QUANTIFICATION OF PHENOLICS, FLAVANOIDS AND ANTIOXIDANT ACTIVITY OF *TAMARINDUS INDICA* L. FROM SELECTED AREAS IN TANZANIA

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A DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN NATURAL PRODUCTS TECHNOLOGY AND VALUE ADDITION OF SOKOINE UNIVERSITY OF AGRICULTURE. MOROGORO, TANZANIA.

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ABSTRACT

The purpose of this study was to establish the quantities and antioxidant activity in fruits and leaves of *Tamarindus indica* L. collected from three agro-ecological zones of Tanzania represented by Morogoro, Tanga and Dodoma regions. Samples were studied for their total phenolic and flavonoid contents as well as their antioxidant activity. The total phenolic content in all extracts of the fruits and leaves were significantly different ($p<0.05$) and ranged from 1994.4±530.77 to 17874.67±5234 mg GAE/100g. Similarly the total flavonoid content in tamarind leaf and fruit extracts ranged from 880±609.45 to 11483.11±2559.67 mg CE /100 g dry weight. There was a significant difference ($p<0.05$) between the antioxidant activity in the leaf (54.39±0.13%) and fruit extracts (40.11±0.03%). Tamarind leaf extracts exhibited significantly higher ($p<0.05$) radical scavenging activity than fruit extracts. The antioxidant activity in fruit extracts expressed in percentage ranged between 29.27±0.06% and 40.11±0.03% while in leaf extracts the activity ranged from 22.33±0.08% to 54.39±0.13%. In a decreasing order the radical scavenging activity from Coastal leaf extracts had the highest activity followed by Eastern leaf extracts and lastly Central leaf extracts. In fruit samples, Coastal fruit extracts had the highest activity followed by Central fruit extracts and Eastern fruit extract being the least active. Ferric reducing power (FRAP) for leaves and fruits ranged between 6968±3655.91 µM Fe (II)/g and 76822.67±23259.9 µM Fe (II)/g dry mass respectively. The values in FRAP assay expressed the corresponding concentration and electron donating antioxidants. Antioxidant activity positively correlated with the total phenolic and flavonoid contents (R^2=0.923). Geographical location and climatic conditions have shown to have profound effects on the amount and activity of antioxidants available in both tamarind fruits and leaves. Findings from the study have indicated that tamarind can be used as a cheap source for antioxidants. However further agronomic studies should be considered to justify the effects of agro-ecological differences on antioxidant activity.

DECLARATION

I, Mourice Mbunde, do hereby declare to the Senate of Sokoine University of Agriculture, that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

_____ _____

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DEDICATION

This work is dedicated to my beloved parents, my late mother Christina Mbunde and my father Peter Nyangabo Mbunde and also to my sister Happiness Peter, and my brother Samwel Shaba for their prayers, love and encouragement all the time of my studies.

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LIST OF ABBREVIATIONS AND ACRONYMS

%	Percentage
<	Less than
>	Greater than
±	Plus or Minus
AFNNET	African Natural Products Network
$AlCl_3$	Aluminium chloride
ANOVA	Analysis of Variance
BHT	Butylated hydroxytoluene
$C_2H_3NaO_2.3H_2O$	Sodium acetate buffer
C_2H_5OH	Ethanol
CE	Catechin equivalents
CH_3COONa	Sodium acetate
CH_3OH	Methanol
DPPH	2, 2-diphenyl-1-picrylhydrazyl
FAO	Food and Agriculture Organization
FCR	Folin-Ciocalteau reagent
$FeSO_4.7H_2O$	Iron sulphate heptahydrate
FRAP	Ferric Reducing Antioxidant Power
g	Gram
GAE	Gallic acid equivalents /100 g of fruit weight
GPS	Global Positioning System
H_2O	Water
HCl	Hydrochloric Acid
Km	Kilometre

L	Litre
LDL	Low Density Lipoprotein
Mg	Milligram
ml	Millilitre
N	Normality
Na_2CO_3	Sodium carbonate
$NaNO_2$	Sodium nitrite
NaOH	Sodium hydroxide
nm	Nanometre
p	Probability Value (for statistical significance)
SD	Standard Deviation
SPP	Species
SUA	Sokoine University of Agriculture
TFC	Total Flavonoid Content
TPC	Total Phenolic Content
TPTZ	2, 4, 6-tripyridyl-s-triazine
USDA	United States Department of Agriculture
μL	Microlitre
μM	Micromole

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Oxidation reactions that occur particularly in the human body are likely to produce free radicals which in turn bring about various disorders including atherosclerosis, arthritis, ischemia and reperfusion injury of many tissues, gastritis and cancer (Seal, 2011). To protect the cells, organs and systems of the body against effects of free radicals, humans have evolved a highly sophisticated and complex antioxidant protection system. This system involves a variety of components, both endogenous and exogenous in origin, that function interactively and synergistically to neutralize free radicals (Percival, 1996). Antioxidants work by preventing the oxidative damage caused by free radicals in the body, as it can react with free radicals, chelate catalytic metals and also by acting as oxygen scavengers. Most of the antioxidant compounds in the body are obtained from external source mainly through consumption of fruits and vegetables. The need for supply of antioxidants becomes even more critical with increased exposure to free radicals from external sources, such as exposure to x-rays, ozone, cigarette smoking, air pollutants and industrial chemicals (Dimitrios, 2006; Kumar, 2011).

Since ancient times, man has been depending on natural sources especially plants for protection against effects of various diseases and improvement of his life style. With technological advancement and recent research findings, it has been revealed that certain non-nutritive chemicals produced by plants such as terpenoids, flavonoids and other phenolic compounds, which were initially thought to be of no importance to human health, possess antioxidant properties (Seal, 2011).

Antioxidant compounds have been searched in several types of plant materials such as vegetables, fruits, leaves, barks and roots in form of crude plant drugs. Polyphenolic compounds, which are dominant in antioxidant activity, are then found to be common in leaves, fruits, stem and barks. In the plants these compounds are important for normal growth development and defence against infection and injury (Seal, 2012; Aires *et al*., 2013). Epidemiological reports suggest that, dietary intake of natural products, especially fruits and vegetables; have proved to have a strong inverse correlation with the risk of developing coronary heart disease and cancers (Lako *et al*., 2007; Zidenberg-Cherr and Heneman, 2008). Presence of antioxidants in natural sources has created an upsurge demand for natural products for the control and treatment of various infections and diseases as some chemically synthesized drugs claim to have undesirable effects (Mayunzu *et al.*, 2011).

Tamarindus indica L. commonly known as tamarind has a long history in traditional medicine throughout Africa and Asia (El-Siddig *et al.*, 2006; Lourith *et al*., 2009). In Tanzania, this species is increasingly being used by the society for juice making or as a vegetable. Modern medical science has also confirmed its laxative and diuretic properties. All morphological parts of *T. indica* are used, from the fruit pulp and seed to the leaves, bark and flowers. Ailments such as diarrhoea, jaundice, ulcers, eye infections and digestive problems can be treated with infusions and pastes and powders from *T. indica* (Khairunnuur *et al.*, 2009; De Caluwé *et al*., 2010). Herbal practices are still widely used in almost every place wherever *T. indica* is accessible (Rudrappa, 2009).

A number of studies have reported the content of some essential nutrients in tamarind having high levels of ascorbic acid, vitamins; A, B and C and organic acids like citric, tartaric and malic; as well as polyphenols and flavonoids, that are the main responsible for

the strong antioxidant; hepatoprotective and antimicrobial activity (Lamien-Meda *et al.*, 2008; Lourith *et al*., 2009; Rodríguez-Amado *et al*., 2012).

1.2 Problem Statement and Justification

Many wild fruits and leaves contain large amounts of antioxidant compounds, which are important in the prevention of various human diseases (Javanmardi *et al*., 2003). Antioxidants property is mainly brought about by the presence of polyphenolic compounds such as anthocyanins, flavonoids, phenolic acids and phenolic diterpenes. *Tamarindus indica* L. is said to contain a large number of polyphenolic compounds with potential for antioxidant activity (Pieta, 1998). However, the quantities of antioxidants may vary with geographical location (Aires *et al*., 2011; Mahmood *et al.,* 2012). Despite the wide utilization and availability of *T. indica* in most parts of Tanzania, little is known on the amount and activity of antioxidants from this plant. Furthermore, there is limited information on comparative analysis of antioxidant compounds available in the wild tamarind from different agro-ecological zones of Tanzania. Therefore, this study was designed to fill the existing knowledge gap. Findings from the study would be useful in providing baseline information about antioxidant and antioxidant capacity of *T. indica*.

1.3 Objectives

1.3.1 Overall objective

To establish the antioxidant activity in different morphological parts of *Tamarindus indica* collected from Central, Eastern and Coastal zones of Tanzania.

1.3.2 Specific objectives

i. To determine the quantity of phenolics and flavonoids contents in leaves and fruits of *T. indica* from Coastal, Eastern and Central zones of Tanzania.

ii. To establish antioxidant activities of extracts from leaves and fruits of *T. indica* from the Coastal, Eastern and Central zones of Tanzania.

1.3.2 Research questions

i. What are the concentrations of phenolics and flavonoids in the leaves and fruits of *T. indica*?

ii. What is the antioxidant activity of extracts from leaves and fruits of *T. indica*?

iii. Is there any variation in antioxidant properties of *T. indica* obtained from different morphological parts of plant and different agro-ecological zones?

1.3.3 Hypotheses

i. H_0: There is no significant difference in phenolic and flavanoid content in extracts of *T. indica* fruits and leaves

H_1: There is a significant difference in phenolic and flavanoid content of *T. indica* fruits and leaves

ii. H_0: There is no difference in phenolic and flavanoid content of *T. indica* fruits and leaves obtained from different agro-ecological zones

H_1: There is the difference in phenolic and flavanoid content of *T. indica* fruits and leaves obtained from different agro-ecological zones

iii. H_0: There is no difference in antioxidant activity of *T. indica* leaves and fruits obtained from different agro-ecological zones

H_1: There is a difference in antioxidant activity of *T. indica* leaves and fruits obtained from different agro-ecological zones

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Overview of *Tamarindus indica*

Tamarindus indica L. belongs to the dicotyledonous family, Fabaceae; Sub-family Caesalpiniaceae, which is the third largest family of flowering plants (El-Siddig *et al.*, 2006). The name tamarind originates from a Persian word 'Tamar-I-hind', meaning date of India. In Tanzania, tamarind is commonly known by several vernacular names such as Swahili (Mkwaju), Maasai (Ol-masamburai), Sukuma (Bushishi), Luo (Chwaa) and in Meru (Muthithi) (Chikamai *et al*., 2005; Orwa *et al*., 2009).

A truly pan-tropical tree tamarind grows well over a wide range of soils and climatic conditions, occurring in low-altitude woodland, savannah and bush, often associated with termite mounds. Tamarind species can also be found growing along streams and riverbanks. It has ability to tolerate fog and saline air in coastal areas, and even monsoon climates, where it has proven its value for plantations (Orwa *et al.*, 2009).

It is found in altitude from 0 to 1,500 m above sea level, at a mean annual temperature (20-33) ^{o}C and mean annual rainfall ranging from 350-2700 mm. Regardless of total annual rainfall, tamarind produces more fruit when subjected to a fairly long, annual dry period. The tree prefers slightly acid (pH 5-5) deep alluvial, well-drained soils of loamy texture (Orwa *et al.*, 2009).

Tamarind species is indigenous to tropical Africa; however, it has a wide geographical distribution ranging from Africa, South Asia, South America up to Caribbean islands. In Tanzania, it is a very adaptive species distributed in several regions, being abundant at the

Coastal zone and in Zanzibar Islands (Chikamai, *et al.*, 2005). The tree growth averages to 20-30 m in height and 1.5-2.0 m in diameter and has a wide spreading crown and a short, stout trunk. It is a slow growing, but long lived, with an average life span of 80-200 years (Christman, 2004; Khairunnuur *et al.*, 2009).

Tamarind species has a long history in terms of use. In primitive and modern medicine, tamarind has played a prominent role. Its fruit pulp is used either for seasoning, as a food component, to flavour confections, curries and sauces, porridge and is a main component in juices, infusion, jam, sweets and certain beverages. Tamarind fruit pulp can also be eaten fresh (El-Siddig *et al*., 2006; De Caluwé *et al*., 2010). The leaves and flowers are known to be used as vegetables and are prepared in a variety of dishes. They are used to make curries, salads, stews and soups in many countries, especially in times of scarcity (Orwa *et al.*, 2009; De Caluwé *et al*., 2010). The pulp can also be used, when mixed with salt, to polish brass, copper and silver. It can be used as a fixative with turmeric and annatto dyes and also serves to coagulate rubber. Extracts from the plant also have an inhibitory effect on plant viruses. The leaves and foliage of tamarind can be used as forage for cattle and the timber for furniture and other tools. Tamarind seed kernel powder (TKP) is a major industrial product, which is used in the sizing of textile, paper and jute (ICUC, 1999).

Tamarind seeds are also edible after soaking in water and boiling to remove the seed coat. Flour from the seed may be made into cake and bread (Orwa *et al.*, 2009). The seed and its extracts can be used in the food processing industry, as an adhesive in the plywood industry and in the tanning industry due to the high tannin content in the seed testa (ICUC, 1999).

Tamarind species is rich in fatty acids and heavy alcohols. Its leaves contain protein and essential amino acids, carbohydrates and minerals such as zinc, magnesium, phosphorus, copper, selenium, and calcium; volatile oils, steroids, resins, mucilage and sugars reported in this species too. Tamarind contains iron, vitamins A, B and C and organic acids like citric, tartaric and malic as well as polyphenols and flavonoids, which are responsible for the strong antioxidant; hepatoprotective and antimicrobial activity of the tamarind leaf extract (Lourith *et al*., 2009; Rodríguez-Amado *et al*., 2012). Extracts from the fruit pulp have shown some molluscicidal activity and have been reported to have potent fungicidal and bactericidal properties (ICUC, 1999).

In terms of medicinal applications, tamarind claims to be used in herbal remedies in many parts of the world. Medicinal uses of tamarind can be found in many cultures and for a wide array of applications (El-Siddig *et al*., 2006). Tamarind fruit and leaves are known for treatment of skin infections. It is also regarded as a digestive, carminative, laxative, expectorant and blood tonic. The American pharmaceutical industry processes 100 tonnes of tamarind pulp annually as a common ingredient in cardiac and blood sugar reducing medicines. Leaves and flowers, dried or boiled, are used as poultices for swollen joints, sprains and boils. The latter are usually applied after grinding leaves and flowers into powder whereby they are used in lotions or infusions. Lotions and extracts made from them are used in treating conjunctivitis, as antiseptics, as vermifuges, treatments for dysentery, jaundice, erysipelas and haemorrhoids and various other ailments (ICUC, 1999; De Caluwé *et al*., 2010).

2.2 Antioxidants

Antioxidants are substances that may protect cells from the damage caused by unstable molecules known as free radicals. An antioxidant is a molecule capable of slowing or

preventing the oxidation of other molecules. Oxidation is a chemical reaction that transfers electrons from a substance to an oxidizing agent. Oxidation reactions can produce free radicals, which start chain reactions that damage cells (Hamid *et al*., 2010). Antioxidants terminate oxidation reactions that occur in the body by removing free radical intermediates and inhibit other oxidation reactions by being oxidized themselves. As a result, antioxidants are often reducing agents such as thiols, ascorbic acid or polyphenols. They exist as vitamins, minerals and other compounds in foods (Sies, 1997).

2.2.1 Classification of antioxidants

According to Hamid *et al.* (2010) antioxidants are categorized into two major groups namely; primary or natural antioxidants and secondary or synthetic antioxidants.

2.2.2 Primary or natural antioxidants

These are the chain breaking antioxidants which react with lipid radicals and convert them into more stable products. This group of antioxidants has phenol structures and includes the following:

Antioxidants minerals- are co-factor of antioxidant enzymes in which their absence will definitely affect metabolism of many macromolecules such as carbohydrates. Examples include selenium, copper, iron and manganese.

Antioxidants vitamins –needed for most body metabolic functions, i.e. vitamin C, vitamin E, vitamin B.

Phytochemicals are phenolic compounds that are neither vitamins nor minerals. They include; flavonoids, which are low molecular weight, polyphenolic compounds responsible for autumnal burst of hues and yellow, orange and red shades in vegetables,

fruits, grains, seeds leaves, flowers and tree barks. They are mainly classified into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones.
The common feature of flavonoid compounds is phenyl benzopyrone skeleton (C_6-C_3-C_6) (Nandave *et al.,* 2005; Sisa *et al*., 2010).

Carotenoids are fat soluble colour in fruits and vegetables. β-carotene is rich in carrot and converted to vitamin A when the body lacks enough of the vitamin. Lycopene, high in tomatoes and zeaxanthin is high in spinach and other dark greens. Herbs and spices-sources include diterpene, rosmariquinone, thyme, nutmeg, clove, black pepper, ginger, garlic and curcumin and derivatives (Hamid *et al.*, 2010).

2.2.3 Secondary or synthetic antioxidants

Secondary antioxidants are also regarded as preventive or class II antioxidants. These offer their antioxidant activity through various mechanisms to slow the rate of oxidation reactions. The main difference with primary antioxidants is that secondary antioxidants do not convert free radicals into stable molecules. They act as chelators for prooxidant or catalyst metal ions, provide H to primary antioxidants, decompose hydroperoxide to non-radical species, deactivate singlet oxygen, absorb ultraviolet radiation, or act as oxygen scavengers. They often enhance the antioxidant activity of primary antioxidants. Examples of secondary antioxidant compound include; Butylated hydroxyl anisole (BHA), Butylated hydroxyrotoluene (BHT), Propyl gallate (PG) and metal chelating agent (EDTA) (Wanasundara and Shahidi, 2005; Hamid *et al.*, 2010).

Generally, plants are rich source of antioxidants brought about by the presence of polyphenolic compounds. Polyphenolic compounds belong to a large heterogeneous group of secondary plant metabolites that are widespread in the plant kingdom. The

antioxidant capacity possessed by phenolic compounds is mainly due to their redox properties, which permit them to act as reducing agents, hydrogen donors, singlet oxygen quenchers or metal chelators. Besides their roles as antioxidants, these compounds exhibit a wide spectrum of medicinal properties, such as anti-allergic, anti-inflammatory, anti-microbial, anti-thrombotic, cardio-protective and vasodilatory effects (Saurabh and Bonde, 2011).

Tamarind fruit being a plant contains a biologically important source of mineral elements and with a high antioxidant capacity associated with high phenolic content that can be considered beneficial to human health. The phenolics include gallic acid equivalent of 626-664 mg per 100g (El-Siddig *et al*., 2006). Khairunnuur *et al*. (2009) reported a wide range of total phenolic content in tamarind parts. Their contents ranged from 1.83±0.02 to 19.21±0.29 mg Gallic Acid Equivalent (GAE)/100g of dried samples, with an average of 9.64 mg (GAE)/100g fresh sample.

2.3 Free Radicals

A free radical is an atom, molecule, or compound that is highly unstable because of its atomic or molecular structure (i.e., the distribution of electrons within the molecule). As a result, free radicals are very reactive as they attempt to pair up with other molecules, atoms, or even individual electrons to create a stable compound. To achieve its stability, free radicals require a hydrogen atom from another molecule, bind to another molecule, or interact in various ways with other free radicals (Wu and Cederbaum, 2003).

Examples of oxygen free radicals are superoxide, hydroxyl, peroxyl ($RO_2^{\bullet}$), alkoxyl ($RO^{\bullet}$), and hydroperoxyl ($HO_2^{\bullet}$) radicals. Nitric oxide and nitrogen dioxide ($^{\bullet}NO_2$) are two nitrogen free radicals. Oxygen and nitrogen free radicals can be converted to other non-

radical reactive species, such as hydrogen peroxide, hypochlorous acid (HOCl), hypobromous acid (HOBr), and peroxynitrite ($ONOO^-$). Reactive oxygen species (ROS), reactive nitrogen species (RNS), and reactive chlorine species are produced in animals and humans under physiologic and pathologic conditions. Thus, ROS and RNS include radical and non-radical species (Fang, 2002).

If free radicals are not inactivated in the body, their chemical reactions can damage all cellular macromolecules including proteins, carbohydrates, lipids and nucleic acids (Kumar, 2011). Their destructive effects result into a wide range of physiological dysfunctions such as atherosclerosis, diabetes, ischemia/reperfusion (I/R) injury, inflammatory diseases (rheumatoid arthritis, inflammatory bowel diseases and pancreatitis), cancers, neurological diseases, hypertension (Aliyu *et al*., 2009; Kumar, 2011).

2.4 Effect of Agro-ecological Factors in Antioxidants

Growth and maturation of plant tissues involve a series of complex reactions, which leads to changes in phytochemistry (Mahmood *et al*., 2012). The content of polyphenols in fruit is affected by the degree of maturity at harvest, genetic differences (cultivar), pre-harvest environmental conditions, and post-harvest storage conditions and processing (Aires *et al*., 2011). It has been documented by various authors that pre-harvest conditions such as climate, temperature, light intensity, soil type, compost, mulching, fertilization, increasing carbon dioxide concentration in the atmosphere, and application of naturally occurring compounds can affect the antioxidant content and antioxidant activity of the harvested fruits (Wang, 2006; Gull *et al*., 2012; Rodríguez-Amado *et al*., 2012). The impact of geographical location on the extraction of total phenol content is also supported by the fact that variety of diverse factors such as worldwide changes in seasonal patterns,

weather episodes, changes in temperature, biotic and abiotic stresses may affect the production of secondary metabolites in plants.

Nutritional stress such as low iron levels can cause increased release of phenolic acids, presumably to help solubilize metals and thereby facilitate their uptake (Marschner, 1991). For instance, a study undertaken by Aires *et al*. (2011) using *Brassica* vegetables revealed that, climatic factors such as sun exposure, soil type, temperature and rainfall are important in interfering chemical biosynthesis bioactive compounds. This in turn interferes the antioxidant properties of *Brassica* vegetables. Gull e*t al*. (2012) also reported that, polyphenolics content is affected by a number of factors, such as agronomic (biological culture, greenhouses or fields, hydroponic culture, fruit yield per tree) or climatic (sun exposure, soil type, rainfall). Besides, the concentration of polyphenols is also influenced by the extent of fruit maturity. Similarly, the bark of *B. retusa* exhibited variations in their phyto-constituents depending upon the geographical location from where they were harvested as stated by Saurabh and Bonde (2011). Based on such variation of existing antioxidant properties, this study intended to determine variation in terms of antioxidants quantities available in the wild tamarind from Central and Coastal locations in Tanzania.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Description of Areas Where Samples Were Collected

This study involved sample collection from three different locations that falls in agro-ecological zones. Coastal zone (Tanga), Eastern Plateau and Mountain Blocks (Morogoro) and Central Plateau (Dodoma). The Coastal zone (Tanga) lies 500-1200 metres above sea level and has been developed over gneissic rocks. The zone has poorly drained flat wide topographical depressions developed on young alluvium and strongly dissected areas of pronounced slopes, often rocky and severely eroded. There are two main types of soils: sandy clay loams and sandy clays, and sands and loamy sands. The zone is mostly infertile and has poor moisture acceptance properties due to a tendency for surface sealing. It experiences bimodal rainfall ranging from 700-1200 mm per annum (USDA, 2005; Handeni, 2008).

An Eastern Plateau and Mountain block, which encompasses Morogoro region (Mvomero district), has undulating plains to dissected hills and mountains as well as moderately fertile clay soil. The region experiences unimodal rainfall ranging from 800-1400 mm (USDA, 2005; Mbogoni and Ley, 2008).

The Central Plateau (Dodoma region) has undulating plains with rocky hills and low scarps. Its soil is drained with low fertility. The rainfall is unimodal and unreliable ranging from 500-800 mm (USDA, 2005).

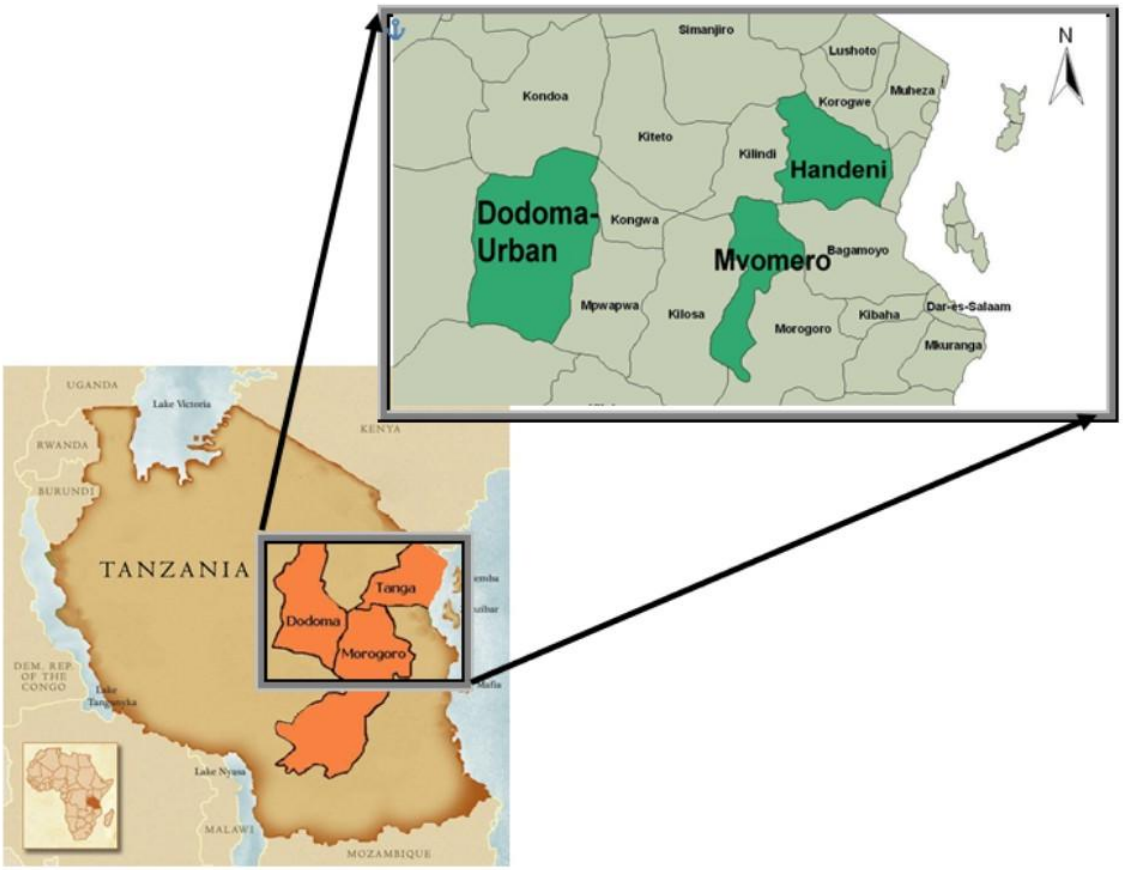

Figure 1: Map of study area showing areas where samples were collected

3.2 Study Design

This study adopted observational study design whereby samples were collected and taken to the laboratory for extraction and analysis of antioxidants. Samples were collected from three (3) villages namely Misima (Tanga), Doma (Morogoro) and Ntyuka (Dodoma) purposively selected from the three zones. Collection of samples was done purposively based on availability of tamarind species with mature fruits in the areas and in each region, five (5) samples (leaves and fruits from five tamarind trees) were taken from one village. The basis for selection of each region was climatic condition, i.e., semi-arid, woodland and coastal climatic conditions.

3.3 Materials

3.3.1 Equipment and apparatus

Whatman no.1 filter paper, UV-visible spectrophotometer (UNICO VIS1200 Version SS-1.24, United Products and Instruments, Inc.), bench centrifuge, buchner funnel, separating funnel (250 ml), beakers (250 ml), volumetric flask (5, 10, 25, 50 ml), measuring cylinder (5, 10, 25, 50 ml), conical flasks (25 ml), cuvettes, appendorf tips, micropipettes.

3.3.2 Chemicals and reagents

Methanol (CH_3OH), ethanol (C_2H_5OH)), hydrochloric acid (HCl), Folin Ciocalteu reagent (FCR), TPTZ (2, 4,6-tripyridyl-s-triazine), iron sulphate heptahydrate ($FeSO_4.7H_2O$), iron chloride ($FeCl_3$), sodium carbonate (Na_2CO_3), sodium hydroxide (NaOH), sodium acetate (CH_3COONa), sodium acetate buffer ($C_2H_3NaO_2.3H_2O$), sodium nitrite ($NaNO_2$), aluminium trichloride ($AlCl_3$), standard Gallic acid, butylated hydroxytoluene (BHT), 1, 1-diphenyl-2-picryl-hydrazyl (DPPH), vitamin C (ascorbic acid) and catechin. All the chemicals used including the solvents were analytical grade and purchased from the University Suppliers.

3.4 Methods

3.4.1 Collection of plant materials

Fresh tamarind leaves and ripened fruits were collected from several populations of tamarind species in the selected agro-ecological zones (Plate 1). At each location where samples were collected Global Positioning System (GPS) was used to mark the coordinates and photograph of the plant taken.

Plate 1: Collection of fruits and leaves from *T. indica*

3.4.2 Extract preparations

The separated leaves were air dried under the shade at room temperature (30^{o}C) and ground to powder using grinding machine. Ten grams (made from 2 g of individual sample) of leaf powder were extracted in 99.9% methanol for 48 hrs at 30-33^{o}C. The fruits were peeled and the pulps separated from the seed (Ashafa *et al*., 2010). Ten percent (10%) pulp extract was prepared by soaking 10 g (made from 2 g of individual sample) of the fresh pulp in 100 ml of 99.9% methanol and mixed thoroughly (Khairunnuur *et al.,* 2009). The mixture was shaken and allowed to stand for 48 hrs. Both fruit and leaf extracts were then filtered through Whatman filter paper No.1. The filtrate was evaporated to dryness under reduced pressure at 40ºC using a rotary evaporator. The crude extract obtained was stored at -20^{o}C until further analysis (Ashafa *et al*., 2010). The plates 2,3, 4 and 5 below shows stages employed during extract preparations.

Plate 2: Drying of *T. indica* leaves

Plate 3: Mature pods of *T. indica*

Plate 4: Tamarind fruits and leaves soaked in methanol (99.9%) for extraction of phenolics and flavonoids compounds

Plate 5: Fruit extract from *T. indica*

3.4.3 Coding of samples

Samples collected from three different zones were coded as can be seen in the Table 1 below.

Table 1: Coding of samples collected from three zones

Code	Agro-Ecological Zone	Source Of Tamarind Extract
LVMR	Eastern zone	Leaves
FRMR	Eastern zone	Fruits
LVDM	Central zone	Leaves
FRDM	Central zone	Fruits
LVTA	Coastal zone	Leaves
FRTA	Coastal zone	Fruits

LVMR=Morogoro leaf extract; FRMR=Morogoro fruit extract; LVDM=Dodoma leaf extract; FRDM=Dodoma leaf extract; LVTA=Tanga leaf extract; FRTA=Tanga fruit extract

3.4.4 Determination of total phenolics content

The concentration of total phenolics was determined according to the method described by Velioglu *et al*. (1998) with some modification whereby the diluted aqueous solution of each extract (0.5 mL) was mixed with Folin Ciocalteu reagent (0.2 N, 2.5 mL). This mixture was allowed to stand at room temperature for 5 minutes and then sodium carbonate solution (75 g/L in water, 2 mL) was added. After 2 hours of incubation, the absorbance was read at 760 nm against water blank. A standard calibration curve was plotted using Gallic acid (0, 25, 50, 75, 100, 125, 150, 175, 200, 225, 250 mg/L). The concentration of samples was calculated from the equation obtained from the standard

curve. The results were expressed as mg of Gallic Acid Equivalents (GAE)/100 g of fruit weight.

3.4.5 Determination of total flavonoid contents

Total flavonoid content was measured using colorimetric assay (Zhishen *et al*., 1999). One ml aliquot of appropriately diluted extract or standard solutions of catechin (5, 10, 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 mg/L) was placed in a test tube, then added 4 ml of double distilled water (ddH_2O) and 0.3 ml (5%) $NaNO_2$. After 5 min, 1.5 ml (2%) $AlCl_3$ was added to the test tube and shaken. The mixture was shaken and 5 min later, 2ml of 1 M NaOH was added to the mixture and shaken well. Absorbance of the mixture, pink in color, was read by spectrophotometer at 510 nm versus the prepared standard. Total flavonoids content in the fruit extract was expressed as mg/100g catechin equivalents (CE) (fresh weight basis). All samples were analyzed in triplicate.

3.4.6 Determination of 1, 1-diphenyl-1-picrylhydrazyl scavenging activity

Antioxidant activity of ethanolic extract of leaves and fruits of tamarind was determined by using the stable DPPH (2, 2-diphenyl-1-picrylhydrazyl) free radical scavenging according to Mensor *et al*., (2001) method with some modification. A 0.1 mM solution of DPPH in methanol was prepared, and 4 ml of this solution was added to 2 ml of the solutions of Butylated hydroxyl toluene (BHT) in methanol at different concentrations (25, 50, 75, 100, 125, 150 mg/L). The mixtures were shaken vigorously and allowed to stand at room temperature for 30 min. Also 4 ml of DPPH was added to 1 ml of sample diluted with 2 ml of methanol in the test tube and the mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was read at 517 nm using a UV-VIS spectrophotometer (UNICO VIS1200 Version SS-1.24). Butylated hydroxyl toluene (BHT) was used as the reference. Lower absorbance values of reaction

mixture indicate higher free radical scavenging activity. The mixture was measured spectrophotometrically at 518 nm. The antioxidant activity (AA) was calculated as below: AA%= 100-[((Absorbance of the sample-Absorbance of the blank)/ (Absorbance of the control)] x100 (Mensor *et al*., 2001)**.**

3.4.7 Determination of ferric reducing antioxidant power (FRAP)

A modified method of Benzie and Strain (1996) was adopted for the Ferric reducing antioxidant power (FRAP assay). The stock solutions included 300 mM acetate buffer (3.1 g CH_3COONa and 16 ml CH_3OOH), pH 3.6, 10 mM TPTZ (2, 4, 6-tripyridyl-s-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6H_2O$ solution. The fresh working solution was prepared in a ratio of (10:1:1) by mixing acetate buffer, TPTZ, and $FeCl_3 \cdot 6H_2O$. The temperature of the solution was raised to 37°C before use. A volume of 100 µl extracts/standard was placed in a test tube and diluted with 300 µl of distilled H_2O then 2.85 ml of the FRAP solution was added and incubated for 30 min. Readings of the colored product (ferrous tripyridyltriazine complex) were taken at 593 nm. The standard curve was between 100 and 700 µl $FeSO_4.7H_2O$. Results were expressed in µM Fe (II)/g dry mass and compared with that of catechin.

3.5 Data Analysis

Results were expressed as mean values ± standard deviation (three replicate experiments). For each variable, treatment means were subjected to Analysis of Variance (ANOVA) and Duncan's Multiple Range Test (DMRT) of the CoHort Costat software version 6.33. Significant differences were reported at $p< 0.05$.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

The fruit and leaves extracts had different antioxidant capacities in relation to the method of estimation. The antioxidant capacity of the different fruit and leaf extracts and the ranking order for each assay is presented in tables 2, 3 and 4 and figures 2 and 3.

Variation observed in the amount of polyphenolic contents in fruits harvested from three different agro-ecological zones can be explained by series of complex biochemical reactions during fruit ripening that affected the formation of phenolics, flavonoids, anthocyanins, carotenoids and other volatile compounds leading to the development of final characteristics and distinct flavour of a mature fruits (Gull *et al*., 2012).

4.2 Total Phenolics Content

The basic mechanism of the method used in phenolic determination (Folin-Ciocalteau assay) is an oxidation/reduction reaction based on the redox properties of antioxidant compounds that can react with the Folin-Ciocalteau Reagent (FCR) enhancing the measurement of phenolic concentration (Norshazila *et al*., 2010). The phenolic content of tamarind extract and the ranking order for each extract is shown in Table 2.

Table 2: Total phenolics content (mg GAE/100g) of tamarind leaf and fruit extracts collected from different agro-ecological zones in Tanzania (n=6)

[2]Code	Agro-Ecological Zone	[1]Total Phenolics content (mg GAE/100g)
LVMR	Eastern zone	17874.67±5234[a]
LVTA	Coastal zone	17799.25±4825.05[a]
LVDM	Central zone	6144.6±2205.23[b]
FRTA	Coastal zone	4755±1699.25[bc]
FRMR	Eastern zone	2073.33±287.39[c]
FRDM	Central zone	1994.4±530.77[c]

[1]Values are mean ± SD of six samples analyzed individually in triplicates. Values within a column with superscripts significantly different at ($p<0.05$) in fruits and leaf extracts. [2]LVMR=Morogoro leaf extract; FRMR=Morogoro fruit extract; LVDM=Dodoma leaf extract; FRDM=Dodoma leaf extract; LVTA=Tanga leaf extract; FRTA=Tanga fruit extract

Results of this study revealed that the level of phenolic compounds in tamarind extracts from the three different agro-ecological zones varied significantly ($p<0.05$) in both leaves and fruits extracts. Generally, among the six sample extracts, the tamarind leaf and fruit extracts from the Coastal zone (17799.25±4825.05 mg GAE/100 g and 4755±1699.25 mg GAE/100 g respectively) demonstrated higher levels of phenolic content compared to the Eastern (17874.67±5234 mg GAE/100 g leaf extracts and 2073.33±287.39 mg GAE/100 g fruit extracts) and Central zones (6144.6±2205.23 mg GAE/100 g leaf extracts and 1994.4±530.77 mg GAE/100 g fruit extracts) samples. In all cases, it was revealed that, there was a high variation in phenolic content between tamarind morphological parts (i.e. fruits and leaves). For instance, tamarind leaf extract from Coastal zone had significantly higher ($p<0.05$) concentration of phenolics (Table 2) compared to fruits from the same

zone. Higher concentration of phenolics in tamarind fruits and leaves has not been reported compared to present study except in seed extracts as reported by Lourith *et al*. (2009) who found the contents in the range of 713.24 mg GAE/100 g to 63,691 mg GAE/100 g depending on the solvent used. Lamien-Meda *et al*. (2008) also reported low content of phenolics tamarind pulp (957.33 ± 13.20 g of GAE/ 100 g of fruit) compared to the present study. Other studies in tamarind fruit done by Khairunnuur *et al*. (2009) reported lower levels of phenolic contents at 19.21±0.29 g GAE/100 g in seed and 2.14±0.05 g GAE/100 g in fruit compared to the findings of this study.

The results further showed that, there was a significant variation ($p<0.05$) in the total phenolic content in the tamarind leaf extract from the Coastal and Central zones (Table 2). No significant difference ($p>0.05$) was observed in the total phenolic content of the tamarind leaf extract from Eastern (Morogoro) and Coastal (Tanga) zone, likewise for fruit extracts from samples collected in Central (Dodoma) and Eastern zones. These findings showed that, tamarind leaves and fruits growing in Tanzania contain high amount of phenolic contents than those reported elsewhere, which suggests Tanzania *T. indica* could have high antioxidant activity.

Presence of higher concentrations of phenolic compounds in leaves compared to those in fully ripe fruits could be explained by the fact that, in the later stages of fruit ripening, different phenolic acids condense to form complex phenolic compounds such as tannins and lignin (Gull *et al*., 2012). Most of the tamarind trees sampled had only younger leaves thus supporting the argument by Rodríguez-Amado *et al*. (2012) who reported that, younger leaves of tamarind possess higher phenolic compounds due to the need of the plant to protect itself from predators' attacks. Plant extracts containing high levels of phenolic compounds may be able to scavenge free radicals such as superoxide anion

radicals and peroxy radicals in the human body and protect human cells or tissues against oxidative stress (Norshazila *et al*., 2010).

4.3 Total Flavonoid Content

The results for the distribution of total flavonoid content (TFC) in tamarind leaves and fruits extracts in relation to geographical regions are presented in Table 3.

Table 3: Total flavonoid contents of tamarind leaves and fruits extracts from different agro-ecological zones in Tanzania (n=6)

[2]Code	Agro-Ecological Zones	[1]Total Flavonoid Content (Mg Ce 100/G Dry Wt)
LVTA	Coastal zone	11483.11±2559.67[a]
LVMR	Eastern zone	9853.33±6588.47[a]
LVDM	Central zone	3957.33±390.82[b]
FRMR	Eastern zone	2146.67±107.7[bc]
FRDM	Central zone	1088±294.23[c]
FRTA	Coastal zone	880±609.45[c]

[1]Values are mean ± SD of six samples analyzed individually in triplicates. Values within a column with superscripts significantly different at ($p<0.05$) in fruits and leaf extracts. [2]LVMR=Morogoro leaf extract; FRMR=Morogoro fruit extract; LVDM=Dodoma leaf extract; FRDM=Dodoma leaf extract; LVTA=Tanga leaf extract; FRTA=Tanga fruit extract

The concentration of flavonoids in tamarind leaf extracts with the exception of those samples collected from Central zone were significantly ($p<0.05$) higher than those of fruit extracts from all zones. In all extracts, fruits harvested from Coastal zone had the lowest content of flavonoids (Tables 3). Tamarind fruits extract from samples collected from

Central and Coastal zone did not show any significant differences in concentration of flavonoids among them ($p>0.05$). Likewise, leaf extracts from samples collected from the Coastal and Eastern zones had no significant difference in flavonoid contents ($p>0.05$). However, a significant difference ($p<0.05$) in flavanoid content was observed between the leaf and fruit extracts from Coastal zone region (Table 3).

The flavonoid content in the present study was found to be higher than values reported from other studies. Lamien-Meda *et al*. (2008) reported lower levels of flavonoids (2.18 ± 0.21 mg QE/100 g) in fruit methanolic extracts and (5.68 ± 0.10 mg QE/100 g) in fruit acetone extracts in tamarind growing in Burkina Faso. Presence of higher concentrations of flavonoid compounds in younger leaves compared to those in fully ripe fruits could be explained by the fact that, in the later stages of fruit ripening different phenolic acids may condense to form complex phenolic compounds such as tannins and lignin. Hence, due to changes of phenolic compounds with maturity, fully-ripe fruit possessed relatively lower amounts of total flavonoid contents (Gull *et al*., 2012). Concentrations of flavonoid compounds in younger leaves compared to the fruits can be explained by the fact that, flavonoids are responsible for color formation thus most of the samples collected were found to have yellow and red color (Plate 6) which indicates higher levels of flavonoids. Differences in flavonoid contents of different samples of tamarind from different locations could also be influenced by the growing conditions, genetic make-up of the species, amount of precipitation, temperature, altitude, soil conditions and availability of nutrients (Jaffery *et al*., 2003; Mahmood *et al*., 2012; Rodríguez-Amado *et al*., 2012). These factors might have affected the concentration of flavonoids in fruit and leaf extracts tested by affecting the composition of their phytochemicals.

On the other hand, it was observed that, tamarind has higher concentration of phenolics than flavonoids. This was evident in both leaf and fruit extracts tested. Tamarind leaf extracts had significantly higher levels of polyphenolic compounds (phenolics) and flavonoids than fruits extracts ($p<0.05$).

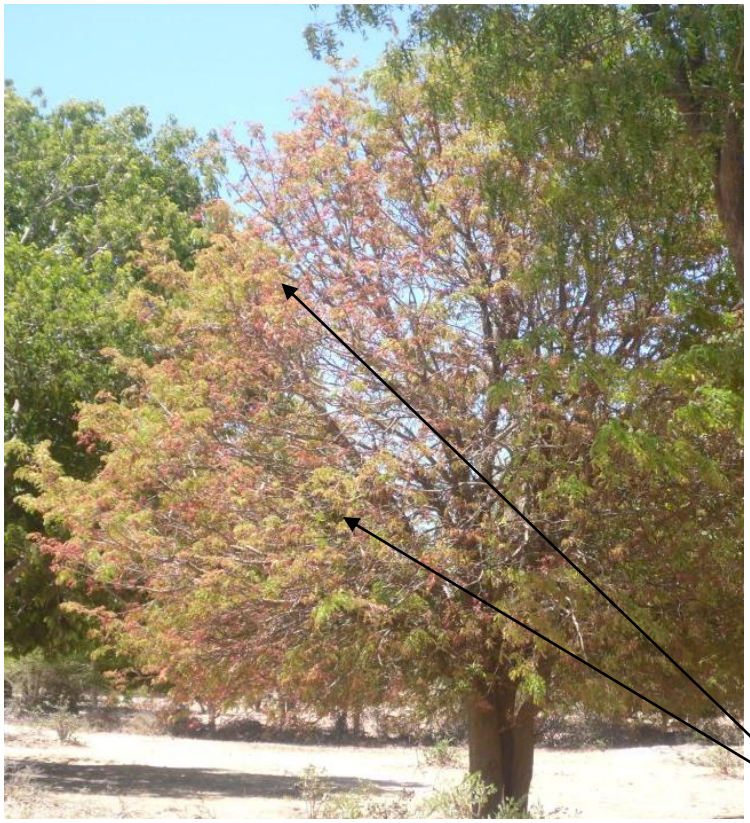

Plate 6: Tamarind plant

4.4 Radical Scavenging Activity

The DPPH test is the oldest indirect method for determining the antioxidant activity which is based on the ability of the stable free radical 2,2-diphenyl-1-picrylhydrazyl to react with hydrogen donors including phenols (Lamien-Meda *et al*., 2008). The bleaching of 2, 2-diphenyl-1-picrylhydrazyl by a test compound is representative of its capacity to scavenge free radicals generated independently from any enzymatic or transition metal based system. Antioxidants compounds available in a sample extracts react with DPPH,

which is a stable free radical to convert it to 1,1-diphenyl-2-(2,4,6-trinitrophenyl) hydrazine (Ali *et al*., 2010).

The bleaching of DPPH solution increases regularly with increasing amount of sample fruit and leaf extracts in a given volume as can be shown in the present study (Figure 3). The bleaching action is mainly attributed to the presence of antioxidant compounds like polyphenols in the solution (Lamien-Meda *et al*., 2008). The antioxidant activity of the fruits and leaves tested was found to vary over the tested samples (Figure 3). It can be seen that, scavenging activity decreased in the following order: LVTA<LVMR<LVDM<FRTA<FRDM<FRMR. This trend implied that, tamarind growing in the Coastal zone had the highest reducing potential compared to tamarind growing in the other zones. This observation reflected the concentration of phenolics and flavonoids observed in the sample extracts from the Coastal zone.

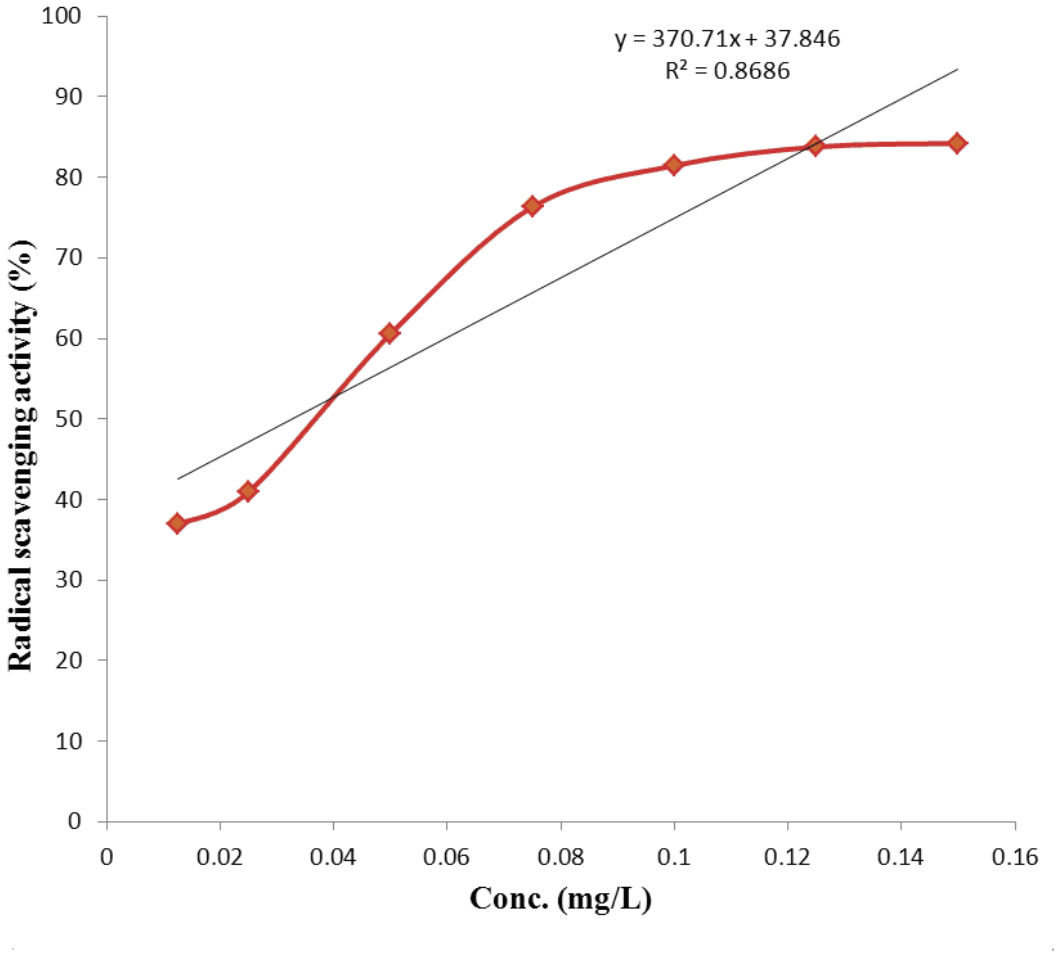

Figure 2: DPPH-free radical scavenging activity (RS %) of butylated hydroxyltoluene (BHT) used as standard

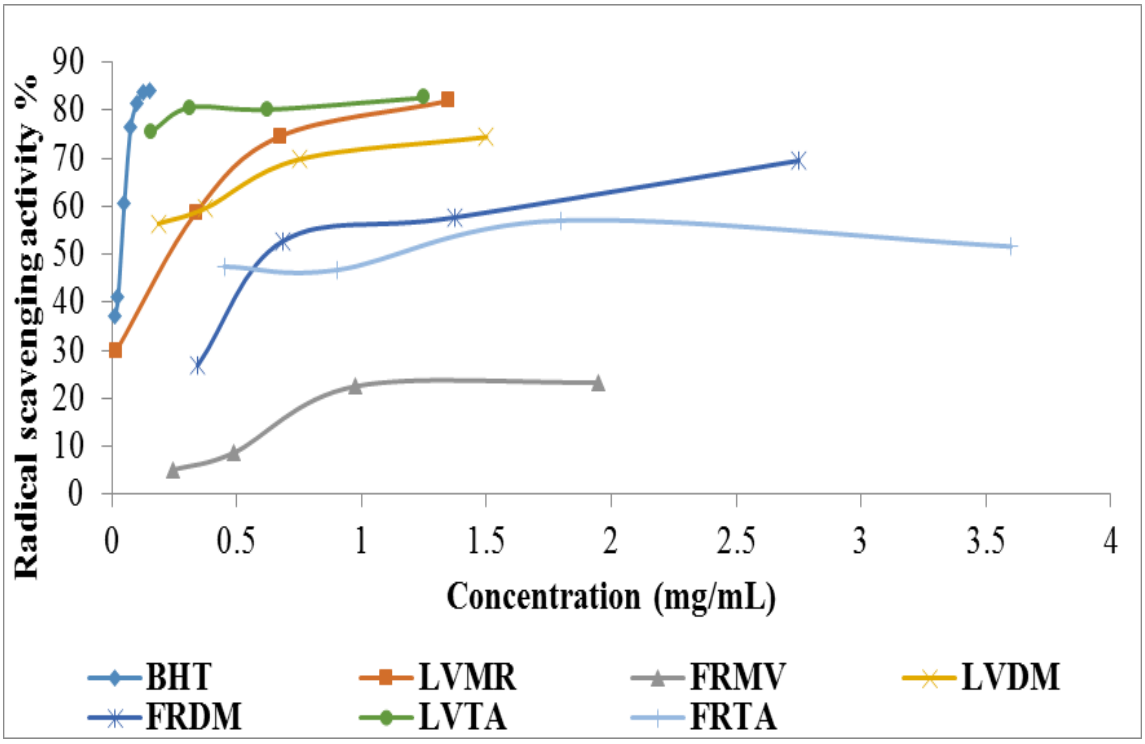

Figure 3: Effect of concentration of tamarind extracts in free radical scavenging activity

BHT= Positive Control; LVMR=Morogoro leaf extract; FRMR=Morogoro fruit extract; LVDM=Dodoma leaf extract; FRDM=Dodoma leaf extract; LVTA=Tanga leaf extract; FRTA=Tanga fruit extract

There was a significant difference ($p<0.05$) in the antioxidant activity of the leaf extracts (54.39±0.13%) and that of the fruit extracts (40.11±0.03%). The antioxidant activity of fruit extracts ranged from 29.27±0.06% to 40.11±0.03% while leaf extracts ranged from 22.33±0.08% to 54.39±0.13%. Radical scavenging activity observed in leaf and fruit extracts were linked with the concentration of phenolics and flavonoids observed in all the extracts, with strong positive correlation between total phenolic content and radical scavenging activity (R^2= 0.923). This correlation suggested that, polyphenols are responsible for antioxidant activity. Lamien-Meda *et al.* (2008) underscored that variation

in radical scavenging ability among the tamarind extracts over the regions can be brought by difference in climate and solvent used.

4.5 Ferric Reducing Activity

The ferric reducing antioxidant potential (FRAP) assay was used to assess the free radical scavenging capacities and the reducing potentials of the antioxidant constituents of the tamarind extracts. FRAP assay is usually based on the reducing power of a compound (antioxidant). It measures the reduction of Fe^{3+} (ferric iron) to Fe^{2+} (ferrous iron). As the ferric ion is reduced to ferrous ion the values in the FRAP assay expresses the corresponding concentration of electron donating antioxidants (Ali *et al*., 2010).

Table 4: Ferric Reducing Antioxidant Power (FRAP) values of tamarind leaf and fruit extracts from different Agro-ecological zones in Tanzania (n=6)

[2]Code	Agro-Ecological Zones	[1]µM Fe (II)/g dry mass
LVTA	Coastal zone	76822.67±23259.9[a]
LVMR	Eastern zone	32776±24506.66[b]
LVDM	Central zone	8199.33±2929.49[c]
FRTA	Coastal zone	6968±3655.91[c]
FRMR	Eastern zone	5328±2945.96[c]
FRDM	Central zone	3860±2377.57[c]

[1]Values are mean ± SD of six samples analyzed individually in triplicates. Values within a column with the superscripts significantly different at ($p<0.05$) in fruits and leaf extracts. [2]LVMR=Morogoro leaf extract; FRMR=Morogoro fruit extract; LVDM=Dodoma leaf extract; FRDM=Dodoma leaf extract; LVTA=Tanga leaf extract; FRTA=Tanga fruit extract

Table 4 shows the FRAP values of tamarind leaf and fruit extracts. The antioxidant activity was found to vary over the extracts from different agro-ecological zones of Tanzania. The trend for decrease in FRAP values or reduction potential among the extracts was LVTA< LVMR< LVDM<FRTA<FRMR<FRDM. When comparing the FRAP values among the extracts, it was found that tamarind leaf extracts collected from Coastal zone had highest FRAP values ($p<0.05$), followed by leaf extracts from Eastern zone (Table 4).

Leaf and fruit extracts obtained from Central zone did not show any difference ($p>0.05$) among them but showed significant difference ($p<0.05$) with the leaves from Coastal and Eastern zone. There was a significant difference in FRAP values (76822.67±23259.9 µM Fe (II)/g dry mass) in leaf extracts from the Coastal zone compared with FRAP values of fruits extracts from the same location ($p<0.05$). There was a correlation between total phenolic and flavonoid content with ferric reducing antioxidant activity ($R^2 = 0.923$ and $R^2 = 0.762$), respectively. These results agreed with Khairunnuur *et al*. (2009), who found a significant difference between FRAP values of tamarind fruit and seed extracts. Impacts of geographical factors in antioxidant property have been demonstrated by variation in FRAP values observed among tamarind extracts obtained from the three agro-ecological zones.

The findings in this study showed that, the amount and activity of antioxidant was higher for samples from Coastal zone (moderate temperature), than in samples from Central zone (extreme temperature). This clearly indicated the effect of agro-ecological zone on the amount and activity of antioxidants available in both tamarind fruits and leaves. The Coastal zone (Tanga) and Eastern zone (Morogoro) have been observed to possess favourable factors that promote production of phytochemicals in tamarind as compared to

the Central zone (Dodoma). These two zones (Tanga and Morogoro region) share some geographical factors such as temperature, soil type, and rainfall (USDA, 2005), unlike Dodoma region which receive extremely low rainfall, has poor soil fertility and average temperatures. According to Gull *et al*. (2012), moderate temperature conditions (25/30°C) are suitable for increasing antioxidant content. The authors further argued that, plants growing in extreme cold (18/12^{o}C) or hot (above 35^{o}C) temperatures, produce fruits and leaves with lower antioxidant content (Gull *et al*., 2012).

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

On the basis of the results, the leaf extracts hold the highest amount of phenolics and flavonoids content compared to fruit extracts. The present findings have again shown that, there is variation in phenolics and flavonoids content in which sample extracts obtained from the Coastal zone contained the highest amount of these polyphenols amongst the three agro-ecological zones.

Leaf extract from Eastern and Coastal zone exhibited significantly higher levels of antioxidant activity compared to extracts from Central zone. It was also found that, the leaf extracts exhibited higher radical scavenging activity than fruits extracts. The scavenging activity was observed to decrease in the following orders LVTA<LVMR<LVDM<FRTA<FRDM<FRMR. Antioxidant activity was found to be positively correlated with the total phenolics and flavonoids contents.

Polyphenols content in all sample extracts were significantly different and the mean DPPH and FRAP in all extracts were also different. This behavior is frequent in natural products, due to variation appears to relate to climate, soil characteristics and phenological stages of the plant, being the fructification stage. Phenolics and flavonoids compounds in leaves of the plants are higher in younger growth stages, probably due to the need of the plant to protect itself from the insects and other predators' attacks.

5.2 Recommendations

Both the tamarind pulp and leaf extracts have shown to be rich in several phytochemicals that act as powerful dietary antioxidants. Therefore, the presence of high amount of

phenolics and flavonoids and their multifaceted actions make the tamarind plant extract a good candidate for exploration of antioxidants. Since, the parts of the plant used in the present study have not yet received much attention as sources of antioxidant due to limited information or lack of commercial/pharmaceutical and nutritional applications in Tanzania; it is recommended that, exploitation should be made in the production of potential antioxidant supplement(s) from *T. indica*.

This study has revealed the effects of geographical factors on the quantity and activity of antioxidants available in tamarind morphological parts however, this calls for further agronomic studies that will also take soil and climatic conditions into account to justify the differences observed in different agro-ecological zones of Tanzania.

Existing research work indicated that, utilization of underexploited sources and better evaluation of ethnic and traditional foods can offer many benefits in the promotion of human health. In order to utilize such sources of antioxidants, to evaluate traditional products, to develop more complete compositional databases and to obtain more accurate antioxidants intake data, further chemical characterization is needed. Conservation of these useful plants from bad human practices, like cutting the trees for firewood, that threatens their survival is also recommended.

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