."

BLOCK "No Blocking"
TREATMENTS Sample
COVARIATE "No Covariate"
ANOVA [PRINT=aovtable,information,means,%cv,contrast; FACT=32; FPROB=yes; PSE=diff,\]
lsd,means; LSDLEVEL=5] Inhibition_%
***** Analysis of variance *****

Variate: Inhibition %

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
<th>pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
<td>7</td>
<td>775.6194</td>
<td>110.8028</td>
<td>293.59</td>
<td>&lt;.001</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>16</td>
<td>6.0384</td>
<td>0.3774</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>781.6578</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* MESSAGE: the following units have large residuals.

*units* 19  1.52  s.e. 0.50

***** Tables of contrasts *****

Variate: Inhibition %

***** Tables of means *****

Variate: Inhibition %

Grand mean  90.75

Sample  
C. bispinosa Rf 0,1125  C. bispinosa Rf 0,375  
94.35  82.56  Catechin

91.91

Sample  
F. sycomorus Rf 0.047  F. sycomorus Rf 0.094  
90.26  90.78  G. Bicolar Rf 0,625

81.56

Sample  
G. Bicolar Rf 0,812  Quercetin

95.40  99.17

*** Standard errors of means ***

Table  Sample
rep.  3  
d.f.  16  
e.s.e. 0.355
*** Standard errors of differences of means ***

Table | Sample
--- | ---
rep. | 3
d.f. | 16
s.e.d. | 0.502

*** Least significant differences of means (5% level) ***

Table | Sample
--- | ---
rep. | 3
d.f. | 16
l.s.d. | 1.063

***** Stratum standard errors and coefficients of variation *****

Variate: Inhibition%

d.f. | s.e. | cv%
--- | --- | ---
16 | 0.614 | 0.7

990 A PLOT [R METHOD=standardized] fitted, normal
991 AGRA PH [M ETHOD=means]
992 DESCRIBE [S ELECTION=mean, sd; G ROUPS=Sample] Inhibition%

Summary statistics for Inhibition %: Sample C. bispinosa Rf 0.1125

Mean = 94.35
Standard deviation = 0.43

Summary statistics for Inhibition %: Sample C. bispinosa Rf 0.375

Mean = 82.56
Standard deviation = 0.45

Summary statistics for Inhibition %: Sample Catechin

Mean = 91.91
Standard deviation = 0.32

Summary statistics for Inhibition %: Sample F. sycomorus Rf 0.047

Mean = 90.26
Standard deviation = 0.44
Summary statistics for Inhibition_%: Sample *F. sycomorus* R<sub>f</sub> 0.094

Mean = 90.78  
Standard deviation = 0.77

Summary statistics for Inhibition_%: Sample *G. bicolar* R<sub>f</sub> 0.625

Mean = 81.56  
Standard deviation = 1.32

Summary statistics for Inhibition_%: Sample *G. bicolar* R<sub>f</sub> 0.812

Mean = 95.40  
Standard deviation = 0.00

Summary statistics for Inhibition_%: Sample Quercetin

Mean = 99.17  
Standard deviation = 0.00

Linear Regression Analysis in SPSS for standards and samples.

Quercetin

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<sup>a</sup> Predictors: (Constant), Concentration

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<sup>a</sup> Predictors: (Constant), Concentration
### ANOVA

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b. Dependent Variable: Inhibition

### Coefficients

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a. Dependent Variable: Inhibition
C. bispinosa Rₚ 0.375

Regression

[DataSet4] C:\Users\NYAMANDI TAWAZ\Documents\C Bispinosa Rₚ 0.375.sav

Variables Entered/Removed

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a. All requested variables entered.

b. Dependent Variable: Inhibition

Model Summary

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a. Predictors: (Constant), Concentration

b. Dependent Variable: Inhibition

ANOVA

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a. Predictors: (Constant), Concentration

b. Dependent Variable: Inhibition

Coefficients

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Coefficients*

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a. Dependent Variable: Inhibition

Catechin

Regression

[DataSet5] C:\Users\NYAMANDI TAWAZ\Documents\Catechin.sav

Variables Entered/Removedb

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a. All requested variables entered.

b. Dependent Variable: Inhibition

Model Summaryb

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a. Predictors: (Constant), Concentration

b. Dependent Variable: Inhibition

ANOVAb

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a. Predictors: (Constant), Concentration

b. Dependent Variable: Inhibition
Coefficients

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a. Dependent Variable: Inhibition

**F. sycomorus Rf 0.094**

**Regression**

[DataSet6] C:\Users\NYAMANDI TAWAZ\Documents\F. sycomorus Rf 0.094.sav

**Model Summary**

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a. Predictors: (Constant), Concentration
b. Dependent Variable: Inhibition

**ANOVA**

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a. Predictors: (Constant), Concentration
b. Dependent Variable: Inhibition
### Coefficients

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a. Dependent Variable: Inhibition

F. sycomorus R² 0.47

### Regression

[Dataset7] C:\Users\NYAMANDI TAWAZ\Documents\F sycosorous R² 0.47.sav

### Variables Entered/Removed

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a. All requested variables entered.

b. Dependent Variable: Inhibition

### Model Summary

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a. Predictors: (Constant), Concentration

b. Dependent Variable: Inhibition

### ANOVA

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#
Residual | 110.070 | 10 | 11.007 |
Total | 2500.308 | 11 |

a. Predictors: (Constant), Concentation
b. Dependent Variable: Inhibition

### Coefficients*

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a. Dependent Variable: Inhibition

*C. bispinosa R_f 0.1125

### Regression

[DataSet8] C:\Users\NYAMANDI TAWAZ\Documents\C bispinosa R_f 0.1125.sav

### Variables Entered/Removed*

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a. All requested variables entered.
b. Dependent Variable: Inhibition
**Model Summary**

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a. Predictors: (Constant), Concentration  

b. Dependent Variable: Inhibition

**ANOVA**

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a. Predictors: (Constant), Concentration  

b. Dependent Variable: Inhibition

**Coefficients**

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a. Dependent Variable: Inhibition

**G. bicolar R_f 0.625**

Regression

[DataSet9] C:\Users\NYAMANDI TAWAZ\Documents\G. bicolar R_f 0.625.sav

**Variables Entered/Removed**

98
### Model Summary

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a. Predictors: (Constant), Concentration  

b. Dependent Variable: Inhibition

### ANOVA

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a. Predictors: (Constant), Concentration  

b. Dependent Variable: Inhibition

### Coefficients

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a. Dependent Variable: Inhibition

---

**G. bicolar R<sub>f</sub> 0.812**
Regression

[DataSet10] C:\Users\NYAMANDI TAWAZ\Documents\G. bicolar Rf 0.812.sav

Variables Entered/Removed\(^a\)

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a. All requested variables entered.
b. Dependent Variable: Inhibition

Model Summary\(^b\)

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a. Predictors: (Constant), Concentration
b. Dependent Variable: Inhibition

ANOVA\(^b\)

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a. Predictors: (Constant), Concentration
b. Dependent Variable: Inhibition

Coefficients\(^a\)

<table>
<thead>
<tr>
<th>Model</th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
<th>95% Confidence Interval for B</th>
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<tr>
<td></td>
<td>B</td>
<td>Std. Error</td>
<td>Beta</td>
</tr>
<tr>
<td>1 (Constant)</td>
<td>65.921</td>
<td>4.326</td>
<td>.828</td>
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<tr>
<td>Concentration</td>
<td>.176</td>
<td>.038</td>
<td>.828</td>
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</table>
Coefficients a

<table>
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<th>Model</th>
<th>Unstandardized Coefficients</th>
<th>Standardized Coefficients</th>
<th>95% Confidence Interval for B</th>
</tr>
</thead>
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<tr>
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<td>B</td>
<td>Std. Error</td>
<td>Beta</td>
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<tr>
<td>(Constant)</td>
<td>65.921</td>
<td>4.326</td>
<td>15.239</td>
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<tr>
<td>Concentration</td>
<td>.176</td>
<td>.038</td>
<td>.828</td>
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a. Dependent Variable: Inhibition

DPPH statistical analysis

898 A PLOT [RMETHOD=simple] fitted, normal, halfnormal, histogram
899 AGRAPH [METHOD=means] Concentration (µg/L)
900 "General Treatment Structure (in Randomized Blocks)."
901 BLOCK Sample
902 TREATMENTS Concentration (µg/L)
903 COVARIATE "No Covariate"
904 ANOVA [PRINT=aovtable, information, means, residuals, %cv; FACT=32; CONTRASTS=15; FPROB=yes;
905 PSE=diff, lsd, means; LSDLEVEL=5] Inhibition_%

905...............................

***** DPPH Analysis of variance *****

Variate: Inhibition_%

Source of variation d.f. s.s. m.s. v.r. F pr.
Sample stratum 6 6259.49 1043.25 16.38
Sample.*Units* stratum
Concentration (µg/L) 5 55341.16 11068.23 173.79 <.001
Residual 114 7260.33 63.69
Total 125 68860.99

* MESSAGE: the following units have large residuals.

Sample Quercetin *units* 1 28.99 s.e. 7.59
Sample Quercetin *units* 2 29.01 s.e. 7.59
Sample Quercetin *units* 3 28.94 s.e. 7.59

***** Tables of residuals *****

Variate: Inhibition_%
Sample residuals  s.e. 7.048  rep. 18

Sample  *units*  residuals  s.e. 7.591  rep. 1

Sample  *units*       1       2       3       4
C. bispinosa  0.1125 +DPPH  11.30  11.28  11.32  -2.31
C. bispinosa  0.375 +DPPH  9.98  9.66  10.05  -9.54
F. sycomorus  0.094 +DPPH  0.93  0.86  0.91  3.93
F. sycomorus  0.47+DPPH  7.74  7.76  7.78  0.15
G. bicolar  0.63 + DPPH  14.60  14.60  14.60  1.06
G. bicolar  0.812 + DPPH  -15.49 -15.47 -15.49  1.24
Quercetin  5.68

Sample  *units*       5       6       7       8
C. bispinosa  0.1125 +DPPH  -2.26 -2.29 -7.87 -7.77
C. bispinosa  0.375 +DPPH  -9.61 -9.56 -6.30 -6.49
F. sycomorus  0.094 +DPPH  3.93  3.93  3.98  3.75
F. sycomorus  0.47+DPPH  0.20  0.18 -11.13 -11.15
G. bicolar  0.63 + DPPH  1.04  1.04  0.81  0.81
G. bicolar  0.812 + DPPH  1.22  1.22  8.04  8.06
Quercetin  5.61  5.61  12.52  12.52

Sample  *units*       9      10      11      12
C. bispinosa  0.1125 +DPPH  -7.82 -4.04 -3.97 -3.97
C. bispinosa  0.375 +DPPH  -6.07  2.35  2.44  2.39
F. sycomorus  0.094 +DPPH  3.89 -1.31 -1.31 -2.47
F. sycomorus  0.47+DPPH -11.13 -1.50 -1.48 -1.53
G. bicolar  0.63 + DPPH  0.79 -5.43 -5.20 -5.27
G. bicolar  0.812 + DPPH  8.04  4.28  4.26  4.28
Quercetin  12.52  5.81  5.84  5.81

Sample  *units*       13     14     15     16
C. bispinosa  0.1125 +DPPH  1.27  1.25  1.25  1.52
C. bispinosa  0.375 +DPPH  0.77  0.81  0.84  2.73
F. sycomorus  0.094 +DPPH -3.10 -3.10 -3.10 -3.90
F. sycomorus  0.47+DPPH  2.08  2.10  2.10  2.61
G. bicolar  0.63 + DPPH -5.39 -5.29 -5.32 -5.98
G. bicolar  0.812 + DPPH  1.47  1.47  1.52  0.44
Quercetin  2.77  2.81  2.79  2.36

Sample  *units*       17     18
C. bispinosa  0.1125 +DPPH  1.54  1.56
C. bispinosa  0.375 +DPPH  2.76  2.78
F. sycomorus  0.094 +DPPH -3.90 -3.90

102
F. sycomorus R, 0.47 + DPH 2.63 2.58
G. bicolar R, 0.63 + DPH -5.67 -5.81
G. bicolar R, 0.812 + DPH 0.46 0.46
Quercetin 2.34 2.39

***** Tables of means *****

Variate: Inhibition_%

Grand mean 66.98

<table>
<thead>
<tr>
<th>Concentration_g_L</th>
<th>26.36</th>
<th>53.86</th>
<th>72.89</th>
<th>79.73</th>
<th>83.85</th>
<th>85.16</th>
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<tr>
<td></td>
<td>62.50</td>
<td>125.00</td>
<td>250.00</td>
<td>500.00</td>
<td>1000.00</td>
<td>2000.00</td>
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</table>

*** Standard errors of means ***

Table  Concentration_g_L
rep.    21
d.f.   114
e.s.e. 1.741

*** Standard errors of differences of means ***

Table  Concentration_g_L
rep.    21
d.f.   114
s.e.d. 2.463

*** Least significant differences of means (5% level) ***

Table  Concentration_g_L
rep.    21
d.f.   114
l.s.d. 4.879

***** Stratum standard errors and coefficients of variation *****

Variate: Inhibition_%

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<td>7.613</td>
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<td>Sample.<em>Units</em></td>
<td>114</td>
<td>7.980</td>
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906  APLT [RMETH=simple] fitted,normal,halfnormal,histogram
907  AGRAPH [METHOD=means] Concentration_g_L

LINEAR REGRESSION ANALYSIS FOR RANGE 0-250µg/L for samples and standard

***** Quercetin Regression Analysis *****

Response variate: Inhibition_%
Fitted terms: Constant, Concentration_g_L

*** Summary of analysis ***

<table>
<thead>
<tr>
<th></th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
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<tbody>
<tr>
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<td>10217.</td>
<td>10216.8</td>
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<td>Residual</td>
<td>7</td>
<td>2053.</td>
<td>293.2</td>
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<td>12269.</td>
<td>1533.7</td>
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Percentage variance accounted for 80.9
Standard error of observations is estimated to be 17.1

*** Estimates of parameters ***

<table>
<thead>
<tr>
<th>estimate</th>
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<th>t pr.</th>
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<tr>
<td>Concentration_g_L</td>
<td>0.4322</td>
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*** Correlations between parameter estimates ***

<table>
<thead>
<tr>
<th>estimate</th>
<th>ref</th>
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<tbody>
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<td>1.000</td>
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<tr>
<td>Concentration_g_L</td>
<td>2</td>
<td>-0.882 1.000</td>
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</table>

943 RCHECK [RMETHOD=deviance; GRAPHICS=high] residual; composite
944 RGRAPH [GRAPHICS=high]
945 RDISPLAY [PRINT=model,summary,correlations,estimates; FPROB=yes; TPROB=yes]
***** G. bicolar Rf 0.63 Regression Analysis *****

Response variate: Inhibition_%
Fitted terms: Constant, Concentration_g_L

*** Summary of analysis ***

<table>
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<th>m.s.</th>
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<th>F pr.</th>
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Percentage variance accounted for 98.7
Standard error of observations is estimated to be 1.59

*** Estimates of parameters ***

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*** Correlations between parameter estimates ***

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<tbody>
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<td>Constant</td>
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<td>1.000</td>
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<tr>
<td>Concentration_g_L</td>
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<td>-0.882 1.000</td>
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READ [print=*;SETNVALUES=yes] _stitle_
PRINT [IPrint=*] _stitle_; Just=Left

Data imported from GenStat Spreadsheet: C:\Users\NYAMANDI TAWAZ\Desktop\Mangena DPPH Regression analyses and Combined graph final.gsh
on: 13-Feb-2008 16:18:53

UNITS [NVALUES=*]
VARIATE [nvalues=126] Concentration_g_L
READ Concentration_g_L

Identifier | Minimum | Mean     | Maximum | Values | Missing |
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<td>62.50</td>
<td>656.3</td>
<td>2000</td>
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Simple Linear Regression

MODEL

Inhibition_%

TERMS Concentration_g_L

FIT [PRINT=model, summary, correlations, estimates; CONSTANT=estimate; FPROB=yes; TPROB=yes]

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F. sycomorus $R_f$ 0.47 Regression Analysis

Response variate: Inhibition_%
Fitted terms: Constant, Concentration_g_L

*** Summary of analysis ***

<table>
<thead>
<tr>
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<th>s.s.</th>
<th>m.s.</th>
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<th>F pr.</th>
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<td>152.47</td>
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Percentage variance accounted for 79.3
Standard error of observations is estimated to be 5.62

*** Estimates of parameters ***

<table>
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*** Correlations between parameter estimates ***

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<tbody>
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<td>Constant</td>
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<td>1.000</td>
</tr>
<tr>
<td>Concentration_g_L</td>
<td>2</td>
<td>-0.882 1.000</td>
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1061 RCHECK [RMETHOD=deviance; GRAPHICS=high] residual; composite
1062 RGRAPH [GRAPHICS=high]
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DPPH Regression analyses and Combined graph final.gsh
on: 13-Feb-2008 16:18:53

UNIT [NVALUES=*]
VARIATE [nvalues=126] Concentration_g_L
READ Concentration_g_L

Identifier  Minimum  Mean  Maximum  Values  Missing
Concentration_g_L  62.50  656.3  2000  126  0

DELETE [redefine=yes] _rest_
READ [print=*,setnvalues=y] _rest_
Restrict Sample,Concentration_g_L,Absorbance,Inhibition_%; _rest_

"Simple Linear Regression"
MODEL Inhibition_%
TERMS Concentration_g_L
FIT [PRINT=model,summary,correlations,estimates; CONSTANT=estimate;
FPROB=yes; TPROB=yes]
Concentration_g_L

-------------------------------

C. bispinosa Rf 0.1125 Regression Analysis

Response variate: Inhibition_%
Fitted terms: Constant, Concentration_g_L

*** Summary of analysis ***

<table>
<thead>
<tr>
<th></th>
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<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
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Percentage variance accounted for 95.5
Standard error of observations is estimated to be 2.51

*** Estimates of parameters ***

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<td>0.1407</td>
<td>0.0107</td>
<td>13.12</td>
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*** Correlations between parameter estimates ***

estimate  ref  correlations

---

107
Constant  1  1.000
Concentration_g_L
   2  -0.882  1.000
   1  2

RCHECK [RMETHOD=deviance; GRAPHICS=high] residual; composite
RGRAPH [GRAPHICS=high]
"Data taken from File: \\C:/Users/NYAMANDI TAWAZ/Desktop/Mangena DPPH Regression analyses and Combined graph final.gsh"

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Data imported from GenStat Spreadsheet: C:\Users\NYAMANDI TAWAZ\Desktop\Mangena DPPH Regression analyses and Combined graph final.gsh on: 13-Feb-2008 16:18:53

UNITS [NVALUES=*]
VARIATE [nvalues=126] Concentration_g_L
READ Concentration_g_L

Identifier   Minimum      Mean   Maximum    Values   Missing
Concentration_g_L   62.50     656.3      2000       126         0

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"Simple Linear Regression"
MODEL Inhibition_%
TERMS Concentration_g_L
FIT [PRINT=model,summary,correlations,estimates; CONSTANT=estimate; FPROB=yes; TPROB=yes]\nConcentration_g_L

******************************************************************************

****C. bispinosa Rf 0.375 Regression Analysis ****

Response variate: Inhibition_%
Fitted terms: Constant, Concentration_g_L

*** Summary of analysis ***

<table>
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<tr>
<th></th>
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<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
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Percentage variance accounted for 99.3

108
Standard error of observations is estimated to be 1.10

*** Estimates of parameters ***

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<th>t pr.</th>
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<tbody>
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*** Correlations between parameter estimates ***

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<th>ref</th>
<th>correlations</th>
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</thead>
<tbody>
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<td>1.000</td>
</tr>
<tr>
<td>Concentration_g_L</td>
<td>2</td>
<td>-0.882 1.000</td>
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1129 RCHECK [RMETHOD=deviance; GRAPHICS=high] residual; composite
1130 RGRAPH [GRAPHICS=high]
1131 "Data taken from File: \
-1132 C:/Users/NYAMANDI TAWAZ/Desktop/Mangena DPPH Regression analyses and
Combined graph final.gsh\ 
-1133 "
1134 DELETE [redefine=yes] _stitle_: TEXT _stitle_
1135 READ [print=*;SETNVALUES=yes] _stitle_
1138 PRINT [IPrint=\*] _stitle_; Just=Left

Data imported from GenStat Spreadsheet: C:\Users\NYAMANDI
TAWAZ\Desktop\Mangena
DPPH Regression analyses and Combined graph final.gsh
on: 13-Feb-2008 16:18:53

1139 UNITS [NVALUES=\*]
1140 VARIATE [nvalues=126] Concentration_g_L
1141 READ Concentration_g_L

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<th>Maximum</th>
<th>Values</th>
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<td>62.50</td>
<td>656.3</td>
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1150 DELETE [redefine=yes] _rest_
1151 READ [print=\*;setnvalues=y] _rest_
1156 RESTRICT Sample,Concentration_g_L,Absorbance,Inhibition_%; _rest_
1157 1158 "Simple Linear Regression"
1159 MODEL Inhibition_%
1160 TERMS Concentration_g_L
1161 FIT [PRINT=model,summary,correlations,estimates; CONSTANT=estimate;
FPROB=yes; TPROB=yes]
1162 Concentration_g_L
### G. bicola Rf 0.812 Regression Analysis

Response variate: Inhibition %  
Fitted terms: Constant, Concentration_g_L

#### Summary of analysis

<table>
<thead>
<tr>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
<td>1</td>
<td>21043.</td>
<td>21042.7</td>
<td>124.47</td>
</tr>
<tr>
<td>Residual</td>
<td>61</td>
<td>10313.</td>
<td>169.1</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>62</td>
<td>31356.</td>
<td>505.7</td>
<td></td>
</tr>
</tbody>
</table>

Percentage variance accounted for 66.6%

Standard error of observations is estimated to be 13.0

* MESSAGE: The residuals do not appear to be random;  
  for example, fitted values in the range 75.5 to 75.5  
  are consistently larger than observed values  
  and fitted values in the range 46.1 to 75.5  
  are consistently smaller than observed values

#### Estimates of parameters

<table>
<thead>
<tr>
<th>estimate</th>
<th>s.e.</th>
<th>t(61)</th>
<th>t pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>16.84</td>
<td>3.48</td>
<td>4.85</td>
</tr>
<tr>
<td>Concentration_g_L</td>
<td>0.2345</td>
<td>0.0210</td>
<td>11.16</td>
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</table>

#### Correlations between parameter estimates

<table>
<thead>
<tr>
<th>estimate</th>
<th>ref</th>
<th>correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>1</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>-0.882</td>
</tr>
</tbody>
</table>

RCHECK [RMETHOD=deviance; GRAPHICS=high] residual; composite
RGRAPH [GRAPHICS=high]
"Data taken from File: C:/Users/NYAMANDI TAWAZ/Desktop/Mangena DPPH Regression analyses and Combined graph final.gsh"
DELETE [redefine=yes] Concentration_g_L
UNITS [NVALUES=*]
FACTOR [modify=yes;nvalues=126;levels=!(62.5,125,250,500,1000,2000);reference=1] Concentration_g_L
READ Concentration_g_L; frepresentation=ordinal

Identifier Values Missing Levels
Concentration_g_L 126 0 6

DELETE [redefine=yes] _rest_
READ [print=*,setnvalues=y] _rest_
RESTRICT Sample,Concentration_g_L,Absorbance,Inhibition_%; _rest_

"Data taken from File: C:/Users/NYAMANDI TAWAZ/Desktop/Mangena DPPH Regression analyses and Combined graph final.gsh"

DELETE [redefine=yes] _stitle_: TEXT _stitle_
READ [print=*,SETNVALUES=yes] _stitle_
PRINT [IPrint=*] _stitle_; Just=Left

Data imported from GenStat Spreadsheet: C:\Users\NYAMANDI TAWAZ\Desktop\Mangena DPPH Regression analyses and Combined graph final.gsh on: 13-Feb-2008 16:18:53

DELETE [redefine=yes] Concentration_g_L
UNITS [NVALUES=*]
VARIATE [nvalues=126] Concentration_g_L
READ Concentration_g_L

Identifier Minimum Mean Maximum Values Missing
Concentration_g_L 62.50 656.3 2000 126 0

DELETE [redefine=yes] _rest_
READ [print=*,setnvalues=y] _rest_
RESTRICT Sample,Concentration_g_L,Absorbance,Inhibition_%; _rest_

"Simple Linear Regression"
MODEL Inhibition_%
TERMS Concentration_g_L
FIT [PRINT=model,summary,correlations,estimates; CONSTANT=estimate; FPROB=yes; TPROB=yes] Concentration_g_L

........................................
***** F. sycomorus R, 0.094 Regression Analysis *****

Response variate: Inhibition_%
Fitted terms: Constant, Concentration_g_L

*** Summary of analysis ***

<table>
<thead>
<tr>
<th></th>
<th>d.f.</th>
<th>s.s.</th>
<th>m.s.</th>
<th>v.r.</th>
<th>F pr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression</td>
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<td>3363.0</td>
<td>3363.02</td>
<td>61.99</td>
<td>&lt;.001</td>
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<tr>
<td>Residual</td>
<td>7</td>
<td>379.8</td>
<td>54.25</td>
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</tr>
<tr>
<td>Total</td>
<td>8</td>
<td>3742.8</td>
<td>467.85</td>
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</tbody>
</table>

Percentage variance accounted for 88.4
Standard error of observations is estimated to be 7.37

*** Estimates of parameters ***

<table>
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<th></th>
<th>estimate</th>
<th>s.e.</th>
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<th>t pr.</th>
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</thead>
<tbody>
<tr>
<td>Constant</td>
<td>25.66</td>
<td>5.21</td>
<td>4.93</td>
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<tr>
<td>Concentration_g_L</td>
<td>0.2480</td>
<td>0.0315</td>
<td>7.87</td>
<td>&lt;.001</td>
</tr>
</tbody>
</table>

*** Correlations between parameter estimates ***

<table>
<thead>
<tr>
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<th>ref</th>
<th>correlations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constant</td>
<td>1</td>
<td>1.000</td>
</tr>
<tr>
<td>Concentration_g_L</td>
<td>2</td>
<td>-0.882 1.000</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
</tbody>
</table>

1223  RCHECK [RMETHOD=deviance; GRAPHICS=high] residual; composite
1224  RGRAPH [GRAPHICS=high]