COMPARATIVE EVALUATION OF THREE FUNDAMENTALLY DIFFERENT ANALYTICAL METHODS FOR ANTIOXIDANT ACTIVITY DETERMINATION WITH REFERENCE TO BUSH TEA (*ATHRIXIA PHYLICOIDES*)

by

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DECLARATION

I declare that the dissertation hereby submitted to the University of Limpopo, for the degree of Master of Science in Chemistry has not previously been submitted by me for a degree at this or any other university; that it is my work in design and in execution, and that all material contained herein has been duly acknowledged.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)
$ABTS^{\cdot+}$	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical
ANOVA	Analysis of variance
CE	Catechin equivalents
CUPRAC	Cupric ion reducing antioxidant capacity
CV	Cyclic voltammetry
$DPPH^{\cdot}$	2,2-diphenyl-1-picrylhydrazyl radical assay
EC_{50}	Median effective concentration
EPR	Electro paramagnetic resonance
FC reagent	Folin-Ciocalteu's reagent
FCR	Folin-Ciocalteu reducing method
FRAP	Ferric reducing ability of plasma assay
FTIR	Fourier transform infrared spectroscopy
GAE	Gallic acid equivalents
GC	Gas chromatography
GCE	Glassy carbon electrode
HAT	Hydrogen atom transfer
HPLC	High performance liquid chromatography
NMR	Nuclear magnetic resonance
ORAC	Oxygen radical absorbance capacity assay
P-value	Probability value
R^2	Coefficient of determination
RNS	Reactive nitrogen species

ROS	Reactive oxygen species
Rpm	Revolutions per minute
SET	Single Electron transfer
TE	Trolox equivalent
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin layer chromatography
TPC	Total phenolic content
TRAP	Total radical – trapping antioxidant parameter
Trolox	6-Hydroxy-2,5,7,8,- tetramethylchromane-2-carboxylic acid

ABSTRACT

In this study, antioxidant activity methodologies were evaluated in terms of analytical performances. The total antioxidant activity from A*thrixia phylicoides* leaves (Bush tea) determined using 2,2-diphenyl-1-picrylhydrazyl radical scavenging ($DPPH^{\bullet}$) method, cupric ion reducing power (CUPRAC) method and cyclic voltammetry (CV). Folin-Ciocalteu method was used to quantify total phenolic content (TPC) in A*thrixia phylicoides* leaves. The influence of chemical and physical parameters on the total phenolic content and antioxidant activity determination were investigated. Results from direct sample and crude sample were compared. Antioxidant activity and phenolic content from *Athrixia phylicoides* leaves were compared with those from commercialised green tea, black tea and rooibos tea using two chosen antioxidant capacity method with acceptable characteristics.

Results from the evaluation of the methods demonstrated excellent recoveries (99 to 103%) consistently, good linearity within the calibration concentration range (R^2 = 0.997) and repeatable low coefficient of variation < 5% were indicative of good precision except for CV method. The average total antioxidant activity of various extracts of A*thrixia phylicoides* leaves ranged from 0.039 to 0.122 mg/mL (EC_{50}), 0.031 to 0.233 mg/mL (EC_{50}) and 339 to 429 mV (anodic potential) for DPPH method, CUPRAC method and CV method, respectively. The total antioxidant activity values for each *Athrixia phylicoides* samples determined by CUPRAC method were higher than the values produced by DPPH and CV methods.

The highest antioxidant activities in the DPPH and CUPRAC methods were found in water extracts (direct sample). However, concentrated samples for DPPH method and CV gave a different trend with the methanol extract (crude sample) displaying

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Chapter 1

INTRODUCTION

1.1. Background

Antioxidants are compounds capable to either delay or inhibit the oxidation process which occur under the influence of atmospheric oxygen or reactive oxygen species (ROS). They have become of critical importance lately, because of their potential in improving health and anti-aging effects as well as prevention of spoilage of food, cosmetics and pharmaceutical compounds [1-3]. Synthetic antioxidants like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) have been used as food additives [3]. However this has been tainted with the adverse effect, leading to an increase in the use of natural antioxidants [4-8].

Overwhelming scientific evidence has been provided showing that antioxidants may prevent oxidative damage and thus protect cells against adverse effects of oxidants [2,9]. It is now clear that in biological cells, oxidants as well as antioxidants may selectively regulate certain signal transduction pathways [10]. Based on these findings the role of nutritional and pharmacological antioxidants turned into a subject of intense research. Strong consumer interest in the relationship between diet and health has created an insatiable demand for a comprehensive insight into nutrition information. Dietary antioxidants including tocopherols, flavonoids, vitamin C and other phenolic compounds are abundantly found from plant material [11].

One plant that is receiving special attention, mainly due to its antioxidant properties is *Athrixia phylicoides* commonly known as bush tea. It is an indigenous South African herbal plant that belongs to the Asteraceae family [12]. Leaves and stalks of this plant are boiled and the extract is drunk as a tea beverage [13]. The antioxidant properties present in bush tea are due to its high polyphenol content [14,15]. Polyphenols or phenolic compounds constitute a large group of secondary metabolites derived from phenylalanine and are widely distributed throughout the Plant Kingdom. Although they typically comprise less than 2% of the fresh weight basis of the plant, phenolic compounds serve diverse functions such as imparting

colour to leaves, attracting or repelling insects, antimicrobial action, antiviral activity, protection from harmful ultraviolet radiation and protection from herbivores [16,17]. Chemically, phenolic compounds are defined as compounds possessing an aromatic ring bearing one or more hydroxyl groups, there are also different variants that occur as their natural derivatives [16,18].

Numerous studies have shown that the antioxidant activity is due to the phenolic composition of food or natural health product [1,2,9]. The consumption of these foods may increase the overall antioxidant status in the body. A recent survey revealed that *Athrixia phylicoides* is still frequently utilised as a tea and medicine by both rural and urban communities in South Africa and will be purchased if commercially available [13,19]. However, the nutritional composition of *Athrixia phylicoides* is not well known and there is limited published scientific data on its chemical composition, toxicity and pharmacological activities which is published.

Different antioxidant activity determination methods have been used directly or indirectly to estimate the antioxidant activity of food, plants, beverages and other samples [20]. The various methods operate by employing completely different principles with some measuring the electric potential of the sample and others monitoring the reducing power of the sample as well as quenching of the synthetic coloured radical or redox-active compound using a spectrophotometer. These methods commonly, apply an appropriate antioxidant standard such as vitamin C (L-ascorbic acid) or Trolox (6-Hydroxy-2,5,7,8,- tetramethylchromane-2-carboxylic acid) to quantify the antioxidant activity.

1.2. Problem statement

Demand for the accurate and reliable determination of antioxidant capacity is gaining importance in most areas within the food industry; therefore several analytical methods and measuring systems have been developed, yet with no outright preferred universal method [21]. Most researchers use not only one, but varying types of methods for determining the total antioxidant activity from a given sample as each method is specific for certain antioxidants or for certain reactions. In addition, the analysis of the antioxidant activities of the extract from food materials or food can

be affected by various factors such as cultivating growth environment (climate, soil and irrigation), method of food manufacturing and even the efficiency of extraction methodology [22-24]. Little is known about the antioxidant concentration of bush tea infusions and their influence on human health. According to the above mentioned statement it is important and scientifically appropriate to compare the analytical performance of fundamentally different types of antioxidant methods, to investigate the effect of extraction methodology on the antioxidant characteristics of *Athrixia phylicoides* and to get to know the quality and quantity of the compounds that play significant roles in developing the antioxidant capacity.

1.3. Purpose of the study

1.3.1. Aim

The overall goal of this study was to evaluate and compare the performance characteristics of three different analytical methods for determination of antioxidant activity of extracts from unprocessed bush tea (A*thrixia phylicoides*) leaves. The aim was to comprehensively characterise the current antioxidant capacity of the investigated herbal plant using three types of antioxidants methods based on different principles: (i) DPPH$^{\bullet}$ is a free radical scavenging antioxidant method, (ii) CUPRAC is a reducing power antioxidant method and (iii) cyclic voltammetry is an oxidation potential antioxidant method.

1.3.2. Objectives

Below are the specific objectives of the study:

1. To determine the total antioxidant activity of bush tea extract using three methods namely DPPH$^{\bullet}$, CUPRAC and CV,

2. To evaluate the performance of three analytical methods with regard to the rate of analysis, accuracy, precision, sensitivity, limit of detection, dynamic range and select the best method,

3. To determine the total antioxidant activity of commercialized green tea, black tea and processed rooibos tea,

4. To use different extraction solvents (water, methanol and acetic acid/water) to investigate the effect of extraction polarity on the total polyphenol content and antioxidant activity.

1.4. Significance of the study

It is reported that all kinds of food containing phenolic compounds usually have high antioxidant activity, which means they may have positive effects on preserving the quality of food and human health when present in high concentration [10]. Several foodstuffs like tea beverages have been proven to act as antidotes in counteracting against the negative effects of free radicals. Bush tea (*Athrixia phylicoides* DC.) and black tea (*Camellia sinensis*) are beverages rich in antioxidants, which make them excellent sources for increased health benefits [24,25]. Green tea has properties that improve cognitive function [26] and decrease body fat composition while increasing one's energy expenditure [27]. These findings point to various polyphenols in tea as a contributing factor for these beneficial results. It is of great interest to the general public, medicinal and nutritional experts, and health and food science researchers to establish the antioxidant capacity and constituents in food.

The total polyphenol amounts determined from the same plant and their corresponding antioxidant may vary widely depending on the extraction conditions applied. Studies indicate that the use of different solvents, solvent extraction times, and temperature conditions for extraction of tea polyphenols can impact the antioxidant activity. Although there have been some reports on effects of temperature and brewing time of *Athrixia phylicoides* [28], the information on the effect of different extraction solvent of varying polarities on *Athrixia phylicoides* is limited.

Current research findings have revealed that the analysis of antioxidant activity using different methods has to date provided inconsistent data leading to varying conclusions. Ethanol extracts of *Athrixia phylicoides* exhibited antioxidant activity comparable to that found in rooibos when using TEAC assay [29]. It has also been

reported that the ethanol extract of *Athrixia phylicoides* displayed antioxidant activity in DPPH free radical assay [14]. Comparison of different methods for the detection of total antioxidant activity from *Athrixia phylicoides* can be carried out and improved for optimum performance to give accurate results. This will significantly contribute to the accurate assessment of potent antioxidant activity. The results thereof will allow a selection of the appropriate analytical procedure for antioxidant activity determination and assist in providing optimum conditions with the greatest concentration of polyphenols. A method that is universal and applicable through the wide spectrum of samples available will be of great importance in either disease control or their prevention. Such a method can be used by nutritionists and in medicine for rating various foodstuff and pharmaceuticals according to their antioxidant activity through a uniform non-contradictory system.

1.5. Layout of the dissertation

The dissertation is organized into five chapters. Continuing from this introductory chapter is the literature review (Chapter 2) in which current research related to the topic of discussion is reviewed. The research methods, including a description of the tea used and procedures in graphical analysis of each experimental are presented in Chapter 3. Chapter 4 reports and discusses the results. The final chapter, Chapter 5 will summarize the conclusions from this research. Following the conclusion will be a short projection of these study findings implications and recommendation for further research.

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Chapter 2

LITERATURE REVIEW

2.1. Redox reactions in biological system

The core of redox reactions stems from the exchange of electrons as specified within a chemical mechanism. Reduction of any chemical species can be defined as an uptake of electrons whereas oxidation refers to the loss of electrons [1]. It follows that, a reducing agent or reductant is a substance that donates electrons and as a consequence causes the reduction of another reactant. An oxidizing agent or oxidant on the other hand is a chemical species that accepts electrons and therefore causing another reactant to be oxidised. Redox reactions are best described as half cells made up of oxidation and reduction whereupon neither can take place without complementary process of another.

Redox reactions form the basis of aerobic respiration whereby atmospheric oxygen is utilised in a chain of chemical reactions to absorb food chemicals which in turn release the chemical energy for living organisms [2]. These life-essential elemental oxygen is also responsible for a number of oxidation processes which have harmful consequences such as:

(i) weathering of non-biological materials [3],

(ii) oxidative stress in plants [4,5],

(iii) food quality deterioration [6,7] and

(iv) human health disorders that are related to oxidative damage of important biological molecules [8].

While reductant and oxidant are scientific terms, in biological systems they are usually referred to as antioxidant and pro-oxidant, respectively [9]. An antioxidant is a substance capable of inhibiting the oxidative processes that take place in different systems under the action of either the molecular oxygen or of the reactive species thereof. Pro-oxidant is a species that causes or promotes oxidation which leads to

oxidative damage of various biomolecules. In general, these pro-oxidants are referred to as reactive forms of oxygen (ROS) and nitrogen (RNS). The important redox reaction mechanisms in biological systems can be represented as shown in Scheme 2.1 [10].

Start or Initiation:

Formation of peroxy ($ROO^{\cdot}$), alkoxy ($RO^{\cdot}$) or alkyl ($R^{\cdot}$) radicals

$$RH \xrightarrow{\text{Initiator}} R^{\cdot} + H \qquad \textbf{2.1}$$

Chain propagation:

$$R^{\cdot} + O_2 \rightarrow ROO^{\cdot} \text{ (fast)} \qquad \textbf{2.2}$$

$$ROO^{\cdot} + RH \rightarrow ROOH + R^{\cdot} \text{ (slow, rate limiting)} \qquad \textbf{2.3}$$

$$RO^{\cdot} + RH \rightarrow ROH + R^{\cdot} \qquad \textbf{2.4}$$

Chain branching:

$$ROOH \rightarrow RO^{\cdot} + OH^{\cdot} \qquad \textbf{2.5}$$

$$2ROOH \rightarrow ROO^{\cdot} + RO^{\cdot} + H_2O \qquad \textbf{2.6}$$

Chain termination:

$$R^{\cdot} + R^{\cdot} \rightarrow \textbf{non-radical products} \qquad \textbf{2.7}$$

$$R^{\cdot} + ROO^{\cdot} \rightarrow \textbf{stable products} \qquad \textbf{2.8}$$

$$ROO^{\cdot} + ROO^{\cdot} \rightarrow \textbf{molecular products} \qquad \textbf{2.9}$$

Scheme 2.1: The modified steps of free radical reaction chain [10].

2.2. Free radicals

2.2.1. What are free radicals?

Free radicals are defined as chemical species that are electronically charged, having a specific permanent oxidation state and are free floating within a particular environment [11]. They are characterized by one or more unpaired electrons in the outer shell of the molecule which render them highly unstable ($M^{n\bar{e}}$, where n is always an odd number, M is chemical species and ē the number of electrons). Examples of different ROS and RNS species are given in Table 2.1 [12]. Electrons

are most stable in pairs, hence the free radicals tend to attach to or receive hydrogen ions from molecules with lower bond dissociation energy like unsaturated fatty acids or phenolic antioxidants.

Table 2.1: The examples of different reactive species [12].

Radicals	Non radicals
Reactive Oxygen species	
Alkoxyl radicals **RO$^{\bullet}$**	Hydrogen peroxide **H_2O_2**
Peroxyl radicals **ROO$^{\bullet}$**	Nitrosoperoxycarbonate **$ONOOCO_2^-$**
Hydroxyl radical HO$^{\bullet}$	Peroxomonocarbonate **$HOOCO_2^-$**
Superoxide anion radical **$O_2^{\bullet-}$**	Singlet oxygen **$^1O_2$**
Singlet oxygen $^1O_2$	Ozone **O_3**
Hydroperoxyl **$HO_2^{\bullet-}$**	Organic peroxides **ROOH**
Reactive Nitrogen species	
Nitric oxide radical **NO$^{\bullet}$**	Dinitrogen trioxide **N_2O_3**
Nitrogen dioxide radical **$NO_2^{\bullet}$**	Peroxynitrite anion **$ONOO^-$**
Nitrate **$NO_3^{\bullet}$**	Peroxynitrous acid **ONOOH**
Nitrosoperoxycarbonate anion **$ONOOCO_2^-$**	
Nitroxyl anion **NO**	

2.2.2. Sources of free radicals

Free radicals can be generated endogenously or exogenously [9]. Those present in the atmosphere as pollutants are generated by exogenous sources such as exposure to heavy metals (e.g. iron and copper), cigarette smoke, ionizing radiation, environmental pollutants, and chemicals such as ethyl alcohol, ozone, halogenated hydrogen and lipid oxidation products in foods [13]. Ozone (O_3), whose presence in

the upper atmosphere is essential in scavenging deleterious UV-irradiation, is also used as disinfection agent by the food industry to destroy food-borne pathogens. Nevertheless, it can oxidize biomolecules yielding the formation of various reactive species. Although the exposure to exogenous sources is relatively high, the exposure to endogenous sources is much more important and extensive, because it is a continuous process. In the human body, free radicals are endogenously produced by catalyzed reactions and other mechanism in mitochondria as well as by neutrophils and macrophages during inflammatory cell activation [14]. Moreover, activated phagocytes produce a variety of reactive oxygen, halogen and nitrogen species that play an important role in the mechanism defence against infectious agents [15].

2.2.3. Effects of free radicals

Depending on the site and the concentration generated, the free radicals are well recognised for playing a dual role, as both beneficial and deleterious effects have been established [16]. Overproduction of free radicals in the human body cause destructive and lethal cellular effects by oxidizing membrane lipids, proteins or nucleic acid, thus inhibits their normal function. Radicals and ROS such as superoxide anion, hydroxyl radical and peroxyl radical have been implicated as mediators of degenerative and chronic deteriorative, inflammatory, autoimmune diseases, diabetes, vascular disease, cancer, brain dysfunction as well as ageing [17]. Additionally, several degradation reactions, as a result of free radicals reacting naturally with the fats in the food matrix, may occur on heating or during long term storage. These oxidation reactions are the major causes of chemical spoilage, resulting in rancidity and /or deterioration of the nutritional quality, colour, flavor, texture and safety of foods [7].

The physiological adverse effect of this free radical has attracted a keen interest in the food industry as well as the health fraternity worldwide, as they have caused mayhem due to their reactivity or toxic effect. To counteract the negative effects of these ROS/RNS species, living cells have developed a complex defence mechanism that converts them to harmless species. Defense mechanisms against free radical induced oxidative stress include preventative mechanism (prevention of oxygen

access), repair mechanism, physical defense and antioxidant defenses [18]. The antioxidant system is divided into enzymatic and non-enzymatic antioxidant.

2.3. Antioxidants

2.3.1. Definition

In biological systems, antioxidant is defined as any substance that when present in low concentrations compared to that of an oxidisable substrates, significantly delay or inhibits a pro-oxidant initiated oxidation of the substrate [19]. The oxidisable substrates may be any molecule found in foods or biological materials including carbohydrates, DNA, lipids and proteins. Antioxidant in foods can be defined as any substance that is added to fats to retard oxidation and prolong wholesomeness and their stability [20]. However a broader meaning is hidden in this simple description, which is rooted in the complex phenomena involved in oxidation – antioxidation process. Antioxidants effect their protection at different stages of lipid oxidation and by different mechanisms.

2.3.2. Classification of antioxidants

Antioxidants represent a class of compounds that vary widely in chemical structure and have varied mechanisms of action. They generally exhibit an effect on oxidation in two ways, namely by scavenging free radicals or by inhibiting/retarding their formation [21,22]. Some antioxidants exhibit more than one mechanism pathway and are often referred as multiple-function antioxidants. A distinction is usually made between short and long term protection of antioxidants owing to their reaction kinetics [18]. According to the mechanism of action antioxidants can be classified broadly as primary antioxidants and secondary antioxidants based on the way the reaction pathway (Table 2.2).

Table 2.2: Mechanism of antioxidant activity [25].

Antioxidant class	Mechanism of antioxidant activity	Examples of antioxidants
Primary antioxidant	Inactivating lipid free radicals	Phenolic compounds
Secondary antioxidants		
Hydroperoxide stabilizers	Preventing decomposition of hydroperoxides into free radicals	Phenolic compounds
Synergists	Promoting activity of primary antioxidant	Citric acid, ascorbic acid
Metal chelators	Binding heavy metals into inactive compounds	Phosphoric acid, compounds of Maillard reaction
Singlet oxygen quenchers	Transforming singlet oxygen into triplet oxygen	Carotenes
Substances reducing	Reducing hydroperoxides in a non-radical way	Proteins, amino acids, hydroperoxides,

2.3.2.1. Primary antioxidants

Primary antioxidants are also known as free radical scavengers (FRS) or chain breaking antioxidants. FRS can either delay the initiation step of lipid peroxidation by scavenging lipid radical or inhibit the propagation step of lipid peroxidation by reacting with peroxyl· or alkoxyl radicals, thus forming antioxidant free radicals. The antioxidant-derived radicals would become stable, or decay to a stable state, or be regenerated by other antioxidants. The reactions of antioxidant towards these radicals can be seen below [18,23]:

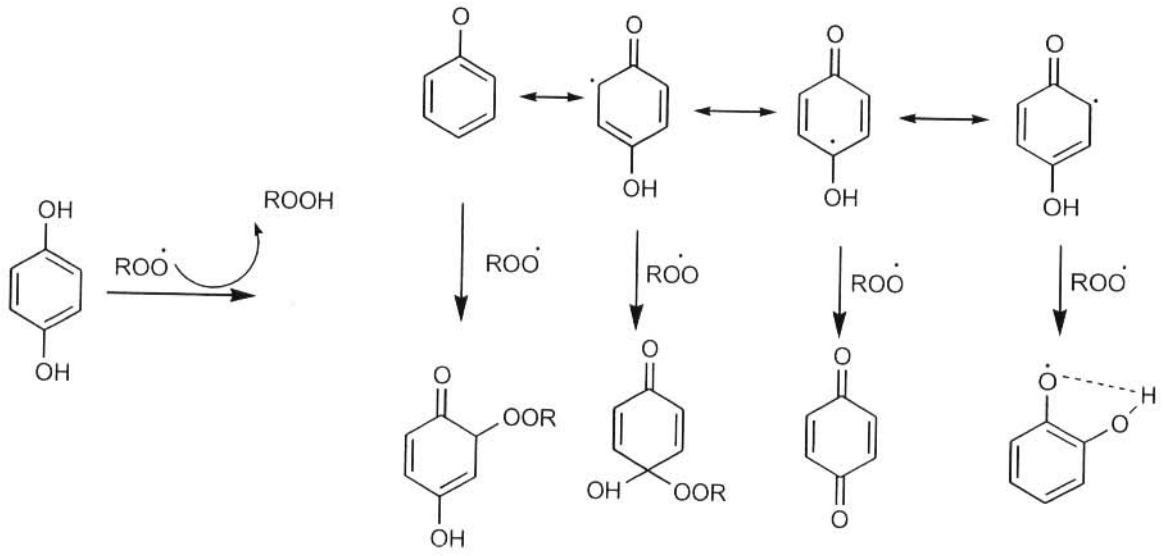
$$R^{\cdot} + FRS \rightarrow RH + FRS^{\cdot} \quad \textbf{2.10}$$

$$ROO^{\cdot} + FRS \rightarrow ROOH + FRS^{\cdot} \quad \textbf{2.11}$$

$RO^{\cdot} + FRS \rightarrow ROH + FRS^{\cdot}$ **2.12**

where, FRS: antioxidant, $R^{\cdot}$: lipid radical, $ROO^{\cdot}$: peroxyl radical and $RO^{\cdot}$: alkoxy radical.

Primary antioxidants are mainly phenolic compounds and reacts predominately with peroxyl radicals. At low concentration phenolic compounds compete efficiently with lipids to deactivate free radicals by donating an electron followed by deprotonation thereby inhibiting formation of rancid flavor in food [20]. Furthermore they form low energy stable radicals which do not react with oxygen to produce peroxides [24]. The latter is due to antioxidant radical stabilization through resonance, delocalization, intramolecular hydrogen bonding or further oxidation (Scheme 2.2) [20].



Scheme 2.2: Antioxidant radical stabilization through resonance delocalization, intramolecular hydrogen bonding or by further oxidation.

2.3.2.2. Secondary antioxidants

Secondary or preventative antioxidants usually enzymes, slow the rate of lipid oxidation by different actions, but do not convert free radicals to more stable products. Reaction mechanisms of secondary antioxidants as described below:

- **Chelate pro-oxidant or catalyst metals and deactivate them**

They either alter metal solubility or its redox potential, or sterically hinder formation of metal-hydroperoxide complex and this prevent further decomposition [24,26,27]. The most efficient metal chelators are compounds that possess lone pairs to donate to the metal ion and orbitals suitably arranged in space so that vacant metal orbitals can be accommodated.

- **Decomposition of hydrogen peroxides to non-radical species**,

These antioxidants deactivate oxidation intermediate such as superoxide anion, hydrogen peroxide or lipid hydroperoxide. For example, superoxide dismutase (SOD) enzyme catalyses superoxide radicals to produce ground state oxygen and hydrogen peroxide, which in turn is decomposed to water by a catalase (CAT) as, exemplified in reaction 2.13 and 2.14. Peroxidase enzymes remove H_2O_2 by using it to oxidise substrate. Glutathine peroxidase family removes H_2O_2 by coupling its reduction to H_2O with oxidation of reduced gluthathione.

$$2\ O_2^{\bullet-} + 2\ H^+ \xrightarrow{SOD} H_2O_2 + O_2 \qquad \textbf{2.13}$$

$$2\ H_2O_2 \xrightarrow{CAT} 2\ H_2O + O_2 \qquad \textbf{2.14}$$

- **Act as oxygen scavengers and reducing agents**.

Oxygen scavengers function by donating hydrogen atoms. Ascorbyl palmatate, ascorbic acid, sodium erythorbate, sulfites are commonly used as oxygen scavengers. These are compounds that react with oxygen and thus remove oxygen in a closed system.

- **Deactivate singlet oxygen**

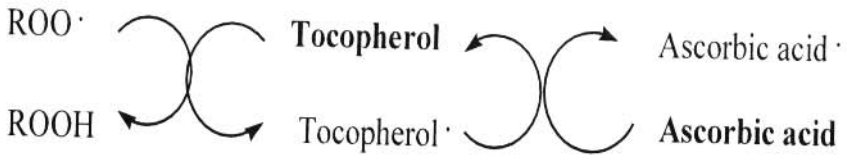
Singlet oxygen can be inactivated by physical (collisional) or chemical quenching (it can add to antioxidant to form endoperoxides), the first mechanism being predominant [28]. Collisional quenching takes place by energy transfer from the $^1O_2$ to the quenching molecule (reaction 2.15). The latter dissipates the acquired energy as emission of heat into the surrounding medium allowing one molecule of the quencher to inactivate many moles of $^1O_2$ (reaction 2.16) [3].

$$^1O_2 + \text{B-carotene} \rightarrow {}^3\text{B-carotene} + {}^3O_2 \qquad \textbf{2.15}$$

$$^3\text{B-carotene} \rightarrow {}^1\text{B-carotene} + \text{Heat} \qquad \textbf{2.16}$$

- **Replenish or provide hydrogen atom to primary antioxidants**

These types of antioxidants are often referred as synergists because they have little or no antioxidant activity of their own however enhance or promote the antioxidant activity of primary antioxidants. Citric acid owes its synergistic effect to metal chelation [29], whereas ascorbic acid has a synergistic action with α-tocopherol. The latter is due to the fact that ascorbic acid is able to restore the antioxidant properties of α-toçopherol by reducing its oxidized form [30] or by donating hydrogen atom to the tocopherol radical (Scheme 2.3) [26]. Also some flavonoids like quercetin and tea catechism regenerate tocopherol from tocopherol radical, thus ensuring a "sparing" effect [31].



Scheme 2.3: Synergistic combination between tocopherol and ascorbate [26].

2.4. Antioxidants in Foods

The food industry suffers significant losses as a result of decreased product shelf-life caused by warmed over flavor development, rancidity and diminished nutritional quality all of which stem from lipid oxidation [21]. The prevention or retardation of

these oxidation processes is essential for the food producer and for all persons involved in the value of chain.

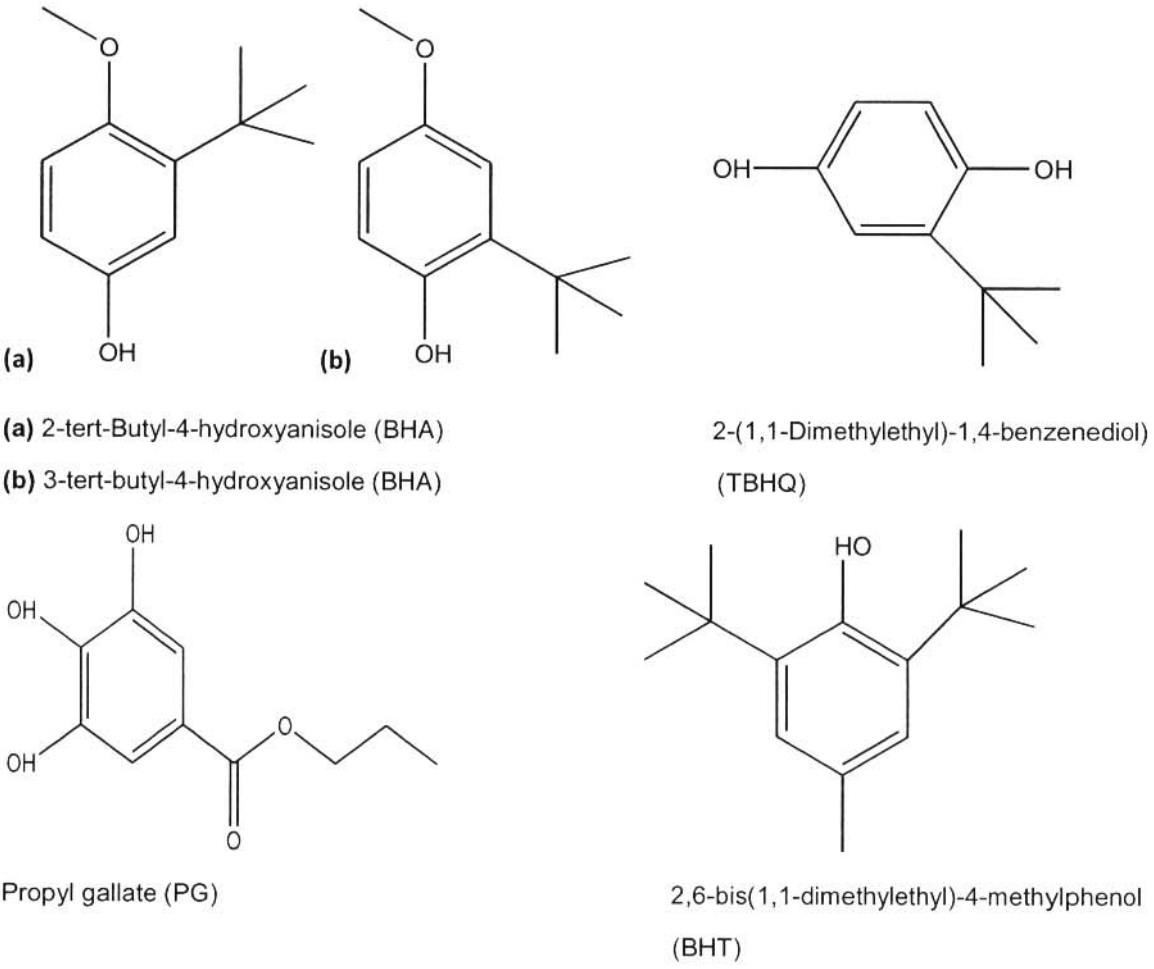
Antioxidants are added in many types of food systems because of their important role in preventing oxidative deterioration of lipids by reacting with free radicals that are part of the oxidation process, chelating metal ions that can initiate autoxidation or being oxygen scavengers [21]. Many compounds are active as antioxidants, but only few are incorporated into food as preservatives because of strict safety regulations. The most commonly used synthetic antioxidants are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ) owing to their effectiveness in a variety of food systems, low cost and wide availability (Scheme 2.4) [32].

Although synthetic antioxidants are extremely effective at slowing oxidation, there have been recent consumer concerns over potential adverse health effects associated with these compounds. Studies have reported that BHT and BHA cause a wide range of health problems including, enlarged liver, increased liver microsomal enzyme activity and conversion of some ingested materials into toxic and carcinogenic substances, especially if they are presents in excessive amounts [33]. Hence an increasing interest in the commercial development of plants as a source of antioxidants that can be used to enhance the properties of food, for both nutritional purposes and for preservation.

It is revealed from literature that the replacement of synthetic antioxidants by natural ones may have several benefits and much of the research on natural antioxidants has focused on many active compounds from various plants. In the majority of cases the active ingredients are of phenolic nature: phenolic acids [34,35], flavonoids [31,34,36] and catechins [37]. Other classes include hydrolysable tannins, proanthocyanidins, lignans as well as biflavones and coumarins [31].

Because polyphenol compounds are widespread in the plant kingdom (app. 5000 – 8000), they are currently attracting the biggest attention [31]. A great deal of natural substances and mixtures have been investigated and recognized as antioxidants e.g. tocopherol (Vitamin E and its derivatives) and extracts from herbs and spices have been proven effective in preserving food and are currently being used in industry as anti-drying agents to prevent oxidation and polymerization of

polyunsaturated fatty acid-rich plant oils [26]. In addition to tocopherol some natural products such as different teas [38,39] and wine [37] have been investigated and recognized as antioxidants.



(a) 2-tert-Butyl-4-hydroxyanisole (BHA)

(b) 3-tert-butyl-4-hydroxyanisole (BHA)

2-(1,1-Dimethylethyl)-1,4-benzenediol) (TBHQ)

Propyl gallate (PG)

2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT)

Scheme 2.4: Commonly used synthetic antioxidants.

2.5. Antioxidants and Health

In addition to protection against quality degradation in food systems, natural antioxidants have positive effects on overall human health. The addition of antioxidants into foods can help boost overall nutritive values with often positive effects on sensory quality [41]. Epidemiological studies have strongly suggested that diet plays an important role in the prevention of chronic diseases [38,42,43].

Polyphenolics, thiols, carotenoids, tocopherols, and glucosinolates commonly found in fruits, vegetables and beverages, have been found to have potential health benefits that are believed to be associated mainly with their antioxidant activity [44]. These compounds provide chemo protective effects to combat oxidative stress in the body and maintain balance between oxidants and antioxidants to improve human health [45,46].

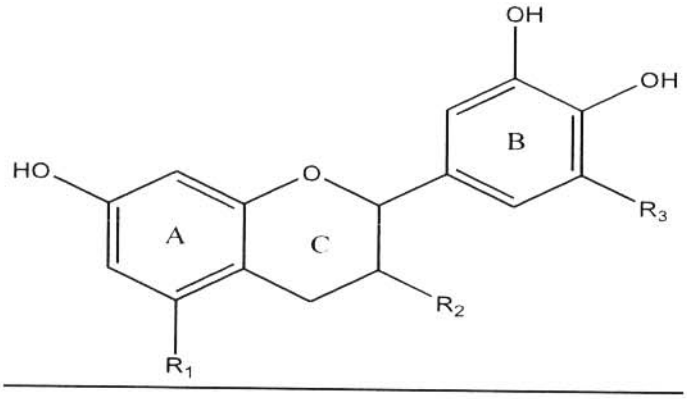
Tea phenolic compounds have been reported to exhibit a very broad spectrum of medicinal activities. Green tea phenolic components inhibit intestinal uptake of glucose through rabbit intestinal epithelial cells and thus may contribute to the reduction of blood glucose levels [47]. Epigallocatechin gallate, a phenolic compound of green tea has been found to possess effective antioxidant properties and can provide protection in vitro against both peroxyl radical and hydroxyl radical-induced oxidation of DNA [48]. This radical scavenging activity suggests that the phenolic compounds may provide protection against carcinogens. Flavonoids reportedly lower the risk of various degenerative diseases associated with aging, such as cancer, cardiovascular diseases, osteoporosis, and neurodegenerative diseases [40].

2.6. Plants as a source of antioxidants

Phenolic compounds are a group of organic compounds having an aromatic ring with one or more hydroxyl groups, and recently have been the subject of considerable scientific and therapeutic interest. Their ability to act as free radical acceptors and chain breakers appear to have played a major role in successful medical treatments of ancient times, and their use has persisted up to now [40]. Most of the major classes of plant polyphenol which are common to all plant sources [49] are listed in Table 2.3.

Table 2.3: The major classes of phenolic compounds in plants.

Number of Carbon atoms	Basic skeleton	Class
6	C_6	Simple phenols, benzoquinones
7	$C_6 - C_1$	Phenolic acids
8	$C_6 - C_2$	Acetophenones, tyrosine derivatives
9	$C_6 - C_3$	Hydroxycinnamic acid, coumarins
10	$C_6 - C_4$	Naphthoquinones
13	$C_6 - C_1 - C_6$	Xanthones
14	$C_6 - C_2 - C_6$	Stilbenes
15	$C_6 - C_3 - C_6$	Flavonoids
18	$(C_6 - C_3)_2$	Lignans
30	$(C_6 - C_3 - C_6)_2$	Biflavonoids

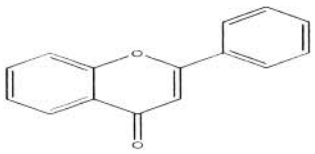


Scheme 2.5: The basic unit of flavonoids.

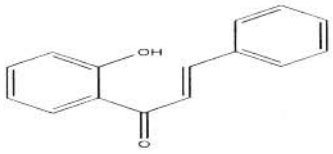
Flavonoids represent a large group of polyphenols that occur naturally in plants and are found in fruits, vegetables, grains, barks, roots, and beverages such as tea and wine [50]. They are referred to as "nutraceuticals" which are defined " as a food or parts of food that provide medical or health benefits including the prevention and

treatment of disease" [51]. Their common structure is that of diphenylpropanes (C_6-C_3-C_6) and consists of two benzene rings linked by an oxygen containing heterocycle (Scheme 2.5).

They are subdivided into several groups differing in the ring C depending on specific hydroxylation and conjugation pattern [34], whether there is a closed ring or oxidation state of the pyran moiety. The subclass consists of chalcones, flavonols, flavones, flavanones and flavanals (Scheme 2.6). Flavones (e.g. apigenin and luteolin) contain one carbonyl group at C-4, double bond between C-2 and C-3, and no hydroxyl at C-3. Flavonols (e.g. quercetin and kaempferol) have a carbonyl group at C-4, double bond between C-2 and C-3 and hydroxyl at C-3. Flavanols (e.g. catechin and epicatechin) have hydroxyl at C-3, lack carbonyl group at C-4 as well as double bond between C-2 and C-3 and, isoflavonoids, the B ring is located in the 3 position of the C ring.

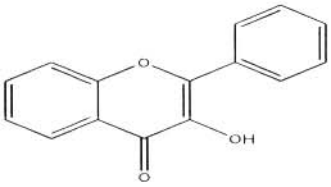


2-phenyl-chromen-4-one (Flavone)

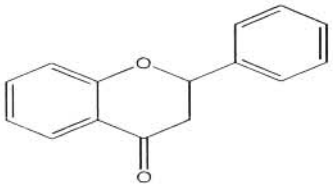


1-(2-hydroxy-phenyl)-3-phenyl-propenone

(Chalcone)

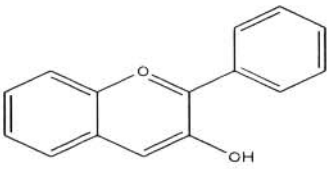


3-hydroxy-2-phenyl-chromen-4-one (Flavonol)

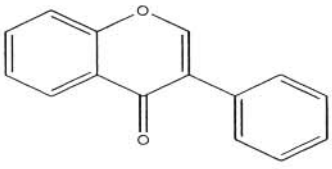


2-phenyl-chroman-4-one

(Flavanone)



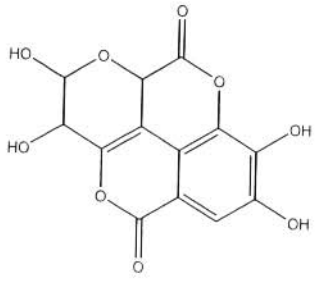
3-hydroxy-2-phenyl-chromenylium (Anthocyanidine



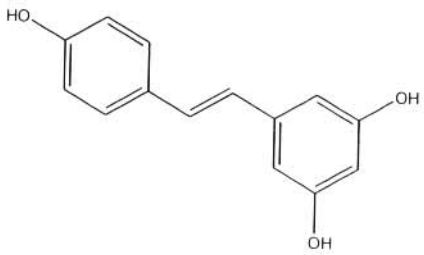
3-phenyl-chronen-4-one (Isoflavone)

Scheme 2.6: Chemical structures of some representative flavonoids.

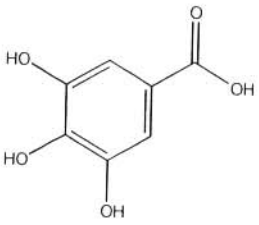
Phenolic polymers and phenolic acids are other examples of major classes of plant polyphenol. Phenolic polymers, commonly known as tannins, are compounds of high molecular weight that are divided into two classes: hydrolysable and condensed tannins. Phenolic acids are a type of organic acids that possess a carboxylic acid bonded to a simple phenol. Hydroxybenzoic and hydroxycinnamic acids are two main groups of phenolic acids, both of which are derived from the nonphenolic molecules benzoic and cinnamic acid [52]. Although the basic skeleton remains the same, the number and position of the hydroxyl groups on the aromatic ring create a variety of compounds. The most common phenolic acids in plants are gallic, caffeic, p-coumaric, vanillic, ferulic and protocatechuic (Scheme 2.7).



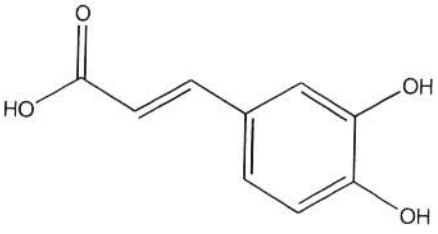
Ellagic acid



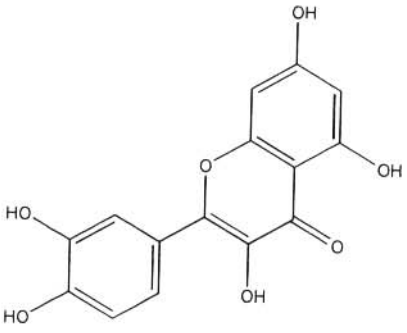
Resveratrol



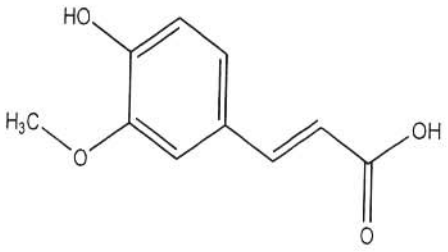
Gallic acid



Caffeic acid



Quercetin



Coumaric acid

Scheme 2.7: Examples of some simple phenol and flavonoids in plants.

2.6.1. Antioxidative activity of polyphenols

The antioxidant activity of phenolic compounds depends primarily on the number and positions of hydroxyl groups and other substituents in the structure [25]. The substitutions on the structure are probably the most significant with respect to the ability of a natural antioxidant to participate in the control of radical reactions and to form resonance-stabilized, natural antioxidant radicals [32]. More specifically, since flavonoids are widely recognized as effective antioxidants [21,31,34,53,54] the following observations have been made:

(1) The degree of hydroxylation and the positions of the hydroxyl groups in the B ring, in particular an ortho-dihydroxyl structure of ring B (catechol group) results in higher activity as it confers higher stability to the aroxyl radical by electron delocalisation or acts as the preferred binding site for trace metals.

(2) The presence of hydroxyl groups at the 3'-, 4'-, and 5'-positions of ring B (a pyrogallol group) has been reported to enhance the antioxidant activity of flavonoids compared to those that have a single hydroxyl group. However, under some conditions, such compounds may act as pro-oxidants, thus counteracting the antioxidant effect. This is consistent with the observation of Seeram and Nair who reported that the conversion of the 3',4'-dihydroxyphenyl to 3',4',5'-trihydroxylphenyl increases the antioxidant activity for anthocyanins but decreases the activity for catechins.

(3) A double bond between C-2 and C-3, conjugated with the 4-oxo group in ring C enhances the radical scavenging capacity of flavonoids.

(4) A double bond between C-2 and C-3, combined with a 3-hydroxyl group, in ring C, also enhances the active radical scavenging capacity of flavonoids, as seen in the case of kaempferol. Substitution of the 3-hydroxyl group results in increase in torsion angle and loss of coplanarity and subsequently reduced antioxidant activity.

(5) Substitution of hydroxyl groups in ring B by methoxyl groups alters the redox potential, which affects the radical scavenging capacity of flavonoids.

2.6.2. Extraction of polyphenols compounds

Antioxidant compounds are usually present in rather low amounts in natural materials. Therefore, sample preparation is of great importance in analysing polyphenols and simple phenols in plants, foods, and liquid samples (including biological fluids and beverages) that contain chemical functional groups with a great diversity regarding polarity, acidity, number of hydroxyl groups and aromatic rings, concentration levels, and complexity of the matrix.

The first step in sample preparation is extraction of bioactive compounds from plant material. The phenolic composition of an extract is influenced by the chemical nature of the phenolic compounds present in the plant, the extraction method used and the presence of interfering compounds [55]. Solvent extractions are the most commonly used procedures to prepare extracts from plant materials due to their ease of use, efficiency, and wide applicability.

2.6.2.1. Factors influencing extraction of polyphenols

There are various factors influencing the extraction of polyphenols, for example the type of solvents with varying polarities, extraction time and temperature, sample-to-solvent ratio as well as on the chemical composition and physical characteristics of the samples.

- Chemical composition and physical characteristics of the samples

The solubility of polyphenols is governed by the chemical nature of the plant sample varying from simple (e.g. phenolic acids, anthocyanins) to highly polymerized substances in different quantities, as well as the polarity of the solvents used. The interaction of phenols with other plant materials such as carbohydrates might cause the formation of considerably insoluble complexes, which has an effect on the extraction procedure and efficiency.

- Polarity of solvents

Different solvents such as methanol, ethanol, propanol, acetone, ethyl acetate, dimethylformamide (DMF) and their combinations can be used for extraction of

polyphenols with distinct proportions of water [18,55]. These solvent systems when coupled with appropriate extraction conditions destroy cell membranes and simultaneously dissolve the polyphenolic compounds [55]. Methanol, ethanol and water are the most widely employed solvents for toxicological, environmental safety and inexpensive features. However, aqueous methanol solutions are one of the most commonly employed solvent for extracting polyphenols compounds since polyphenolic compounds are quite stable in these methanol solutions. For example, flavones and flavonols were reported to be stable in methanol for greater than three months at 4°C [56]. In addition, aqueous methanol have the ability to isolate a broader spectrum of apolar and polar compounds from the material, while the higher molecular weight flavanols are better extracted with aqueous acetone [57]. Hot or boiling water has also been used to extract flavonoids and polyphenolic compounds from plant material such as tea [57,58].

- Extraction time and temperature

Temperature and time intervals of each of extraction steps in the extraction process reflect the conflicting actions of solubilisation and analyte degradation by oxidation [57]. An increase in the extraction temperature can promote higher analyte solubility by increasing both solubility and mass transfer rate. In addition, the viscosity and the surface tension of the solvents are decreased at higher temperature, which helps the solvents to reach the sample matrices, improving the extraction rate. However, many phenolic compounds are easily hydrolysed and oxidized. Long extraction times and high temperature increase the chance of oxidation of phenols which decrease the yield of phenols in the extracts. The extraction time is thought as an efficient parameter for extraction of polyphenols.

- The ratio of solvent-to-sample and the particle size of the sample

The extraction of polyphenols from plant material might also be affected by the ratio of solvent-to-sample and the particle size of the sample. Increasing solvent-to-solid ratio was found to work positively for enhancing phenol yields [59]. However, an equilibrium between the use of high and low solvent-to-solid ratios, involving a balance between high costs and solvent wastes and avoidance of saturation effects, respectively, has to be found to obtain an optimized value [60]. Lowering particle size also enhances the yield of phenolic compounds [61].

2.7. *Athrixia phylicoides*

Bush tea (A*thrixia phylicoides*) is an indigenous South African herbal plant that belongs to the Asteraceae family [62]. The genus name a*thrixia* is derived from the Greek word *thrix* meaning hair, which refers to the leaves. The specific epithet *phylicoides* means it resembles *phylica*. It is an aromatic shrub with leafy stems, fine dark green leaves found in the mountainous areas, grassland and forests of the eastern parts of South Africa (Limpopo, Free State, Kwazulu Natal and Eastern Cape) and Swaziland [63].

Leaves and stalks of this shrub are boiled and the extract is used as a herbal tea and medicinal decoction [64,65]. In traditional medicine, A*thrixia phylicoides* infusions are used to treat sores and boils and also as blood purifiers [64]. In addition, when boiled over sufficient period, the roots extract serves as a purgative and cough remedy [64]. Outside medicinal uses, the plants shrubs are used for making brooms [62].

There is already growing evidence that tea polyphenols reduce the risk of heart disease and cancer in humans [66]. Studies on the polyphenol content and antioxidant activity of bush tea extracts shows that this is a potential source of antioxidant–rich extracts for the nutraceutical and cosmeceutical industry [67]. Bush tea ethanol extracts showed similar antioxidant activity to rooibos tea, while being less active than black tea [68]. In addition, Padayachee reported that the anti-oxidant activity of the aqueous extract of *Athrixia phylicoides* (IC_{50} = 14.01 ± 2.68 µg/mL) was greater than those of rooibos and Ceylon (black) tea (IC_{50} > 25.00 µg/mL); comparable to green rooibos and honeybush tea ((IC_{50} = 18.01 ± 4.06 µg/mL; 18.02 ± 4.27 µg/mL, respectively), but less active than green tea (IC_{50} = 9.64 ± 0.96 µg/mL) [69]. A unique methoxylated flavonol was isolated from bush tea for the first time whereas the major phenolics in the bush tea aqueous extracts are hydroxycinnamic acids [65]. It has been reported that the ethanol extracts of bush tea have antioxidant and cytotoxic activities, and no caffeine contents or pyrrolizidine alkaloids were detected [68].

The polyphenolic profile of teas is affected by the variations in processing methods as well as the geographic location of the tea plant and the taste of the infused tea [70]. Black and green tea originates from the *Camellia sinensis* plant however their differences come from the methods by which they are processed. Rooibos tea

originates from *Aspalathus linearis* plant. The flavonoids found in tea, specifically catechins and tannins, have strong antioxidant properties. In green tea, all catechin compounds are intentionally left intact, but part of these in oolong tea and almost all in black tea are oxidized to theaflavines, thearugubins. Hence green tea reported to have highest catechin content and thus stronger antioxidant activity than that of two other teas. Black tea is commonly consumed in the western world as "English" tea whereas the consumption of green tea and oolong tea are favoured in Asian countries and brewed traditionally without any addition of sweeteners. Rooibos tea is a South African beverage commercially used as an herbal tea or tisane. It is rich in polyphenols and is a rare source of the dietary dihydrochalcones, aspalathin and nothofagin.

2.8. Antioxidant analysis background

The antioxidant activity which is the capability of the compound or mixture to inhibit the production of oxidative intermediates and final products, thus preventing oxidative damage and / or deterioration has been evaluated in various matrices, such as plasma, beverages, vegetables and fruits as well as pure compounds [28,71]. Numerous methods and techniques for assessment of lipid oxidation and evaluation of antioxidant activity have been reported [20]. The majority of the methods rely on objective chemical or physical processes and various instrumentation techniques that include: chromatographic like TLC, GC, and HPLC; spectroscopic like UV/Vis, IR NMR, EPR and electrochemical ones like polarography and voltammetry.

These analytical methods for evaluation of antioxidant capacity of pure compounds or complex matrices are applied to investigate the structure-activity relationship [72], to separate and detect specific components and to determine their contribution to the total antioxidant composition [17]. In food product assessment of antioxidant capacity is of utmost importance to determine the antioxidant effectiveness for food protection against oxidative damage, for food quality monitoring over a product shelf life, and for commercialization of nutritional-added-value products. While in the case of biological samples (e.g. plasma, serum, urine), measurement of antioxidant status is

essential for diagnostic and treatment monitoring, especially during supplementation trials for boosting plasmatic antioxidant levels [73].

Although there is a great multiplicity of methods used for antioxidant capacity, none of these methods provide an ideal individual, approved standardised method. This situation is due to several reasons. Firstly, the term antioxidant capacity cannot be measured directly but rather by the effects of antioxidant in controlling the extent of oxidation such as i) inhibition of generation and scavenging capacity against ROS/RNS, ii) reducing capacity, iii) metal chelating capacity; iv) activity of antioxidative enzymes. Secondly, different antioxidants act by different mechanisms and even the same compound can have different ways of actuation. Therefore, to assess and describe the total antioxidant activity of any sample, more than one analytical method must be applied [20].

2.8.1. Methods for measurement of antioxidant activity

An increased interest in information about antioxidant potentials of phenolic rich matrices has led to the development of a wide array of assays for determination of antioxidant capacity. Most assays for the antioxidant activity are based on the ability of the antioxidant to either scavenge free radicals that promote oxidation or prevent formation of lipid oxidation products such as peroxides and carbonyl compounds [74]. On the basis of the chemical reaction mechanisms involved, the assays can be roughly divided into two categories: hydrogen atom reaction and single electron transfer reaction based assays.

2.8.1.1. Hydrogen atom transfer (HAT) based method

HAT based assay measure the ability of an antioxidant to quench free radicals ($X^{\cdot}$ = any radical) by hydrogen atom donation (AH = any H donor). The reaction mechanism is shown below:

$$X^{\cdot} + AH \rightarrow XH + A^{\cdot} \qquad \textbf{2.17}$$

The methods involve antioxidant, reactive species that are usually free radicals (radical and non-radical) and probe (oxidisable substrate). The damage to the substrate could be inhibited or delayed in the presence of antioxidants. The inhibition time or inhibition percentage or both is measured and related to total antioxidant capacity (TAC) of a sample. The total antioxidant capacity of tested compounds is dependent on: (i) the rate of reaction between them and the reactive species, (ii) the rate of reaction between the probe and the reactive species and (iii) the concentration ratio between antioxidant and probe. The HAT based methods that have received much attention for their applicability in determining total antioxidant capacity are ORAC (Oxygen Radical Absorbance Capacity Assay) and TRAP (Total radical-trapping antioxidant parameter).

2.8.1.1.1. Oxygen Radical Absorbance Capacity Assay (ORAC)

ORAC measures antioxidant inhibition of peroxyl-radical induced oxidations and reflects classical radical chain breaking antioxidant activity by hydrogen atom transfer [75]. The first version of the ORAC assay employed B-phycoerythrin (B-PE, a protein isolated from *Porphyridium cruentum*) as the fluorescent probe and 2,2'-azobis-(2-amidino-propane)dihydrochloride (AAPH). The loss of fluorescence was an indicator of the extent of the decomposition, from its reaction with the peroxyl radical. The use of B-PE in antioxidant assays has some limitations such having large interbatch differences, photo bleaching of B-PE after exposure to the excitation light, and interaction with polyphenols by nonspecific protein binding. These factors cause inconsistency in assay results and false low values. This procedure has been used to evaluate the antioxidant activity of foods, serum and other biological fluids [75,76]. The ORAC assay provides a controllable source of peroxyl and hydroxyl radicals that model reactions of antioxidants with lipids in both and physiological systems and it can be adapted to detect both hydrophilic and hydrophobic antioxidant by altering the radical source and solvent. These methods are time consuming and need special equipment and technical skills for the analysis.

2.8.1.1.2. Total radical-trapping antioxidant parameter (TRAP)

This procedure is based on the measurement of oxygen consumption within a process in which there is a controlled lipid oxidation promoted by the thermal decomposition of (AAPH) 2,2 A-Azobis (2-amidinopropane) [73]. The units used are adopted as the number of µmoles of peroxyl radicals trapped by 1 l of plasma. The measurement of serum TRAP was directly proportional to the elapsed time that a particular serum would resist induced oxidation.

This is generally performed by monitoring oxygen consumed in a thermostated oxygen electrode cell during oxidation of linoleate by free radicals. One of the greatest shortcomings of this method is that the oxygen electrode is not sustainable over the rate of analysis. The latest version uses chemiluminescence (CL) as the end-point indicator. In its simplest form this involves the quenching of CL by addition of antioxidant over a period that is directly proportional to the radical trapping ability of the antioxidant.

Results can be standardized by addition of Trolox to the sample after consumption of natural antioxidants to produce a second induction period. As in most analytical procedures stoichiometric relationships for pure antioxidants will vary, for example (Trolox, 2.0; ascorbate, 1.5; urate, 1.7) and these must then be used for proper adjustment of experimental values. This is a very slow method and unreliable. TRAP assay may be used as an alternative method where accuracy is not critical.

2.8.1.2. Single Electron Transfer (SET) based method

SET based measure the ability of a compound antioxidant to transfer one electron to reduce stable radicals, metals or carbonyls ions (oxidant). The reaction mechanism is shown below:

$$X^{\cdot} + AH \rightarrow X^{-} + AH^{\cdot+} \qquad \textbf{2.18}$$

$$H_2O + AH^{\cdot+} \leftrightarrow A^{\cdot} + H_3O^{+} \qquad \textbf{2.19}$$

$$X^{-} + H_3O^{+} \rightarrow XH + H_2O \qquad \textbf{2.20}$$

$$M(III) + AH \rightarrow AH^{+} + M(II) \quad \textbf{2.21}$$

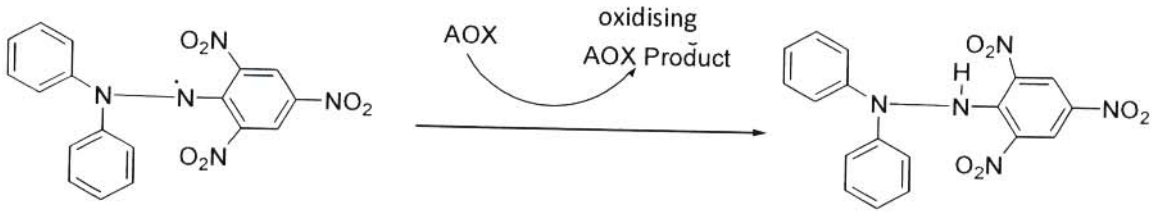
These assays involve two components in the initial reaction mixture: the antioxidant compounds and the reactive species, which may also be the probe for reaction monitoring. The reduction of the reactive species is accompanied by a change on its absorption spectra at the visible region. Therefore, degree of colour change indicates the scavenging potential of the sample is proportional to the antioxidant/reducing capacity. The reactivity in SET is based on ionization potential and on deprotonation of the reactive functional group in a particular pH milieu [74]. Assays utilising the SET mechanism includes FCR (Folin-Ciocalteu reducing method), FRAP (Ferric reducing ability of plasma assay), TEAC (Trolox equivalent antioxidant assay), $DPPH^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay) as well as electrochemical methods like CV (Cyclic voltammetry), DPV (Differential pulse voltammetry), etc. and their analytical features are summarised in Table 2.4.

Table 2.4: In vitro scavenging capacity assays against non-biological radicals and evaluation of total reducing capacity.

Assay	Principle	Quantification	Reference
DPPH$^{\bullet}$	DPPH$^{\bullet}$ radical is reduced by antioxidant causing absorbance at 515 – 520 nm	EC_{50}, RSE, (μM) Trolox equivalent	[77]
CUPRAC	Neocuproine Cu(II) chelate cation is reduced by antioxidant causing absorbance increase at 450 nm	Trolox equivalent	[86]
Folin-Ciocalteu	Tungstate-molybdate complexes are reduced by antioxidant causing absorbance increase at 750 -760 nm(mg/L)	Gallic acid equivalent,	[87]
Cyclic voltammetry	The intensity of anodic current is increased due to to oxidation of antioxidant compounds at the surface of the electrode	Oxidation potential ($E_{1/2}$) intensity of the anodic current (I_a), area under the anodic wave (S)	[83]

RSE, radical scavenging efficiency; EC_{50}, sample concentration that inhibit 50% of the blank analytical signal; DPPH, 2,2-diphenyl-1-picrylhydrazyl (DPPH) Radical Scavenging Capacity Assay; CV, Cyclic Voltammetry; CUPRAC, Cupric Ion Reducing Antioxidant Capacity.

2.8.1.2.1. Free radical scavenging capacity method
2,2-diphenyl-1-picrylhydrazyl (DPPH˙) Radical Scavenging Capacity



Scheme 2.8: Structure of 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) radical reaction.

This method is based on the measurement of the reducing ability of antioxidants on 2,2-diphenyl-1-picrylhydrazyl (DPPH˙) radical [76]. The DPPH radical is one of the few stable, commercially available organic nitrogen radical, which bears a deep purple colour and does not have to be generated before analysis. In this assay, the purple chromogen radical is reduced by antioxidant or reducing compounds to the corresponding pale yellow hydrazine (Scheme 2.8). Upon reduction due to the presence of the test compound (antioxidant), DPPH radical loses colour and the reaction progress is monitored by a spectrophotometer optimized at a range from 515 to 528 nm. Generally, the results are reported as the efficient concentration (EC_{50}), which is the amount of antioxidant that causes a decrease in the initial DPPH concentration by 50% [74]. The percentage of the DPPH remaining is calculated using equation 2.22 proposed by Brand-Williams [77].

$$\% \ DPPH_{rem} = 100 \times [DPPH_{rem} / DPPH]_{T=0} \qquad \textbf{2.22}$$

The other two parameters to express antioxidant are "T_{EC50}" time needed to reach the steady state with EC_{50} concentration calculated from the kinetic curve and "antiradical efficiency AE" as used by C. Sanchez-Moreno, from equation $AE = (1/EC_{50})\ T_{EC50}$ [78]. However, "antiradical efficiency AE" is more discriminative than T_{EC50} and more useful because it takes into account the reaction time. Another conceptually similar parameter designated as radical scavenging efficiency (RSE) was suggested by De Beer and is calculated as the ratio of the reaction rate (obtained during the first minute) and the EC_{50} value [79].

The DPPH can be used for solid or liquid samples and it is not specific to any particular antioxidant component, but applies to the overall antioxidant capacity of the sample. The documented drawback for this method will be the reduction of absorbance of DPPH radical at 517 nm after reaction with an antioxidant of interest due to light, oxygen, pH and the type of solvent employed. Stearic inaccessibility and narrow linear range of absorbance versus concentration [80]. Another disadvantage that is under debate is the fact that the decolourisation from the more intense colour is difficult to follow in a precise sequence. The smaller absorbance decrease the less proportional the antioxidant activity can be measured.

Despite the limitations mentioned above, the DPPH is considered because it is a rapid, technically simple and inexpensive method widely used to evaluate the antioxidant activity in food and test the ability of compounds to act as free radical scavengers or hydrogen donors using UV-vis spectrophotometer. The DPPH radical compound is stable, commercially available and does not have to be generated before assay like $ABTS^{\bullet+}$.

2.8.1.2.2. Oxidation potential method

Cyclic Voltammetry

Electrochemical properties of pure compounds, foods and biological samples may be used for the evaluation of their reducing antioxidant capacity since the electric oxidation potential has conceptually relation with the expected antioxidant capacity. The cyclic voltammetry technique is one of the electrochemical method that has been adapted to evaluate the overall reducing capacity of low molecular weight antioxidant in plasma [81], tissue homogenates and plant extracts [82]. It is based on the analysis of the anodic current (AC) waveform, which is a function of the reductive potential of a given compound in the sample and/or a mixture of compounds.

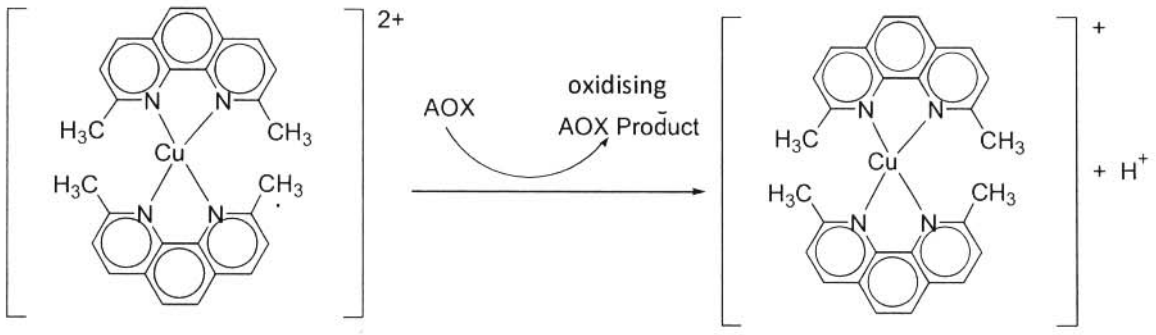
The total antioxidant capacity of the sample is analysed from two combined sets of parameters obtained through the CV tracings: a function combining two sets of parameters. The first is the biological oxidation potentials (Ops), characterized by the $E_{1/2}$ value which reflect the specific reducing capacity, the lower the $E_{1/2}$, the higher the ability of the tested sample to donate electrons to the working electrode. The second is the intensity of the AC current (Ia) and the area under the anodic wave (S).

Both Ia and S are related to the concentration of the reducing species present in the sample. Nevertheless, as an anodic wave in complex matrix such as biological and food samples integration yields a value equivalent to the total antioxidant activity of each of the components. It was proposed that using the area under the AC wave (S) rather than the I_a is a better parameter reflecting the total capacity of a sample [83].

The CV methodology allows rapid screening of the electrochemical profile of samples and is especially suitable for screening studies. Furthermore, the CV profile can be obtained in aqueous medium as well as in organic solvents like acetonitrile, water/acetonitrile, and acetonitrile/methanol mixtures provided that there are redox-active components and enough electrolytes in the solution to support redox reactions on the electrode surface. These assays based on the electrochemical properties of the compound/sample do not require the use of reactive compounds, since it is based on electrochemical behaviour and, consequently on their chemical-physical properties. Moreover, turbid and/or intensely coloured samples can be determined without prior sample preparation.

The shortcoming of these methodology is related to the fact that some biologically relevant antioxidants (e.g. glutathione, cysteine and other thiol-containing compounds) show a low response when glass carbon electrodes are applied. In this case, other electrodes such as an Au/Hg electrode are needed for glutathione measurement [84]. Furthermore, an important practical limitation is that the working electrode has to be frequently cleaned to remove residues of sample from its surface and to maintain its sensitivity. For instance, Blasco described the procedure for cleaning a glassy carbon electrode [85]. Hence, between each work session the electrode was cleaned by physical, chemical and electrochemical treatments, while during the session the electrode was electrochemically cleaned, whenever necessary, using cyclic voltammetry.

2.8.1.2.3. Reducing power methods
Cupric Ion Reducing Antioxidant Capacity (CUPRAC)



Scheme 2.9: Reaction mechanism for CUPRAC method.

The CUPRAC method is based on the reduction of Cu(II) to Cu(I) through the action of electron donating antioxidants (Scheme 2.9). Neocuproine Cu(II) chelate cation is used as chromogenic reagent which is reduced in the presence of *n*-electon reductant antioxidant to cuprous neocuproine chelate [Cu(I)Nc] showing maximum light absorption at 450 nm. A dilution curve generated by Trolox standard is used to convert sample absorbance to Trolox equivalents (TEAC). The analytical response versus the concentration curves is perfectly linear over a wide range, unlike those of other methods like DPPH and TEAC yielding polynomial curves. The redox reaction giving rise to a coloured chelate Cu(I)Nc is relatively not affected by parameters such as air, sunlight, humidity and pH to a certain extend.

The method can concurrently measure hydrophilic and lipophilic antioxidants unlike FCR and DPPH [86]. All classes of antioxidants, including thiols are detected with little interference from reactive radicals and the reaction kinetics is faster [74]. The cupric reagent is selective towards sugars and citric acid because it has a lower redox potential than that of the ferric-ferrous couple in the presence of phenanthroline ligands.

Folin Ciocalteu reducing method (FCR)

The chemistry behind the FCR method relies on the theory of a single electron transfer (SET) reaction which involves two components in the reaction mixture, antioxidants and oxidants (also probe) [87]. The method is based on the fact that antioxidants ionize completely under alkaline conditions, and can be readily oxidized by the Folin-Ciocalteu (FC) reagent. The FC reagent is an oxidant that abstracts an electron from the antioxidant, causing a colour change which is easy to monitor with a spectrophotometer optimized at 765 nm. The degree of the colour change is proportional to the antioxidant concentration. The reaction endpoint is reached when colour changes stops [74]. Generally, gallic acid is used as the reference standard compound and results are expressed as gallic acid equivalents (GAE).

The method is convenient, sensitive and precise and commonly used to determine the total phenolic content of foods. It also produces predictable results on a wide range of phenolic [74,87]. Colour development Folin-Ciocalteu reagent is generally the preferred method for measuring phenolic because most delivered antioxidant contains large amount of polyphenols. Recent studies have shown that total phenols determined by this method can be correlated to antioxidant activity determined by different methods particularly TEAC and DPPH [72].

2.9. Data handling

Data evaluation allows the extraction of useful information in order to form conclusions about the input representing the concentration of the sample under investigation and the output representing the experimental response from the instrument used. It allows qualitative and quantitative analysis of the sample.

An analytical result is deemed to be reliable when it has been shown to be sufficiently accurate so that any decision based on it can be taken with confidence and on its merit. For example, if an experimental measurement is carried out repeatable on the same sample, more often than not different individual values are frequently obtained. The measurement is thus considered to be a random variable. The method must therefore be validated in order to derive useful information from

the experimental data and to draw robust conclusions about the validity of the method.

This section highlights the criteria used for evaluating analytical methods termed figure of merits as listed in Table 2.5.

Table 2.5: Figures of Merit for Analytical Methods.

Criterion	Figure of Merit
1. Precision	Absolute standard deviation, Relative standard deviation, coefficient of variation and Variance
2. Accuracy	Absolute systematic error and relative systematic error
3. Sensitivity	calibration sensitivity and analytical sensitivity
4. Detection Limit	Blank plus three times standard deviation of the blank
5. Dynamic range	Concentration limit of quantitation(LOQ) to concentration limit of linearity (LOL)
6. Selectivity	Coefficient of selectivity

2.9.1. Figures of merit for Analytical methods

2.9.1.1. Precision

Precision refers to closeness of agreement between a series of measurements obtained from multiple sampling of the same or similar homogeneous sample under specified conditions. The specified conditions can be repeatability conditions of measurement, intermediate precision conditions of measurement or reproducibility conditions of measurement [88]. The precision can be assessed by expressing numerically the random error or the degree of dispersion of a set of individual measurements by means of the standard deviation (s), the variance or coefficient of variation ($\%CV$) and percentage relative standard deviation ($\%RSD$).

Repeatability of a measurement method which is also termed "intra-day assay precision" is the agreement between the results of successive measurements of the

same measurand (analyte) carried out under the same conditions of measurement such as instrumental, preparation and geographical over a short period of time [88]. Repeatability is dependent on analyte concentration. It is determined at different levels of concentrations (low, medium and high) prepared in triplicate and covering the whole analytical range under study (3 levels x 3 replicates per level = 9).

The coefficient variation which is frequently stated as a percentage (*%CV*) is an estimate of the standard deviation of a population from sample of *n* results divided by the mean [90]. The lower the *%CV*, the more precise the method. Equations for calculating standard deviation and coefficient variation are shown below: x_i is the measurement made, *x* with a bar on top is the average of all measurements and *N* is the total number of measurements made.

$$s = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}(x_i - \overline{x})^2} \qquad \textbf{2.24}$$

$$CV = \frac{s}{\overline{x}} \times 100 \qquad \textbf{2.25}$$

In order to examine the repeatability of the method the average (mean) and standard deviation of a set of ten measurements (for each concentration level) made on the same day and under the same conditions, is determined.

Reproducibility is the closeness of the agreement between the results of measurements of successive measurements of the same measurand (analyte) carried out under changed conditions of measurement [89]. It is the largest measure of precision (worst precision) normally encountered in a measurement method. Changed conditions could mean changing temperature at which the analysis is done, having different analysts perform the analysis or performing the analysis of the same sample in different laboratories using different instruments. The reproducibility limit, *R*, is defined as "the value less than or equal to which the absolute difference between two test results obtained under reproducibility conditions may be expected to be with a probability of 95% [91].

Intermediate precision which is also termed "inter-day variation" refers to the agreement between the results from within laboratory variations due to random events such as different days, different analysts and use of different equipments. An

intermediate measure of reproducibility was identified for the purpose of intra laboratory method validation where it was decided to test the reproducibility by performing the analysis of the same sample on different days.

2.9.1.2. Accuracy or Bias

The bias of a measurement may be described as the degree to which the method yields results that are consistently different from the sample's true value due to the systematic error inherent in the method and [89,92]. Accuracy is the closeness of an experimental measurement or result to the true or accepted value [93]. Accuracy estimates the extent to which systematic errors affect a particular method and can be determined by several approaches such as:

(i) comparison to reference material (measuring the analyte in a particular reference material and comparing the result with the certified value).

(ii) recovery of the analyte spiked into blank matrix (measuring the analyte in blank matrix samples spiked with known analytical concentrations and determining the percentage).

(iii) standard addition to the analyte (determining the analytical concentration in the sample by means of standard addition technique).

It is not unusual for the accuracy to be indeterminate because of the incoming sample that is not identified with a true value, hence the use of equation 2.26 in assessing accuracy.

$$\text{Recovery (\%)} = \frac{Xs - X}{Xadd} \times 100 \qquad \textbf{2.26}$$

where: X_s = mean result of spiked samples, X = mean result of unspiked samples and X_{add} = amount of added analyte.

2.9.1.3. Sensitivity

Sensitivity of a method (or an instrument) is a measure of its ability to discriminate between small differences in analyte concentrations at a desired confidence level. The simplest measure of sensitivity is the slope of the calibration curve in the concentration range of interest. This is referred as the calibration sensitivity. Usually the calibration for instruments are linear and are given by an equation of the form

$$S = m_c + S_{bl} \qquad \textbf{2.27}$$

where S is the signal at concentration c and S_{bl} is the blank (i.e. signal in the absence of analyte. The m is the slope of the calibration curve and hence the sensitivity.

However, when sample preparation is involved, recovery of these steps has to be factored in. For example, during extraction, only a fraction proportional to the extraction efficiency (r) is available for analysis .Then equation 2.27 reduces to

$$S = mr_c + S_{tbl} \qquad \textbf{2.28}$$

Now the sensitivity is mr rather than m. The higher recovery the higher the sensitivity. The blank is also modified by the extraction efficiency in the sample preparation step; S_{tbl} refers to the blank that arises from total contribution from sample preparation and analysis. Since the precision decrease at low concentrations, the ability to distinguish between small concentration differences also decreases. Therefore, sensitivity as a function of precision is measured by analytical sensitivity, which is expressed below as follows [94]:

$$A = mr/S_s \qquad \textbf{2.29}$$

where S_s is the standard deviation based on sample preparation and analysis. Due to its dependence on S_s, analytical sensitivity varies with concentration.

2.9.1.4. Limit of detection (LOD)

Limit of detection is defined as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified. Different criteria are used for

evaluating the LOD. The LOD may be estimated by visual evaluation, signal-to-noise ratio, response standard deviation and slope or based on *%RSD*.

The signal to noise relationship is determined by comparing the analytical signals at known low concentrations with those samples up to an analytical concentration that produces a signal equivalent to three times the standard deviation of the blank sample ($3.3 \times \sigma_{blank} / S$).

2.9.1.5. Limit of quantification (LOQ)

The limit of quantification is defined as the lowest concentration or amount of analyte that can be determined with an acceptable level of accuracy and precision. Similar to LOD, LOQ is evaluated by using different approaches, that is:

(1) visual evaluation: samples with known analytical concentration are prepared and the minimum level at which the analyte can be quantified with an acceptable level of uncertainity is established.

(2) signal/noise ratio: the signals of samples with known analytical concentrations are compared with those of blank samples up to an analytical concentration that produces signal equivalent to 10 times the standard deviation of the blank ($10 \times \sigma_{blank}/S$) in a more general context is defined as the lowest amount of analyte that can be reproducibly quantified above the LOD ($LOQ = 10\ \sigma_{blank} / S$)

(3) standard deviation/slope ratio ($LOQ = 10\ \sigma_{blank}/S$): the parameters are calculated in the same manner as LOD.

2.9.1.6. Linearity and range

Linearity is the ability of an analytical procedure to produce test results which are proportional to the concentration (amount) of the analyte in the sample within a given concentration range, either directly or by means of well-defined mathematical transformation. It can be assessed by performing single measurements at several analyte concentrations. The linearity of a method measure how well a calibration plot

of analytical response *vs.* analyte concentration approximates a straight line or how well the data fit to the linear equation:

$$y = mx + c \qquad \textbf{2.30}$$

where: y = analyte response/measured signal, x = analyte concentration of sample, m = slope of a line fit to the data (tangent), is a measure for the sensitivity of the procedure; the steeper the slope the more sensitive the procedure and c = intercept of a line fit to the data.

The range of an analytical procedure is normally derived from the linearity studies and is the interval between the upper and lower concentrations (amounts) of the analyte in the sample (including these concentrations) that can be determined with acceptable precision, accuracy and linearity [95]. The linear (or calibration, or dynamic) range corresponds to the valid interval of functional dependence of the signal on concentration [96]. If there is any doubt of the linearity, a t-test should be performed to test for significant non-linearity.

The working (or analytical) range which is wider than the linear range describes the interval between the lowest (limit of detection) and the highest concentration where the signal can be related to the concentration for the evaluation of random and systematic errors.

2.9.1.7. Selectivity and Specificity

The measurement of an analyte may be disturbed by the presence of other components. The measurement is then non-specific for the analyte under investigation. Selectivity is defined as the ability to accurately quantify the compound of interest, also in the presence of other compounds [96]. Specificity is considered to be the ultimate in selectivity; it means that no interferences are supposed to occur. Specificity takes the degree of interference from other sample constituents into account and therefore ensures that a signal's response is due to a single compound without interferences. Selectivity can be measured by spiking known levels of impurities into a sample with a known amount of the analyte of interests, by

comparison of the slopes obtained by the standard addition and the external standard methods.

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Chapter 3

EXPERIMENTAL STUDY

3.1. Introduction

In this chapter, reagents, standards and samples that were used throughout the work are described. Several aspects related to the antioxidant activity determination methods, namely the optimization procedures and the statistical treatment used to assess the quality of the results are also described in detail in the following sections.

3.2. Chemicals

All reagents were of analytical grade unless otherwise indicated. The following chemicals were purchased from Sigma-Aldrich Company, Johannesburg, South Africa: Trolox (6-Hydroxy-2,5,7,8,- tetramethylchromane-2-carboxylic acid), quercetin, (+)-catechin hydrate, monohydrate gallic acid, anhydrous sodium carbonate, sodium acetate, copper (II) chloride dihydrate, ammonium acetate, DPPH$^{\bullet}$ (2,2-diphenyl-1-picrylhydrazyl), neocuproine (2,9-dimethyl-1,10-phenanthroline), Folin-Ciocalteu (FC) phenol reagent, and alumina powder (PK-4 polishing kit).

All solvents used were HPLC grade and were obtained from Sigma-Aldrich Company, Johannesburg, South Africa: methanol, acetic acid and ethanol.

The water used in this research, labelled as dH_2O (deionized water) was produced from a Millipore Milli-QTM water system (Millipore Corporation, Milford, MA) supplied by LASEC, Cape Town, South Africa.

3.3. General instrumental equipment

Scientific Laboratory Equipment Company (LASEC) Cape Town, SA supplied the following: A Varian Cary 300 UV-VIS spectrophotometer (Shimadzu Scientific Instruments, Columbia, MD), Epsilon Bioanalytical Systems Inc. (BASI) potentiostat

using 3 electrode system, pH meter, Grand mixer (GM 200 Retsch Grindomix), Vortex 2 Genies (Scientific Industries (SI), USA, model no G560E), Meter CP 64 Sartorius weighing balance, Rotary evaporator (Buchi, Switzerland).

LABOTEC, Johannesburg, SA supplied the following: Bench top Hettich Zentrifugen Rotanda 360A and Heidolph MR 3001 K magnetic stirrer.

3.4. Methods

Experimental work was performed in three different phases. The first phase was the pre-treatment of *Athrixia phylicoides* leaves and extraction of antioxidants (polyphenols) from the pre-treated leaves. This part included the determination of *Athrixia phylicoides* phenolic compounds. Moreover, the effects of extraction solvents, infusion time and sample to solvent ratio changes on the spectrophotometric analysis of polyphenolic compounds were also involved. The second phase was the comparison of three fundamentally different methods for measuring antioxidant capacity. In this phase, the methods for the characterization of bioactive (polyphenolic) compounds and the methods used for the determination of antioxidative properties of the bioactive compounds are given. Moreover the analytical performance characteristics of each method were evaluated. The last phase belongs to the application of the better method(s) for comparison of antioxidant activity of different tea samples (commercial teas vs unprocessed *Athrixia phylicoides* leaves). The experimental procedure followed in this study is schematically represented in Figure 3.1.

3.4.1. Phase 1: pre-treatment, extraction and polyphenol content

3.4.1.1. Pre-treatment of plant materials

The tea samples that were used for this study are processed rooibos tea, English tea and Chinese tea distributed for commercial purpose and unprocessed *Athrixia phylicoides* (bush tea) leaves. The aerial parts of *Athrixia phylicoides* were bought from street vendors. The leaves were washed with deionized water and allowed to

air dry under shade under ambient conditions. The taxonomy of *Athrixia phylicoides* sample was authenticated by Dr. B Egan. The dried leaves were then ground with a grinding mill and were kept in dark in well-closed container at 4°C.

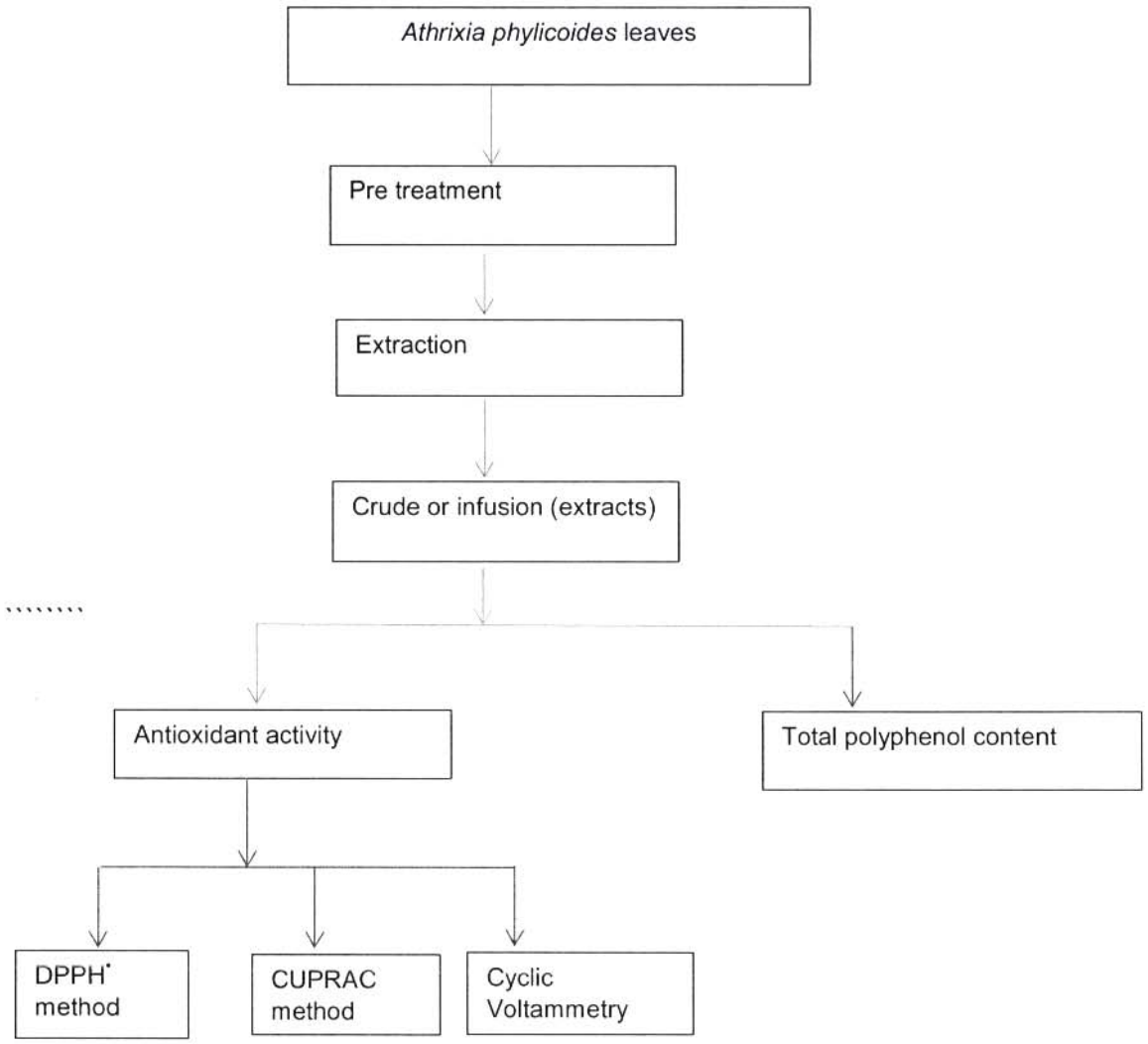

Figure 3.1: Experimental procedure of the study.

3.4.1.2. Extraction studies of polyphenols from *Athrixia phylicoides* leaves

Extraction as described in this work is the isolation or removal of a particular analyte either from a complex matrix or a designated sample. The solvent type is the most important factor affecting the efficiency of liquid solid extraction. For this reason, three different types of solvents were combined in varying ratios or percentage viz (acetic acid/water, methanol/water and deionized water) to investigate the best extraction combined solvent to extract bioactive compounds from A*thrixia phylicoides*

leaves. The extracts from all extraction procedure were treated in two ways: the filtrate obtained and analysed in the form of infusion (direct sample) and concentrating the infusion using a rotary evaporator or air-drying and keeping the solid at 4°C until analysis (crude sample).

Methanol / water (70:30, v/v) extract was obtained by weighing the *Athrixia phylicoides* leaf powder of 0.2000 ± 0.001 g and transferring it to extraction tube, and then 5.00 mL of the solvent mixture was added. The extract was heated at 70°C in the water bath for 10 min with constant mixing in the vortex mixer at 5 min intervals. After cooling to room temperature the extract was centrifuged at 200 rpm for 10 min in order to enhance cell breakage and to separate the particles from the extract. The supernatant was then decanted into a graduated tube. The extraction step was repeated twice as way to avoid loss of essential components. Both extracts were mixed and the volume adjusted to 10.00 mL with the methanol/water mixture.

Acetic acid / water (30:70, v/v) extract was obtained following the same procedure used in methanol extract. Both extracts were mixed and the volume adjusted to 10.00 mL with the acetic acid/water mixture.

Deionized water was also used to extract the polyphenols from *Athrixia phylicoides* leaves. The rationale here was to mimic the real conditions during the consumption of this hot beverage in households. Moreover, the effect of infusion time and sample to solvent ratio on total polyphenol content and antioxidant constituents was investigated.

To determine the effect of infusion time, aqueous extracts were obtained by weighing 2.000 ± 0.001 g of dried *Athrixia phylicoides* leaves into 500 mL beaker. To the beaker 250.00 mL boiled dH_2O was added, heated and stirred on the magnetic strirrer at 100°C for 3, 5 and 10 min, respectively. The mixture was strained through a tea strainer followed by filter paper (Whatman GF/C).

The other aqueous extracts were prepared by weighing 2.000 ± 0.001 g of dried *Athrixia phylicoides* leaves into a 500 mL beaker and infused in (100.00, 200.00 and 300.00) mL of dH_2O at 100°C followed by stirring with a magnetic stirrer. After 5 min steeping at room temperature, the extracts were strained through a tea strainer followed by filter paper (Whatman GF/C).

3.4.1.3. Determination of total polyphenol content

The total phenols content of extracts were estimated by a colorimetric assay proposed by Singleton, which is based on the reduction of phosphotungstic acid complex by aromatic phenolic groups to blue-coloured products [17]. In brief, 1.00 mL of appropriately diluted sample was mixed with 5.00 mL of Folin-Ciocalteu reagent (1:10, v/v, diluted with dH_2O) and 4.00 mL of 15% (w/v) sodium carbonate in a 20 mL test tube. The contents were vortexed for 15 seconds and then left to stand at room temperature for 1 hour before the absorbance against the reagent blank at 765 nm was measured on UV-visible spectrophotometer. The total phenolic content was determined from a gallic acid calibration curve prepared and analysed concurrently with the different extracts. All samples were analysed in triplicate.

3.4.2. Phase 2: Analytical methods for antioxidant activity

3.4.2.1. DPPH Radical Scavenging Activity method

A modification of the method of Brand-Williams was used [18]. The samples of *Athrixia phylicodes* leaves were prepared in the different extracting solvent at three to ten concentrations ranging from 25.00 to 250.00 mg/L to produce DPPH radical scavenging levels ranging from approximately 10 to 95%. Fresh solutions were prepared each day of analysis. A 0.50 mL aliquot of the sample solution was added to 2.00 mL of DPPH (0.06 mM) in a 20.00 mL test tube then transferred to 1 mL cuvette. A blank solution was prepared by adding 0.5 mL of the respective extraction solvent to 2.00 mL DPPH solution. Samples were stored at room temperature (22 ± 3°C) in the dark for 30 minutes. The decrease in absorbance was determined at 517 nm using a spectrophotometer until the absorbance stabilized. Trolox, quercetin and catechin were utilized as controls and treated in the method as the sample extracts measured at 517 nm. The (%) inhibition of DPPH radical was calculated from the absorbance data using the equation below according to Yen and Duh [19]:

$$\% \text{ inhibition against DPPH} = [(AB - AA)/AB) \times 100$$

where AB and AA are the absorbances of blank sample and test material.

3.4.2.2. Copper reducing antioxidant capacity (CUPRAC) method

In order to determine the cupric ion (Cu^{2+}) reducing ability of *Athrixia phylicoides* extracts, the method proposed by Apak was used with slight modification [20]. Briefly, 1.00 mL $CuCl_2$ solution (0.01 M), 1.00 mL ethanolic neocuproine solution (0.0075 M) and 1.00 mL ammonium acetate buffer solution (1 M) were added to each test tube, respectively. Then different concentration of *Athrixia phylicoides* extracts (25 - 250 mg/L dissolved in the respective solvents) or standard were added to test tube, separately. Finally, the total volume was adjusted to 4.1 mL with dH_2O, and mixed well. The tubes were stoppered, kept at room temperature and stored away from light for 30 min. Absorbance against a reagent blank was measured at 450 nm. Increased absorbance of the reaction mixture indicates increase reduction capability. Trolox, quercetin and catechin were used as the positive controls.

3.4.2.3. Cyclic voltammetry

Voltammetric measurements were carried out in a standard three - electrode electrochemical cell with a glassy-carbon (GC) electrode of 3 mm diameter, a saturated calomel reference (SCE) electrode and platinum (Pt) gauze auxiliary electrode. Prior to each run, the surface of the glassy carbon electrode was polished on a polishing pad with alumina powder (0.05 µm), rinsed with distilled water and degreased in ethanol in ultrasonic bath. Using a procedure described by Piljac-Zegarac, samples were prepared by diluting *Athrixia phylicoides* extracts 50 times in 0.1 M sodium acetate - acetic acid buffer, pH = 3 [21]. The samples in the electrochemical cell were de-aerated by purging with high purity nitrogen during the measurements. The potentials were recorded against saturated calomel reference electrode used in conjunction with a platinum counter electrode, placed in 50 mL volume cell together with glassy-carbon working electrode. The scan was taken in the potential range between 0 and 800 mV with a scan rate 100 $mV.S^{-1}$. Prior to each measurement solutions in the diluted extracts solution background currents were measured in the acetate buffer alone, and subtracted from the currents measured in the extracts solution. In order to minimize the adsorption of antioxidants on the electrode surface measurement was performed instantly after the immersion

into the solution. Cyclic voltammograms were also recorded for the Trolox in the concentration range (10 – 70 µM). All measurements were done in, at least triplicates.

3.4.2.4. Evaluation of performance parameters for the analytical methods

3.4.2.4.1. Robustness

The robustness of the two methods (CUPRAC and DPPH) were evaluated. The preparation of analytical solutions (DPPH radical, Cu (II) neocuproine complex, Trolox) was modified to produce deliberate small condition changes. Each tested robustness parameter was evaluated in triplicate. The stability of Trolox stock solution was evaluated by storing different Trolox stock solution (1000 µM) aliquots at room temperature (RT) and at 8°C for a period of 24 hours before being diluted (100 µM) and analysed. Results were compared to those obtained from analysis with fresh Trolox stock solution and evaluated for significant differences. The thermal decomposition of DPPH radical, Cu (II) neocuproine complex was evaluated at different times of incubation (15 min, 30 min, 60 min and 120 min). Results were evaluated for significant differences.

3.4.2.4.2. Linearity and range

It is mandatory that calibration standards should encompass the entire analytical range and be evenly distributed across it [22]. Generally a minimum of five to eight calibration points are required, however it is recommended to use fewer rather than more calibration levels and perform more replicates. Trolox calibration standards ranging from 10 µM – 100 µM were prepared in methanol. The standards and blank sample were all analysed in triplicates and the results obtained were used to evaluate and establish the linear range for the assays. The percentage relative standard deviation of the responses were plotted against the respective concentration levels, and the concentration range in which the %RSD is equal or less than 5% was used to establish the linear range. Additionally the extracts were each appropriately diluted spanning four concentration levels within the expected linear working range. Their responses were evaluated for linearity with acceptable

accuracy and precision by appropriate statistical methods such as least squares linear regression.

3.4.2.4.3. Accuracy and precision

The methods ability to measure antioxidant capacity of a sample with accuracy and precision was determined by recovery studies using the standard addition recovery method. The standard addition method was performed by the addition of varying concentrations (within the linear range) of Trolox calibration standards (10 µM, 20 µM and 30 µM) to appropriately diluted A*thrixia phylicoides* extracts and a low concentration of Trolox sample (5 µM) which had already been analysed prior to the Trolox standard addition. The measured concentration was compared to the expected concentration of the Trolox spiked samples and percentage recovery was calculated.

3.4.2.4.4. Limit of detection and quantification

A series of blank samples (n = 8) were assayed on three consecutive days in triplicate and the standard deviations determined. Limit of detection and limit of quantification were determined from the standard deviation of response (y-intercept) of the blank samples and slope (from Trolox calibration curve) of the regression equation using:

$$\text{LOD} = \frac{3.3\sigma}{S} \qquad \text{LOQ} = \frac{10\sigma}{S}$$

3.4.3. Phase 3: Comparison of total antioxidant capacity of athrixia phylicoides with different teas sample

The influence of solvent polarity (water (1) 1:125 sample (g) to solvent (mL) ratio and (2) 1:150 sample (g) to solvent (mL) ratio; 70% methanol and 30% acetic acid) on total polyphenol content (TPC) and antioxidant activity were studied on samples for comparison. Samples used were A*thrixia phylicoides* leaves (APL) and different

processed teas viz. *C. sinensis*, Chinese green tea (CGT) and Joko black tea (JBT), *A. linearis,* Laager rooibos tea (LRT). The antioxidant capacities and total phenolic content of extracts obtained from the three solvent forms were assessed by Cupric Ion Reducing Antioxidant Capacity (CUPRAC), 2,2-diphenyl-1-picrylhydrazyl Radical Scavenging Capacity (DPPH) and Folin-Ciocalteu reducing method (FCR).

3.4.4. Statistical analysis

The mean, standard deviation, and coefficient of variance (COV) were determined for all data. The statistical software package was used to analyse data. Analysis of variance (ANOVA) was used to ascertain whether the means between sample/standard experimental groups were significantly different at a 95% confidence level ($p < 0.05$ considered/ indicated significant differences). The paired t-test was used to show differences between two samples/standard experimental groups.

3.5. References

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Chapter 4

RESULTS AND DISCUSSION

4.1. Introduction

This chapter compromises four sections. The first and second sections discusses the findings of the research, with emphasis on the effect of extraction solvent on the total polyphenol content and antioxidant activity as well as the determination antioxidant capacity of *Athrixia phylicoides* leaves using DPPH$^{\bullet}$, CUPRAC and CV respectively. The third section evaluates the methodologies used with regard to the rate of analysis, accuracy, precision, sensitivity, limit of detection, dynamic range and selects the best method. In the final section the total phenolic content and antioxidant activity of A*thrixia phylicoides* leaves in comparison with different processed teas (green tea, black tea and rooibos tea) is determined using FCR, CUPRAC and DPPH.

4.2. Extraction Studies

4.2.1. Influence of solvent type on extraction of bioactive compounds from *Athrixia phylicoides* leaves

In this study a comparison of the effect of solvent properties on the total polyphenol content and antioxidant capacity of A*thrixia phylicoides* leaves was done. The three solvent forms used were aqueous methanol, aqueous acetic acid and pure distilled water. The data for the total polyphenol content (TPC) and antioxidant capacity of A*thrixia phylicoides* extracts are presented in **Tables 4.1, 4.2 and 4.3**. The results were expressed as means ± standard deviation (SD). Any significant differences between the solvents and samples were determined by one-way ANOVA, followed by Tukey's test for multiple comparison considering differences statistically significant at $P < 0.05$. Trolox, catechin and gallic acid were used as reference compounds.

The polyphenols have been reported to be the important antioxidant compounds in tea extracts by a number of researchers [1 - 3]. It is not clear which solvent system is more effective in extracting phenolic content of different materials. In general polyphenols in plants are polar compounds, which are usually extracted with polar solvents such as methanol, ethanol, propanol, acetone in their absolute and with different proportions of water. The extracting solvents significantly affected the total phenolic content of *Athrixia phylicoides* leaves (Figure 4.1). The TPC in terms of gallic acid equivalent (GAE) and catechin equivalent (CE) for all the *Athrixia phylicoides* extracts ranged from 2.66 to 170 mg/100g and from 3.88 to 296 mg/100g, respectively. The catechin equivalent amounts of extracts were twice the gallic acid equivalent, the reason is the two reference antioxidant compounds differ in polarity and one may have a phenolic group common with the main active plant constituent.

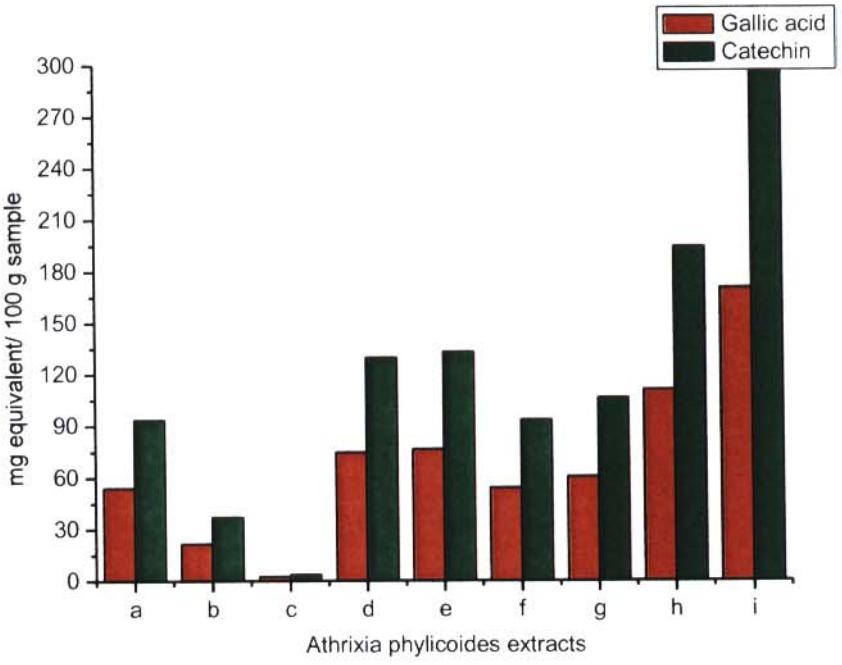


Figure 4.1: Comparison of the effect of the extraction solvent on total polyphenol content of *Athrixia phylicoides* extracts expressed as gallic acid equivalent and catechin equivalent. (a) – (c) solvent types (water, methanol and acetic acid, respectively), (d) – (f) infusion time (3 min, 5 min and 10min, respectively), (g) – (i) sample:solvent ratio (1:50, 1:10 and 1:150, respectively).

When the results of the different extracting solvents used in Table 4.1 were compared, water extractant gave the highest level of phenolic content (54.09 ± 0.78 mg GAE/100 g sample) followed by 70% methanol (21.54 ± 0.35 mg GAE/100 g sample). The lowest amounts of total phenolic content were obtained with 30% acetic acid (2.66 ± 0.22 mg GAE/100 g sample). This can be attributed to different affinities of the extraction solvent in terms of their extraction conditions, such as polarity of extracting solvents, and temperature [4]. It is clear from these results that there was a decreasing trend proportional to diminishing dielectric constant (ε) of each solvent (water, = 80, methanol, = 33, acetic acid, = 6.15) and higher content of phenolics was obtained with an increase in polarity of solvent used. Therefore water proved to be a very effective solvent for the extraction of polyphenols from A*thrixia phylicoides* leaves.

Table 4.1: Comparison of the effect of solvent on total polyphenol content and antioxidant activity from A*thrixia phylicoides* leaves.

Extracts	**TPC** (mgGAE/mgCE)	**DPPH** ($^{1}EC_{50}$, mg/mL) or TEAC value	**CUPRAC** ($^{2}EC_{50}$, mg/mL) or TEAC value	**CV** (Epa, mV)
Water	$^{a}54.09\pm0.78^{1a}$ $^{b}93.61\pm1.38^{1b}$	$^{c}0.084$ or 0.62^{2c} $^{d}0.126$ or 0.41^{1d}	$^{c}0.031$ or 7.23^{1c} $^{d}0.213$ or 1.05^{1d}	$^{c}429^{1c}$
Methanol	$^{a}21.54\pm0.35^{2a}$ $^{b}37.28\pm0.63^{2b}$	$^{c}0.039$ or 1.33^{1c} $^{d}0.170$ or 0.30^{2d}	$^{c}0.093$ or 2.41^{2c} $^{d}0.224$ or 1.00^{2d}	$^{c}490^{2c}$
Acetic acid	$^{a}2.66\pm0.13^{3a}$ $^{b}3.88\pm0.23^{3b}$	$^{c}0.122$ or 0.43^{3c} $^{d}0.202$ or 0.26^{3d}	$^{c}0.124$ or 1.81^{3c} $^{d}0.233$ or 0.96^{3d}	nd

Values followed by same number in a column are not statistically significant from each other at $P < 0.05$ measured by ANOVA one way. [a]expressed as mg gallic acid equivalent/ 100g sample, [b]expressed as mg catechin equivalent/ 100g sample, [c]analysis performed on samples after removing the solvent; [d]analysis performed on samples on the same day, $^{1}EC_{50}$ - the extract concentration able to inhibit 50% of the used DPPH amount, $^{2}EC_{50}$ – the extract concentration at absorbance 0.5, E_{pa} – anodic peak potential, nd – not detected.

The extracting solvent also influenced the antioxidant capacity methods but not in the same way (Table 4.1). The direct analysis of DPPH and CUPRAC as well as the concentrated analysis of CUPRAC and CV gave the same trend as the TPC. In addition the extracting solvent influence was weakest in all direct analysis compared to the corresponding concentrated analysis. Interestingly the concentrated methanol extract in DPPH did not correlate with the corresponding methanol extract of TPC.

Table 4.2: Comparison of the effect of infusion time on total polyphenol content and antioxidant activity from *Athrixia phylicoides* leaves.

Aqueous extracts	**TPC** (mgGAE/mgCE)	**DPPH** ($^1EC_{50}$, mg/mL) or TEAC value	**CUPRAC** ($^2EC_{50}$, mg/mL) or TEAC value	**CV** (Epa, mV)
3 min	$^{a}74.77\pm2.2^{2a}$ $^{b}129.76\pm3.9^{2b}$	$^{c}0.066$ or 0.79^{1c}	$^{c}0.073$ or 3.07^{2c}	$^{c}513^{2c}$
5 min	$^{a}76.45\pm1.36^{1a}$ $^{b}133.14\pm2.40^{1b}$	$^{c}0.098$ or 0.53^{2c} $^{d}0.144$ or 0.36^{2d}	$^{c}0.128$ or 1.75^{3c} $^{d}0.270$ or 0.83^{2d}	$^{c}429^{1c}$
10 min	$^{a}54.09\pm0.78^{3a}$ $^{b}93.61\pm1.38^{3b}$	$^{c}0.084$ or 0.62^{2c} $^{d}0.126$ or 0.41^{1d}	$^{c}0.031$ or 7.23^{1c} $^{d}0.213$ or 1.05^{1d}	$^{c}429^{1c}$

Values followed by same number in a column are not statistically significant from each other at $P < 0.05$ measured by ANOVA one way. [a]expressed as mg gallic acid equivalent/ 100g sample, [b]expressed as mg catechin equivalent/ 100g sample, [c]analysis performed on samples after removing the solvent; [d]analysis performed on samples on the same day, $^1EC_{50}$ - the extract concentration able to inhibit 50% of the used DPPH amount, $^2EC_{50}$ – the extract concentration at absorbance 0.5, E_{pa} – anodic peak potential.

Total polyphenol content in addition to solvent type, particularly polarity is also influenced by infusion time and mass sample to solvent ratio. Three infusion periods: (3, 5, and 10) min were evaluated using deionised water as a solvent in order to determine the optimum extraction time for obtaining peak antioxidants concentration

without making the procedure too long for routine use (Table 4.2). The TPC from *Athrixia phylicoides* extracts increased with longer steeping time up to 5 min (from 3 min (74.77 ± 2.24) to 5 min (76.45 ± 1.36) mg GAE/100g sample) then decreased (at 10 min, 54.09 ± 0.78 mg GAE/100g sample). Results obtained from this study are in total agreement with the work previously done, confirming that TPC from tea is correlated with infusion time [5,6]. Additionally, Cheong and Perva-Uzunalić showed that prolonged infusion time at high temperature can lead to degration of phenolic compounds, hence the decrease in the extracts total phenolic content [7,8].

Other researchers found that the extraction of the phytochemical compounds, namely flavonoid was completed at approximately 4 min which were in contrast with results from this study (Figure 4.1) [9,10]. In this study expressing results with another type of flavonoid reference compound, the 5 min steeping time gave the highest TPC (133.14 ± 2.40 mg CE/100 g). The steeping time beyond 5 min caused a decrease in TPC (93.61 ± 1.38 mg CE/100g sample).

The total antioxidant activity of *Athrixia phylicoides* leaves prepared by different infusion conditions found with DPPH, CUPRAC and CV are shown in Table 4.2. The results showed a different tendency among the antioxidant capacity methods and to the TPC. In the CUPRAC and CV method, the EC_{50} and Ep_a values were higher in 10 min extract than in 3 min and 5 min extracts. The two methods measure mainly the reducing ability of the extracts and have important difference in their response towards antioxidant. In DPPH method which measures the hydrogen donating ability of the extract towards DPPH radical, the EC_{50} value was higher in 3 min extract compared to 5 min and 10 min extracts. The inconsistencies in the trends could be most likely attributed to the differences in experimental methodology used for each method, and also to some unrelated reactions, such as polymerization, that probably occur in the reaction mixtures.

Various ratios of sample mass to extraction solvent volume were evaluated (from 1 g: 50 mL (2%) to 1 g: 150 mL (0.66%), (Table 4.3). Among the various water-based systems, the 1:150 ratio extract was the most efficient, with higher total phenolic content values obtained (170.07 ± 4.64 mg GAE/g sample). There is a general trend where sample mass to solvent ratio is proportional to the total phenolic content and antioxidant capacity obtained. This is consistent with the principles of mass transfer

where the driving force during mass transfer is the concentration gradient between the solid and the bulk of the liquid, which is greater when a higher solvent-to-solid ratio is used [11].

Table 4.3: Comparison of the effect of sample to solvent ratio on total polyphenol content and antioxidant activity from *Athrixia phylicoides* leaves.

Aqueous extracts	TPC (mgGAE/mgCE)	DPPH ($^1EC_{50}$, mg/mL) or TEAC value	CUPRAC ($^2EC_{50}$, mg/mL) or TEAC value	CV (Epa, mV)
1:50	$^{a}60.59\pm2.19^{3a}$ $^{b}106.19\pm3.87^{2c}$	$^{c}0.057$ or 0.91^{2c} $^{d}0.172$ or 0.30^{2d}	$^{c}0.065$ or 3.50^{1c} $^{d}0.311$ or 0.71^{2d}	$^{c}429^{3c}$
1:100	$^{a}110.70\pm3.02^{2a}$ $^{b}194.23\pm5.37^{2b}$	$^{c}0.041$ or 1.27^{1c} $^{d}0.194$ or 0.27^{3d}	$^{c}0.064$ or 3.45^{1c} $^{d}0.314$ or 0.72^{2d}	$^{c}339^{1c}$
1:150	$^{a}170.07\pm4.64^{1a}$ $^{b}296.42\pm8.16^{1b}$	$^{c}0.039$ or 1.33^{1c} $^{d}0.151$ or 0.34^{1d}	$^{c}0.065$ or 3.45^{1c} $^{d}0.258$ or 0.87^{1d}	$^{c}378^{2c}$

Values followed by same number in a column are not statistically significant from each other at $P < 0.05$ measured by ANOVA one way. [a]expressed as mg gallic acid equivalent/ 100g sample, [b]expressed as mg catechin equivalent/ 100g sample, [c]analysis performed on samples after removing the solvent; [d]analysis performed on samples on the same day, $^1EC_{50}$ - the extract concentration able to inhibit 50% of the used DPPH amount, $^2EC_{50}$ – the extract concentration at absorbance 0.5, E_{pa} – anodic peak potential.

The results obtained for sample to solvent ratio effect on the antioxidant capacity of *Athrixia phylicoides* leaves are shown in Table 4.3. The sample to solvent ratio influence was weakest in the DPPH and CV method (direct analysis) as well as in CUPRAC method (direct and concentrated analysis). From EC_{50} values obtained, it can be concluded that 1:150 ratio extract were more effective in DPPH radical scavenging, however in CUPRAC method all the extracts displayed similar reducing

potential. The low oxidation exhibited by 1:50 extract is associated with the greater strength of the extract for the electron donation at the glassy carbon electrode.

4.3. Analytical methods

4.3.1. DPPH radical scavenging method

Phenolic compounds exhibit their antioxidant activity through their radical scavenging effect via different pathways. Radical scavenging activity is very important owing to the delirious role of free radicals in biological systems and generally proceeds via hydrogen atom transfer or donation of electrons [12]. DPPH radical scavenging method was performed to estimate the free radical scavenging capacity of *Athrixia phylicoides* extracts. Solvents used for polyphenol extraction had significant effect on the DPPH scavenging capacity determination from *Athrixia phylicoides* extracts.

DPPH method has been widely used in antioxidant capacity studies of plant extracts [1]. The method is based on the reduction of alcoholic DPPH solution at 517 nm in the presence of an hydrogen donating antioxidant [14] and polyphenols have been reported to be potent hydrogen donors to the DPPH radical [15] because of their favourable structural chemistry [16].

The *Athrixia phylicoides* extracts exhibited a concentration dependent antiradical activity by inhibiting the DPPH radical (Figures 4.2, 4.3 and 4.4). At each competitive concentration points the concentrated extracts regardless of the type of solvent used displayed a higher scavenging activity than the direct extracts, this may be due to the sensitivity of the method. Dawidowicz, Musa, Kedare and Singh, reported that the sensitivity of DPPH method is affected by factors such as the type and amount of solvent used, presence and concentration of hydrogen and metal ion, freshness of DPPH reagent, Lewis bases as well as oxygen [17 - 19].

All the extracts were able to reduce $DPPH^{\cdot}$ radical (visible deep purple colour) to the yellow – coloured non radical diphenylpicrylhydrazine (DPPH-H). At the lowest concentration of 0.075 mg/mL, the scavenging abilities of concentrated extracts water, 70% methanol and 30% acetic acid were (48.17, 92.82, and 29.76)%, respectively (Table B 1.3). The reaction mechanism of DPPH method is based on an

electron transfer reaction and the scavenging capacity is influenced by the solvent and the pH of the reaction [20], hence the methanol extract displayed high DPPH scavenging activity. The reference compounds quercetin, catechin and Trolox scavenged (40.98, 40.78 and 30.84)% of DPPH radicals at 0.075 mg/mL, respectively (Table B 1.1). All the standards were diluted ten times compared to the extracts, this proves that they are stronger DPPH radical scavenger than the extracts.

The methanol and water extracts exhibited the highest radical scavenging activity than the acetic acid extract regardless of the method of analysis, this might be the resultant of extracts containing good amounts of phenolic compounds, which show antioxidant activity due to their redox properties (absorbing and neutralising free radicals, quenching single and triple oxygen or decomposing peroxide). Furthermore, the less effectiveness in scavenging ability of acetic acid extract might be due to the fact that the extract contains a lower percentage of polyphenols which did not combine or complex easily with DPPH radicals.

All the reference compounds showed no flattening of the graph which is an indication of a complete reaction (Figure 4.2, 4.3 and 4.4). Brand-Williams found that the reaction time of antioxidant and DPPH radicals could be classified into three reaction kinetic types [21]. Many experiments assessed the scavenging ability on DPPH radicals after 30 min, hence all experiments were incubated in darkness for 30 min.

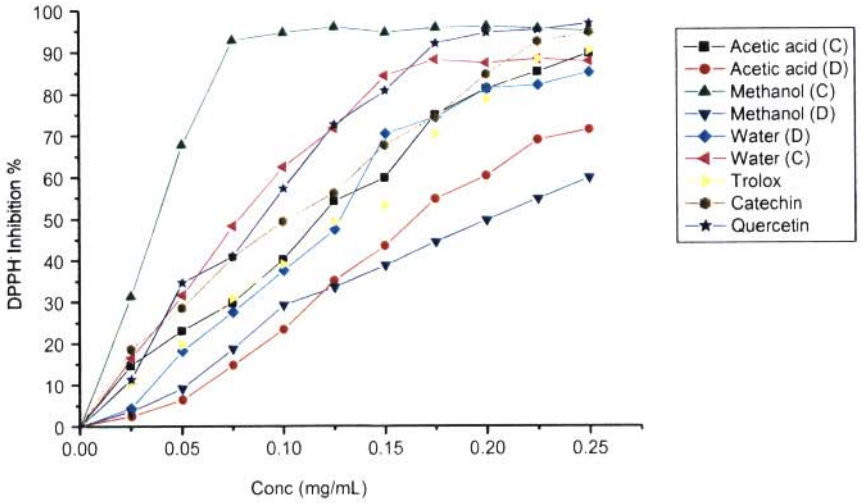


Figure 4.2: **DPPH radical scavenging activities of aqueous, methanol and acetic acid *Athrixia phylicodes* extracts at different concentrations compared with standards. (C) analysis performed on samples after removing the solvent; (D) analysis performed on samples on the same day.**

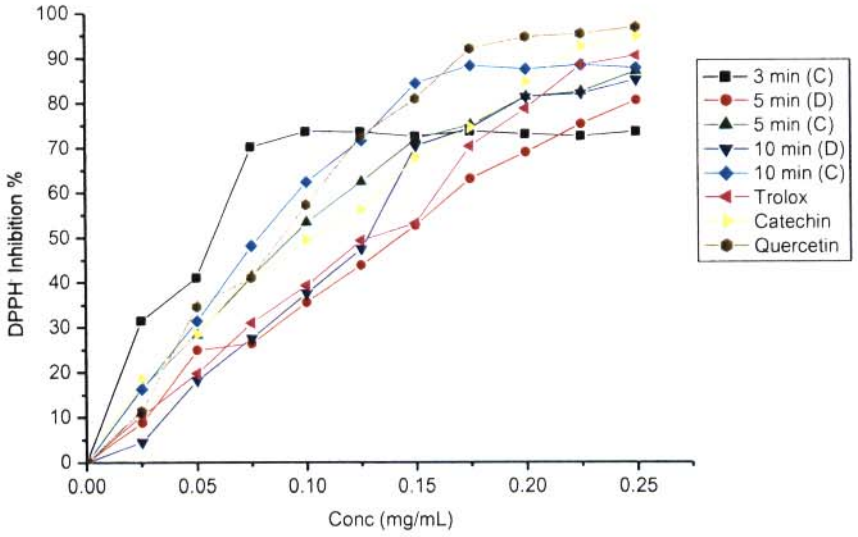


Figure 4.3: **DPPH radical scavenging activities of aqueous *Athrixia phylicodes* extracts (varying the infusion time) at different concentrations compared with standards. (C) analysis performed on samples after removing the solvent; (D) analysis performed on samples on the same day.**

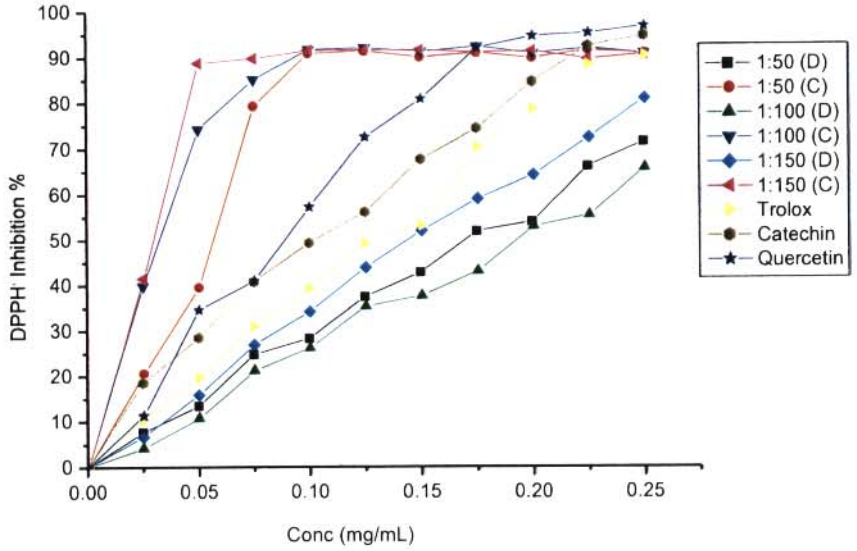


Figure 4.4: DPPH radical scavenging activities of aqueous *Athrixia phylicodes* extracts (varying sample to solvent ratio) at different concentrations compared with standards. (C) analysis performed on samples after removing the solvent; (D) analysis performed on samples on the same day.

The relative antioxidant capacity of all extracts comparing to the reference compound (Trolox) was defined as follows: the ratio of EC_{50} (Trolox)/EC_{50}(extract). Therefore the results of the study were normalised and expressed as TEAC values (mmol Trolox equivalent per gram sample). The EC_{50} is a parameter representing the extract concentration able to inhibit 50% of the used DPPH amount and it determines the effectiveness of the extracts, the lower the EC_{50} the higher is the antioxidant capacity [21]. There were large variations in the DPPH scavenging capacities among the *Athrixia phylicoides* extracts which indicate that the extraction solvent influenced significantly ($P < 0.05$) the DPPH scavenging activity.

Table 4.4: Comparison of EC_{50} and TEAC values of standards (Trolox, quercetin and catechin) obtained using DPPH method and CUPRAC method.

Standards	DPPH method				CUPRAC method			
	[1]EC_{50} (mg/mL)	[3]TEAC	[4]TEAC	[5]TEAC	[2]EC_{50} (mg/mL)	[3]TEAC	[4]TEAC	[5]TEAC
Trolox	0.013[3]	1.00	2.29	2.92	0.056[3]	1.00	0.83	0.98
Quercetin	0.009[1]	5.78	1.00	4.21	0.014[1]	16.0	1.00	3.94
Catechin	0.011[2]	4.73	2.71	1.00	0.016[2]	14.0	2.89	1.00

Values followed by same number in a column are not statistically significant from each other at $P < 0.05$ measured by ANOVA one way. [1]EC_{50} - the extract concentration able to inhibit 50% of the used DPPH amount, [2]EC_{50} – the extract concentration at absorbance 0.5, [3]TEAC value expressed as mmol Trolox equivalent/ g sample, [4]TEAC value expressed as mmol quercetin equivalent/ g sample, [5]TEAC value expressed as mmol catechin equivalent/ g sample.

Among the standards, quercetin gave the highest antioxidant capacity with EC_{50} value (0.009 mg/mL) followed by catechin (0.011 mg/mL) with the Trolox being the least by EC_{50} = 0.013 mg/mL (Table 4.4). The resulting order can be explained by the structure of the phenolic compounds presents in the standard. Studies of relationship between the chemical structure of phenolic compounds and their capacity to scavenge free radicals have shown that the scavenging activity depends on the number, position and nature of the substituents of the B and C cycles and on the degree of polymerisation [22 - 25].

Although there was no significant difference of the EC_{50} values (0.039 mg/mL) for methanol and water extract in the concentrated analysis the water extract exhibited considerably high antioxidant capacity in the direct analysis with EC_{50} value of 0.012 mg/mL (Table 4.1). It can be concluded that the extracts obtained using high polarity solvents were considerably more effective DPPH radical scavengers than those using less polar solvents, indicating that antioxidant or active compounds of different polarity could be present in *Athrixia phylicoides*. Change in solvent polarity alters the solvent ability to dissolve a selected group of antioxidant compounds and influences

the antioxidant capacity estimation [26]. Mavundza and Padayachee studied the antioxidant activity of A*thrixia phylicoides* in ethanol and aqueous extract, respectively [27,28]. The results revealed that aqueous extract (EC_{50} = 14.01 µg/mL) and ethanol extract (EC_{50} = 10.64 µg/mL) displayed some degree of antioxidant activity.

The study revealed that tea infused for 3 min exhibited the highest antioxidant activity (EC_{50} = 0.066), followed by tea infused for 10 min (EC_{50} = 0.084) and 5 min (EC_{50} = 0.098) which exhibited no significant difference (Table 4.2). The results are not in agreement with those previously reported [29]. They found that an infusion time of 5 min with hot water is optimal for extracting tea antioxidants (ordinary and green tea), but after 5 min tea antioxidants either precipitate or form micelles which in turn reduce the antioxidant capacity and total polyphenol content of the extract. The further steeping above 3 min caused the antioxidant capacity of the extracts to decrease significantly ($p<0.05$). According to Campanella and Pinelo, the decrease in the antioxidant capacity could be explained by the strong tendency of polyphenols to undergo polymerization reactions [29,30]. When the degree of polymerization exceeds a critical value, the increased molecular complexity and steric hindrance reduce the availability of hydroxyl groups in reactions with radicals, which cause a decreased in the antioxidant activity.

The effects of sample to solvent ratio on DPPH radical scavenging capacity are given in Table 4.3. A high sample to solvent ratio was found to be favourable in DPPH radical scavenging capacities for concentrated analysis however for direct analysis it was insignificant. Limited studies were conducted on the effect of sample to solvent ratio ranging from 1:50 (g/mL) to 1:150 (g/mL) on antioxidant capacity. Perva –Uzunalić and Vuong reported that increasing the sample to solvent ratio increased the extraction efficiency of catechin (antioxidants) [8,31]. Therefore from the results of this study, it can be deduced that antioxidant capacity increases with the increase of solid to solvent ratio until reaching an optimum level.

For the extracts studied, it was ascertained that all the concentrated extracts displayed superior scavenging capacity (EC_{50} = 0.039 – 0.122 mg/mL) compared to their respective direct extracts (EC_{50} = 0.126 – 0.202 mg/mL). The highest activities in both method of analysis *viz*: (concentrated and direct) were observed for methanol

extracts and aqueous extracts. The antioxidant activity of acetic acid extracts (EC_{50} = 0.122 and 0.202 mg/mL, crude and direct samples, respectively) is significantly lower than all the others.

4.3.2. Cupric ion reducing capacity method (CUPRAC)

Although a reductant is not necessarily an antioxidant, however an antioxidant is commonly a reductant [32]. The reducing capacity of a sample is regarded as a significant indicator of its potential antioxidant activity [33].

Cupric ion reducing capacity method (CUPRAC) was used to evaluate reducing power of A*thrixia phylicoides* extracts because of its advantage such as wider scope of application and less interaction over FRAP. The method uses bis (neocuproine) copper (II) chloride (Cu(II)-Nc) as the chromogenic oxidising reagents, which reacts with n-electron reductant (polyphenolic antioxidant) in the following manner:

$nCu(Nc)_2^{2+}$ + 2n-electron reductant → $nCu(Nc)_2^{+}$ + n-electron oxidise product + nH^{+}.

As the reactive polyphenolic antioxidants are oxidised to the corresponding quinones, Cu(II)-Nc is reduced to highly blue coloured Cu(I)-Nc chelate showing maximum absorption at 450 nm. The univalent-charged CUPRAC chromophore ($Cu(Nc)_2^{+}$) is soluble in both aqueous and organic solvents, enabling the simultaneous determination of hydrophilic and lipophilic antioxidants.

Figures 4.5, 4.6 and 4.7 display relationship between concentration and absorbance, which indicate that the A*thrixia phylicoides* extracts and the positive controls (Trolox, quercetin and catechin) can reduce the Cu(II)-Nc to Cu(I)-Nc effectively. Moreover the reducing power values were directly proportional to sample concentration within the tested range. As the concentration increased there was always a positive response, a fact that was expected since the more concentration sample the greater the number of reducing units. The lower is the curve, the less reducing potential the sample has. The results show that Trolox has less reducing potential however it is not the case as all the reference standards were diluted ten times.

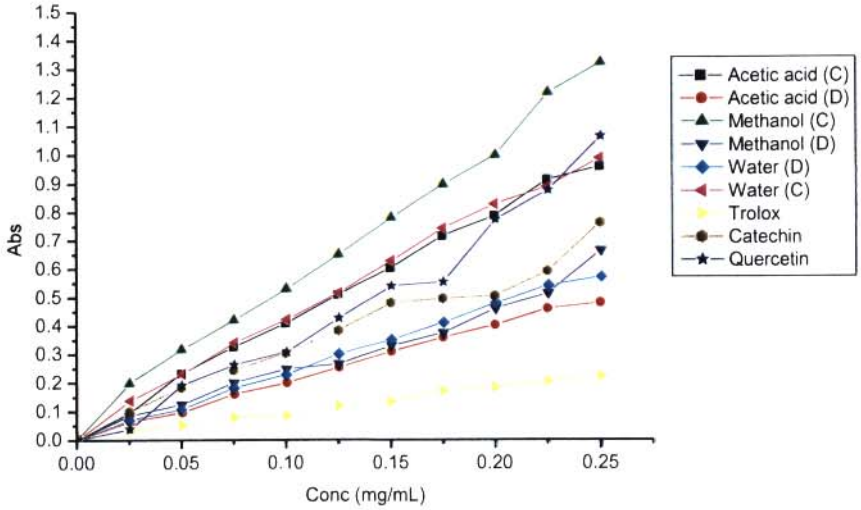


Figure 4.5: Cupric reducing power of aqueous, methanol and acetic acid A*thrixia phylicodes* extracts at different concentrations compared with standards. (C) analysis performed on samples after removing the solvent; (D) analysis performed on samples on the same day.

In the concentrated analysis, methanol extract showed the highest reducing potential than those of water extract and acetic acid extract, which are not significantly different (Figure 4.5). Celik investigated the solvent effect for selected antioxidants using CUPRAC method and it was shown that the antioxidant behaviour of phenolic compounds show variations based on solvent type and polarity, reaction mechanism, solubility parameters which was observed in this study [34].

The extract 3 min has shown to have the highest reducing potential with higher slope (6.52 absorbance unit per mg/mL) compared to all extracts (Figure 4.6). Extracts 5 min (C) and 10 min (C) are significantly not different and exhibited the reducing potential with slope of 3.70 and 3.91 absorbance unit per mg/mL, respectively. The lowest reducing potential was exhibited by the directly analysed 5 min extract (1.85 absorbance unit per mg/mL). These results reveal that the aqueous extract could act as electron donors and could also react with free radicals by converting them to more stable products and terminating the radical chain reaction.

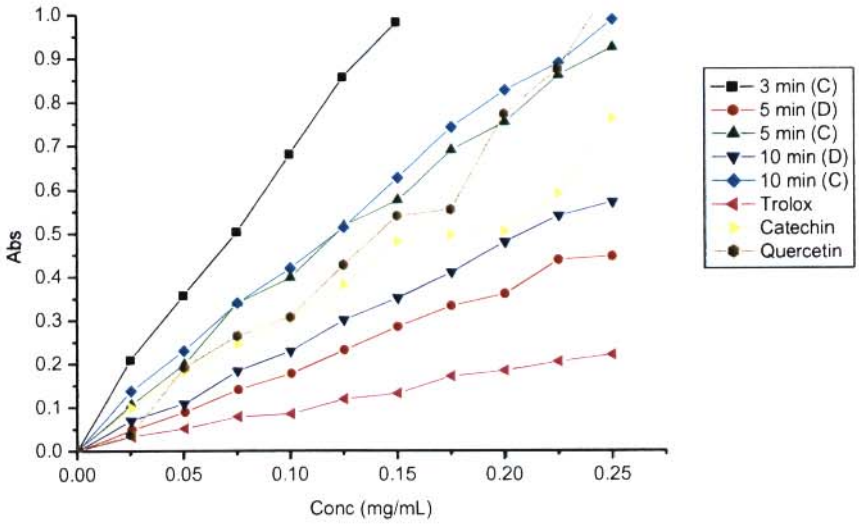


Figure 4.6: Cupric reducing power of aqueous *Athrixia phylicodes* extracts (varying the infusion time) at different concentrations compared with standards. (C) analysis performed on samples after removing the solvent; (D) analysis performed on samples on the same day.

The concentration response curves (Figure 4.7) for all concentrated aqueous extracts were similar and exhibited higher slopes (7.28 – 7.89 absorbance unit per mg/mL) than the respective directs extracts which also exhibited similar lower slopes (1.58 – 1.95 absorbance unit per mg/mL). Interestingly the two standards (quercetin and catechin) were significantly indifferent and in between the lowest and the highest reducing potential extracts. The observed similar reducing potential of the two reference compounds can be ascribed to common **3'4'**-catechol structure of the B-ring which is considered as important structural characteristics for antioxidant potency [16]. Significant indifferent among the various direct and concentrated extracts might be due to the presence of the same reductants (*i.e.*, antioxidants (flavonoids and phenolic acids) responsible for the reduction of the cupric ion (Cu^{2+}) complex to the cuprous form (Cu^{+}).

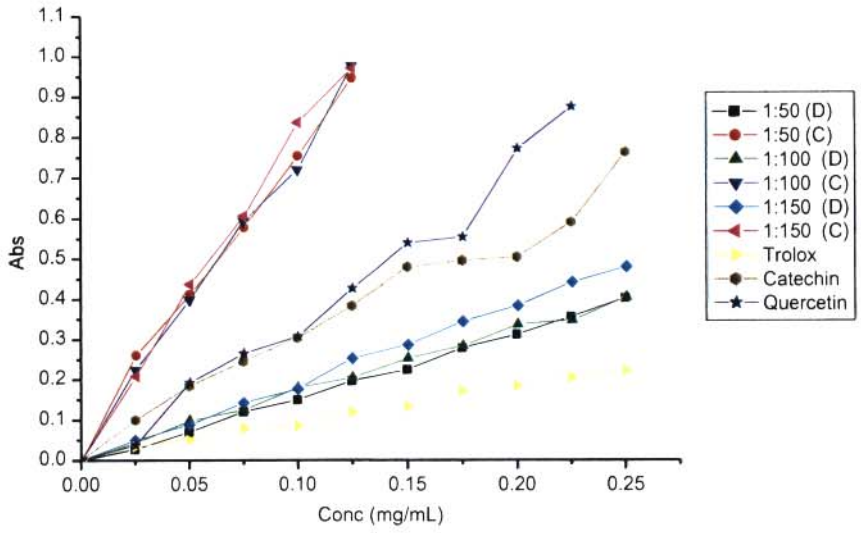


Figure 4.7: Cupric reducing power of aqueous *Athrixia phylicodes* extracts (varying sample to solvent ratio) at different concentrations compared with standards. (C) analysis performed on samples after removing the solvent; (D) analysis performed on samples on the same day.

The reducing EC_{50} values and the slopes of the extracts are presented in Tables 4.1, 4.2 and 4.3 and Tables C 1.2, 1.3 and 1.4, respectively. The EC_{50} in this method is a parameter representing the extract concentration at absorbance of 0.5. The reducing power of all the extracts were less than all the standards (Trolox, quercetin and catechin with EC_{50} = 0.056, 0.014 and 0.016 mg/mL, respectively).

Among the aqueous extracts varying the sample to solvent ratio, it was evident that the crude 1:150 ratio extract displaying the highest reducing potential, will have the lowest EC_{50} (0.061 mg/mL), which would then be explained by the fact that this extract releases more of the antioxidant than other ratios (Table 4.3). The solubility chemistry thereof stems from the compatibility of the solvent and the chemical conformation of the sample. The reducing power of extract 3 min infusion with EC_{50} value of 0.073 mg/mL was more effective than the other extracts in varying the effect infusion time (Table 4.1).

Cupric ion reducing capacity of the extracts of A*thrixia phylicoides* were evaluated for the first time in this study and expressed as TEAC values (Tables 4.1, 4.2 and 4.3).

For this reason there is no data on TEAC values of A*thrixia phylicoides*, the results were just compared to the data on TEAC values of different plant species. Apak investigated the total antioxidant activity (TAC) values of different medicinal and food herbs using Folin Ciocalteu, ABTS and CUPRAC method [35]. The TEAC values were between 0.99 – 1.63 mmol Trolox equivalent per gram sample and total phenolics content of herbal teas with the cupric total antioxidant capacities giving linear curves with correlation coefficient of 0.966. The results from this study were low compared to their findings. The overall results indicate that the concentrated analysis gave the highest reducing potential than the direct analysis.

4.3.3. Cyclic Voltammetry

The phenolic groups of flavonoids can be electrochemically oxidised and show an oxidation or reduction peak in cyclic voltammetry measurements [36]. Thus, redox potential of flavonoids determined by cyclic voltammetry have been utilised as a measure of antioxidant capacity [37]. The lower the potential of oxidation, the more pówerful the compound as a reducing agent [48]. Cyclic voltammetry was used to characterise and quantify polyphenols in A*thrixia phylicoides* leaves and Trolox was used as antioxidant standard to express the antioxidant capacity. In order to cover all groups of antioxidant compounds the cyclic voltammograms were acquired in acetate buffer pH 3 in the range of 100 to 800 mV at a scanning rate of 100 $mV.s^{-1}$.

Cyclic voltammogram of Trolox solutions, differentiating in their concentrations are given in Figure 4.8. The voltammograms exhibited one well defined anodic oxidation peak in the potential range of 250mV- 300 mV *vs* SCE and is shifted to more positive values with increasing concentration of Trolox. According to the work done by some researchers this peak can be ascribed to the oxidation of 3'4'-dihydroxy moiety at B ring (catechol moiety) [39,40]. Trolox also shows reduction peaks at 212.3 mV to (cathodic peak potential, E_{pc}). The reduction potentials (E^{o}) calculated were 248.85 mV to which are the average of the oxidation and reduction potentials [41].

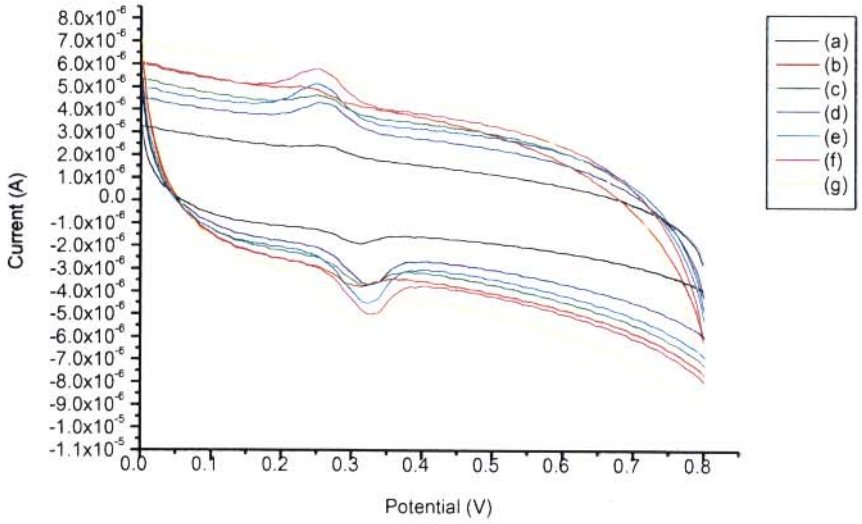


Figure 4.8: Cyclic voltammograms of different Trolox concentration within the range 10-70 µM in acetate buffer (pH 3), scan rate 100 mV/s. (a) 10 µM, (b) 20 µM (c) 30 µM (d) 40 µM ,(e) 50 µM, (f) 60 µM (g) 70 µM.

The calibration curve was constructed by plotting the total charge under anodic wave as function of increasing concentration of Trolox. The calibration graph (Figure D 1.1) shows linear in the concentration range from 10 – 70 µM Trolox and is described by the equation: total charge (Q) = 0.00178x + 1.0116, where total charge of anodic peak represent y and x is the concentration of Trolox expressed in µM (correlation coefficient, R^2 = 0.9943). Poor linearity was observed at concentration of 60 µM where response values showed deviation from the line of best fit. The linearity is limited within a low concentration range making it difficult to study the antioxidant capacity or to compare the capacities within high concentration. The instrumental precision expressed as a coefficient of variation was 15.2% and the precision of the method was 20.5%. The precision of the method determined by repeatability is not satisfactory as it gave the RSD values of above 10 %. The acceptable RSD should be below 10% [42]. The limit of detection (LOD) and quantification (LOQ) were calculated from 3.3 σ/S and 10 σ/S, respectively where σ is the standard deviation of the y-intercept of regression line and S is the slope of the calibration curve. The smallest concentration of the Trolox standard (10 µM) was used as blank, because the blank did not show any signal. Moreover the calculated detection (LOD = 0.53

μMTE) and quantification (LOQ = 1.88 μMTE) limits confirm linearity concentration range for antioxidant capacity determination.

Cyclic voltammograms of A*thrixia phylicoides* extracts were recorded and the effect of concentration range (low or high) of substrate on the peak potential response was studied (Figures D 1.2 – 1.9). The peak current is expected to increase linearly with concentration, however these results show that the current for some of the extract at higher concentration was less than the response for the lower concentrations. This might be as a result of electrode contamination due to oxidation products adsorbing onto the electrode surface [39]. In addition the intensities of the voltammogram increases as the substrate concentration increases and the linear representation of the potential peak with the logarithm of the concentration shows a slope ranging between 0.0052 to 0.7622 (Table D2 - 4). As a result of higher sensibility, the peak potentials of the oxidation signals shifted in all cases either towards positive or negative values. The linear dependence of the potential peak values with the slope obtained and the reversibility of the peak are in agreement with the electron transfer which takes place at the platinum electrode followed by a redox chemical reaction.

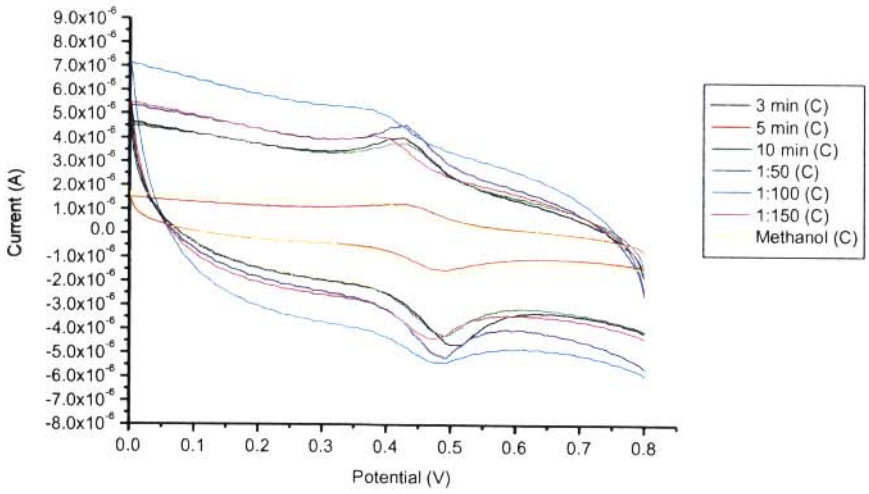


Figure 4.9: Cyclic voltammograms of *Athrixia phylicoides* extracts measured at 100 mV/s in pH 3 acetate buffer. (C) analysis performed on samples after removing the solvent.

The results of concentration effect on the potential showed that concentration 0.004 mg/mL have the peak current densities ranging from 1.411 to 5.43 μA which is below the value of the Trolox (5.85 μA) and can be used for quantification of antioxidant capacity of the extracts. The cyclic voltammetry responses and electrochemical parameters of representative A*thrixia phylicoides* extracts (0.004 mg/mL) are presented in Figure 4.9 and Table 4.5. The cyclic voltammograms of extracts exhibit at least two oxidation peaks in the anodic wave and one in the cathodic wave. Most of the second peaks produced were appearing as a shoulder due to the response of different polyphenolic compounds in *Athrixia phylicoides* extracts available in smaller quantities. The first anodic peaks were broadened due to the response of several antioxidants with different oxidation potentials (400 – 600 mV) and these peaks can be ascribed to the oxidation of the monophenol group or the meta-diphenols on the A ring of flavonoids, actually phenolic acids [39]. These monohydrophenolic compounds have methoxy substituents making them to donate the electrons faster than the monohydroxyphenols without methoxy groups.

Table 4.5: Electrochemical parameters of the voltamograms recorded for the active compounds in *Athrixia phylicoides* extract.

Parameter	*Athrixia phylicoides* Extracts						
	3 min	5 min	10 min	1:50	1:100	1:150	methanol
E_{pa}/mV[a]	422	429	429	429	351	378	372
E_{pc}/mV[a]	513	489	493	492	493	473	490
ΔE/mV	77	58	84	60	105	70	131
E_{mid}/mV	38.5	29	42	30	52.5	35	65.5
I_{pa}/μA	4.061	1.411	5.43	4.592	5.102	3.929	0.786
I_{pc}/ μA	-4.651	-1.870	-6.080	-5.16	-5.366	-4.538	-1.723
I_{pa}/I_{pc}	0.87	0.75	0.89	0.89	0.95	0.87	0.46
Q_{800}	0.434	0.859	1.918	0.493	0.564	1.653	0.846
TEAC/μM	nd	nd	509	nd	nd	360.	nd

Conditions: concentrated *Athrixia phylicoides* extracts (0.004 mg/mL) in acetate buffer pH 3 taken to 800 mV at scan rate of 100 mV/s. **Abbreviations**: E_{pa} - oxidation peak potential; E_{pc} - reduction peak potential; $\Delta E = E_{pa} - E_{pc}$; $Emid = (E_{pa} - E_{pc})/2$; I_{pa} - anodic peak current; I_{pc} - cathodic peak current, nd not detected, TEAC, antioxidant capacity expressed as trolox mmol equivalent per gram of sample, nd – not detected.

Several criteria can be utilized to confirm a single reversible electron transfer to characterize the phenolics as reducing agents. The ratio of the cathodic peak current to the anodic peak current I_{pa}/I_{pc} of 1 and the peak potential separation ΔEp of 57 mV depending on the switching potential defines the degree of reversibility of the redox process [39]. ΔEp values of extracts (5 min, 1:50 and 1:150) were calculated as 58, 60 and 70 mV, respectively from the cyclic voltammogram and these values are quite closer to the 70 mV. So, electrochemical behaviour of these extracts can be defined to have a reversible electron transfer mechanism. However, the ratio I_{pa}/I_{pc} of extracts (3 min, 10 min and methanol) is ranging from 0.46 – 0.95, indicating that the

reaction is quasi-reversible implying that the oxidised form is gradually converting to some other chemical form, such as dimer.

The findings from this study were compared with antioxidant capacity reported by other authors. Studies on diluted teas have shown that the peak current less than 3 μA is generally required to reach a range in which current is directly proportional to the concentration of phenolic present [38]. From the different extracts studied only the water 1:150 extract displayed the peak current less than 3 μA. Kilmartin and Roginsky reported the oxidation peak of different teas with 230 mV and Piljac reported 440 mV for fruit tea infusions [38,43,44]. Among the water extracts with reference to infusion time and sample to solvent ratio, 3 min (422 mV) and 1:150 (351 mV) had the highest total antioxidant activity, respectively. Regarding the comparison of the water and methanol extracts, significant differences was observed with the 1:100 water extract (351 mV) being the only water extract that had highest antioxidant activity than the methanol extract (372 mV).

Oxidation of the the ortho-dihydroxy-phenol group results in the formation of a stable quinone, reduced in the reversed scan appearing as a cathodic peak around 350 mV, however the extracts in this study were reduced at 499 mV. Owing to the relatively low peak potential that corresponds to the reducing ability of this group of compounds, and the high intensity of the current peak corresponding to the compounds concentration it may not be concluded that the monohydroxyphenols with methoxy groups are the contributor to the overall antioxidant potential of *Athrixia phylicoides* leaves. Differences in the relative concentration of low oxidation potential (high activity) and high oxidation potential (low activity) antioxidants in the analysed samples have affected the location of the principal peak. There is insufficient evidence from these results to indicate which of the solvent is more effective for extraction of low oxidation potential antioxidants.

4.4. Method evaluation

4.4.1. Robustness

Tables 4.6 & 4.7 illustrate the evaluation of robustness of the DPPH and CUPRAC respectively with regard to Trolox stability and decomposition DPPH radical and Cu(II) neocuproine cation complex. The validation results demonstrated robustness of the DPPH and CUPRAC method when small variations in Trolox preparation, decomposition of DPPH radical and Cu(II)neocuproine complex were applied.

Table 4.6: Evaluation of robustness of DPPH method.

	Trolox stability			Reproducibility of incubation time			
	Fresh	4°C	RT	15 min	30 min	60 min	120 min
Abs	0.048[1]	0.076[1]	0.115[2]	0.092[3]	0.082[3]	0.102[4]	0.086[3]
SD	0.001	0.002	0.003	0.001	0.001	0.001	0.002
%RSD	0.20	0.30	0.51	0.11	0.20	0.24	0.32

Values are means ± standard deviation (SD). Values followed by same number in the row are not statistically significant from each other at $P < 0.05$ measured by ANOVA one way. **Abbreviations:** Abs – absorbance at 517 nm, %RSD - relative standard deviation.

Trolox stability in DPPH method showed no significant different between the absorbance values for freshly prepared solution and one stored at 4°C for 24 hrs. Additionally, significantly ($p < 0.05$) higher values were observed for Trolox solution stored at room temperature. The decomposition of DPPH radical at each incubation period showed significant variations, with incubation period of 30 min giving the highest antioxidant capacity (Table 4.6). Ozcelik showed that the absorbance of DPPH at 517 nm in methanol and acetone decreased by 20 and 35% for a120 min at 25°C under light, respectively; however it did not change significantly for 150 min in the dark [45].

Trolox standard freshly prepared for CUPRAC method gave the highest absorbance value as compared to 24 hrs stored standard at room temperature and 4°C (Table 4.7). However the was no significant difference between room temperature and refrigerated stored Trolox solution. This confirmed that the stock solution is affected by storage conditions. With regard to the effect of reaction incubation period on the CUPRAC method, when the assay was performed at incubation time above or below 30 min, significant variations in absorbance values were observed (Table 4.8).

Table 4.7: **Evaluation of robustness of CUPRAC method.**

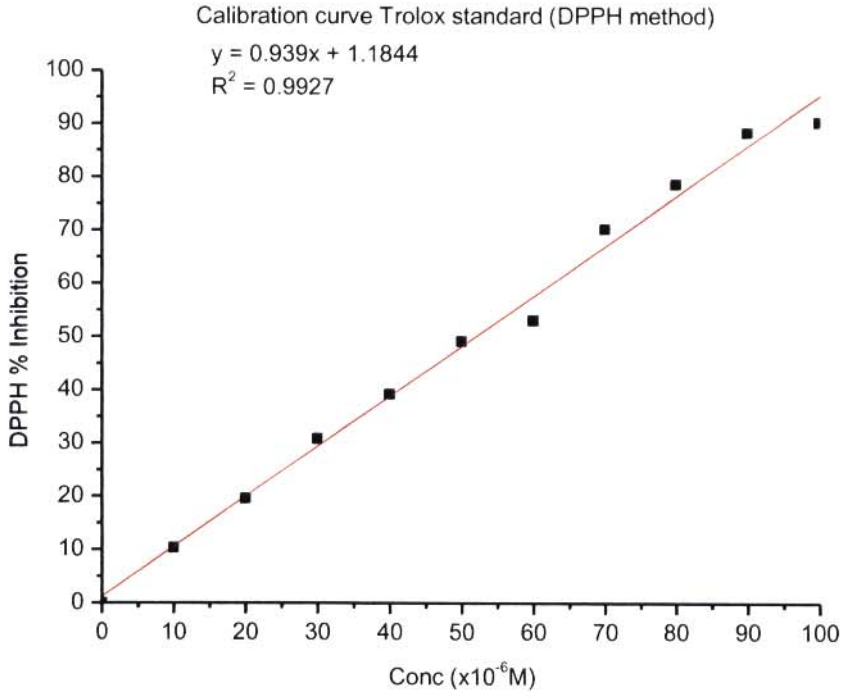
	Trolox stability			Reproducibility of incubation time			
	Fresh	RT	4°C	15 min	30 min	60 min	120 min
Abs	0.219[1]	0.206[2]	0.204[2]	0.202[3]	0.252[5]	0.223[4]	0.229[3]
SD	0.004	0.003	0.002	0.026	0.002	0.008	0.002
%RSD	1.84	1.22	0.75	10.40	0.99	3.74	0.91

Values are means ± standard deviation (SD). Values followed by same letter in the row are not statistically significant from each other at $P < 0.05$ measured by ANOVA one way. **Abbreviations:** Abs – absorbance at 450 nm, %RSD - relative standard deviation.

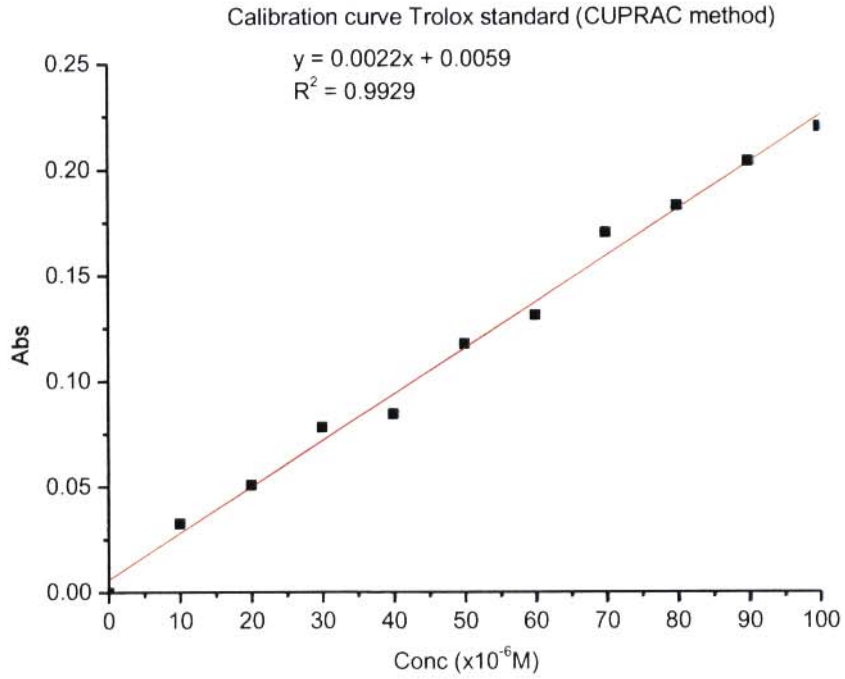
Results of the decomposition of the oxidant of the two methods confirmed that the method is affected by incubation time, which was indicated by significant ($p < 0.05$) differences among the absorbance values. When using the DPPH method, the small absorbance value indicates higher activity whilst for the CUPRAC method the small absorbance value shows lower reducing power. Sochor J reported that the stability of Trolox standard solution is 24 hrs at 4°C [46]. The results of the robustness study demonstrated and confirmed that DPPH and CUPRAC remained robust, despite small changes in method parameters.

4.4.2. Linearity and range

Figure 4.10 illustrates the linearity of the Trolox standards for DPPH and CUPRAC methods respectively; this is simply the working range. In figure 4.10 (a), the 60 µM was the limit of linear response (LOLR) whilst in Figure 4.10 (b) was at 40µM. Poor linearity was observed at higher concentrations where response values showed a deviation from the line of best fit. Additionally, Figure 4.10 (c) DPPH method illustrates an increased imprecision at lower (10 µM) and higher (60 µM) concentrations and a good precision from 20 µM to 50 µM on the Trolox standard curve. Hence, a linear calibration range made up of five Trolox standards (10 µM to 50 µM) was chosen for routine analysis. With regards to CUPRAC method a good precision for the Trolox standards ranged between 10 µM and 90 µM.



(a)



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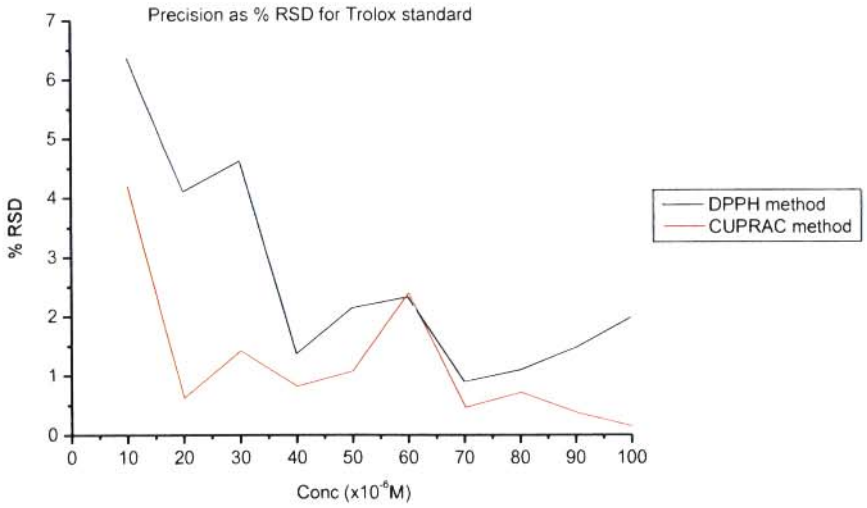


(c)

Figure 4.10: **Linearity and precision of Trolox standards (a) DPPH method (linearity of Trolox standard samples (10 – 100 µM). The limit of linear response (LOLR) occurred at 60 µM. (b) CUPRAC method (linearity of Trolox standard samples (10 – 100 µM). The limit of linear response (LOLR) occurred at 40 µM (c) Precision (repeatability) of Trolox standard (10 – 100 µM) expressed as coefficient of variation. Imprecision increases at lower and higher concentrations.**

In establishing a linear range for a method, both accuracy and precision must be demonstrated to be acceptable at all points on the calibration curve. The results demonstrated good accuracy and precision for Trolox standards. The linearity of the DPPH and CUPRAC methods was tested in the concentration range of 10 to 50 µM and 10 to 90 µM, respectively (Figure 4.10.a & b). Both methods demonstrated to be linear, with correlation coefficient higher than 0.99, moreover allowed evaluation of antioxidant extract capacity with ease.

Table 4.8: Accuracy, precision and linearity of Trolox standard obtained using DPPH and CUPRAC method.

CUPRAC METHOD					DPPH METHOD				
Trolox standard (µmol/L)	**$_1$Intermediate Precision**	**$_2$Accuracy (µmol/L)**	**REC**	**R^2**	**Trolox standard (µmol/L)**	**$_1$Intermediate Precision**	**$_2$Accuracy (µmol/L)**	**REC**	**R^2**
10	4.19	11.38	113.77	0.970	10	4.19	10.25	102.48	0.999
30	1.40	31.09	103.62		20	0.61	19.75	98.76	
60	1.06	48.33	96.67		30	1.40	31.24	104.14	
70	0.45	71.23	101.76		40	0.81	39.75	99.38	
90	0.13	85.87	95.41		50	1.06	49.96	99.93	

Results for all Trolox standards obtained from analysis over three days were as follows; $_1$Precision within individual runs (n = 3). $_2$Accuracy values expressed as means ± SD of three determinations (n = 3) and concentration calculated using y = mx + c. **Abbreviations**: REC - recovery percentage; R^2 - mean correlation coefficient.

4.4.3. Accuracy and precision

Table 4.8 gives the following parameters: accuracy, precision and linearity of the Trolox standards using DPPH and CUPRAC method. Good repeatability for individual runs was observed ranging from 0.61 to 4.19% with an accuracy of 99.38 to 104.14% expressed as percentage recovery for DPPH method and precision of 1.09 to 2.33% and accuracy of 93.51 to 103.80% for CUPRAC method. As the concentration increases, the repeatability of the two methods becomes better, as was expected. The comparison of the precision between DPPH and CUPRAC method was evaluated through the RSD values, and it may be observed that the RSD values of CUPRAC were lower than that of DPPH. Moreover the RSD for all the methods were below 5% and confirmed the high precision of the methods.

Tables 4.9 and 4.10 summarise the accuracy and precision of the DPPH and CUPRAC methods using standard addition recovery method. All samples and standards demonstrated an acceptable precision (<5%) which was expressed as the coefficient of variance. The accuracy expressed as percentage recovery for the DPPH and CUPRAC method was found to be from 77 to 131% and from 96 to 110%, respectively. All samples and standards displayed a linear response ($R^2 \geq 0.815$) for all Trolox standard additions.

Table 4.9: Standard addition recovery method for validating accuracy and precision: DPPH method.

Sample	Trolox spike (µmol TE/L)	Expected (µmolTE/L)	Measured (µmol TE/L)	REC	COV	R^2
Methanol extract (22.87±2.79)	10	32.87	39.82	121	0.78	0.8529
	20	42.87	43.96	102.54	0.44	
	30	52.87	69.31	131.01	0.26	
Acetic Extract (22.02±3.07)	10	32.02	31.03	96.91	6.06	0.8147
	20	42.02	32.55	77.45	6.73	
	30	52.02	48.51	93.25	2.24	
Trolox (5.02±1.04)	10	15.02	12.17	81.13	4.88	0.996
	20	25.02	24.39	97.56	10.46	
	30	35.02	34.19	97.67	0.60	

TEAC values in columns are means ± SD of three determinations (n = 3). **Abbreviations**: REC - recovery percentage; R^2 - mean correlation coefficient; COV - coefficient of variance.

Table 4.10: Standard addition recovery method for validating accuracy and precision: CUPRAC method.

Sample	Trolox spike (μmolTE/L	Expected (μmolTE/L)	Measured (μmol TE/L)	REC	COV	R^2
Methanol	10	85.13	88.87	104.39	0.71	0.929
extract	20	95.13	98.32	103.35	1.17	
(75.13±2.62)	30	105.13	101.65	96.69	0.83	
Acetic	10	142	149.06	104.97	0.74	0.950
Extract	20	152	156.83	103.18	0.35	
(132±8.63)	30	162	160.17	98.87	0.34	
Trolox	10	15.00	16.53	110.2	14.10	0.998
(5±1.04)	20	25.00	26.35	105.4	6.40	
	30	35.00	34.68	99.09	3.83	

TEAC values in columns are means ± SD of three determinations (n = 3). **Abbreviations:** REC - recovery percentage; R^2 - mean correlation coefficient; COV - coefficient of variance.

4.4.4. Limit of detection and limit of quantification

The LOD of an analytical procedure is the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value, and the LOQ is the lowest amount of analyte in sample that can be quantitatively with defined precision under stated experimental conditions [42]. The LOD and LOQ for DPPH were 8.75 and 26.53 μM Trolox, respectively, and for CUPRAC these limits were 3.61 and 10.92 μM, respectively (Table 4.11 and 4.12). These results suggest that both methods are sensitive with DPPH with high sensitivity. The results are not in agreement with those previously published [47].

Table 4.11: LOD and LOQ of the DPPH method using Trolox standard.

	Abs blank	COV	Slope	LOD_1 (μmolTE/ L)	LOQ_2 (μmolTE/ L)
Day 1	0.487 ± 0.008	1.63	0.0046	5.74	17.39
Day 2	0.569 ±0.019	3.39	0.0048	13.06	39.58
Day 3	0.542 ± 0.005	0.86	0.0047	7.46	22.61
Average	0.533 ± 0.011	1.96	0.0047	8.75	26.53

Values in columns are the means of 8 determinations of the blank sample. $_1$LOD calculated using formula LOD = [3.3 x σ/slope]; $_2$LOQ calculated using formula LOQ = [10.0 x σ/slope]; COV - coefficient of variance.

Table 4.12: LOD and LOQ of the CUPRAC method using Trolox standard.

	Abs blank	COV	Slope	LOD_1 (μmolTE/L)	LOQ_2 (μmolTE/L)
Day 1	0.0215 ± 0.0019	8.89	0.0021	2.99	9.05
Day 2	0.0318 ±0.0032	9.89	0.0024	4.4	13.33
Day 3	0.0395 ± 0.0026	6.64	0.0025	3.43	10.40
Average	0.0309 ± 0.0026	8.48	0.0023	3.61	10.92

Values in columns are the means of 8 determinations of the blank sample. $_1$LOD calculated using formula LOD = [3.3 x σ/slope]; $_2$LOQ calculated using formula LOQ = [10.0 x σ/slope]; COV - coefficient of variance.

4.5. Comparison of methods

The values of analytical performance parameters reported for both methods are presented in Table 4.13. From the comparison of the values of both LOD and LOQ, the most sensitive method is CV followed by CUPRAC and DPPH being the least. In addition both methods present the widest range of linearity, showing that they are

more suitable for quantifying directly samples with higher concentrations. The linearity of CV even though it was wider than the DPPH method, it is limited to small concentration range and it was observed that at high concentration range the oxidation potential peak become distorted making it difficult to quantify the analyte. Regarding precision, DPPH and CUPRAC had the highest values, whereas CV was the least precise (highest RSD values). The analytical recovery percentage for CUPRAC and DPPH were close to 100% ± 5% which suggests that all the analyte in the solution can be quantified. Finally, to investigate whether any relationship exists among the antioxidant capacities obtained from the three methods, results were correlated and poor significant correlation was obtained ($r_{CV/DPPH}$ = 0.260, $r_{CV/CUPRAC}$ = 0.235 and $r_{CUPRAC/DPPH}$ = 0.136). The results obtained using CV method is not comparable to CUPRAC and DPPH methods. For that reason CUPRAC and DPPH seem to be suitable for this purpose.

Table 4.13: Statistical parameters for analytical performance of antioxidant determination employing different methods.

Statistical parameter	DPPH	CUPRAC	CV
Linear range (μM)	10 – 50	10 – 90	10-70
Precision (%RSD)	0.61 – 4.19	1.09 – 2.33	20.5
Accuracy (% recovery)	99.38 – 104.14	93.51 – 103.80	88.54 – 98.23
LOD (μM TE)	8.75	3.61	0.53
LOQ (μM TE)	26.53	10.92	1.88
Slope	0.939	0.0022	0.0018

4.6. Comparative determination of total polyphenol content and antioxidant activity of *Athrixia phylicoides* and commercial teas

Antioxidants can deactivate reactive substances by two major mechanisms, namely electron transfer and hydrogen atom transfer [48]. These two mechanisms are affected by antioxidant structure, properties, solubility and partition coefficient and solvent type. The study was carried out to determine how antioxidant activity varies in infusions of commercial teas viz *C. sinuses* (Chinese green tea (CGT) and Joko black tea (JBT)), *A lineras* (Laager rooibos tea (LRT), fermented) as compared to *Athrixia phylicoides* leaves. Since no single measurement of antioxidant capacity method is sufficient and there are many different antioxidant compounds presence in plants, Cupric Ion Reducing Antioxidant Capacity (CUPRAC), 2,2-diphenyl-1-picrylhydrazyl Radical Scavenging Capacity (DPPH) methods were studied to identify antioxidant capacity of the different tea species [32]. In addition the effect of extraction solvent on the total polyphenol content and antioxidant activity of the extracts was investigated. Significant differences were observed among various teas in comparison to *Athrixia phylicoides* leaves ($P < 0.05$).

Total polyphenol content

Phenolic compounds are one of the most effective antioxidative constituents that contribute to the antioxidant activity of plant food [49]. Phenolic compounds in tea have been identified as responsible for scavenging free radical, partly due to their electron reduction potential, i.e. the ability to act as hydrogen or electron donors [50]. Catechins characterised as flavonoids are well known to be major components in phenolics of various teas [38]. However the ways of preparation methods and cultivation processes have a marked influence on the total phenolic content in infusions [8,11,51,52,53].

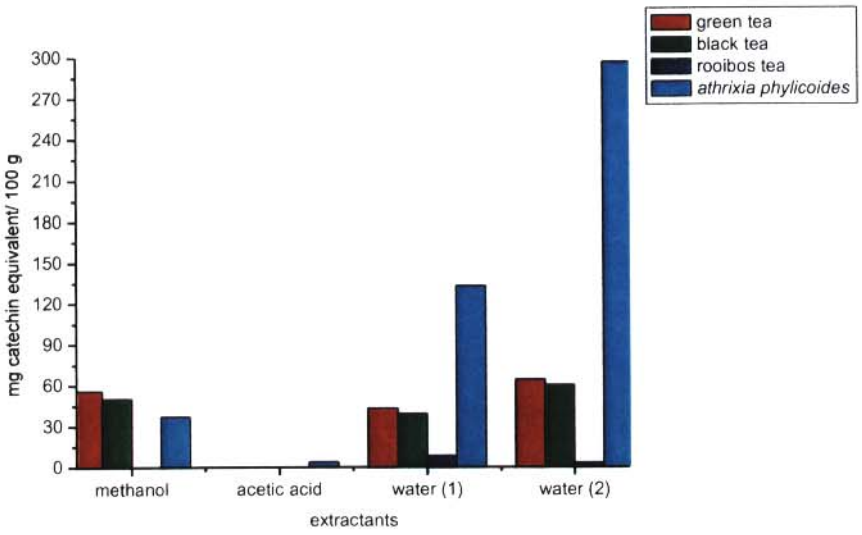


Figure 4.11: Comparison of the *Athrixia phylicoides* and different teas extracted with different solvents.

Figure 4.11 shows the TPC of water, methanol and acetic acid extracts of the commercial teas compared to *Athrixia phylicoides* leaves. Water, methanol and acetic acid extracts from *Athrixia phylicoides* leaves had highest levels of TPC than extracts from commercial teas. Chinese green tea displayed highest level of TPC in water, methanol and acetic acid extracts among all the commercial teas samples.

In this work, TPC levels in *Athrixia phylicoides* leaves were (170.07 mg GAE/100 g) for water extract, 21.54 mg GAE/100 g and 2.66 mg GAE/100 g (Table 4.15). These values fall within the wide range of TPC levels reported for *Athrixia phylicoides* leaves in the literature although in some instances there are differences in sample preparation and extraction method. TPC levels reported for *Athrixia phylicoides* leaves include 56.59 for water extract, decoction 43.63 for methanol extract and 36.73 for ethanol extract [54], 30.57 for water extract, 31.92 for 50% methanol extract and 31.36 for ethanol extract [55].

The extract water (1) of *Athrixia phylicoides* showed higher TPC as compared to extract water (2), however an opposite trend was observed for *C. sinesis* (CGT and JBT) and *A. linearis* (LRT). It is postulated that at higher water to solid ratios, a greater concentration gradient exists between phenolics trapped inside the solid

particles and those located at the surface, consequently leading to accelerated extraction kinetics [56]. The TPC results obtained for water extracts of *Athrixia phylicoides* are consistent with previously published studies on other extracted materials [31,57]. The previous study by Von Gadow, reported 34.9, 33.9 and 35.6% of total water soluble solids of green, black and rooibos tea, respectively, which is comparable to the findings for the water extracts of this study excluding water extract of LRT [15].

Comparing commercial teas, CGT (37.93 mg GAE/100 g) had the highest TPC, closely followed by JBT (35.60 mg GAE/100 g) and LRT (6.37 mg GAE/100g) being the least. These findings are in accordance with other researchers who found the same trend distribution of total phenolics for the same type of infusion (green tea > black tea > rooibos tea) [58 - 60]. The higher polyphenol content of green tea as compared to black and rooibos tea might be due to the presence of flavonoids (catechins) which are presence in high amount [61]. Though these flavonoids are also present in black and rooibos tea, fermentation results inevitably reduce these flavonoids. Del Rio reported that the proportion of catechin in green tea phenolics was 77.1%, which was reduced to 3.3% in black tea phenolics [62]. In addition, the amount of thearibigins increased by 54.8% in black tea, which was not detected before fermentation of fresh leaves of *C. sinensis*.

Among commercial teas, the TPC values found in literature are 19.12 mg GAE/100 g for the water extract and 20.56 mg GAE/100 g for the methanol extract of the *C sinensis* species [63], which are less than the 19.12 mg GAE/100 g for the water extract and 20.56 mg GAE/100 g for the methanol extract of Chinese green tea found in this study. Chan also reported TPC value range of 11370 – 14120 mg GAE/100 g and 6060 - 8490 mg GAE/100 g for the water extracts of green tea and black tea, respectively which is higher than 11370 – 14120 mg GAE/100 g and 6060 - 8490 mg GAE/100 g reported in this study [59].

The TPC values of water extracts were higher than those of methanol and acetic acid extracts, except for LRT which expectantly had higher methanol extract TPC value. In general, water seems to have mediated a more effective extraction of polyphenols from the teas than methanol and acetic acid. Several researchers have

reported the following optimal total phenol content from tea plant material, water [64], methanol [65] and 50% acetone [53].

Table 4.14: Total polyphenol contents and antioxidant activity of *Athrixia phylicoides* and different teas extracted with different solvents.

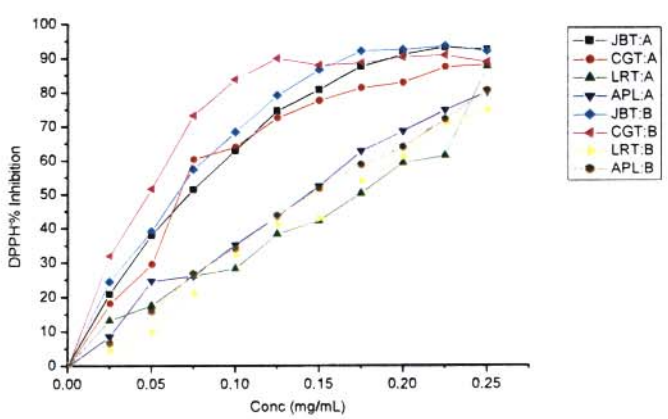
	Type of Extract			
	Water (1)	Water (2)	Methanol	Acetic acid
Green Chinese				
[a]TPC	37.93 ± 0.28	32.97 ± 0.24	26.16 ± 0.28	nd
[b]TPC	43.36 ± 0.57	64.21 ± 0.50	55.91 ± 0.42	nd
[c]DPPH	0.081	0.049	0.057	0.134
[d]CUPRAC	0.148	0.130	0.138	0.355
Joko Black				
[a]TPC	35.60 ± 1.02	29.83 ± 1.63	23.84 ± 0.75	nd
[b]TPC	39.31 ± 1.33	60.15 ± 1.81	50.37 ± 2.89	nd
[c]DPPH	0.079	0.074	0.089	0.104
[d]CUPRAC	0.164	0.149	0.168	0.322
Laager Rooibos				
[a]TPC	3.59 ± 0.28	1.09 ± 0.23	6.37 ± 0.49	nd
[b]TPC	8.34 ± 0.86	3.42 ± 0.5	nd	nd
[c]DPPH	1.710	0.164	0.206	nd
[d]CUPRAC	0.387	0.357	0.374	0.612
Atrixia phylicoides				
[a]TPC	76.45 ± 1.36	170.07 ± 4.64	64.21 ± 0.50	60.15 ± 1.81
[b]TPC	133.14 ± 2.40	296.42 ± 8.16	37.28 ± 0.63	3.88 ± 0.23
[c]DPPH	0.144	0.151	0.170	0.202
[d]CUPRAC	0.270	0.258	0.224	0.233

[a]Expressed as mg gallic acid equivalent/ 100 g sample, [b]Expressed as mg catechin equivalent/ 100 g sample, [c]Expressed as the extract concentration in mg/mL able to inhibit 50% of the used DPPH amount, [d]Expressed as the extract concentration (mg/mL) at absorbance 0.5, nd – not detected.

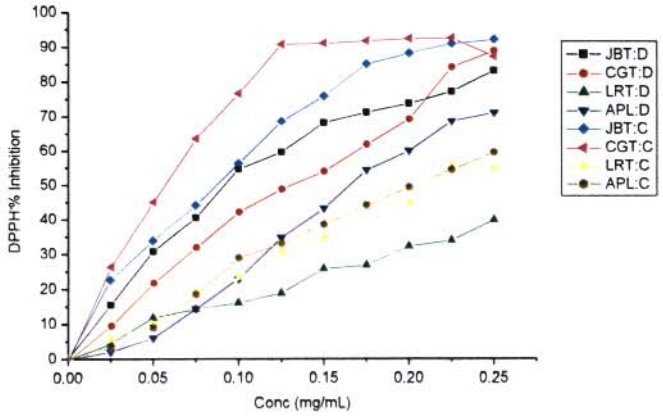
Antioxidant activity

The antioxidant capacity expressed for both water, methanol and acetic acid extracts with EC_{50} values ranging between 0.049 – 0.233 and 0.130 – 0.612 mg/mL for the DPPH and CUPRAC methods, respectively (Table 4.14). Unlike the TPC results, both water, methanol and acetic acid extracts from commercial teas excluding rooibos had higher antioxidant capacity than extracts from APL as measured by the two antioxidant capacity methods. Comparing the methanol and acetic acid extracts, green tea had highest antioxidant capacity for each method compared to the other teas. However, for the water extracts of black and green tea, the antioxidant capacity according to the DPPH method was insignificant. The antioxidant capacity was also expressed in terms of reference compound equivalent (Table 4.15).

DPPH radical scavenging activity and cupric reducing power of the extracts were determined and the percentage inhibition for every tested concentration as well as the slopes of each sample were calculated (Tables A.2.1. to A.2.6, of appendix 2). Figures 4.12 (a & b) indicated concentration dependent radical scavenging activities of the extracts in comparison. A steady state scavenging capacity was only shown by CGT water (2) extract and methanol extract at concentration above 0.1 mg/mL. The dose-response curves for reducing powers of APL as compared to commercial teas extracts are presented in Figure 4.13 (a & b).

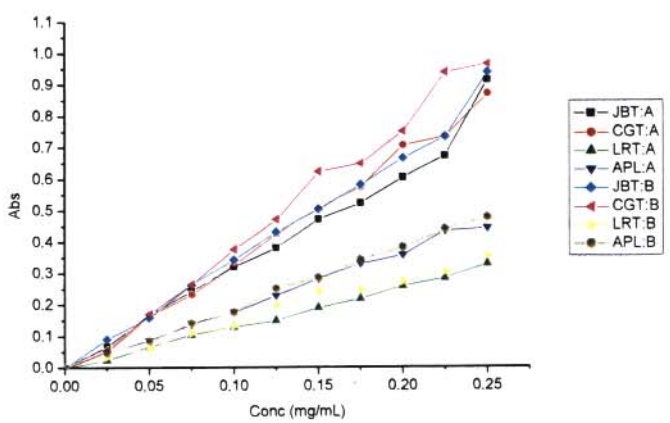


(a)

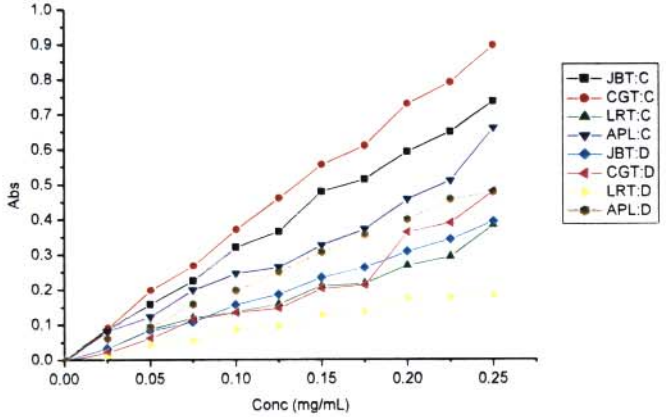


(b)

Figure 4.12: (a & b) DPPH radical scavenging activities of various tea extracts in different solvent forms at different concentrations compared with A*thrixia phylicodes* extract. CGT – Chinese green tea, JBT – Joko black tea, LRT – Laager rooibos tea, APL – *Athrixia phylicoides*. A – water(1), B – water(2), C – methanol, D – acetic acid.



(a)



(b)

Figure 4.13: (a & b) Cupric reducing power of various tea extracts in different solvent forms at different concentrations compared with A*thrixia phylicodes* extracts. CGT – Chinese green tea, JBT – Joko black tea, LRT – Laager rooibos tea, APL – A*thrixia phylicoides* leaves. A – water(1), B – water(2), C – methanol, D – acetic acid.

The antioxidant capacity using the DPPH method for water extracts of APL (0.151 mg/mL) were lower than the CGT (0.049 mg/mL) and JBT (0.074 mg/mL), however compared well with LRT (0.164 mg/mL). Compared to this study Padayachee reported that the antioxidant capacity for water extract of APL are greater than black tea and unfermented rooibos tea (EC_{50} > 0.025 mg/mL), comparable to green rooibos tea and honeybush tea (EC_{50} = 0.01801 and 0.01802 mg/mL, respectively), however lower than green tea (EC_{50} = 0.00964 mg/mL) [28]. Nevertheless, the antioxidant capacity of APL and commercial teas used in this study is within the EC_{50} value range 25 – 50 mg/mL reported for various teas (roasted, green, oolong and black teas) [66]. In literature Du Toit reported antioxidant capacities of LRT among other teas with EC_{50} value 0.85mg/mL (water extract) [67].

The overall results from this study indicates that Chinese green tea had the highest hydrogen donating capacity, closely followed Joko black tea and *Athrixia phylicoides* leaves, while Laager rooibos tea was the weakest of all. In literature it was found that antioxidant capacity by DPPH radical decrease in this order: green tea > oolong tea> black tea > rooibos tea [15,68], although some studies show that black tea are better than green ones [64,69] while others report the absence of any significant differences [1,70].

Dissimilar antioxidant activity of A*thrixia phylicoi*des (APL) compared to *C. sinuses* (CGT and JBT) and *A. lineris* (LRT) may thus be related to the differences in their polyphenolic profiles. Standley investigated the influence of processing stages, and demonstrated that fermentation had a greatest effect on DPPH radical scavenging abilities resulting in decreased radical scavenging ability [71]. The DPPH radical scavenging activity of *C. sinuses* (Chinese green and Joko black teas) has been attributed to the actions of polyphenolic compounds, mainly catechins and their oxidised products, i.e. the theaflavins and thearubigins. This is also supported by the antioxidant activity results from this study (Table 4.16) where the catechin equivalent for green tea is higher than the other teas. Thearibigins and theaflavins also have hydroxyl group considered necessary for free radical scavenging activity [72], however are less effective than catechins [73]. Hence, the antioxidant scavenging activity of black tea is more or less than that of green tea [74].

The trend for the antioxidant capacity assessed by CUPRAC did not vary markedly from the DPPH scavenging capacity results, although not in total accordance with it (Table 4.14). The antioxidant capacity for methanol extract of CGT (EC_{50} = 0.057 mg/mL) is significantly greater than the water extracts of JBT (EC_{50} = 0.074 mg/mL & 0.079 mg/mL). The antioxidant capacities of teas reported in this study shows that Chinese green and Joko black teas gave Trolox equivalent of 0.63 – 1.72 and 0.70 – 1.50, respectively, while Laager rooibos tea and A*thrixia phylicodes* gave 0.37 – 0.58 and 0.33 – 0.87, respectively. Apak had reported 0.4 -1.1 mmol TR/g for black and green tea (infusion prepared from dried leaves) and 0.8 – 1.16 mmol TR/g (prepared from tea bags), which are generally in accord with or slightly lower than the values given in Table 4.15 (0.63 and 1.72 mmol TR/g, respectively) [35].

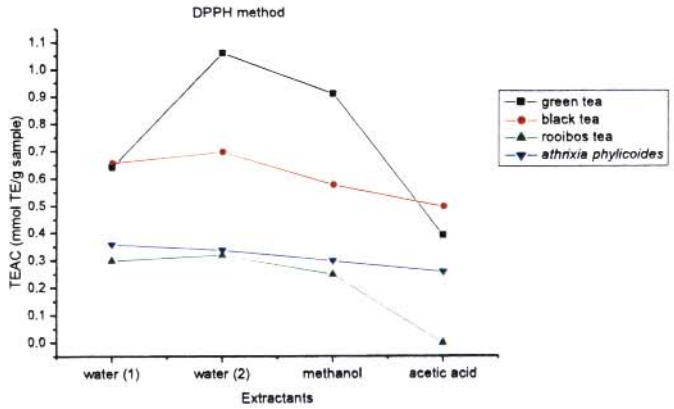
Effects of solvent on antioxidant capacity methods

Change in solvent polarity alters its ability to dissolve a selected group of antioxidants compounds and influences the antioxidant activity estimation [26]. Solvents with high hydrogen bond accepting ability interfere with the stabilization of the catechol through intermolecular H- bonding. This effect together with steric hindrance for approach of the oxidising radical will reduce the rate constant for H-atom abstraction [75]. The results indicate that DPPH method is affected to a greater extent by the solvent polarity whereas the effect from the CUPRAC method was not as pronounced (Figure 4.14 (a & b). DPPH method operates on the basis of hydrogen atom donation from phenolic compound whereas CUPRAC operation is based on outer- sphere electron transfer by a coordinative saturated metal complex involving minimal re-orientation of uniform ligands around the central metal ion in the formation of a transient intermediate during electron transfer.

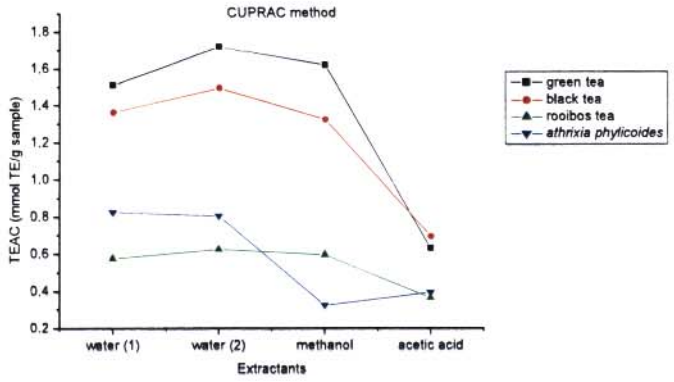
Table 4.15: The total antioxidant activities of *Athrixia phylicoides* extracts compared with various tea extracts as measured by the DPPH method and CUPRAC method expressed in different standard equivalent values.

	[1]TEAC (mmol TE/g sample)				[2]TEAC (mmol CE/g sample)			
	Water (1)	Water (2)	methanol	Acetic acid	Water (1)	Water (2)	methanol	Acetic acid
Green Chinese								
[a]DPPH	0.64	1.06	0.91	0.39	0.47	0.77	0.66	0.28
[b]CUPRAC	1.51	1.72	1.62	0.63	0.37	0.42	0.40	0.16
Joko Black								
[a]DPPH	0.66	0.70	0.58	0.50	0.48	0.51	0.43	0.36
[b]CUPRAC	1.37	1.50	1.33	0.70	0.34	0.37	0.33	0.17
Laager Rooibos								
[c]DPPH	0.30	0.32	0.25	Nd	0.22	0.23	0.18	nd
[d]CUPRAC	0.58	0.63	0.60	0.37	0.14	0.15	0.15	0.09
Athrixia phylicoides								
[c]DPPH	0.36	0.34	0.30	0.26	0.26	0.25	0.21	0.18
[d]CUPRAC	0.83	0.87	0.33	0.40	0.20	0.21	0.25	0.24

[1]Expressed as mmol Trolox equivalent/ g sample, [2]Expressed as mmol catechin equivalent/ g sample [c]Expressed as the extract concentration in mg/mL able to inhibit 50% of the used DPPH amount, [d]Expressed as the extract concentration (mg/mL) at absorbance 0.5.



(a)



(b)

Figure 4.14: Solvent effect on the total antioxidant capacity of various tea extracts compared with A*thrixia phylicodes* extracts using DPPH and CUPRAC method.

Table 4.16: Correlation coefficients, R, for relationships between values obtained from each method.

Correlation coefficient	CGT	JBT	LRT	APL
TPC_{GAE}/TPC_{CE}	0.7556	0.721	0.0012	1
$TPC_{GAE}/CUPRAC_{TE}$	0.8508	0.8959	0.3183	0.6764
$TPC_{GAE}/DPPH_{TE}$	0.4651	0.8121	0.2386	0.5226
$TPC_{CE}/CUPRAC_{TE}$	0.9821	0.9469	0.1727	0.6766
$TPC_{CE}/DPPH_{TE}$	0.8995	0.6987	0.3519	0.5239
$DPPH_{TE}/CUPRAC_{TE}$	0.8055	0.8291	0.9574	0.7594

Values are means ± standard deviations of triplicate measurement. **Abbreviations**: CGT - Chinese green tea, JBT - Joko black tea, LRT - Laager rooibos tea, APL - *Athrixia phylicoides* leaves, TE - Trolox equivalent, CE - Catechin equivalent, GAE - Gallic acid equivalent.

If all data from the different teas are considered, no relationship among CUPRAC, DPPH values and phenol content is observed. Hence the total antioxidant capacities of the extracts of different teas were correlated with the total contents of polyphenols separately (Table 4.16). The results show that the correlation between TPC and the antioxidant activity of the herbal extracts was weak to highly significant depending on the type of tea and antioxidant capacity method. There are no previous reports of TPC correlated with CUPRAC method. With regard to DPPH method a good correlation (R^2 = 0.989) was observed for black teas [76] and weak correlation for herbal teas (R^2 = 0.53) [68]. The highly significant correlation of TPC and antioxidant confirm that polyphenols are likely contributed to radical scavenging activity of these plant extract [77]. However some studies show that the radical scavenging capacity cannot be predicted on the basis of its TPC [78] and this was proven by the study done reporting that the polyphenols present in *Athrixia phylicoides* are not responsible for the antioxidant activity [54]. In support of the studies done previously, weak correlation between total phenol with antioxidant capacity was observed in the study suggesting that the polyphenol compounds (catechin and gallic acid) are not

the primary contributors to antioxidant capacity in LRT (R^2_{DPPH} = 0.239, R^2_{CUPRAC} = 0.318) and APL (R^2_{DPPH} = 0.522, R^2_{CUPRAC} = 0.676) extracts. In the present study, the CUPRAC method yielded higher antioxidant capacity than DPPH method. Moreover highly significant positive correlations (R^2 = 0.759 – 0.957) was observed between CUPRAC and DPPH in all teas. This implied that polyphenolics in the extracts had both hydrogen and electron donating abilities; the efficiencies depended on the polyphenolic compositions in the extracts.

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Chapter 5

CONCLUSIONS AND RECOMENDATIONS

5.1. Conclusions

(i) Do the different methods for antioxidative determination viz; DPPH, CUPRAC and CV show consistency, correlation and accuracy?

The reported values of analytical performance indicate that DPPH, CUPRAC and CV are suitable for the determination of total antioxidant capacity in *Athrixia phylicoides*. However, the degree of the antioxidant capacity differed for the same extracts in the different methods even though all methods follow single electron antioxidant mechanism. As a result of this it was however determined that the ratio in sensitivity is constant leading to the critical observation that with a correction factor, the methods would yield consistently correlated results.

(ii) Is there one particular method whose analytical performance is superior to all other methods?

Although the best results were obtained by CV with exception to precision, after evaluating the performance characteristics of each method "DPPH and CUPRAC" appeared to be good alternatives. With small concentration cyclic voltammogram was perfect and easy to extract analytical information. The oxidation peak at high concentration become distorted making it difficult to quantify the analyte. CV yielded compromised precision and a small linearity range. CUPRAC and DPPH method supports the quantification of larger range of antioxidants and are simple and quite fast with acceptable RSD values. CUPRAC method seems to be the most sensitive

and reliable antioxidant capacity method as is not affected by various factors such as pH.

(iii) What parameters of the identified method can be altered to enhance optimal performance?

The results indicate that CV and DPPH method is affected to a greater extent by the solvent polarity whereas the effect from the CUPRAC method was not as pronounced. The use of a solvent that is between polar and nonpolar would make it easy to have a method whose range is wide enough to be applicable for both types of solvents, without greatly affecting the analytical output. A pH that is slightly acidic would be appropriate for all three chosen methods. A relatively small deviation of temperature above room temperature (± 4°C) would greatly enhance the functionality of the respective methods.

(iv) How versatile will the identified method be?

CUPRAC does not depend on the solvent, and so it can be applied to a wide variety of samples irrespective of the solvent in question. The DPPH method is strongly depended on the polarity of the solvent. A limitation that adversely affect its overall performance across the wide spectrum of samples.

(v) Can the economics and environmental impact be improved?

The economics and environmental impact can be improved by scaling down on the sample and chemical reagents. For UV/Vis absorption studies as applied in both CUPRAC and DPPH methods, the instrumentation is competitively priced as compared to the more expensive cyclic voltammetry.

(vi) How reliable will the identified method be?

Reliability depends on the precision and accuracy of which CUPRAC gave the best precision. A fact that makes it a more reliable method for antioxidant capacity determination by yielding consistent results throughout.

(vii) Does the solvent polarity, infusion time and sample to solvent ratio have an effect on the total polyphenol content and antioxidant activity?

The leaves of A*thrixia phylicoides* plant are source of polyphenols and the extraction efficiency of these compounds strongly depends on the solvent polarity, infusion time and sample to solvent ratio. This was shown by the different TPC and antioxidant capacities determined from each method. The study confirmed that water is the most effective among the solvents tested, greater TPC and antioxidant capacities were achieved with directly analysed aqueous extracts of 5 min infusion time and 1:150 ratio. For crude samples, it is difficult to conclude because of contrasting results.

(vii) Do analytical results depend on the nature of the sample?

The types and amounts of polyphenols present in tea differs depending on the variety of leaf, growing environment, processing, manufacturing, particle size of ground tea-leaves and infusion preparation. Based on results from the study, *Athrixia phylicoides* leaves TPC and antioxidant capacity (EC_{50} = 0.151 mg/mL) was less than that of green rooibos and Joko black tea (EC_{50} = 0.049 mg/mL and 0.074 mg/mL, respectively) but comparable to rooibos tea (EC_{50} = 0.164 mg/mL).

5.2. Recommendations for future work

This study needs to be further developed by investigating phenolic compound profiles including individual flavonoids and tannin contents in *Athrixia phylicoides* antioxidant extracts. Since the study was mainly focused in the total antioxidants in *Athrixia phylicoides*, these approaches can be valuable.

To design a hybrid method that would encompass best values and applicability across the wide sample spectrum. This may be achieved by having a synergic (merging) of CUPRAC and DPPH, CUPRAC and CV, DPPH and CV, or a combination of all three methods.

To come up with a method for pre-treatment of samples to be used in line for analytical purpose. This will be an automated system where the pre-treated sample will be fed into the individual method systems.

Future isolation and identification of the specific compounds may lead to value added products along with new or novel bioactive compounds for use in the food or pharmaceutical industries.

APPENDICES

Appendix A: Total polyphenol content

Folin- Ciocalteu method was used to determine the total polyphenol content of the extracts. Gallic acid and catechin were used as standard and results were calculated based on the standard curve such the one presented here.

Calibration Curve of Gallic Acid Standard

0.500 mg/mL stock standard of gallic acid was prepared by firstly dissolving 250 mg of gallic acid monohydrate in dH_2O and then diluting to 500 mL with distilled water. The solution was kept in the 4°C. The standard concentrations that were prepared for calibration curve are 0.01, 0.02, 0.03, 0.04 and 0.05 mg/mL. The calibration curve was constructed by plotting the absorbance at 765 nm versus standard concentration. Calibration curve had correlation coefficients of 0.995.

Table A 1.1: Absorbance of gallic acid as standard.

Conc (mg/mL)	Absorbance
0.00	0.000
0.01	0.224
0.02	0.369
0.03	0.517
0.04	0.661
0.05	0.85

calibration curve was constructed by plotting the absorbance at 765 nm (A_{765}) versus standard concentration. Calibration curve had correlation coefficients of 0.995

Table A 1.2: Absorbance of catechin as standard.

Conc (mg/mL)	Absorbance
0.00	0.000
0.015	0.078
0.030	0.224
0.045	0.384
0.060	0.525
0.075	0.667

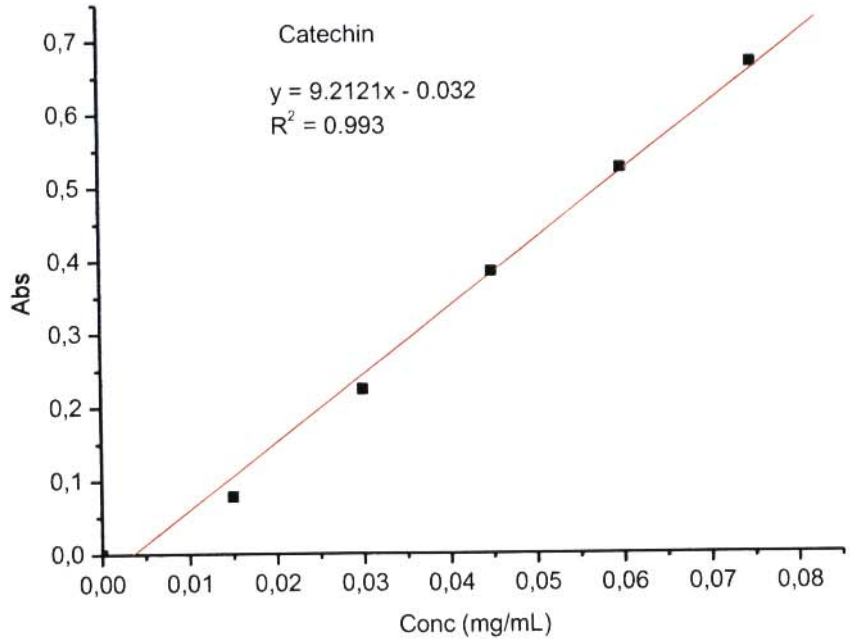

Figure A 1.2: Calibration curve of catechin standard which is used for expression of total polyphenol contents as catechin equivalents.

Appendix B: DPPH method

Calibration Curve of Standards (Trolox, quercetin and catechin)

A working DPPH solution of 0.06 mM was prepared by adding 9.8 ± 0.2 mg of DPPH to a 50 mL volumetric flask and diluting to volume with methanol.

Quercetin, catechin and Trolox standard solutions of 1000 µM were prepared by dissolving 75.560 mg, 10.5 mg and 62.573 mg of quercetin, catechin and Trolox, respectively in 250 mL of pure methanol. Various standard solutions ranging between 100 and 1000 µM were prepared, respectively in methanol by serial dilution of the 1000 µM stock solution.

For establishing calibration curve, a standard solution (0.1 mL) was mixed with methanolic DPPH working solution (3.9 mL). The absorbance for each standard solution was measured at 517 nm after incubation in the dark for 30 min. The blank was prepared as the standard and read spectrophotometrically. A plot of the % DPPH radical scavenging versus concentration of each standard was prepared. The (%) inhibition of DPPH radical was calculated from the absorbance data according to Yen and Duh.

% inhibition against DPPH = [(AB – AA)/AB) x 100

Where AB and AA are the absorbances of blank sample and test material @517 nm after 30 min

Regression equations had correlation coefficients ≥ 0.99. Regression equations were as follows: quercetin (y = 5441.6x + 0.1268), catechin (y = 3938.2x + 6.4077) and Trolox (y = 3883.3x + 0.2281). The concentration at 50% radical inhibition (EC_{50}) was determined from the linear regression equation and was expressed as mg solids/mL DPPH solution. The antioxidant activity of the sample was reported as EC_{50}, hence the higher the EC_{50} value the higher the radical scavenging activity.

Table B 1.1: Percentage inhibition of DPPH by varying concentration of Trolox, quercetin and catechin.

Conc (mg/mL)	Trolox		Quercetin		Catechin	
	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition
0.000	0.481	0.00	0.497	0.00	0.546	0.00
0.025	0.431	10.33	0.441	11.27	0.445	18.56
0.050	0.387	19.61	0.325	34.54	0.391	28.45
0.075	0.334	30.84	0.293	40.98	0.323	40.78
0.100	0.293	39.15	0.213	57.14	0.277	49.27
0.125	0.245	49.13	0.137	72.50	0.240	56.04
0.150	0.226	53.01	0.096	80.75	0.177	67.58
0.175	0.143	70.20	0.040	91.95	0.140	74.36
0.200	0.103	78.59	0.027	94.57	0.084	84.55
0.225	0.056	88.29	0.024	95.24	0.042	92.37
0.250	0.047	90.30	0.016	96.71	0.029	94.69

Table B 1.2: Percentage inhibition of DPPH by *Athrixia phylicoides* water, methanol and acetic acid extracts (direct) at different concentrations.

Conc (mg/mL)	Water		Methanol		Acetic acid	
	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition
0.000	0.487	0.00	0.562	0.00	0.486	0.00
0.025	0.465	4.45	0.542	3.62	0.475	2.33
0.050	0.398	18.21	0.511	9.13	0.456	6.24
0.075	0.353	27.52	0.457	18.68	0.415	14.61
0.100	0.304	37.51	0.398	29.12	0.373	23.25
0.125	0.256	47.36	0.374	33.39	0.316	34.98
0.150	0.144	70.36	0.344	38.73	0.275	43.42
0.175	0.125	74.26	0.313	44.37	0.220	54.66
0.200	0.092	81.18	0.283	49.59	0.193	60.29
0.225	0.088	82.00	0.255	54.63	0.151	68.86
0.250	0.073	85.01	0.226	59.73	0.139	71.33

Table B 1.3: Percentage inhibition of DPPH by *Athrixia phylicoides* water, methanol and acetic acid extracts (concentrated) at different concentrations.

Conc (mg/mL)	Water		Methanol		Acetic acid	
	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition
0.000	0.665	0.00	0.348	0.00		0.00
0.025	0.556	16.34	0.239	31.32	0.613	14.62
0.050	0.456	31.43	0.112	67.72	0.553	22.98
0.075	0.345	48.17	0.025	92.82	0.504	29.76
0.100	0.251	62.31	0.019	94.64	0.430	40.16
0.125	0.189	71.63	0.014	95.88	0.329	54.13
0.150	0.105	84.26	0.019	94.64	0.289	59.80
0.175	0.079	88.12	0.015	95.69	0.180	74.93
0.200	0.084	87.37	0.014	95.98	0.135	81.15
0.225	0.078	88.27	0.016	95.50	0.106	85.24
0.250	0.082	87.72	0.018	94.92	0.074	89.69

Table B 1.4: Percentage inhibition of DPPH by *Athrixia phylicoides* water extract (direct) at different concentrations varying infusion time.

Conc (mg/mL)	5 min		10 min	
	Abs	% Inhibition	Abs	% Inhibition
0.000	0.559	0.00	0.487	0.00
0.025	0.511	8.65	0.465	4.45
0.050	0.421	24.75	0.398	18.21
0.075	0.412	26.30	0.353	27.52
0.100	0.361	35.42	0.304	37.51
0.125	0.315	43.65	0.256	47.36
0.150	0.265	52.59	0.144	70.36
0.175	0.207	62.97	0.125	74.26
0.200	0.174	68.87	0.092	81.18
0.225	0.139	75.13	0.088	82.00
0.250	0.110	80.38	0.073	85.01

Table B 1.5: Percentage inhibition of DPPH by *Athrixia phylicoides* water extract (concentrated) at different concentrations varying infusion time.

Conc (mg/mL)	3 min		5 min		10 min	
	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition
0.000	0.525	0.00	0.665	0.00	0.665	0.00
0.025	0.316	31.48	0.554	16.74	0.556	16.34
0.050	0.252	41.11	0.477	28.27	0.456	31.43
0.075	0.058	70.23	0.390	41.30	0.345	48.17
0.100	0.036	73.58	0.311	53.23	0.251	62.31
0.125	0.036	73.48	0.251	62.31	0.189	71.63
0.150	0.043	72.48	0.187	71.93	0.105	84.26
0.175	0.035	73.68	0.167	74.89	0.079	88.12
0.200	0.039	73.03	0.125	81.20	0.084	87.37
0.225	0.043	72.53	0.117	82.41	0.078	88.27
0.250	0.036	73.53	0.087	86.93	0.082	87.72

Table B 1.6: Percentage inhibition of DPPH by *Athrixia phylicoides* water extract (direct) at different concentrations varying sample to solvent ratio.

Conc (mg/mL)	1:50		1:100		1:150	
	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition
0.000	0.524	0.00	0.503	0.00	0.514	0.00
0.025	0.484	7.64	0.482	4.18	0.479	6.74
0.050	0.454	13.42	0.449	10.74	0.432	15.89
0.075	0.394	24.75	0.396	21.21	0.376	26.91
0.100	0.376	28.24	0.371	26.18	0.338	34.18
0.125	0.328	37.47	0.326	35.26	0.288	43.90
0.150	0.300	42.75	0.314	37.58	0.247	51.88
0.175	0.252	51.85	0.287	42.94	0.211	58.89
0.200	0.241	53.94	0.238	52.75	0.184	64.14
0.225	0.178	66.09	0.225	55.27	0.142	72.37
0.250	0.149	71.50	0.173	65.67	0.098	80.87

Table B 1.7: Percentage inhibition of DPPH by *Athrixia phylicoides* water extract (concentrated) at different concentrations varying sample to solvent ratio.

Conc (mg/mL)	1:50 (concentrated)		1:100 (concentrated)		1:150 (concentrate)	
	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition
0.000	0.525	0.00	0.531	0.00	0.531	0.00
0.025	0.417	20.51	0.319	39.93	0.311	41.43
0.050	0.318	39.43	0.137	74.26	0.060	88.64
0.075	0.109	79.24	0.079	85.12	0.055	89.71
0.100	0.048	90.79	0.044	91.71	0.045	91.46
0.125	0.045	91.37	0.042	92.03	0.045	91.59
0.150	0.053	89.97	0.047	91.15	0.045	91.53
0.175	0.048	90.92	0.041	92.22	0.048	91.02
0.200	0.053	89.84	0.049	90.77	0.046	91.34
0.225	0.045	91.49	0.043	91.90	0.055	89.58
0.250	0.049	90.73	0.049	90.84	0.055	90.58

Table B 1.8: Percentage inhibition of DPPH by commercial teas and *Athrixia phylicoides* water (A) extract (direct) at different concentrations.

Conc (mg/mL)	Chinese Green tea		Joko Black tea		Laager Rooibos tea		*Athrixia phylicoides*	
	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition
0.000	0.538	0.00	0.581	0.00	0.517	0.00	0.559	0.00
0.025	0.439	18.40	0.460	20.83	0.448	13.35	0.511	8.65
0.050	0.432	19.70	0.360	37.98	0.425	17.73	0.421	24.75
0.075	0.212	60.59	0.282	51.41	0.381	26.31	0.412	26.30
0.100	0.192	64.25	0.216	62.88	0.370	28.37	0.361	35.42
0.125	0.146	72.92	0.147	74.64	0.318	38.43	0.315	43.65
0.150	0.119	77.94	0.111	80.84	0.298	42.42	0.265	52.59
0.175	0.099	81.60	0.072	87.66	0.256	50.42	0.207	62.97
0.200	0.066	83.15	0.052	91.11	0.209	59.51	0.174	68.87
0.225	0.066	87.79	0.039	93.34	0.198	61.70	0.139	75.13
0.250	0.063	88.35	0.043	92.66	0.063	87.88	0.110	80.38

Table B 1.9: Percentage inhibition of DPPH by commercial teas and *Athrixia phylicoides* water (B) extract (direct) at different concentrations.

Conc (mg/mL)	Chinese Green tea		Joko Black tea		Laager Rooibos tea		*Athrixia phylicoides*	
	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition
0.000	0.538	0.00	0.581	0.00	0.517	0.00	0.514	0
0.025	0.365	32.16	0.439	24.50	0.494	4.51	0.479	6.74
0.050	0.259	51.86	0.353	39.19	0.466	9.93	0.432	15.89
0.075	0.142	73.54	0.247	57.43	0.407	21.21	0.376	26.91
0.100	0.086	84.08	0.183	68.50	0.349	32.50	0.338	34.18
0.125	0.053	90.21	0.120	79.35	0.303	41.39	0.288	43.90
0.150	0.063	88.23	0.077	86.75	0.293	43.26	0.247	51.88
0.175	0.060	88.91	0.045	92.25	0.237	54.16	0.211	58.89
0.200	0.051	90.58	0.042	92.71	0.200	61.32	0.184	64.14
0.225	0.048	91.14	0.036	93.80	0.148	71.31	0.142	72.37
0.250	0.058	89.16	0.045	92.25	0.128	75.24	0.098	80.87

Table B 1.10: Percentage inhibition of DPPH by commercial teas and *Athrixia phylicoides* methanol extract (direct) at different concentrations.

Conc (mg/mL)	Chinese Green tea		Joko Black tea		Laager Rooibos tea		*Athrixia phylicoides*	
	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition
0.000	0.543	0.00	0.543	0.00	0.543	0.00	0.562	0.00
0.025	0.398	26.70	0.419	22.84	0.511	5.95	0.542	3.62
0.050	0.297	45.30	0.358	34.13	0.486	10.44	0.511	9.13
0.075	0.196	63.90	0.303	44.26	0.437	19.40	0.457	18.68
0.100	0.125	76.92	0.236	56.48	0.415	23.63	0.398	29.12
0.125	0.048	91.10	0.170	68.75	0.375	30.94	0.374	33.39
0.150	0.047	91.41	0.130	76.12	0.353	35.05	0.344	38.73
0.175	0.043	92.02	0.079	85.39	0.300	44.75	0.313	44.37
0.200	0.039	92.76	0.062	88.52	0.298	45.06	0.283	49.59
0.225	0.038	92.94	0.047	91.28	0.239	56.05	0.255	54.63
0.250	0.067	87.66	0.041	92.45	0.243	55.19	0.226	59.73

Table B 1.11: Percentage inhibition of DPPH by commercial teas and *Athrixia phylicoides* acetic acid extract (direct) at different concentrations.

Conc (mg/mL)	Chinese Green tea		Joko Black tea		Laager Rooibos tea		*Athrixia phylicoides*	
	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition	Abs	% Inhibition
0.000	0.470	0	0.470	0	0.470	0	0.486	0.00
0.025	0.424	9.86	0.397	15.46	0.448	4.61	0.475	2.33
0.050	0.366	22.06	0.325	30.78	0.413	12.13	0.456	6.24
0.075	0.318	32.27	0.280	40.50	0.402	14.54	0.415	14.61
0.100	0.271	42.41	0.213	54.61	0.393	16.31	0.373	23.25
0.125	0.239	49.08	0.190	59.65	0.380	19.08	0.316	34.98
0.150	0.215	54.33	0.150	68.16	0.347	26.10	0.275	43.42
0.175	0.178	62.20	0.135	71.21	0.343	27.09	0.220	54.66
0.200	0.143	69.57	0.123	73.76	0.317	32.55	0.193	60.29
0.225	0.073	84.54	0.107	77.23	0.309	34.18	0.151	68.86
0.250	0.051	89.22	0.079	83.26	0.282	40.07	0.139	71.33

Table B 1.12: Correlation coefficients and regression equations used for calculating antioxidant capacity (EC_{50} value) of the extracts (DPPH method).

Samples	Equation and R^2 (direct)	Equation and R^2 (concentrated)
Trolox	y = 388.33x + 0.2281; 0.995	
Quercetin	y = 544.16x + 0.1268; 0.994	
Catechin	y = 393.82x + 6.4077; 0.990	
Methanol	y = 544.16x + 0.1268 0.994	y = 1259.4x + 0.7376 0.9954
Acetic acid	y = 544.16x + 0.1268 0.994	y = 387.07x + 2.7315 0.9909
Water	y = 425.38x – 3.5685 0.9938	y = 563.19x + 2.6369 0.9929
3 min	y = 735.84x + 1.4369 0.995	
5 min	y = 342.06x + 0.6225 0.998	y = 474.11x + 3.5535 0.992
10 min	y = 425.38x – 3.5685 0.9938	y = 563.19x + 2.6369 0.9929
1:50	y = 544.16x + 0.1268 0.994	y = 544.16x + 0.1268 0.994
1:100	y = 544.16x + 0.1268 0.994	y = 544.16x + 0.1268 0.994
1:150	y = 544.16x + 0.1268 0.994	y = 544.16x + 0.1268 0.994

Appendix C: CUPRAC method

Calibration Curve of Standards (trolox, quercetin and catechin)

A $1.0x10^{-2}$ M copper(II) chloride solution was prepared from $CuCl_2 \cdot 2H_2O$ (0.4262 g) dissolved in distilled H_2O and diluted to 250 mL with additional water. Ammonium acetate (NH_4Ac) buffer at pH=7.0 was prepared by dissolving NH_4Ac (19.27 g) in distilled water and diluting to 250 mL. Neocuproine (Nc) solution ($7.5x10^{-3}$ M) was prepared by dissolving Nc (0.039 g) in absolute ethanol, and diluting to 25 mL with ethanol. All standard solutions of synthetic antioxidants, namely Trolox, Catechin and Quercetin were freshly prepared in methanol at 1 mM ($1.0x10^{-3}$ M) concentration prior to measurement and diluted to the desired concentration suitable for absorbance measurement within the applicability range of Beer's law.

To a test tube were added Cu(II), Nc, and NH_4Ac buffer solutions (1.00 mL each). Antioxidant standard solution (0.60 mL) and distilled H_2O (0.50 mL) were added to the initial mixture so as to make the final volume 4.1 mL. The tubes were stoppered, and after 30 min, the absorbance at 450 nm was recorded against a reagent blank using spectrophotometer. The standard calibration curves of each antioxidant compound was constructed in this manner as absorbance *versus* concentration, and the molar absorptivity of the CUPRAC method for each antioxidant was found from the slope of the calibration line concerned. Regression equations had correlation coefficients ≥ 0.99. Regression equations were as follows: Quercetin ($y = 3.990x - 0.0305$), Catechin ($y = 3.0371x + 0.014$) and Trolox ($y = 0.879x + 0.0059$). The reducing power was expressed as concentration at absorbance 0.5.

Table C 1.1: Cupric reducing power of Trolox, Catechin and Quercetin at different concentrations.

Conc (mg/mL)	Trolox	Quercetin	Catechin
	Abs	Abs	Abs
0.000	0.000	0.000	0.000
0.025	0.033	0.037	0.099
0.050	0.051	0.191	0.184
0.075	0.078	0.264	0.245
0.100	0.084	0.307	0.303
0.125	0.118	0.426	0.382
0.150	0.131	0.539	0.480
0.175	0.170	0.553	0.495
0.200	0.183	0.772	0.504
0.225	0.204	0.875	0.590
0.250	0.220	1.063	0.762
Intercept	0.0059	-0.0463	0.018
Slope	0.878	3.990	2.934

Table C 1.3: Cupric reducing power of aqueous *Athrixia phylicodes* extracts (direct and concentrated) varying the infusion time at different concentrations.

Conc (mg/mL)	Concentrated			direct	
	3 min	5 min	10 min	5min	10 min
	Abs	Abs	Abs	Abs	Abs
0.000	0.000	0.000	0.000	0.000	0.000
0.025	0.208	0.048	0.105	0.069	0.137
0.050	0.357	0.089	0.199	0.108	0.230
0.075	0.503	0.140	0.340	0.184	0.340
0.100	0.680	0.177	0.399	0.229	0.420
0.125	0.856	0.230	0.517	0.301	0.514
0.150	0.982	0.285	0.576	0.351	0.627
0.175	1.143	0.332	0.690	0.410	0.742
0.200	1.291	0.360	0.754	0.479	0.826
0.225	1.462	0.438	0.861	0.539	0.888
0.250	1.517	0.446	0.923	0.570	0.987
Intercept	0.0329	0.0176	0.021	-0.0002	0.0021
Slope	6.931	4.105	4.413	1.848	2.383

Table C 1.4: Cupric reducing power of aqueous *Athrixia phylicodes* extracts (direct and concentrated) varying sample to solvent ratio at different concentrations.

Conc (mg/mL)	Direct			Concentrated		
	1:50	1:100	1:150	1:50	1:100	1:150
	Abs	Abs	Abs	Abs	Abs	Abs
0.000	0.000	0.000	0.000	0.000	0.000	0.000
0.025	0.027	0.042	0.049	0.303	0.224	0.206
0.050	0.069	0.097	0.087	0.411	0.397	0.436
0.075	0.120	0.124	0.142	0.577	0.594	0.605
0.100	0.149	0.179	0.177	0.752	0.719	0.835
0.125	0.196	0.204	0.253	0.945	0.976	0.969
0.150	0.225	0.253	0.286	1.097	1.160	1.228
0.175	0.278	0.283	0.345	1.263	1.301	1.362
0.200	0.312	0.336	0.384	1.410	1.364	1.575
0.225	0.356	0.347	0.442	1.567	1.599	1.654
0.250	0.402	0.401	0.479	1.698	1.744	1.840
intercept	-0.008	0.0087	-0.0038	0.0812	0.0535	0.0503
slope	1.617	1.5798	1.9522	6.6386	6.9012	7.3873

Table C 1.5: Cupric reducing power of aqueous (A) extracts of *Athrixia phylicodes* leaves and commercial teas analysed directly.

Conc (mg/mL)	Green tea	Black tea	Rooibos tea	*Athrixia phylicoides*
	Abs	Abs	Abs	Abs
0.000	0	0	0.000	0.000
0.025	0.054	0.066	0.025	0.069
0.050	0.167	0.167	0.068	0.108
0.075	0.235	0.241	0.106	0.184
0.100	0.330	0.320	0.131	0.229
0.125	0.429	0.381	0.150	0.301
0.150	0.509	0.471	0.192	0.351
0.175	0.579	0.522	0.219	0.410
0.200	0.710	0.604	0.261	0.479
0.225	0.738	0.672	0.286	0.539
0.250	0.875	0.914	0.331	0.570
Intercept	-0.016	0.0079	-0.011	-0.0002
Slope	3.4912	2.9936	1.2954	1.848

Table C 1.6: Cupric reducing power of aqueous (B) extracts of *Athrixia phylicodes* leaves and commercial teas analysed directly.

Conc (mg/mL)	Green tea	Black tea	Rooibos tea	*Athrixia phylicoides*
	Abs	Abs	Abs	Abs
0.000	0.000	0.000	0.000	0.000
0.025	0.059	0.092	0.036	0.049
0.050	0.175	0.159	0.064	0.087
0.075	0.268	0.264	0.112	0.142
0.100	0.379	0.344	0.134	0.177
0.125	0.474	0.433	0.202	0.253
0.150	0.626	0.505	0.243	0.286
0.175	0.652	0.583	0.248	0.345
0.200	0.755	0.667	0.274	0.384
0.225	0.942	0.736	0.305	0.442
0.250	0.968	0.941	0.357	0.479
Intercept	-0.0194	0.0077	-0.0006	-0.0038
Slope	3.9042	3.2935	1.3963	1.9522

Table C 1.7: Cupric reducing power of the methanol extracts of *Athrixia phylicodes* leaves and commercial teas analysed directly.

Conc (mg/mL)	Green tea	Black tea	Rooibos tea	*Athrixia phylicoides*
	Abs	Abs	Abs	Abs
0.000	0.000	0.000	0.000	0.000
0.025	0.093	0.087	0.035	0.084
0.050	0.202	0.158	0.089	0.125
0.075	0.272	0.225	0.121	0.201
0.100	0.375	0.321	0.139	0.248
0.125	0.464	0.366	0.161	0.266
0.150	0.560	0.479	0.212	0.329
0.175	0.614	0.515	0.219	0.375
0.200	0.734	0.593	0.270	0.460
0.225	0.795	0.650	0.296	0.514
0.250	0.900	0.738	0.386	0.663
Intercept	0.0112	0.0127	0.0101	0.0173
Slope	3.5535	2.9025	1.2889	2.159

Table C 1.8: Cupric reducing power of the acetic acid extracts *Athrixia phylicodes* leaves and commercial teas analysed directly.

Conc (mg/mL)	Green tea	Black tea	Rooibos tea	*Athrixia phylicoides*
	Abs	Abs	Abs	Abs
0.000	0.000	0.000	0.000	0.000
0.025	0.025	0.035	0.010	0.062
0.050	0.066	0.086	0.044	0.096
0.075	0.117	0.109	0.057	0.160
0.100	0.138	0.159	0.088	0.199
0.125	0.150	0.189	0.098	0.252
0.150	0.206	0.237	0.129	0.308
0.175	0.216	0.264	0.138	0.357
0.200	0.368	0.311	0.178	0.401
0.225	0.394	0.345	0.178	0.459
0.250	0.484	0.396	0.185	0.480
Intercept	-0.0022	-0.001	-0.0057	0.0063
Slope	1.4141	1.5572	0.8961	1.966

Table C 1.9: Correlation coefficients and regression equations used for calculating antioxidant capacity (concentration at absorbance 0.5) of the extracts (CUPRAC method).

Samples	Equation and R^2 (direct)	Equation and R^2 (concentrated)
Trolox	y = 0.878x + 0.0059; 0.993	
Quercetin	y = 9.990x – 0.0463; 0.990	
Catechin	y = 2.934x + 0.018; 0.994	
Methanol	y = 2.159x + 0.0173 0.990	y = 5.063x + 0.00334 0.995
Acetic acid	y = 1.966x + 0.0063 0.997	y = 3.893x + 0.0175 0.997
3 min		y = 6.931x + 0.0329 0.952
5 min	y = 1.848x – 0.0002 0.996	y = 4.105x + 0.0176 0.959
10 min	y = 2.383x + 0.0021 0.996	y = 4.412x + 0.0221 0.948
1:50	y = 1.617x – 0.008 0.998	y = 6.639x + 0.0812 0.996
1:100	y = 1.580x + 0.0087 0.995	y = 6.901x + 0.0535 0.994
1:150	y = 1.952x – 0.0038 0.998	y = 7.387x + 0.0503 0.995

Appendix D: Cyclic voltammetry

Standard Trolox solution was used in cyclic voltammetry experiments. A cyclic voltammogram was obtained and this voltammogram was investigated in order to learn about the redox properties of Trolox. Oxidizibility of a compound has been used as a measure of antioxidant property and this can be determined by measuring an oxidation potential in cyclic voltammetry. Except oxidation potential, a huge number of parameters can be found from the cyclic voltammograms, such as reduction potential, switching potential, anodic peak current and cathodic peak current. A lot of information about the redox properties of polyphenols can be obtained from the investigation of these parameters.

Calibration Curve of Trolox Standard

Acetic acid and sodium acetate solutions of 0.1 M were prepared by adding 5.73 mL acetic acid and dissolving 8.203 g of sodium acetate, respectively thereafter diluting separately to 1 L volumetric flasks using distilled water. An acetate buffer (0.1 M) was prepared by mixing 982.30 mL acetic acid (0.1 M) and 17.70 mL of sodium acetate (0.1 M), adjusted to pH 3.

Trolox 1000 µM stock standard was prepared by firstly dissolving 62.57 mg of Trolox in acetate buffer pH 3 and then diluting to 250 mL. The standard concentrations that were prepared for calibration curve are 10.00, 20.00, 30.00, 40.00, 50.00, 60.00 and 70.00 µM. The calibration curve was constructed by plotting the total charge taken in the potential range between 0 and 800 mV with scan rate 100 mV/s versus standard concentration. Calibration curve had correlation coefficients of 0.995

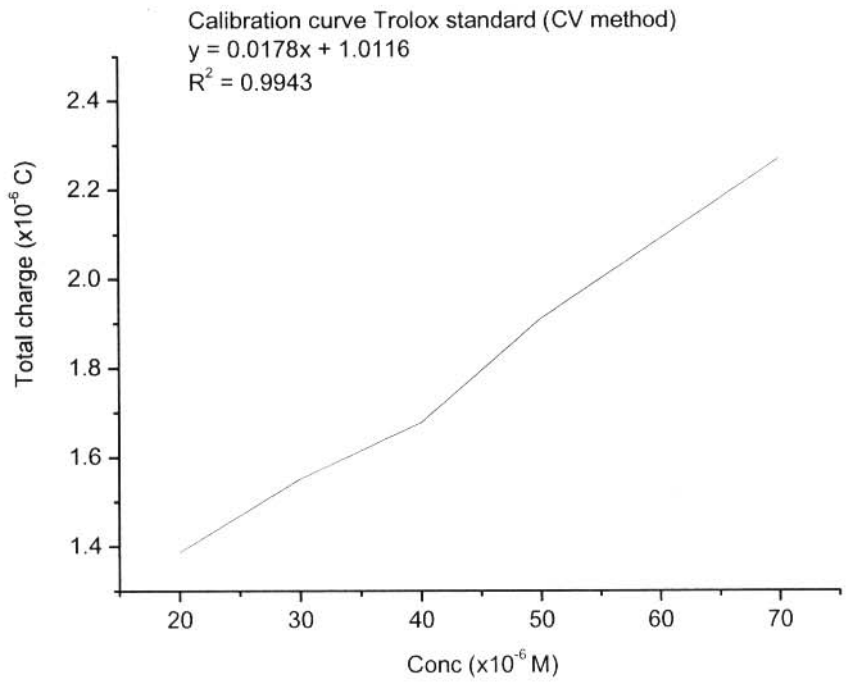

Figure D 1.1: Linearity of Trolox standard samples (20 – 70 µM).

Table D 1.1: Cyclic peak parameters obtained for Trolox at different concentration.

Conc (µM)	Potential (mV)	Total charge (Q)
20.00	225	1.386
30.00	250	1.551
40.00	250	1.677
50.00	249	1.910
70.00	223	2.268

Table D 1.2: Cyclic peak parameters obtained for the effect of anodic (Ea) peak potential of methanol and aqueous *Athrixia phylicoides* extracts at different concentration.

Conc (mg/mL)	log conc	methanol		water	
		Potential (mV)	Total charge(Q)	Potential (mV)	Total charge(Q)
0.002	0.301	364	1.143	nd	nd
0.004	0.602	359	0.694	428	0.983
0.006	0.778	347	1.652	404	1.154
0.008	0.903	350	1.086	404	1.909
0.010	1.000	351	1.996	404	1.699
Intercept		370.33		459.58	
Slope		-22.499		-60.405	
R^2		0.7622		0.721	

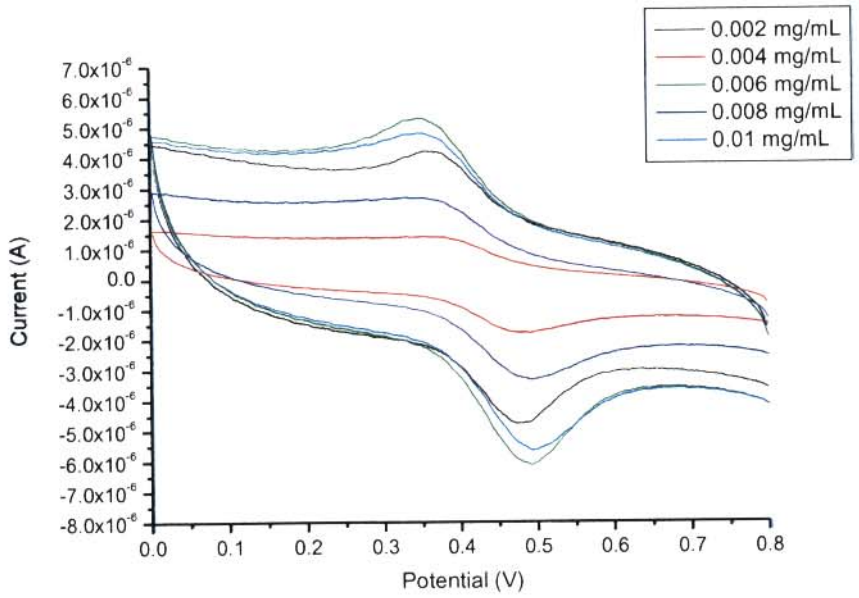

Figure D 1.2: Cyclic voltammogram obtained for the methanol extracts *of Athrixia phylicoides* at different concentration at scan rate 100mv/sec.

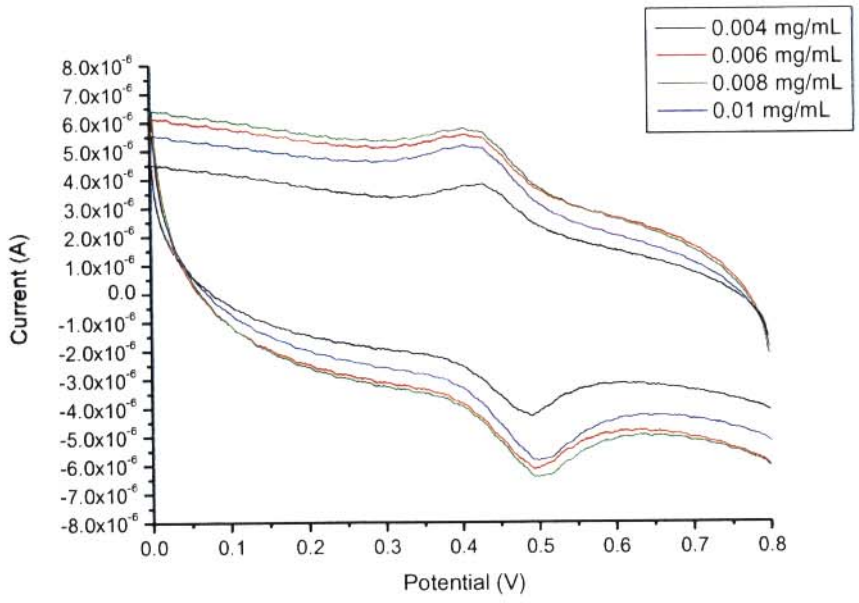

Figure D 1.3: Cyclic voltammogram obtained for the aqueous extract of *Athrixia phylicoides* at different concentration at scan rate 100mv/sec.

Table D 1.3: Cyclic peak parameters obtained for the effect of anodic (Ea) peak potential of aqueous *Athrixia phylicoides* extracts varying infusion time at different concentration.

Conc (mg/mL)	log conc	3 min		5 min		10 min	
		Potential (mV)	Total charge(Q)	Potential (mV)	Total charge(Q)	Potential (mV)	Total charge(Q)
0.002	0.301	365	1.353	287	0.825		
0.004	0.602	405	1.382	429	0.210	428	0.983
0.006	0.778	429	0.530	403	0.907	404	1.154
0.008	0.903	403	1.832	430	0.307	404	1.909
0.010	1.000	351	1.781	404	0.955	404	1.699
intercept		384.55		272.46		459.58	
slope		8.3426		164.99		-60.405	
R^2		0.0052		0.5924		0.721	

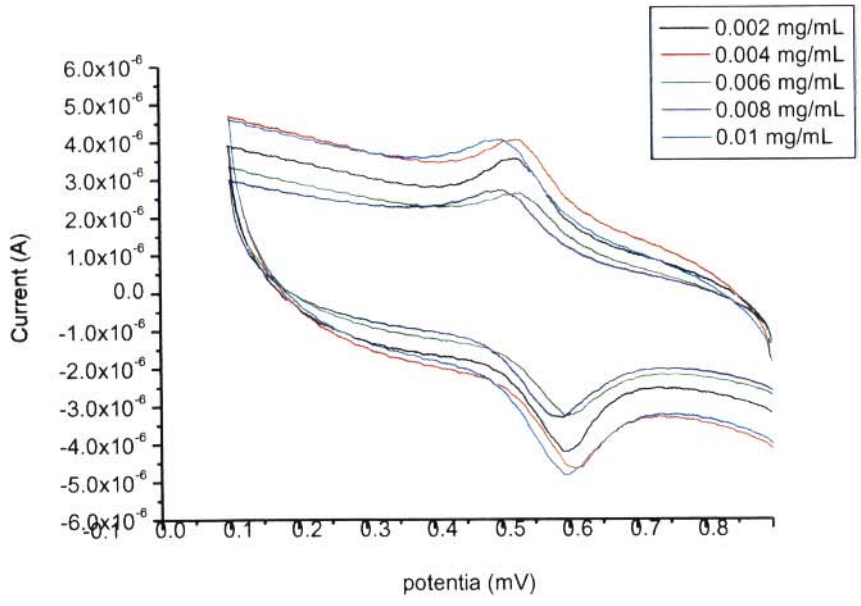

Figure D 1.4: Cyclic voltammogram obtained for the 3 min aqueous extract of *Athrixia phylicoides* at different concentration at scan rate 100mv/sec.

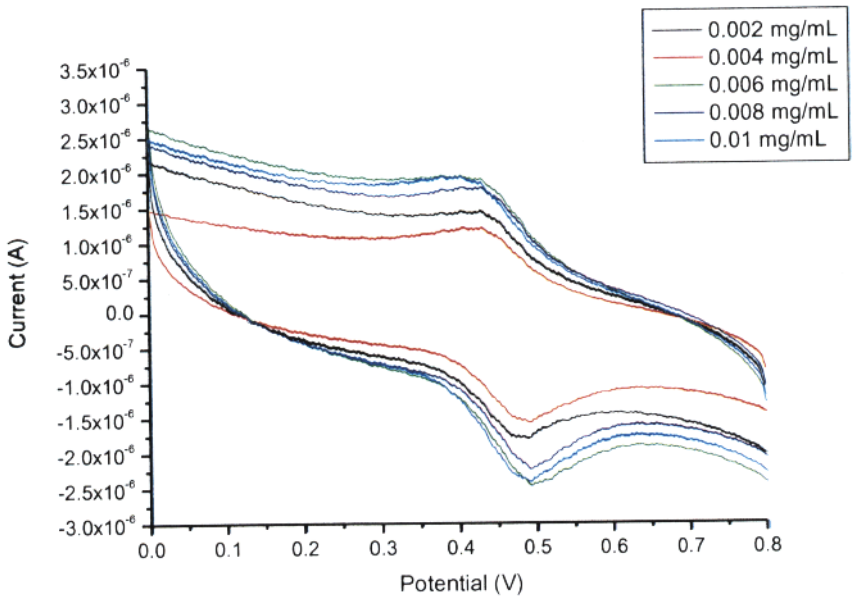

Figure D 1.5: Cyclic voltammogram obtained for the 5 min aqueous extracts of *Athrixia phylicoides* at different concentration at scan rate 100mv/sec.

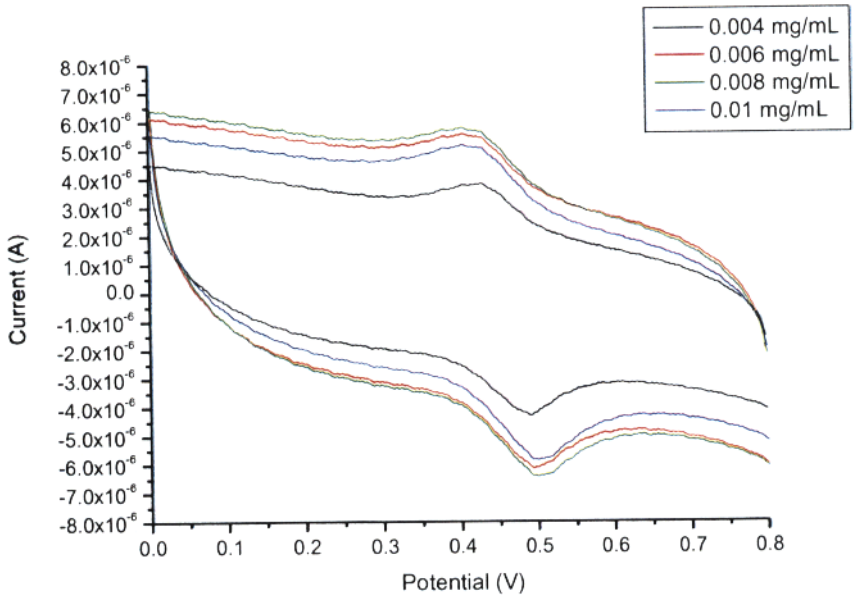

Figure D 1.6: Cyclic voltammogram obtained for the 10 min aqueous extracts of *Athrixia phylicoides* at different concentration at scan rate 100mv/sec.

Table D 1.4: Cyclic peak parameters obtained for the effect of anodic (Ea) peak potential of aqueous *Athrixia phylicoides* extracts varying sample to solvent at different concentration.

Conc (mg/mL)	log conc	1:50		1:100		1:150	
		Potential (mV)	Total charge(Q)	Potential (mV)	Total charge(Q)	Potential (mV)	Total charge(Q)
0.002	0.301	429	0.393	nd	nd	378	1.440
0.004	0.602	429	0.550	339	0.612	378	1.170
0.006	0.778	403	1.328	nd	nd	378	1.450
0.008	0.903	404	2.050	378	0.380	352	2.047
0.010	1.000	404	2.984	341	1.974	358	1.790
intercept		445.15		322.89		393.59	
Slope		-43.85		35.333		34.585	
R^2		0.7672		0.109		0.5615	

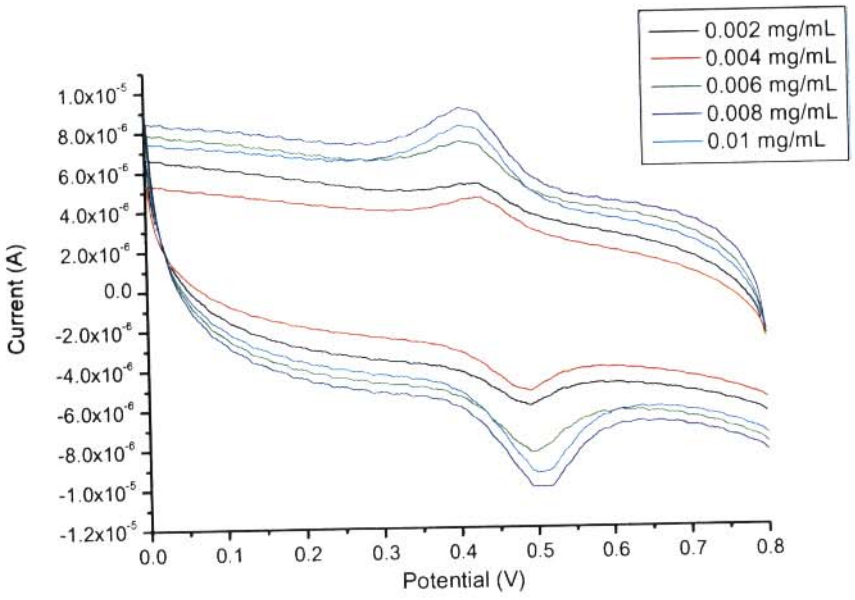

Figure D 1.7: Cyclic voltammogram obtained for the 1:50 aqueous extracts of *Athrixia phylicoides* at different concentration at scan rate 100mv/sec.

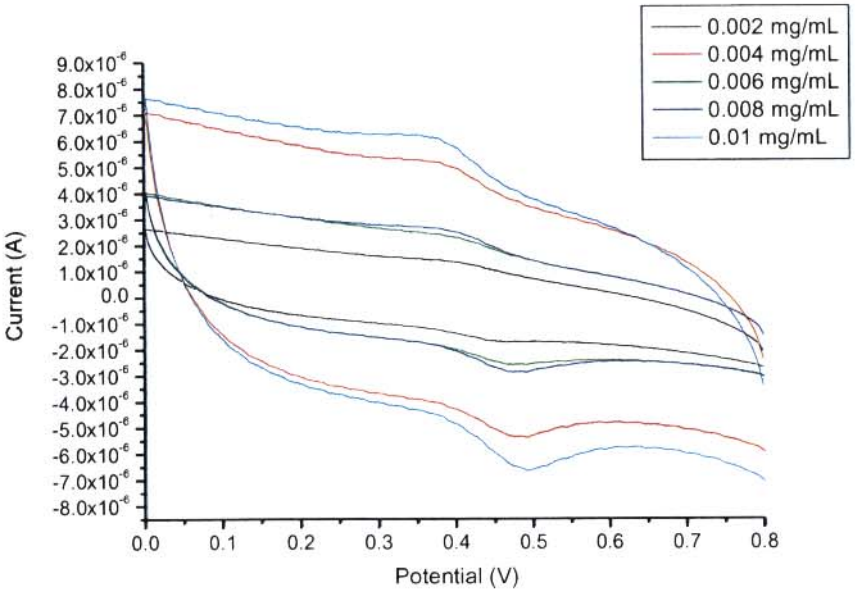

Figure D 1.8: Cyclic voltammogram obtained for the 1:100 aqueous extracts of *Athrixia phylicoides* at different concentration at scan rate 100mv/sec.

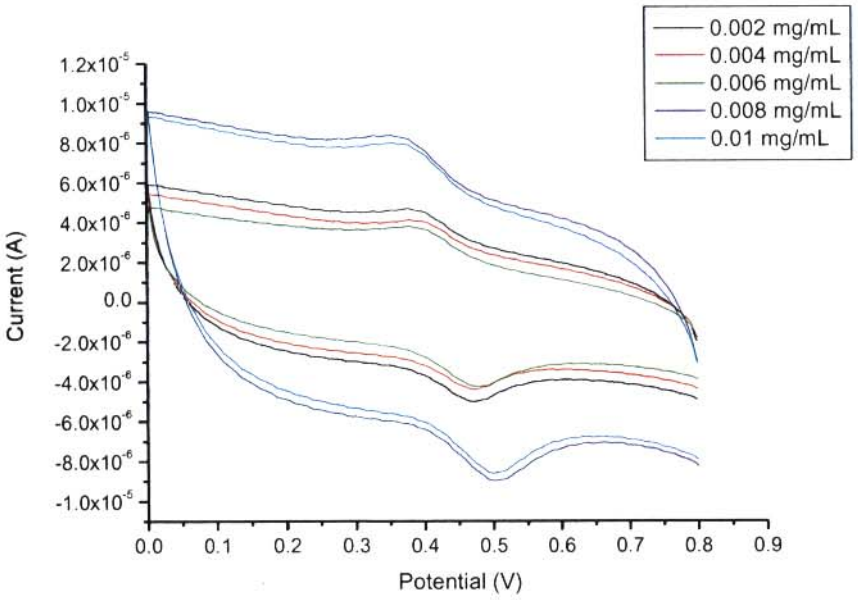

Figure D 1.9: Cyclic voltammogram obtained for the 1:150 aqueous extracts of *Athrixia phylicoides* at different concentration at scan rate 100mv/sec.