UNIVERSITY OF ZULULAND

Effect of the methanolic extract of *Cassia abbreviata* in the oxidative stress caused by overcrowding in indigenous chickens

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Effect of the methanolic extract of *Cassia abbreviata* in the oxidative stress induced by overcrowding in indigenous chickens

Dissertation submitted in fulfillment of the requirements for the Degree of Master of Science in Agriculture (Animal Science)

Department of Agriculture

Faculty of Science and Agriculture

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By

Martha Cebile Jobe

November 2016

DEDICATION

To my family (Jobe)

Sonto Lucia (Mom), Siphosakhe (Brother) and Ntombenhle (Sister)

DECLARATION

I, **Martha Cebile Jobe**, declare that:

- This dissertation contains my original work, and where the work of others has been used as a source, it is acknowledged accordingly.
- The experimental work and procedures described in this dissertation were conducted by me under the supervision of Professor Kunene and Professor Opoku.
- This dissertation has not been previously submitted in any University for particular degree fulfillments.
- All assistance towards the production of this work and all the references contained herein have been duly accredited.

Student name: Martha Cebile Jobe

Signature: ____________________ **Date:** ________________________

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The Lord is my Shepherd I shall not want (Psalms 23).

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ABSTRACT

Indigenous chickens are the largest livestock species that are widely domesticated by rural farmers with poor management practices. Poor management affects the growth performance and health of chickens in various ways. Due to high demand and poor management of chickens, overcrowding has become one of the influencing factors that retard growth. Overcrowding results in metabolic disturbances, causing excessive free radicals production that leads to oxidative stress. The oxidative stress can be managed by introducing radical scavengers, the antioxidants. Interestingly, natural antioxidants from medicinal plants are being adopted for use mainly because the synthetic antioxidants have been observed to have side effects. The aim of this work was to assess the effect of the stocking density on the growth performance of indigenous chickens and to evaluate the effect of *Cassia abbreviata* extract on the oxidative stress in chickens caused by overcrowding.

Phytochemical screening of *Cassia abbreviata* revealed the presence of alkaloids, flavonoids, terpenoids, tannins and saponins. Methanolic stem extract showed strong scavenging of 2,2'-azino-bis-(3-ethlybenzothiozoline-6-sulphonic acid), 2,2-diphenyl-1-picrylhydrozly, superoxide anion and hydroxyl radical; however poor scavenging of nitric oxide was observed. Reduction potential of the plant extract was dose dependent; the iron chelating activity was poor.

The stocking density measures the number of chickens kept in a particular area and in this study, the number of chickens kept in a house had a great effect on the growth rate and body weight of chickens. In the 8th week the mean body weight at low stocking density was 2.743 ± 0.216 kg and for high stocking density it was 1.637 ± 0.004 kg.

The serum levels of the antioxidant enzymes superoxide dismutase (SOD) and catalase (CAT) were significantly higher ($p<0.05$) in chickens receiving the extract of *Cassia abbreviata* compared to the control group. The concentration for extract had an effect on the serum levels. The growth rate of the chickens was also higher in the *Cassia abbreviata* treated groups than that of the control chickens ($p<0.05$). *Cassia abbreviata* was able to inhibit lipid peroxidation as the malondiadehyde (MDA) content was significantly lower in the treated groups. Apparently, the plant's extract stimulated growth in chickens. It is contingent that the plant extract exhibited antioxidant activity that inhibits the oxidative damages in overcrowded chickens. Histological results revealed that high dosages cause damages in the liver, thus concentration should be considered.

Key words: *Cassia abbreviata*, indigenous chickens, antioxidant activity, oxidative stress, growth

ABBREVIATIONS

ABTS	2,2’-azino-bis-(3-ethylbenzothiazoline-6-sulfonate
ALT	Alanine aminotransferase
AA	Ascorbic acid
AST	Aspartate aminotransferase
BHA	Butylated hydroxyl-anisole
BHT	Butylated hydroxyl toluene
DPPH	2,2-Diphenyl-1-picryl-hydrozyl
CA	Citric acid
EDTA	Ethylediaminetetra-acetic acid
HSD	High stocking density
LSD	Low stocking density
MDA	Malonaldehyde
NO	Nitric oxide radical
NSD	Normal stocking density
OH	Hydroxyl radical
O2	Superoxide anion
SD	Stocking density
TBA	Thiobarbituric acid

CHAPTER ONE

INTRODUCTION

South Africa has four indigenous chicken breeds, namely, Venda, Ovambo, Naked neck and Potchefstroom koekoek (Grobbelaar *et al.*, 2010). The chickens are used as a source of proteins (particularly meat and eggs), but they have slower growth rate than commercial breeds, making them not to be considered as a significant occupation for rural farmers (Tadelle and Ogle 2000). They nonetheless possess important characteristics of thermo-tolerance, disease resistance and adaptability. However, these traits are threatened by changes in the production system, particularly the environment (Hoffman, 2010; Lara and Rostagno, 2013).

One of the changes is stocking density, which determines the number of chickens kept in a given space. Overcrowding is one of the factors that highly contribute to growth and health care. Overcrowding causes metabolic disturbances that release reactive oxygen species (Lin *et al.*, 2006). Reactive oxygen species (ROS) are compounds that are very reactive in nature. Oxidants and free radicals are certainly produced by the majority of physiological and metabolic processes (Shini *et al.*, 2010). In living systems, a positive role is played by free radicals in energy production, phagocytosis, cell growth regulation and synthesis of biologically important compounds (Halliwell, 2006).

The imbalance between oxidants and antioxidants in favour of the latter damages the cellular tissues and is termed oxidative stress. Antioxidants have a physiological role to prevent the oxidative damage of cellular constituents that arises from the consequences of free radical chemical reaction (Gulcin *et al.*, 2007). The use of

synthetic antioxidants, like BHA, is effective in fatty foods and toxicologically safe for use. However, there are serious concerns over the side effects of these synthetic antioxidants due to their carcinogenic potential (Grice, 1988). As a result there has been a general desire to replace the synthetic with natural antioxidants (Jayaprakasha and Jaganmohan, 2010).

Many plants, including *C. abbreviata,* have been reported to possess antioxidant activities (Mongalo and Mafoko, 2013). In this study, the *in vitro* scavenging activity of biological free radicals by extracts of *C. abbreviata* was investigated; the study extended to the investigation of the *in vivo* effectiveness of the plant's extract in oxidative stress induced by overcrowding in indigenous chickens.

1.1 Scope of the work and objectives

Stocking density has become one of the challenging factors that negatively affect the poultry production, especially the high stocking density. It retards growth and can cause metabolic diseases. However, some of the diseases can be cured using medicinal plants available in the household. Hence, less knowledge is available on the compounds plants possess and their effectiveness in curing diseases. It is also apparent that synthetic medication has some side effects. *C. abbreviata* has been one of the plants used for various aliments in rural areas and this study aim to discover and establish its effectiveness as a medicinal plant in oxidative stressed chickens. Screening and testing of plants used by rural farmers will give scientific validation and recommendation on the use of plant and this could help in ethno-veterinary practices.

The objectives of the study were to:

- Investigate the influence of stocking density on the growth performance of indigenous chickens.
- Identify the plant and prepare voucher specimens.
- Investigate the phytochemical constituents and antioxidant activity of the plant.
- Establish the level of stress caused by stocking density in indigenous chickens.
- Determine the effect of different doses of the plant extract on growth rate, antioxidant and liver function enzyme activities.

1.2 Structure of the dissertation

Chapter 1 presents the introduction to the study, which leads to the focus of the research problem, as well as the aim and objectives of the study.

Chapter 2 provides the comprehensive literature review based on the indigenous chickens reviews and free radicals, and antioxidant properties available in medicinal plants, with some selected plants that have been used to treat oxidative stress as well as the pharmacological activity of the plant to be used in this study.

Chapter 3 presents the effect of the stocking density on the growth of the chickens using different stock sizes.

Chapter 4 deals with the *in vitro* free radicals scavenging activity of the *C. abbreviata*.

Chapter 5 presents the *in vivo* effect of the plant in oxidative stressed chickens.

Chapter 6 provides a general discussion of results within the context of the literature and provides a link between the research results and conclusions arising out of the research.

CHAPTER TWO

LITERATURE REVIEW

2.1 Characteristics and description of indigenous chickens of South Africa

Indigenous chickens (Fig. 2.1) are characterised as having small body size, slow growth rate, with different colours of plumage, and of dual purpose type with variable body conformation and physical characteristics (Dana *et al*., 2010). Body weight of indigenous chickens is variable and lacks uniformity in growth. They are active, lively and fond of fighting (aggressive), especially when intensively reared. The hens have the instinct of broodiness. Meat from indigenous chickens is often thought to be delicious and is a favourite in many developing countries (Wattanachant *et al*., 2005). The meat, or carcass, of indigenous chickens has less fat than commercial broilers and is relatively tasty, dry and well adapted to the prolonged African way of cooking (Moreki, 2012).

Figure 2.1: Picture showing various morphological features of indigenous chickens

2.2 General overview of indigenous chicken production

Poultry contributes the largest proportion of animal food source (Permin and Pederson, 2010). Indigenous chickens are known for their ability to adapt to a production system characterised by continuous exposure to the incidence of diseases, inadequate quantity and quality of feeding, and poor housing and health care (Justus *et al.*, 2013). They have remained largely genetically uncharacterised and unimproved because of greater interest in the commercial breeds (Akinoluwa *et al.*, 2012).

Poor management of indigenous chickens leads to various stresses that result in the increase of susceptibility to diseases and suppresses performance. However, in some areas extension services have disseminated management interventions such as feed supplementation, vaccination, brooding, and chick rearing systems to improve the productivity of indigenous chickens (Justus *et al.*, 2013). Lokhade *et al.* (2009) observed that an increase in overcrowding reduces the efficient utilisation of feed, and results in diseases and reduced productivity. For various reasons some farmers still use ethno medicinal plants to treat poultry diseases (Justus *et al.*, 2013).

2.3 Effect of stocking density on poultry production

Stocking density refers to the number of chickens kept in a particular pen or chicken house and is calculated by the number of birds per square meter. Stocking density is an important factor in determining productivity. High stocking density has been reported to have a high percentage of cannibalism, high competition for feed and therefore reduced feed intake, and results in low body weight and very slow growth rate (Dawkins *et al.*, 2004). High stocking density has also been reported to cause high levels of litter moisture and ammonia in the chicken house, which affect birds'

health (Ohh *et al.*, 2002). It also results in overcrowding stress and is related to mortalities, leg problems and behavioral changes (Gomes *et al.*, 2014). Raghavan *et al.* (2012) observed that stressed chickens have a numerically lower enzyme activity that is sometimes attributable to a reduction in feed consumption, and lowers the carcass weight.

2.4.1 The physiology of stress

Stress is any deviation that results from changes in the environmental or normal conditions. Stress is caused by various factors that include nutritional imbalances, poor handling, transportation and immobilisation. Social stress is caused by overcrowding and poor uniformity among the chickens in the house. Psychological stress is a result of fear and harsh caretakers, while pathological stress results from the exposure of chickens to a place with poor biosecurity which thus increases the invasion of infectious diseases (Banday and Untoo, 2012; Lokhande *et al.*, 2009). In environmental stress, heat is the major stressor that affects the chicken production system, where a temperature exceeding the thermoneutral zone leads to an elevated core temperature and consequently initiates a number of responses leading to the neutralisation of heat induced metabolic changes, which therefore affects the enzyme activities (Gonzalez-Esquerra and Lesson, 2006). Prolonged stress leads to dramatic physiological changes in the chickens' organs (Melesse *et al.*, 2011).

This change contributes to the performance and health of the animal due to limited body resources (energy and proteins) (Gomes *et al.*, 2014). The effect or influence of stress in chickens depends on the exposure period. The detrimental effect may occur due to long-term stress, or vice versa, and chickens show stress syndromes

as long-term stress causes impaired metabolism (Banday and Untoo, 2012). Short-term stress affects the neurogenic glands and long-term- stress affects the endocrine system (Mohan, 2014). The short-term stress involves the first line of defence against any antigen by an animal secreting catecholamine from the adrenal medulla (Lay *et al.*, 2011). Long-term stress involves an endocrine system that is at the resistance stage with adrenal glucocorticoids or corticosterones released resulting to glucose from carbohydrate, lipids and proteins reserves. The corticosterone results in many diseases associated with long-term stress such as metabolic rearrangement and antibody suppression. The last stage is the exhaustion stage that occurs when the bird does not recover from the stressor due to inadequate body reserves and hormones (Mohan, 2014).

2.4.2 Effect of overcrowding stress in chickens

Stress results in decreased performance because the chickens tend to fight against the stimuli. Mumma *et al.* (2006) reported that during stress more energy is required as the animal tries to adapt to the stressor, thus growth is reduced since energy normally used for growth is redirected to adaptive responses. Heat stress is the key concern in the poultry industry and it occurs when a negative balance exists between the net energy released to the environment and the amount of heat energy produced by the chicken. However, overcrowding caused by high stocking density increases the total plasma corticosteroids in chickens (Coble, 2013). Conversely, Davis *et al.* (2006) found that high stocking density did not affect corticosteroid levels in hens. It has been reported by Lokhade *et al.* (2009) that high stocking densities increase exposure to disease-causing organisms.

2.5 Generation of free radicals in the body

Free radicals are the reactive oxygen species that contain an unpaired electron in their outer shell. The reactivity of radicals has been reported to be very strong though they are less stable than non-radical species (Lobo *et al.*, 2010). Free radicals are formed from molecules through chemical bond breakage such that each fragment keeps one electron, by cleavage of a radical, to give another radical, and also via redox reactions (Young and Woodside, 2001). The free radicals found in nature are hydroxyl, superoxide, nitric oxide, nitrogen dioxide, peroxyl and lipid peroxyl. However, there are other oxidants that can be easily converted to free radicals reactions and these are hydrogen peroxide, singlet oxygen, hypochlorous acid and lipid peroxide (Nimse and Pal, 2015). Biological free radicals are thus highly unstable molecules that have electrons available to react with various organic substrates such as lipids, proteins, and DNA. Free radical formation can be either enzymatic (respiratory chain) or non-enzymatic (oxidative phosphorylation) (Pharm-Huy *et al.*, 2008).

2.6 Oxidative stress in animals

Oxidative stress is metabolic impairment accompanied by the excessive release of reactive oxygen species in the body aggravated by a drastic drop in antioxidants. Choudhary and Devi (2014) reported that oxidative stress damages the membranes of vital organelles such as mitochondria and DNA, in search of their missing molecules. The reactive oxygen species in humans lead to pathogenesis of cardiovascular disease, hypertension, arthrosclerosis and chronic inflammatory disease, as reported by Pavana *et al.* (2009). The reaction of free radicals with enzymes, receptors and ion pumps inactivates the normal cell and body functioning,

hence they target the unsaturated bond in lipids (Punchard and Kelly, 1997). The change in lipid composition alters membrane permeability, impairs the functions of proteins and lipid-dependent enzymes, leading to alterations of cell volume and hemolysis (Halliwell, 1996).

2.7 Antioxidants and their ability to scavenge free radicals

Several mechanisms are produced by the body to counteract oxidative stress by producing antioxidants, either naturally generated (endogenous antioxidants) or externally supplied through foods (exogenous antioxidants). The roles of antioxidants are to neutralise the excess of free radicals, to protect the cells against their toxic effects, and to contribute to disease prevention. Antioxidants are the molecules that contain defence mechanisms that inhibit oxidation of molecules (Prior *et al*., 2005; Valko *et al*., 2006). In nature, there are thousands of compounds possessing antioxidant properties.

Antioxidants are non-enzymatic or enzymatic, with non-enzymatic antioxidants being water soluble ascorbate, glutathione, uric acid and ubiquinone, and enzymatic antioxidants include glutathione peroxidase, catalase and superoxide dismutase. Antioxidants terminate reactions initiated by reactive oxygen species (Guan, 2007). Free radical activity can be assessed by determining endogenous antioxidant levels and by detecting free radicals using electron spin resonance analysis. The concentration and cellular activities of non-enzymatic and enzymatic antioxidants are determined in the plasma and cells (Griffiths *et al*., 2002). The antioxidant enzymes that are synthesised in the body require metal co-factors. A wide range of antioxidant activities is often found in plants (Kumar *et al*., 2010).

2.8 Ethno-veterinary practices in livestock

Nowadays, more attention has been paid to the use of medicinal plants to treat infections and diseases. Plants possess very important compounds (phytochemicals) that are vital to animal health and these compounds are responsible for various medicinal activities attributed to medicinal plants (Machaba, 2014). Medicinal plants contribute about 50% of clinical use and phytochemicals can be developed as drugs directly (Kee *et al*., 2008). Although modern medicine has retained more popularity, it has however been less effective and sometimes has side effects (Simelane, 2014).

2.9 Plants used in treating various sicknesses in chickens

In rural communities, farmers use their indigenous knowledge to treat chickens against various diseases. *Azadirachta indica* has been used to treat endoparasites by administering it in drinking water. *Kalanchoe crenata* has been used to treat coccidiosis. Pawpaw leaves and *Adansonia digitata* have been used to treat diarrhoea. Pepper has been used to treat a cough. *Borreria verticilata* has been used to treat birds' locomotion problems and *Annona senegalensis* or sliced garlic has been used to protect chickens from snake-bites (Gueye, 2014). *Aloe vera* has become the mostly used plant in treating a wide range of diseases in both humans and animals. It has been used to treat coccidiosis in chickens because it contains anthraquinones. It has become easily accessible and is locally available in communal areas (Mwale *et al*., 2005).

In addition, farmers have come up with the idea of controlling ticks, lice and red ants by using *Butyrospermun parkii* oil (Schmeltzer and Gurib-Fakim, 2008). Moreover, medicinal plants and their extracts are widely used in animal nutrition to improve fatty acid composition as well as cholesterol content of meat products (Golzadeh *et al*.,

2012). However, some plants have been observed to have seasonal preferences regarding their use, because of the variation in the chemical constituents between seasons and agro-ecological regions. During the dry season plants have been observed to a have high concentration of anthraquinones, thereby becoming more effective as healing agents (Mwale *et al*., 2005).

2.10 Preparations and application of ethno medicines

Farmers use different parts of plants to treat diseases in chickens. Roots are the most used for health care management while wood is the least. Furthermore, leaves, bark, fruits and seeds are also used (Sri Balaji and Vikrama Chakravarthi, 2010). Various methods of preparation and application are used where the harvested parts are ground for different routes of administration. Farmers administer ethno medicines through drenching, fumigation, spray and injection (Toyang *et al*., 2007). Drenching involves administering medicines in a liquid form through the mouth using a spoon or dropper; fumigation (smoke) is used to kill insects; and lastly medicines can be administered by mixing them with feed or water (Moreki, 2012).

2.11 Possible remedies from plants that alleviate oxidative stress

The extract of *S. aleraceus* and *P. nitida* contains antioxidants that have been found to have the potential of scavenging free radicals in diabetic rats. The diabetic condition directly relates to the oxidative stress (Teugwa *et al*., 2013). The piper plant species was also assessed as having an antioxidant potential catalase. Glutathione peroxidase and glucose-6-phosphatase dehydrogenase activities are predominant in piper *Longum linn.* The umbelliferae family species has been used as a memory enhancing, strength promoting, wound healing, anti-anxiety and anti-

stress. *Centella asiatica* has shown a protective effect against oxidative damage caused by lead acetate (Ponnusamy *et al*., 2008).

2.12 *C. abbreviata* as a medicinal plant

Figure 2.2: A picture of *C. abbreviata* showing its stem, bark and ripe fruit (Google pictures, visited: 11.04.2015)

C. abbreviata is a shrub with light brown bark, rounded crown and yellowish leaves. The plant has 5 to 12 pairs of compound leaves with cylindrical shaped brown black pods. It has yellow, sweet-scented, large, loose flowers that become brown-veined with age; fruit is long cylindrical dark brown and with a hanging pod (Mongalo, 2013). *C. abbreviata* is widely distributed in some provinces of South Africa, Botswana, and Zimbabwe. *C. abbreviata* has been reported to be a herbal plant that cures many diseases and exhibits antibacterial, anthelmintic, antiviral, antifungal, antimalarial, antidiabetic and antioxidant activities (Mongalo, 2013). The ethanol root extract has been observed to exhibit anthocyanins, anthranoids, anthraquinones, polyphenols and tannins (Leteane *et al*., 2013). *C. abbreviata* in tropical Africa is known to treat venereal disease, pneumonia, malaria, snakebites, diarrhoea, bilharzia, and

gastrointestinal disorders (Kawanga, 2005; Maroyi, 2013; Mongalo, 2013). Also, the plant parts are reported to be used for washing blood from miscarried women, as an aphrodisiac, as a purgative and to relieve abdominal pains (Sekepe *et al.*, 2013).

The crushed roots mixed with hot water have been used to treat constipation in humans (Maroyi, 2013). The extract of *C. abbreviata* has been observed to increase antiplasmodial activity in rats and a drop in blood pressure is observed when crude extract injection is administered, but it is dose- dependent. A significant inhibition against gram positive and gram negative bacteria number is revealed in the methanol, acetone and water extract of the *C. abbreviata* stem bark but the root extract showed only modest levels of cytotoxicity (Schmeltzer and Gurib-Fakim, 2008).

2.12.1 Antioxidant activities of *C. abbreviata*

Methanol extract of *C. abbreviata* exhibits high antioxidant activities (Mongalo and Mafoko, 2013). The extract of stem bark exhibited IC_{50} of 1.87±0.25mg/100ml against 2,2-diphenyl-1-picrylhydrazyl (DPPH), which is a good scavenging characteristic of free radicals. Also bark extract of *C. abbreviata* exhibited IC_{50} of <7.8ug/ml against 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid) ABTS (Alabri *et al.*, 2014).

2.12.2 Toxicology of *C. abbreviata*

Methanol extract of the root exhibits high toxicity in a brine shrimp lethality test with LC_{50} of 12.7 µg/ml at 95% confidence limit (Moshi *et al.*, 2007). However, ethanol extracts of the same plant part are less toxic, with an IC_{50} of 39.6 with similar confidence limit (Moshi *et al.*, 2006). Aqueous extract of the stem bark exhibits no

toxicity on the growth of the fibrotic model when applied in a dose range of 0.001 to 1000 µg/ml, the toxicity arises only at 1800 µg/ml (Krishman, 2005).

CHAPTER 3

Effect of stocking density on the growth performance of indigenous chickens reared in an intensive system

Abstract

Stocking density is the measure of how many chickens are kept in a given space of a house. High stocking density, called overcrowding, induces harmful effects that act as a predisposing factor in the reduction of production and poultry performance. The main objective of this part of the study was to evaluate the effect of stocking density on the growth parameters of indigenous chickens reared up to 16weeks of age under an intensive system. A total of 140 four weeks old indigenous chicks were randomly assigned to three stocking densities SD_1, SD_2 and SD_3 , being 5 chickens/m^2, 10 chickens/ m^2 and 20 chickens/ m^2, respectively; each group had three equal groups. Chickens were kept in a house with free access to water, chicken mash and yellow maize were provided in the morning and afternoon. Vaccination against Newcastle disease and Gumboro disease was done prior to the start of the experiment. The parameter recorded was body weight and the growth rate was calculated on a weekly basis. The chickens kept in low stocking density had high body weight of 2.743 ± 0.216 kg and for high stocking density body weight was 1.637 ± 0.004 kg in the 8th week. No significant change was observed in the growth rate of chickens on a weekly basis, but during week four of the experiment an increase was observed in the growth rate. It was concluded that stocking density had an influence on the growth performance of chickens. High stocking densities retard growth, low and normal stocking densities are greatly recommended for indigenous productions to reach the high body weight in a minimum time.

Keywords: Indigenous chickens, stocking density, growth, body weight

3.1 Introduction

Indigenous chickens (*Gallus domesticus*) are the type of poultry that is widely domesticated by rural farmers (Masuku, 2011). Indigenous chickens are mainly kept for meat consumption purposes. In the past, chickens were exposed to scavenging systems for feed and had minimal supplementary feed. There was no provision for housing, thus they were characterised by low input and low output (Thwala, 2012). However, it is argued that smallholder farmers take indigenous poultry production as a great source of living, but they face the challenge of improving productivity of their flock, which could have financial benefit and promote food security as well as achieve market potential. Nowadays, consumers prefer organically produced food that is produced systemically (Ondwasy *et al*., 2006).

Indigenous chickens are raised in the poorest social strata where they utilise less nutritious feed. The system of poultry farming is extensive, where chickens are left to scavenge to meet their nutritional needs (Moreki, 2012). To a lesser extent semi-intensive farming is practised whereby housing is usually of the required standard and supplementation is provided (Sonaiya *et al.,* 1995). However, poor management that is occupying the indigenous chicken production leads to low productivity. Such management includes lack of extension services, technical skills and information and, more importantly, the negligence by farmers (Badhaso, 2012).

Indigenous chickens have a slower growth rate than commercial breeds and this is one of the reasons why such breeds are not considered for market purposes, compete unfairly with broiler chicken farmers (Tadelle and Ogle, 2000). Normally, a chicken takes about eight months to reach a point of sale (Siyaya, 2014).

Among many factors that contribute to low performance and reduced growth is stocking density that measures how many chickens must be kept in a house. The stocking density is a great welfare concern and has influence on sales of poultry production. A good stocking density ensures the comfort, and physical wellbeing of chickens raised and enhances productivity, thus ensuring that proper returns on the farmer's investment are maintained (Skrbic *et al*., 2009). High stocking density has a significant influence on the performance of chickens in that it negatively affects the body weight and thus growth rate is reduced (Gomes *et al*., 2014). However, the recommended stocking density is based on body weight per given floor. Moreover, the body weight appears to determine the chicken's performance and its well-being/welfare yet is not taken into consideration in poultry farming (Puron *et al*., 1995; Feddes *et al*., 2002; Dozier *et al*., 2006). However, the body weight of indigenous chickens is 2.5 times less than exotic breeds (the broilers), making them less popular for marketing (Chulayo *et al*., 2011).

The indigenous poultry industry is gaining importance in South Africa due to changing social strategies. Although indigenous chickens are becoming economically important, their production still results in economic losses because of improper management (Padhi, 2016). Among these management errors, overcrowding of chickens is the most important because it induces stress which leads to great economic loss. This work was designed to test the effect of stocking density on the weight and growth rate of chickens.

3.2 Materials and methods

3.2.1 Study area and animals

The university of Zululand is situated at Empangeni in the Northern KZN, at latitude 28.8415^o S and longitudes 31.8263^o E. The study was conducted at the University of Zululand poultry farm. House preparations were done before the experiment commenced. The experiment started after the Ethical Committee approved the use and care of chickens at the University of Zululand (UZREC 171110-030 PGM 2014/124, see appendix C). The guidelines for the proper care of animals and conducting animal experiments were followed. Chicks were hatched after incubating the eggs for 21 days and after seven days chicks were vaccinated against Newcastle and Gumboro disease. At four weeks old, the chicks were used for the experiment.

3.2.2 Animal model and stocking density

One hundred and forty (140), four weeks old indigenous chickens hatched at UNIZULU were used for the experiment. The chickens were maintained under standard housing conditions and allowed water and feed *ad libitum.* Chickens were divided into three groups of stocking density: high- 20/m^2, medium - 10/m^2 and low - 5/m^2, each group with three replicates in a one square meter house for 12 weeks.

3.2.3 Data collection

Body weight was recorded on a weekly basis using the same weighing scale for all the groups. The body weight was presented in tabular form and the growth rate was calculated using the GR= (BW$_{current}$ - BW$_{previous)}$ /7

GR-growth rate

BW$_{current}$ –body weight before

$BW_{previous}$ –body weight after

3.2.4 Statistical analysis

The data was analysed using SPSS (SPSS, 2012) followed by Post Hoc. The weekly average for all the body weights was calculated. All the data were expressed as mean ± standard error of the mean. It was considered significant at $p \leq 0.05$.

3.3 Results

3.3.1 Growth performance of chickens

The effect of stocking density on the body weight of chickens is shown in Table 4.1. In week one, chickens in the high SD had less body weight (0.207 ± 0.003kg) when compared to the normal group (control 0.418 ± 0.019kg) and 75% in weight (0.337 ± 0.087kg) of the low SD group. The chickens kept in low SD reached one kilogram in week 5 while chickens kept at high SD had 0.577 ± 0.278kg at the same age. At week 8, the chickens had different weights ($p \leq 0.01$); 2.743± 0.216 kg, 2.014 ± 0.113kg at low SD and normal SD respectively. The chickens at the high SD weighed the least 1.637 ± 0.040kg.

Figure 3.1 shows the growth rate of chickens kept in different stocking densities for eight weeks. The growth rate initially increased in the chickens kept in less and normal SD in the first three weeks of the experiment. The sharp decrease was observed as the chickens were growing 4th week to the 8th week. But for the high SD, growth rate was very slow within the weeks of the experiment.

Table 3.1: The mean and standard error of the body weight in kilograms for four weeks old chickens kept in different stocking densities for eight weeks.

Experimental weeks	Low SD (5/ m^2)	Normal SD (10/ m^2)	High SD (20/ m^2)
Week 1	$0.337^{a} \pm 0.087^{1}$	$0.418^{a} \pm 0.019^{1}$	$0.207^{a} \pm 0.003^{2}$
Week 2	$0.431^{a} \pm 0.069^{1}$	$0.471^{a} \pm 0.036^{1}$	$0.331^{a} \pm 0.005^{2}$
Week 3	$0.751^{b} \pm 0.067^{1}$	$0.642^{a} \pm 0.040^{1}$	$0.438^{a} \pm 0.008^{2}$
Week 4	$1.158^{c} \pm 0.077^{1}$	$1.29^{b} \pm 0.109^{1}$	$0.577^{a} \pm 0.278^{2}$
Week 5	$1.446^{c} \pm 0.044^{1}$	$1.411^{b} \pm 0.042^{1}$	$0.752^{b} \pm 0.018^{2}$
Week 6	$1.70^{cd} \pm 0.249^{1}$	$1.452^{c} \pm 0.071^{2}$	$1.356^{c} \pm 0.064^{2}$
Week 7	$2.070^{d} \pm 0.175^{1}$	$1.77^{c} \pm 0.109^{2}$	$1.397^{c} \pm 0.028^{3}$
Week 8	$2.743^{e} \pm 0.216^{1}$	$2.014^{d} \pm 0.113^{2}$	$1.637^{c} \pm 0.040^{3}$

Means with the same alphabet superscripts within the column are significantly different (P<0.05)

Means with the same numerical superscripts within the rows are significantly different (p<0.05)

SD-Stocking density

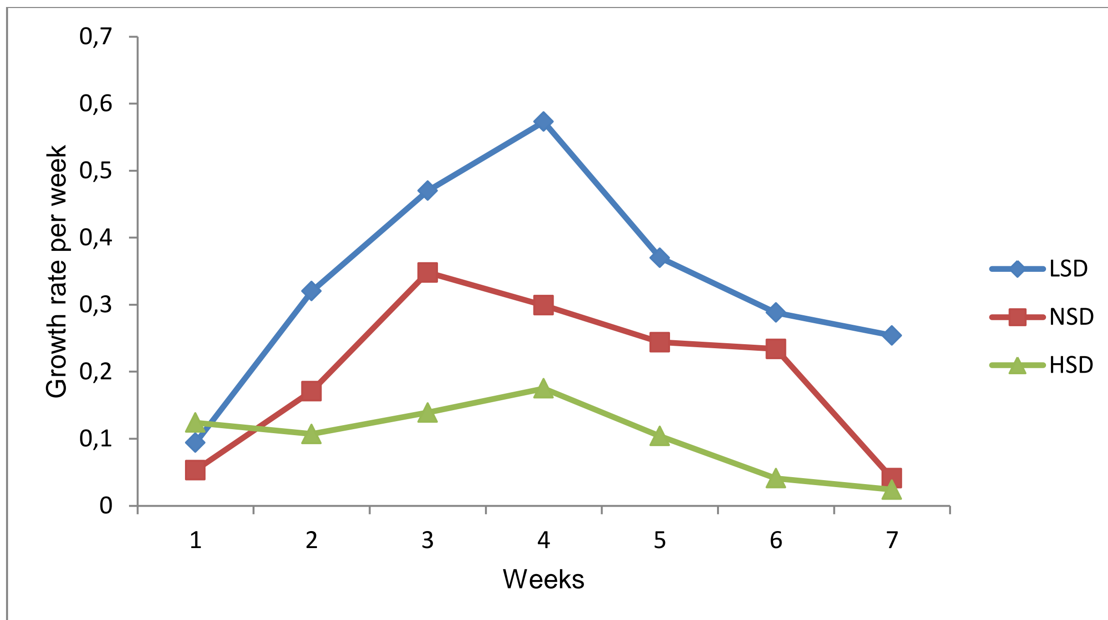

Figure 3.1: The growth rate of chickens in three different stocking densities in a one square metre house for eight weeks.

3.4 Discussion

The body weights of chickens in a low and normal stocking density were similar and the difference was realised after the 5th week of the experiment when chickens were nine weeks old. Significant differences ($p<0.05$) in body weight between the low and normal occurred from the 6th week of the study until the last week. These results are consistent with the findings reported by Lyasere *et al.* (2012) that increased stocking density reduces growth of the chickens. Sekeroglu *et al.* (2011) raised 308 Ross broilers under three stocking densities (9, 13 and 17 chickens/m^2) and observed that the growth rate at a density of 13 chickens/m^2 was higher than that of the other two stocking densities during the 2nd and 3rd weeks of age. The authors also found that birds reared at stocking density of 17 chickens/m^2 had the lowest growth rate of all the three groups (9, 13 and 17 chickens/m^2) during the 4th, 5th and 6th weeks.

However, a difference was realised between the body weights of the chickens in the normal and high stocking density. Body weight of chickens in high stocking density was lower than the normal and low stocking density from the beginning until the end of the experiment. There was no significant difference ($p>0.05$) at the first two weeks of the study and the difference was observed at 10th week. These results are in line with the findings made by Dozier *et al.* (2005) that overcrowding lowered the body weight of chickens. The effect of stocking density was further confirmed by Skrbic *et al.* (2009) on body mass and also on consumption and feed conversion, with the authors specifically concluding that the final body mass of chickens is dependent on the applied stocking density.

3.5 Conclusion

Stocking density has a serious effect on the body weight of indigenous chickens at all growth phases. However, low stocking density does not have a great influence on body weight gain thus, a normal stocking density can be considered by farmers. Optimal stocking density should be taken into consideration to avoid the delay of maturity weight in chickens. The results from this study have indicated that the lower the stocking density, the higher the returns are to the farmer.

CHAPTER FOUR

The *in vitro* antioxidant activity of the *Cassia abbreviata* methanolic extract

Abstract

Cassia abbreviata is a medicinally important plant in the Zulu tradition. It has been earlier reported to exhibit free radicals (ABTS and DPPH) scavenging activities. This chapter aims to investigate the presence of phytochemicals, total phenolic and flavonoids contents. Also to further determine the scavenging of biological free radical activity of the plant. Extracts of the stem bark of *Cassia abbreviata* were screened for phytochemicals; a portion of the plant was extracted with methanol and the extract characterised in various *in vitro* models for DPPH, ABTS, superoxide anion, hydroxyl and nitric oxide free radical scavenging activity. The reducing power and iron chelating activity were also investigated. Phenols and alkaloids were present in the plant. *Cassia abbreviata* showed more scavenging ability of DPPH, ABTS, hydroxyl and superoxide free radicals (IC_{50}< 0.05); the reduction potential was concentration dependent. The findings suggest that *Cassia abbreviata* has a promising antioxidant activity as its methanolic extract contains bioactive compounds and it justifies its medicinal use in the treatment of diseases.

Keywords: *Cassia abbreviata*, scavenging activity, phytochemicals, free radicals

4.1 Introduction

Oxidants are highly reactive compounds that consist of an unpaired electron and are produced during normal metabolic functions or introduced from the environment. Free radical accumulations cause cellular damage, and contribute more in health conditions including aging process and cancer. Thus antioxidants counteract these free radicals to prevent damages (Pawar and Surana, 2010). Lipid peroxidation can be easily initiated by exogenous sources of free radicals in the membrane.

Antioxidants are molecules that have great ability to protect the body from oxidative damage caused by free radicals (Ozsoy *et al.*, 2008). However, a lot of interest is paid to natural antioxidants that are present in medicinal plants; the polyphenols (Silvia *et al.*, 2005). The effectiveness of antioxidants has been reported to be more in polyphenols that possess free radical scavenging activity chemistry. This arises from high reactivity and electron donors, radical stabilisation and delocalisation of unpaired electrons and metal chelating ability (Shukla *et al.*, 2009). Enzymatic and non-enzymatic antioxidants are produced by the body to protect damage induced by reactive oxygen species (ROS). The innate defence may not be enough for oxidative stress, hence certain amounts of exogenous antioxidants are required to maintain an antioxidant level to balance ROS (De Beer *et al.*, 2002).

Antioxidants significantly delay oxidation of oxidizable compounds at low concentration (Deepak *et al.*, 2015). The *Caesalpina decapetala* has been reported to have antioxidant activity (Pawar and Surana, 2010). Synthetic antioxidants (BHT and BHA) are very effective and used for industrial processing but they possess side effects and are toxic to health (Anagnostopoulou *et al.*, 2006). Many plants have been identified as having antioxidant activity (Kitts *et al.*, 2000; Lee and Shibamoto,

2000). Bioactive phenols such as bioflavonoids are known to be effective as free radical scavengers (Langley-Evans, 2000). The various plants, herbs and spices) that have been tested to have antioxidant potentials include *Ocimum sanctum*, *Piper cubeba Linn*., *Allium sativum Linn*., *Terminalia bellerica, Camellia sinensis Linn., Zingiber officinale* and several Indian and Chinese plants. Among tea plants, black and green tea has been observed to be very rich in polyphenolic compounds (Hertog *et al*., 1993; Langley-Evans, 2000; Lie and Xie, 2000). The study focuses on the *C. abbreviata* plant.

C. abbreviata is a shrub that is found in the tropical regions of Africa and is widely known for its various biological and pharmacological activities (Mongalo, 2013). The shrub serves as basic primary health care in traditional medicine, compromising a rational use of resources: The stem bark is used to treat stomach ache and malaria and is sometimes used to treat blood vomits, dysentery, venereal diseases, snake bites, post-partum pains and menstrual cycle problems (Bruschi *et al*., 2011). Roots and barks are also used to treat abdominal pains and uterus problems (Mojeremane *et al*., 2005).

The stem bark extracts of the plant have been reported to possess antioxidant activities (Mongalo, 2013); the methanolic extracts were tested for the scavenging of various free radicals (DPPH and ABTS). However, scavenging activities of biological radicals (such as hydroxyl radical, superoxide anion, and nitrate radical), metal chelating activity, and the reduction potential of the plant have never been reported. These radicals have become the new targets to minimise the oxidative chain. This chapter aims to determine the antioxidant activities that *C. abbreviata* exhibits, and *C. abbreviata* was tested for its ability to inhibit free radicals in oxidative stressed chickens induced by overcrowding later in the study.

4.2 Materials and methods

4.2.1 Materials

All the chemicals and synthetic radicals (DPPH and ABTS), assay kits (SOD and CAT) and all the reagents were purchased from Sigma-Aldrich, St Louis, MO, USA. Equipment used for preparation and determination were obtained from the Biochemistry laboratory at the University of Zululand.

4.2.2 Plant materials

The stem bark of *C. abbreviata* was harvested at Biaba, which is located in the Limpopo province, South Africa. The plant was identified by qualified personnel in the Department of Botany, in the University of Zululand and a voucher specimen (Ramulodi and Jobe MC/01Unizul) was kept in the Herbarium in the same department. Stem barks were chopped, air-dried and ground to powder (2mm mesh). The powder was stored in a brown bottle at room temperature until use.

4.2.3 Phytochemical screening

Qualitative tests were done for saponins, alkaloids, phenols, terpenoids and flavonoids for the plant material using different established methods described by Odebiyi and Sofowara (1978) and Harbone (1973). Various phytochemicals present in the plant were determined with precipitates formation and colour changes upon the corresponding tests present (Mosa *et al.,* 2012).

4.2.4 Plant extraction

The powdered plant material was extracted with methanol 1:5w/v by incubating on the orbital shaker (150rpm, room temperature) for 24 hours. The mixture was filtered

through Whatman No.1 filter paper and the filtrate was concentrated by a rotary evaporator in a vacuum.

4.2.5 Total Phenolic compound

The total phenolic content of *C. abbreviata* was determined using the Folin-Ciocalteu reagent method (Kahkonen *et al.*, 1999). Crude extract (0.5 ml) was mixed and incubated with 1.5 ml Folin-Ciocalteu reagent and 1.2 ml of 7.5% sodium carbonate solution. The absorbance of the colour developed was read at 765 nm against a blank containing Folin-Ciocalteu and sodium carbonate solution. The total phenolic content was expressed as gallic acid equivalent using the standard curve generated with gallic acid and expressed as mg/g dry materials.

4.2.6 Total flavonoid content

The method by Ordonez *et al.* (2006) was used to determine total flavonoid content of the plant extract. The extract (0.5 ml) was mixed and incubated with alcoholic aluminium chloride (2 %, 0.5 ml). Absorbance of the yellow coloured mixtures was read at 420 nm against a blank containing alcoholic aluminium chloride solution. The extract's total flavonoid content was determined as quercetin equivalent (QE) from the calibration curve of quercetin and expressed as mg/g dry plant material.

4.2.7 In vitro scavenging activities

4.2.7.1 ABTS

ABTS (7mM) was allowed to stand for 16 hours in the dark after adding 2.45 mM of potassium persulfate. Thereafter, ABTS was diluted sixty times using methanol. One millimetre of various concentrations (0-5mg/100ml) of the *C. abbreviata* extract was

mixed with 1 ml of the diluted ABTS. The absorbance was read at 734 nm after 6min incubation.

4.2.7.2 DPPH

Radical scavenging activity of DPPH by the *C. abbreviata* extract was determined following the method of Brand-Williams *et al.* (1995) with a few modifications. Two millimetres of DPPH (0.2 nM) radical solution in methanol was mixed with 2 ml of various concentrations (0-5 mg/ml) of plant extract. The mixture was shaken and 30-60min was allowed for the solution to mix thoroughly. The scavenging radical activity was read at 517 nm absorbance.

4.2.7.3 Superoxide anion scavenging activity

The method of Nagai *et al*. (2001), used for determining superoxide dismutase activity, was adopted with slight modification. A mixture of 0.02 ml each of 0.15% bovine serum albumin, 3 mM Xanthine, 3 mM EDTA, sodium carbonate buffer (50mM, pH 10.5), 0.75 mM NBT and plant extract (0-5mg/100ml) was incubated, with 0.02 ml of Xanthine oxidase (6µm) for 20min at 25°C. Six micrometres of $CuCl_2$ was used to monitor the production of blue formazon at 560nm absorbance.

4.2.7.4 Hydroxyl radical scavenging activity

Benzoic acid hydroxylation (Osawa *et al.,* 1997) was used to determine hydroxyl radical activity. A reaction mixture containing 0.2 ml each of 10 mM EDTA, 10 mM $FeSO_4.7H_20$, a different concentration (0-5mg/100ml) plant extract, 10mM DNA and phosphate buffer was incubated after adding 200 µl of 10 mM H_2O_2 solution at 37°C for 2hrs. After incubation, 1 ml TCA (2.8%) and TBA (1%) were added, the mixture was boiled and then allowed to cool on ice. Absorbance was read at 520 nm.

4.2.7.5 Nitric oxide radical

A mixture made of two millilitres of 10 mM sodium nitroprusside, 0.5 ml phosphate buffer saline (pH 7.4, 0.01 M) and 0.5 ml plant extract at different concentrations (0-5 mg/100ml) was incubated at 25 ^{0}C for 150 min. After incubation, 0.5 ml of nitrite solution was pipetted and mixed with 1 ml sulfalinic acid reagent (0.33 % in 20 % glacial acid). The mixture was allowed to stand for 5 min then 1 ml of naphthylenediamine dichloride (1%) was added. After 30 min the absorbance was read at 540nm.

4.2.7.6 Chelating activity

The *C. abbreviata* metal ion chelating activity was tested on Fe^{2+} using the method reported by Decker and Welch, (1990). De-ionized, distilled water (3.75 ml) was used to dilute 1 ml of the plant extract at different concentrations. To the solutions, 0.1 ml of $FeCl_2$ (2 mM) and 0.2 ml of 5 mM ferrozine were added. Chelating activity was determined at 562nm absorbance. Standards used were EDTA and citric acid.

4.2.7.7 Reducing power activity

The method described by Oyaizu (1986) was followed to measure the reducing power of the *C. abbreviata* extracts. One millilitre of the plant extract in different concentrations was mixed with 2.5 ml of phosphate buffer (0.2 M pH 6.6) and 1% potassium ferricyanide. Ten percent of TCA was added to terminate the reaction after 20min and the mixture was centrifuged at 1000rpm for 10min to obtain 2.5 ml of the supernatant. The supernatant was diluted with 2.5 ml distilled water and 0.5 ml of 1% $FeCl_3$ was added. The reducing activity was read at 700 nm; the higher the absorbance value, the greater the reducing power of the extract.

4.3 Calculating the inhibitory concentration activity of the plant

The experiments were repeated two times. Means and standard error of the mean were calculated. Unless otherwise stated, Ascorbic acid and BHT were used as standards. The percentage inhibition activity of the extract for each parameter was calculated from

$\%inhibitory = (A_0 - A_1)/A_0 \times 100$

where

A_o = Absorbance of the control that is fully oxidized

A_1 = Absorbance of the plant extract.

The inhibition concentration with 50% inhibition (IC_{50}) was determined using the statistical package Origin 6.1.

4.4 Data analysis

The data were statistically expressed as mean ± standard deviation. One- way analysis of variance (ANOVA) was used for analysis followed by Dunett post hoc and GraphPad Prism version 4 tests. The differences were considered significant at $p \leq 0.05$.

4.5 Results

4.5.1 Percentage yield

The methanol extract of *C. abbreviata* yielded 20.45% *(w/w relative to dried material).*

4.5.2 Phytochemical screening

The results of the phytochemical screening of *C. abbreviata* revealed mainly the presence of terpenoids, saponins, flavonoids, alkaloids; no steroids were observed to be present in the plant extract (Table 4.1).

Table 4.1: Phytochemical present in *C. abbreviata*

Alkaloids	+
Steroids	-
Terpenoids	++
Saponins	+
Tannins	+
Flavonoids	+

++ High concentration recorded with heavy precipitate

+ Low concentration recorded on slight opaqueness

- Not detected

4.5.3 Total phenolic and flavonoid content

C. abbreviata phenolic and flavonoid contents (measured as gallic acid and quercetin equivalents, respectively) are presented in Figure 4.1. Apparently, the plant contains more of phenolic compounds (3.7 mg/g) than flavonoids (2.3mg/g).

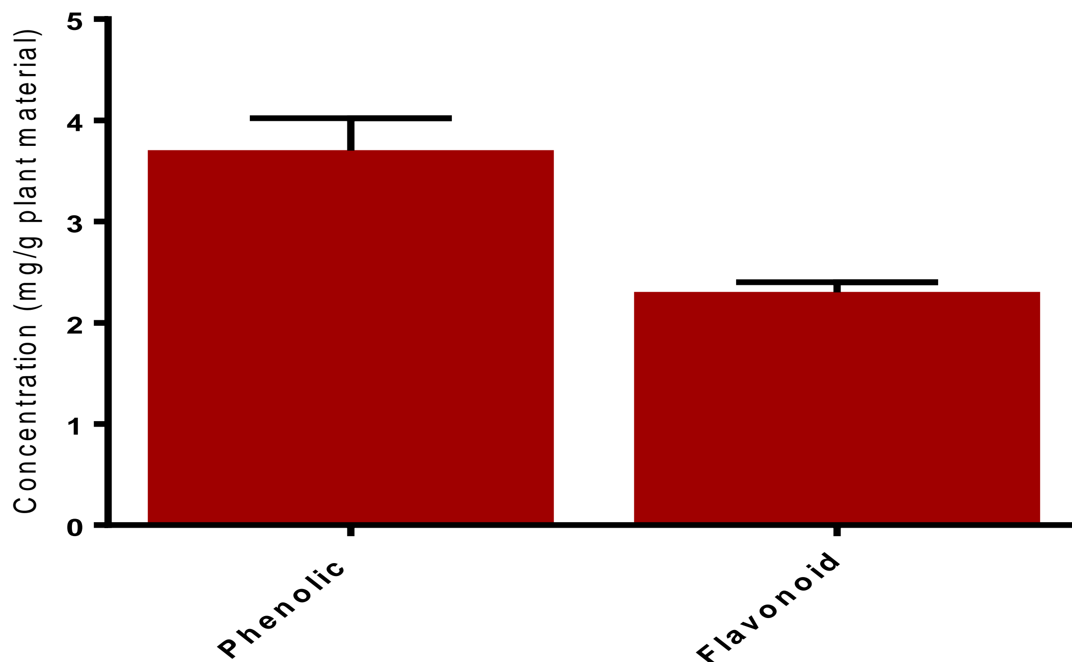

Figure 4.1: The total phenolic (gallic acid) and flavonoid (quercetin) content of the *C. abbreviata* extract.

4.5.4 Antioxidant activity

The ability of *C. abbreviata* stem extract to scavenge synthetic radicals (DPPH, ABTS) and biological radicals (O_2-,$^{\bullet}$HO and NO) is shown in Table 4.2. The results indicated that the increase in the scavenging ability of the *C. abbreviata* was concentration- dependent, although it varies with the degree of efficiency.

The methanolic extract of *C. abbreviata* stem bark exhibited over 98% of the radical scavenging of DPPH activity at 1mg/ml with the antioxidant activity IC_{50} of 1.9 mg/ml. The activity was doubled when compared to the positive controls standards (3.65 mg/ml AA and 3.42 mg/ml BHT). The ABTS antioxidant activity was 0.9mg/ml at a concentration of 0.03 mg/ml and this activity was less than that of the standards 0.25mg/ml AA and 1.30 mg/ml BHA respectively.

The results of the scavenging activity of *C. abbreviata* at various concentrations (0-5 mg/ml) and standard antioxidants (ascorbic acid, BHA and BHT) against superoxide anions and hydroxyl radical indicated a high scavenging activity of 60% and 70% respectively. The activity of nitric oxide was very low (less than 50%). However, *C. abrreviata* was found to be significantly much better in superoxide anion radical scavenging than ascorbic acid.

Table 4.2: Percentage of *C. abbreviata* in scavenging activity of free radicals and the IC_{50} (mg/ml) against standards (AA, BHT and BHA).

	DPPH	ABTS	O^{2-}	$^{\cdot}OH$	NO
1	98.0 ± 0.04	09.0 ± 0.06	29.0 ± 0.01	10.0 ± 0.01	3.0 ± 0.12
2	90.0 ± 0.01	47.6 ± 0.02	37.0 ± 0.01	52.0 ± 0.02	8.9 ± 0.15
3	72.3 ± 0.06	50.0 ± 0.06	60.0 ± 0.04	60.0 ± 0.02	8.9 ± 0.21
4	66.0 ± 0.05	53.3 ± 0.12	59.0 ± 0.02	70.0 ± 0.06	15.3 ± 0.19
5	52.3 ± 0.09	63.2 ± 0.32	40.0 ± 0.01	71.0 ± 0.08	20.6 ± 0.26
IC_{50}	2.3	0.9	0.2	0.01	>5
AA (IC_{50})	-	-	3.49		
BHT (IC_{50})	3.62	0.8	-		3.65
BHA (IC_{50})	2.56	1.3	-		

IC_{50}-Inhibitory concentration; O^{2-} -Superoxide anion radical scavenging; $^{\cdot}OH$ –Hydroxyl radical scavenging; NO –Nitric oxide radical scavenging. Values are expressed as mean ± SEM at n=2.

4.5.5 Reducing power

The reduction potential of *C. abbreviata* stem extract is shown in Figure 4.2 in comparison with the BHA and ascorbic acid. The results revealed that the activity of the extract was dose- dependent and better than the standards; However, the reduction potential of *C. abbreviata* was slightly similar to BHA at 0.05mg/ml; *C. abbreviata* extract (0.524±0.22), BHA (0.562±0.039) and ascorbic acid (0.314±0.03), respectively. It is apparent that the extract has a higher ability to donate hydrogen to accumulating unpaired electrons than the standards tested (BHA and AA.

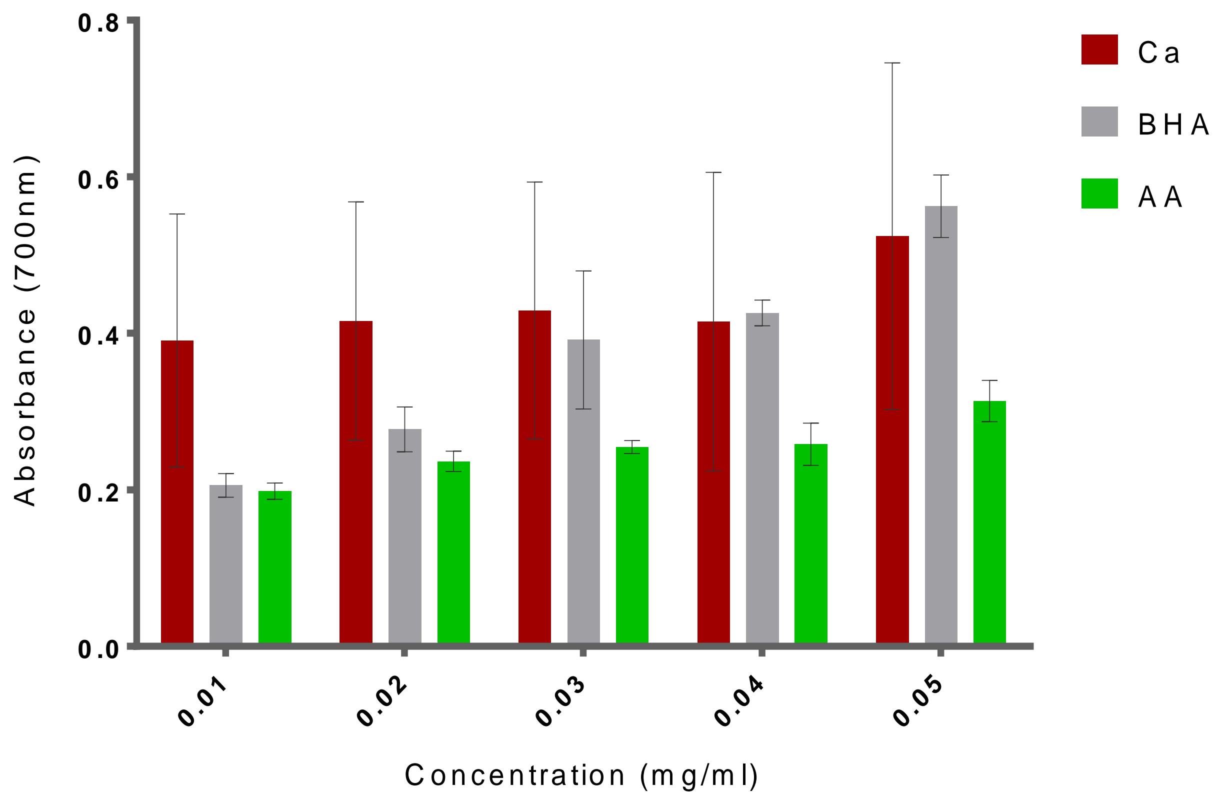

Figure 4.2: Reduction potential of *C. abbreviata (Ca)* extract, Ascorbic acid (AA) and Butylated hydroxyl anisole (BHA), (Mean ±SEM, n=2**).**

4.5.6 Chelating activity

The inhibition of the red coloured ferrozine and formation of ferrous complex were used to assay chelating activity of ferrous ion. Figure 4.3 shows the results obtained, indicating the chelating activity of the extract. The chelating activity of *C. abbreviata* extract was lower than the standards (EDTA and citric acid). *C. abbreviata* chelated 36% iron ions at 0.02mg/ml, where it reached the plateau at a concentration. Citric acid showed higher iron chelating activity than EDTA at a dose dependent manner, at 5mg/ml, it was 60% and 53% respectively.

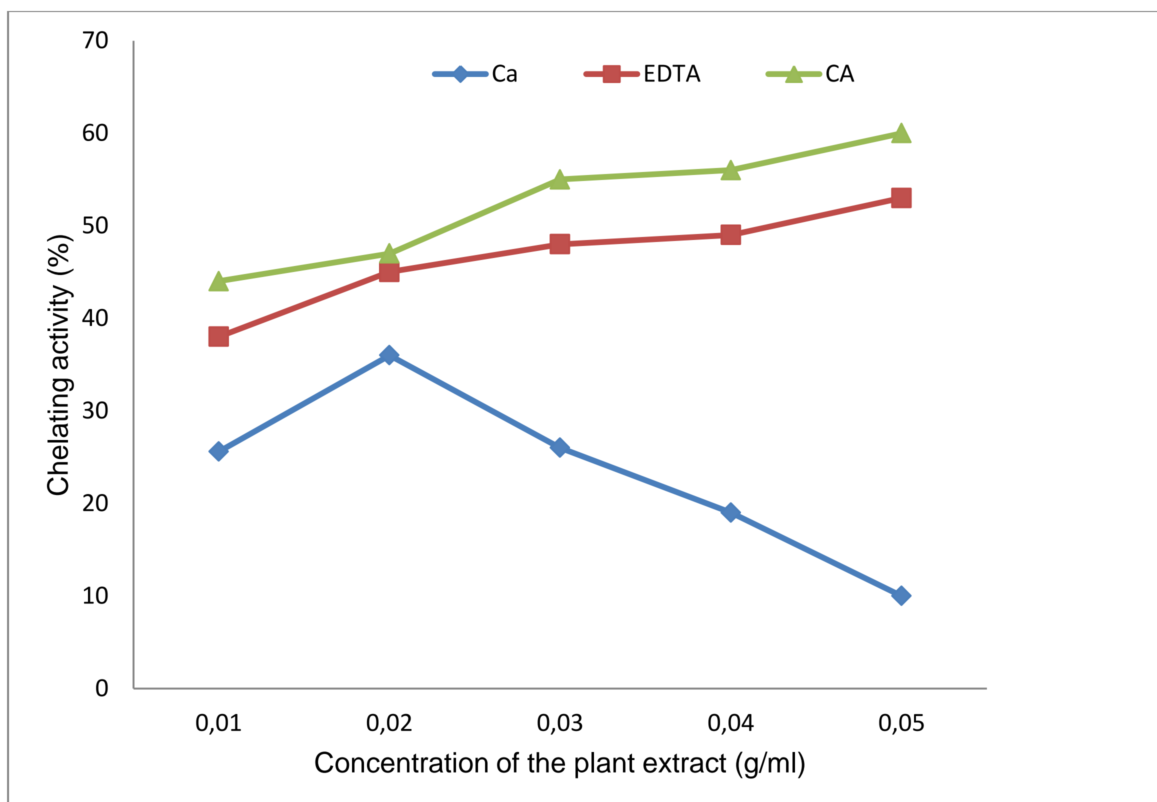

Figure 4.3: Chelating activity of *C. abbreviata (Ca)* stem extract and the standards EDTA and citric acid (n=2, mean ± SEM).

4.6 Discussion

Apparently, *C. abbreviata* is a good free radical scavenger, and can be used against free radicals as a primary antioxidant to prevent oxidative damages (Moure *et al.*, 2001). The phenolic content of *C. abbreviata* revealed its high antioxidant potential, which was also supported by the presence of phytochemicals.

Superoxide anion and hydrogen peroxide are free radicals that are easily converted into highly reactive species after reacting with nitric oxide to form hydroxyl and peroxynitrite radicals. These molecules are capable of attacking sensitive cellular targets causing inhibition and accelerated degradation. Superoxide anion radicals are the predominant radicals that are produced in poultry and are generated during stress. This radical is scavenged at high concentrations. Hydroxyl radicals are very reactive and they have a short half-life, however they are scavenged at high concentrations (Simelane, 2010). The ability of *C. abbreviata* to scavenge these radicals has been evaluated in this study and the results show that the plant exhibited mainly superoxide anion and hydroxyl radicals.

The antioxidant activity of *C. abbreviata* has been found to be correlated with its redox properties. As has been reported by Pin-Der-Duh (1998), the plant's redox properties are associated with the antioxidants. The high reduction potential exhibited by *C. abbreviata* reveals the presence of reductants. Apparently, reductants break radical chains through hydrogen atom donation (Gordon, 1990; Pin-Der-Duh, 1998). Furthermore, reductants have also been reported to prevent peroxide formation by reacting with certain precursors of peroxide (Rathee *et al.,* 2007). Certain compounds present in *C. abbreviata* act as primary and secondary reductants thereby inhibiting lipid peroxidation.

Chelating agents that form a bond with metal (Gordon, 1990) are effective as secondary antioxidants because they decrease redox potential, thereby stabilising the oxidised form of the metal ion. *C. abbreviata* in this study showed weak chelating activity.

4.7 Conclusion

C. abbreviata has been observed to exhibit antioxidant properties. It is apparent that it could be used to increase the action of natural dietary antioxidants. However, proper precautions must apply regarding dosage as a large concentration has been found in the study to be hepato-toxic with elevated ALT activity latter.

CHAPTER FIVE

Effect of *Cassia abbreviata* extracts on the oxidative stress induced by overcrowding indigenous chickens

Abstract

Oxidative stress is one of the most influential conditions in animals' health. Several factors can lead to the excessive accumulation of free radicals in the chicken's body. This lowers the antioxidant activities that inhibit the oxidative chain. The study aims at establishing the effectiveness of *Cassia abbreviata* in alleviating the oxidative stress caused by overcrowding the indigenous chickens. The stress was induced by overcrowding where three stocking densities were established with four replicates at; 5, 10 and 20 chickens per square meter. One subgroup was a control group while the other groups received 50, 200 and 500 mg/kg body weight of the extract, respectively. The liver samples were examined for histopathological activity. The results revealed that the plant extract exhibited antioxidant activity: The Superoxide dismutase and catalase activities were enhanced, while the malonaldehyde content was lowered in the overcrowded chickens given the plant extract at high concentrations. Liver function enzymes were not significantly affected by the plant. Overcrowding induced stress and *Cassia abbreviata* had a positive effect against oxidative stress in the indigenous chickens. However, high dosages cause damages, proper precautions must be taken into consideration.

Keywords: *Cassia abbreviata*, oxidative stress, antioxidant activity, indigenous chickens, overcrowding

5.1 Introduction

Overcrowding is a contributing factor to diseases among chickens, due to decreased space and limited walking. The change in the physiological condition is believed to affect the metabolic activities and oxidative stress function as the serum concentration of glutathione peroxidase production is increased (Simitzis *et al.*, 2012). Stress induces harmful responses that alter general health and productivity and results in immunosuppression (Lethey *et al.*, 2003). The most common cause of immunosuppression in poultry nowadays is a combination of factors such as stress, nutrition, overcrowding and management (Isohe and Lilehoj, 1992). The stress results in oxidation of cellular levels which in turn can have negative effects on chicken growth and health (World poultry, 2010).

Overcrowding stress stimulates excessive levels of reactive oxygen species (ROS), which results in imbalances between oxidation and antioxidants. Such imbalances lead to lipid peroxidation and oxidative damage of biomolecules like proteins and DNA (Droge, 2002; Halliwell and Gutteridge, 1989). ROS are free radicals that contain one or more unpaired electrons for oxygen (Ayo *et al.*, 2011). The body generates this free radical due to an endogenous metabolic process or an environmental stress condition. Free radicals can be harmful at high concentration, but they also possess beneficial effects at low levels for cellular responses and immune functions (Pham-Huy *et al.*, 2008). In poultry, impaired muscle membrane integrity in breast muscle is speculated to be related to changed redox balance (Sandercock *et al.*, 2001). Poultry stress responses are due to hypothalamic pituitary-adrenal axis and orthosympathetic nervous system activation. Studies have

shown that the mimicked exogenous corticosterone of stressed chickens induces lipid peroxidation (Lin *et al*., 2004). Moreover, it is known that oxidative stress in poultry decreases immunity and antioxidant status and can result in poor growth rate and production (Christaki, 2012; Joachim *et al*., 2010).

Cells have several antioxidant molecules that are produced by the body endogenously such as carotenoids and ascorbate (Yun-Zhong *et al*., 2002). Apart from these antioxidants, they are also antioxidant enzymes such as catalase, superoxide dismutase, and glutathione peroxidase that are involved in the removal of free radicals (Surai, 2015).

Antioxidants counteract the effect of free radicals, often in combination with the addition of extra minerals and vitamins to the diet. Among all dietary factors, antioxidants have gained special importance for growth, survival and maintenance, and the productive and reproductive health of chickens (Surai, 2002). Antioxidants hinder oxidation thus, reduce the oxidative stress that causes cell death or damage. Normally, compounds having antioxidant properties and meeting the demands of the antioxidant defence system or directly interfering with free radicals can restore the balance of oxidants/ antioxidants.

Natural antioxidants found in plants may alleviate oxidative stress (Keshavamurthy *et al.,* 2013) and their use satisfies the growing interest of poultry consumers. Medicinal plants contain organic compounds such as alkaloids, flavonoids, steroids, carbohydrates and terpenoids that are capable of inhibiting oxidation; phenolics possess a good capacity to scavenge free radicals, due to their redox properties (Larsomn, 1988). Traditional medicine has been used in the olden days for curing diseases and infections (Caceres *et al*., 1991; Nweze *et al*., 2004; Vineela and

Elizabeth, 2005). Different plant parts have also been used for the treatment of various ailments. The *Balanites aegyptiaca* plant has been reported to be used in skin disease treatment and as a stomach ache and jaundice remedy (Hammiche and Maiza, 2006) and Typoid fever (Doughari *et al.,* 2007). Inflammation treatment also contains *Balanites aegyptiaca roots* (Kubmarawa *et al.,* 2007). *B. aegyptiaca* contains steroidal saponins (Asaolu, 2002).

Using medicinal plants (as used in traditional practices) to keep animals healthy and productive and to treat and control diseases constitutes ethno-veterinary medicine. Traditional medicines are the available health care for animal health by rural farmers because they are easily accessible and not expensive (Kunene and Fossey, 2006). The high cost of pharmaceutical products and lack of access to veterinary services are significant reasons for farmers to use non-conventional medicines.

Apparently, overcrowding causes a series of physiological and metabolic changes in chickens as stated earlier in the introduction. This part of the study therefore hypothesised that physiological changes in overcrowded chickens are also involved in oxidative stress induction. The C*. abbreviata* plant has been tested for its *in vitro* free radical scavenging activity (see chapter 3), thus this study aimed at determining the *in vivo* effectiveness of the plant in chickens with oxidative stress; the status of the antioxidant and liver enzymes superoxide dismutase, catalase, aspartate aminotransferase and alanine aminotransferase was be monitored.

5.2 Materials and methods

5.2.1 Study site

The chickens were raised at the University of Zululand (UNIZULU) poultry unit. The biochemical assays were done in the Biochemistry laboratory at UNIZULU and histopathology done at the Vet Diagnotix lab, Pietermaritzburg (PMB).

5.2.2 Animals and design

One hundred and forty, four week old chickens were used in the experiment. The chickens were maintained under standard conditions (25 °C temperature in summer); they had access to feed (chicken mash and crushed yellow maize at 2:1, meadows feed co.) and water *ad libitum*. The chickens were divided into three groups viz. overcrowded, normal and low group with 20, 10, and 5 chickens, respectively. Each group had four replicates, making twelve groups in total, i.e. 20x4, 10x4 and 5x4 chickens per square metre. The chickens were exposed to overcrowding for 8 weeks to establish stress levels, after which the *C. abbreviata* was administered for four weeks.

5.2.3 Plant preparation for medication

The stress was induced (by overcrowding) for eight weeks and then the plant extract was administered. Three concentrations per body weight (BW) (50mg/kg BW, 200mg/kg BW and 500mg/kg BW) of plant extract (*C. abbreviata*) were prepared and separately administered orally (with a gavage syringe) to the chickens. In each group, there was a control and three replicates that received the extract at 50, 200 and 500mg/kg BW.

5.2.4 Sample collection

Blood samples were collected from the wing vein of four chickens per group from each group at the 1st, 4th and 8th week in the presence of a veterinarian. Serum was obtained from the whole blood after centrifuging and stored at -20°C for further analysis. At the end of the experiment two chickens were sacrificed from each group to collect the liver samples for histology. Livers were kept in 10% formalin and analysis was done at the Veterinary Diagnostic lab, PMB, by a qualified pathologist having no prior knowledge to which groups they belonged. This method allowed for unbiased description of the histological lesions which may be present or absent in the samples.

5.2.5 Biochemical determinations

The antioxidant activities Superoxide dismutase (SOD) and Catalase (CAT) and liver function Aspartate transaminase (AST) and Alanine transaminase (ALT) enzymes were all estimated using their respective commercial assay kit, according to the manufacturer's instructions. Lipid peroxidation was also estimated by Malaldehyde (MDA) content using an assay kit. Absorbance for all the enzymatic inhibitions were read with a Biotek® (ELx808 ul) plate reader equipped withGen5 software.

5.2.6 Statistical analysis

Data was expressed as mean ± mean standard error of the mean. All data were analysed using the statistical package for social sciences SPSS (SPSS, 2012). Statistical differences were determined using One Way Analysis of Variance (ANOVA) followed by the Tukey post hoc test. The values were considered significant at $p \leq 0.05$.

5.3 Results

5.3.1 Effect of the plant extract concentration on the growth rate of chickens in different stocking densities

Figure 5.1 shows the mean growth rate of chickens in the three stocking densities. It is apparent that the plant's extract significantly stimulated growth ($p<0.03$), and that the growth of the chickens at the different densities was concentration-dependent.

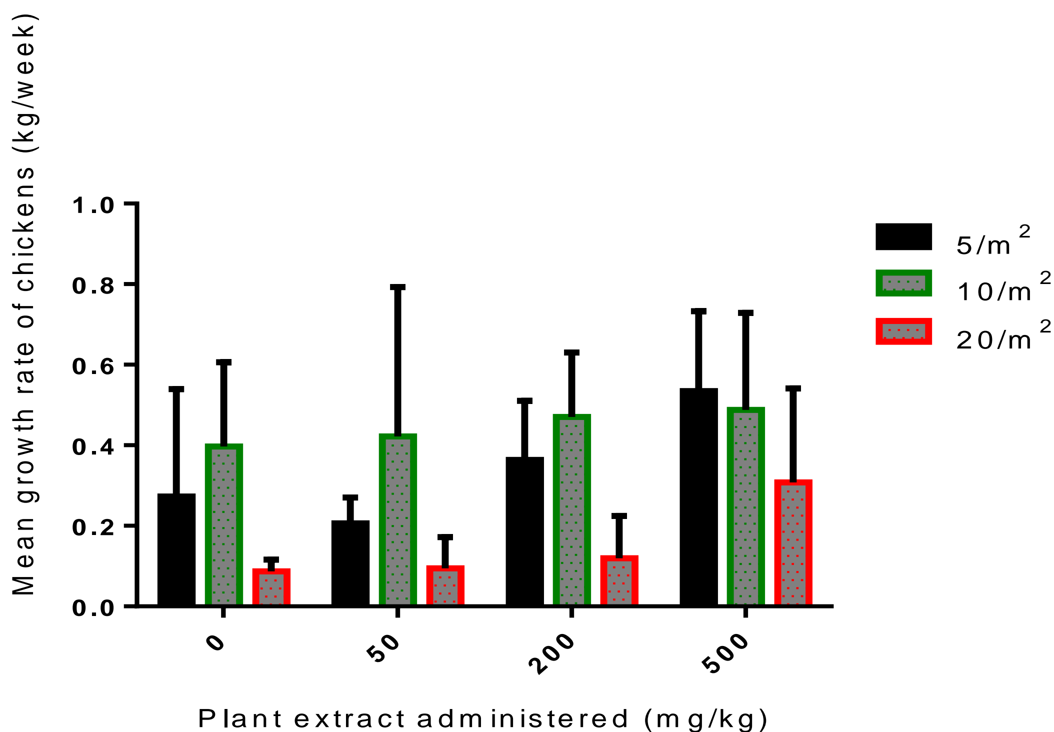

Figure 5.1: Effect of *C. abbreviata* on the growth rate (kilogram/week) of chickens given different concentrations of plant extract at the 3 different stocking densities.

5.3.2 Effect of plant extract on antioxidant enzymes (Superoxide dismutase and Catalase) level

The status of the serum enzymatic antioxidants (SOD and CAT) is shown in Figures 5.2 and 5.3, respectively. The SOD activity showed a dose- dependent manner for 5

and 10 densities; the treated groups showed a significant increase in SOD level on all plant concentrations (p<0.025), when compared to the control chickens at the same stocking densities. However, the increase in concentrations from 0 to 200 mg/kg did not produce a significant change in SOD for the chickens in the highest stocking density. The increase was realised at 500 mg/kg concentration. CAT activity was, however, high in the stocking density of 20 chickens per square metre when compared to the normal group of 10 chickens per square metre. Less CAT activity was observed for less SD of 5 chickens per square metre; it also reveals that it was negatively affected by the concentration. There was a marked decrease of CAT level in chickens treated with 200 and 500 mg/kg when compared to 50 mg/kg.

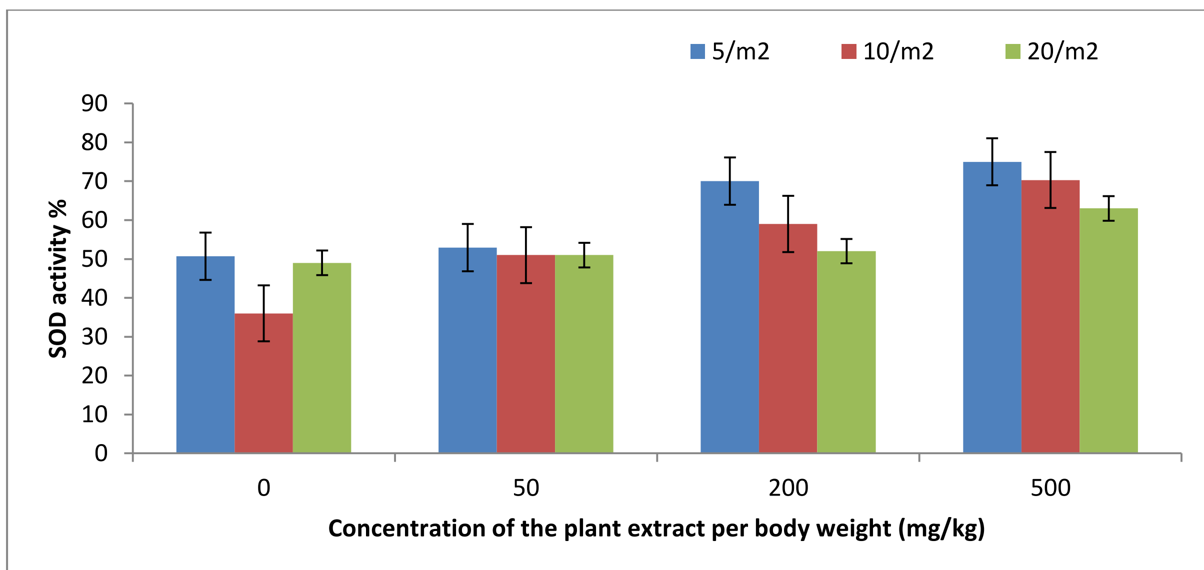

Figure 5.2: Effect of *C. abbreviata* on SOD activity in the blood serum for chickens kept in three stocking densities and receiving extract at 0mg/kg, 50mg/kg/ 200mg/kg and 500mg/kg.

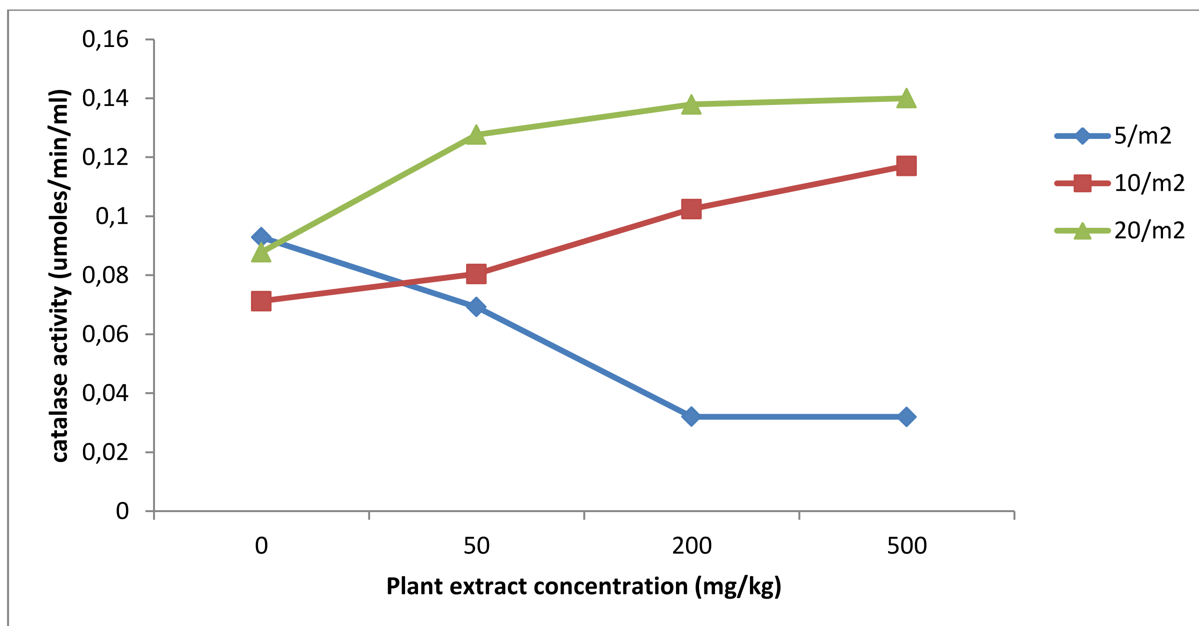

Figure 5.3: Effect of *C. abbreviata* on CAT activity in the blood serum for chickens kept in three stocking densities and receiving extract at 0mg/kg, 50mg/kg/ 200mg/kg and 500mg/kg.

5.3.3 Effect of plant extract on MDA content

The results shown in Figure 5.4 indicate that the MDA content for the treated groups against the control. The MDA content for the treated groups were significantly lower ($p<0.03$) than the control group. Moreover, a decrease was dose dependent for all the stocking densities. It was apparent, that the MDA content was slightly higher for the stressed chickens (20 chickens per square meter) in all the concentrations than in the normal (10chickens/m^2) and low 5chickens/m^2) stocking density.

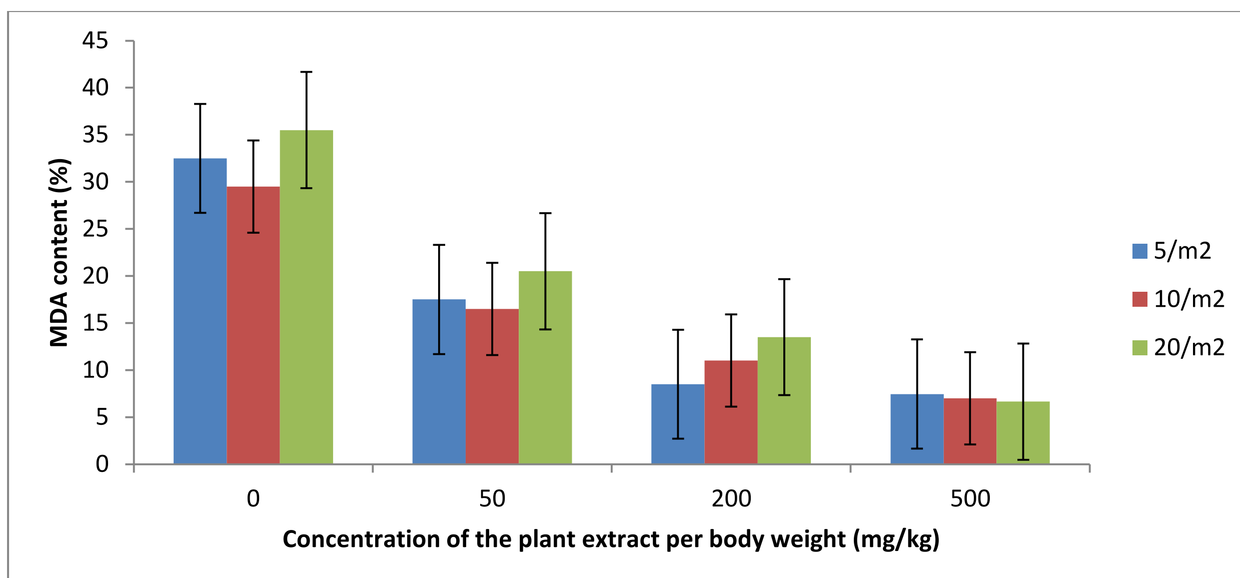

Figure 5.4: The MDA content of *C. abbreviata* in the blood serum for chickens kept in three stocking densities and receiving extract at 0mg/kg, 50mg/kg/ 200mg/kg and 500mg/kg.

5.3.4 Effects of plant extract on liver function enzymes (AST and ALT)

The results of the AST and ALT analysis are presented in Table 5.1. The ALT of extract treated groups was higher than the AST activity. AST activity decreased with the increase of plant extract concentration and was lower in the high stocking density (overcrowded) group compared to control, the normal group. ALT and AST activity was significantly lower by 12% and 45% compared to control ($p<0.05$).

Table 5.1: Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST) in serum of chickens kept in three stocking densities treated with various plant concentrations (n=2, X ±SEM).

Groups / Conc. (mg/kg)	Parameters					
	ALT			AST		
	5/m²	**10/m²**	**20/m²**	**5/m²**	**10//m²**	**20/m²**
Control	49.09±0.002	43.74±0.001	40.01±0.00	3.85±0.25	3.52±0.26	2.16±0.20
50	18.45±0.032	10.62±0.03	6.70±0.025	3.55±0.87	3.24±0.5	2.17±0.36
200	50.81±0.1	36.85±0.05	30.45±0.042	3.47±0.15	3.52±0.06	2.16±0.00
500	51.26±0.12	41.94±0.25	34.70±0.20	3.35±0.123	3.53±0.175	1.18±0.002

5.3.5 Histology of liver tissues

The histopathological examination of liver tissues confirmed the biochemical results of the chickens treated with *C. abbreviata*. The microscopic examination of the liver samples showed no changes in relation to the stocking density, however marked changes were consistently observed in the livers of chickens given different concentrations of the extract.

Figure 5.5 (i) shows the liver sample of the control group with branching and anatomical cords radiating from the central vein with vesicular nuclei. Hepatocytes were within the normal limits with multifocal lymphocytic hepatitis and triaditis.

Figure 5.5 (ii) and (iii) show mild congestion in the lymphocytic hepatitis. Necrotic changes and hydropic swellings of hepatocytes were also observed. However, in high concentration Figure 5.5 (iv), the hepatocytes were within the normal limits and sinusoids discernible. A few small focal areas of necropurulent hepatitis with scant accompanying epithelioid cells, macrophages and lymphocytes were observed. No significant difference was observed in all groups, which therefore suggests that the plant did well in its biochemical activities.

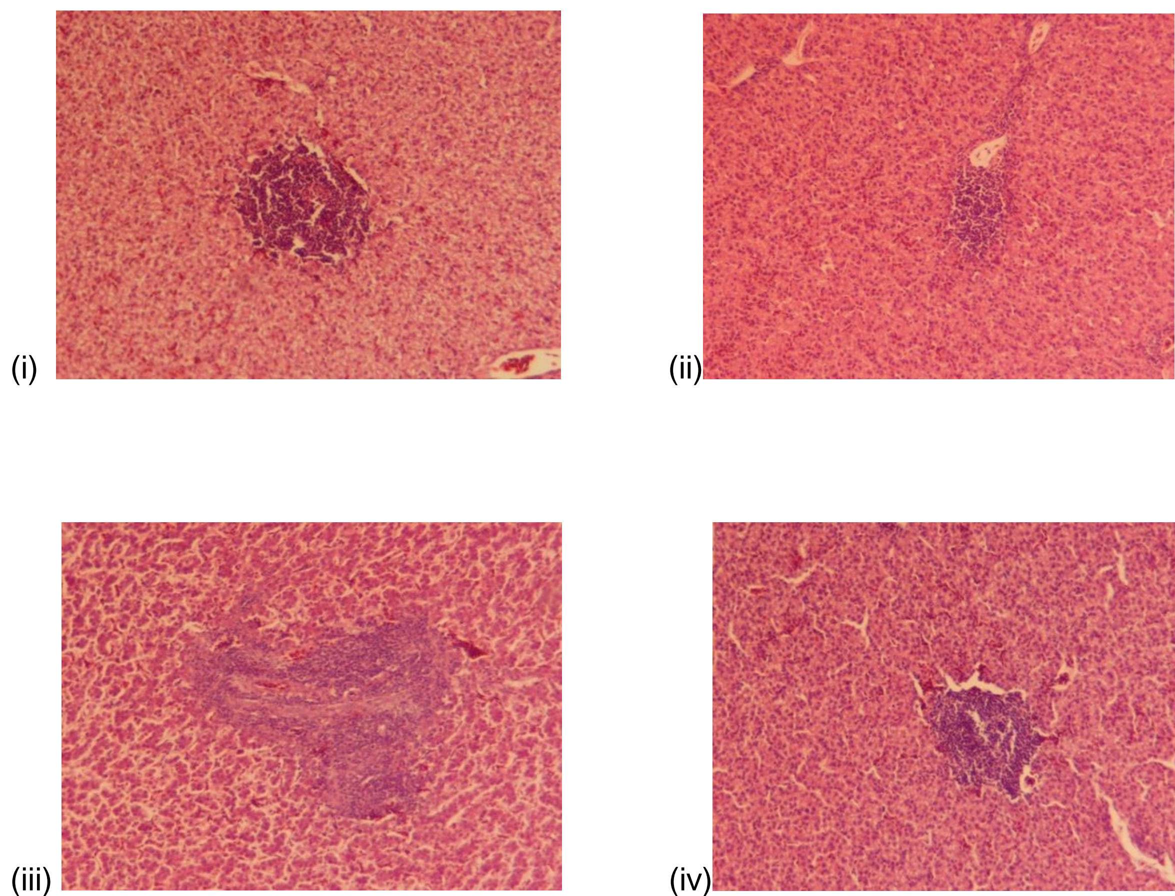

Figure 5.5: Histopathological changes of the liver at different plant concentration, (i) control normal hepatocytes (ii) 50mg/kg), (iii) 200mg/kg) and (iv) 500mg/kg.

5.4 Discussion

The effect of *C. abbreviata* on the oxidative stress caused by overcrowding in indigenous chickens will now be discussed.

Animal growth is generally influenced by genotype, nutrition, hormone, tissue-specific regulatory factors, and other aspects of animal's environment (Simelane, 2010). In a stress- free environment with adequate space and/or ventilation, growth increases until a genetically determined upper limit is reached (Kingori *et al*., 2007). It is apparent that the plant *(C. abbreviata)* enhanced the growth of oxidative stressed chickens, as the physiological conditions were maintained.

It is known that ROS are highly toxic and can produce a variety of pathological changes through lipid peroxidation and DNA damage. There is a wide variety of enzymatic and non-enzymatic antioxidants existing in the body. The enzymatic antioxidant SOD converts superoxide to hydrogen peroxide and molecular oxygen. Generally, elevated SOD might reflect a response to oxidative stress and then may predict a state of excess reactive oxygen species in body. In this study, total SOD activity in the serum of chickens kept in high stocking was significantly higher than that of the control group.

SOD can protect cells from oxidative injury by clearing superoxide anions. A small increase of free radicals induces antioxidant expression (McArdle and Jackson, 2000). The activity of SOD and the concentration of MDA are, therefore, commonly combined in the investigation of the extent of damage to the whole body. In the present study, the activity of SOD was enhanced during plant administration,

indicating the *in vivo* antioxidant properties of *C. abbreviata*. The activity of CAT is the next defence that is responsible for converting hydrogen peroxide into water and oxygen (Surai *et al*., 2003). The removal of hydrogen peroxide protects cells against oxidative damage caused by hydrogen peroxide toxicity (Deepak *et al*., 2015). It is apparent that *C. abbreviata* activates antioxidant enzymes, as CAT found in serum of the extract treated groups was higher than in the control groups. The antioxidant activity of *C. abbreviata* exerted the first line of defence against the free radicals that were accumulating in the oxidative stressed chickens. It is apparent that the exposure to overcrowding increased lipid peroxidation, as indicated by the MDA concentration. The content of MDA in serum reflects indirectly the extent of lipid peroxidation and overproduction of ROS in the body. There was no significant difference ($p>0.05$) in the MDA content of chickens receiving the plant at different concentrations, although a decrease in the MDA content was observed in the treated groups induced with oxidative stress. This was due to high inhibition of ROS by the antioxidant enzymes enhanced by *C. abbreviata.* Similar findings were reported by Sahin *et al*. (2009) that antioxidants from plants reduce lipid peroxidation as they protect the body from free radical scavenging effects.

AST and ALT are similar liver enzymes that are less specific for liver disease as they are produced in muscles and can be elevated in other conditions such as heart attack (Sha *et al*., 2015). However, high levels of serum AST and ALT indicate liver damage, with ALT more specific for liver injury than AST (Broundward *et al*., 2001). This is well supported by the results obtained in this study where the ALT activity was high, meaning that the plant has the toxicity activity. Mongalo (2013) found that the methanolic extract of *C. abbreviata* exhibits high toxicity in the brine shrimp

lethality test. The histological changes in the liver samples confirmed the biochemical results and revealed that *C. abbreviata* given to chickens resulted in significant improvement in the liver tissues. It is apparent that high concentrations cause damage to the liver. (It has been reported by other researchers that *C. abbreviata* contains toxicological compounds).

5.5 Conclusion

Overcrowding causes oxidative stress in indigenous chickens which thus has significant influence on the chicken's health and growth. *C. abbreviata* has been observed to be an effective plant with antioxidant activities that could be used as an antioxidant to enhance the health and growth of chickens. However, it is advised that *C. abbreviata* be used with caution as it seems to be toxic to the liver at high concentrations. The recommended dosages can range between fifty and two hundred milligrams per kilogram of body weight.

CHAPTER 6

General discussion

The stocking density greatly affected the growth performance of chickens. At high stocking density growth of chickens was very low when compared to the chickens kept at low and normal stocking density. However, low and normal stocking density had no major impact on growth, so normal stocking density can be utilised by farmers as it will minimise inputs such as housing, feeding troughs and watering troughs. Estevez (2009) suggested that the recommended stocking density could be increased without any major adverse effects on body weight. High cannibalism was observed in chickens kept at high stocking density. These findings are in line with Simitzis *et al.* (2012) who reported that cannibalism occurs more when chickens are overcrowded. Also, this led to competition for access to feed and water as the superior chickens were chasing the lesser ones. However, the provision of green leaves minimised or controlled the cannibalism. The aggressive behaviour of chickens in the house led to spillage of water, which wet the litter floor. Similar results were reported by Yakubu *et al.* (2009) that high stocking density increases disturbance and conflict between chickens. Wet litter increases the occurrence of disease. Moreover, the metabolic system is disturbed and excessive accumulation of reactive compounds are realised.

The present study aimed at determining how stock size influences the growth of chickens. The findings complement those that have been done on indigenous chickens and broiler production elsewhere (Dozier *et al.,* 2005). Farmers use different medicinal plants to cure diseases affecting chickens, however most of the medicinal plants have not been scientifically validated for use, meaning that despite

their effectiveness the knowledge is left undocumented. This study evaluated the pharmacological and antioxidant activities of the *C. abbreviata* which showed very positive results of scavenging free radicals in the oxidative stressed chickens. Different concentrations showed a significant effectiveness against free radicals as the antioxidant enzymes level was found in the blood serum of the treated chickens. However, a high concentration can cause liver damage because of it toxicity. More evidence of damage was observed in the histopathology of the livers as treated groups showed various hepatic changes.

Stocking density has a serious effect on the growth of chickens at all phases. Less stocking density increases the body weight of chickens but high stocking density delays or retards growth of chickens. Thus, optimal stocking density should be taken into consideration to avoid the delay of maturity weight in chickens. High stocking density or overcrowding causes oxidative stress in indigenous chickens, which thus has significant impacts in the chicken's health and growth. *C. abbreviata* has been observed to be an effective plant with antioxidant activities that could be used as an antioxidant to enhance the health and growth of chicken. However, it is advised that *C. abbreviata* be used with caution, as it seems to be toxic to the liver at high concentrations. Medicinal plants must be utilized by livestock farmers because of their effectiveness with no side effects provided proper precautions are followed.

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Suggestion for further study

Test the:

- Effect of the plant on the meat or carcass quality such as flavour, aroma and off-flavour descriptors.
- Influence of various stressors on the oxidative damage of tissues
- Influence of plants in treating the damaged tissues.

APPENDIX A

Reagent details

A.1 Dragendorff's reagent

0.85 g of Bismuth nitrate was dissolved in 40 ml of distilled water and 10 ml of glacial Acetic acid, followed by the addition of 8 g potassium iodide dissolved in 20 ml of water.

A.2 Mayer's reagent

1.358 g of silver chloride was dissolved in 60 ml of distilled water and poured into a solution of 5 g of KI in 10 ml of distilled water and sufficient water added to make 100 ml.

A.3 Sulphanilic acid reagent

0.33 % of Sulphanilic acid was prepared in 20 % glacial acetic acid.

A.4 2,2'-azino-bis- (3-ethylbenzothiazoline-6-sulfonate)

7mM of ABTS* was prepared in water and an equivalent amount of 2.45 mM Potassium persulfate was added; the mixture was incubated at room temperature in the dark for 16h, which resulted in the production of radical cation ABTS*. The ABTS* was diluted (1:60 methanol, v/v).

A.5 Phosphate buffer (pH 7.4)

40 ml of 0.2 M of potassium hydroxide was prepared and mixed with 50 ml of 0.2 M Potassium dihydrogen phosphate. The mixture was made up to 100 ml.

A.6 Sodium borate buffer (pH 8)

2.473 g of Boric acid was dissolved in 1000 ml distilled water. 2.012 g of sodium borate dissolved in 250 ml of distilled water. Then 166.67 ml of sodium borate was mixed together with 1000 ml of boric acid.

A.7 TBA

50 ml of glacial acetic acid and 50 ml of distilled water was mixed. 1 g of TBA was added into the solution and the solution was made up to 100 ml with distilled water.

A.8 Formalin

1.75 g of sodium dihydrogen orthophosphate ($NaH_2PO_42H_2O$) and 3.25 g of di-sodium hydrogen orthophosphate (Na_2HP0_4) was dissolved in 25 ml of boiling water. 50 ml of 40 per cent formalin was added and the resulting mixture was made up to 400 ml with distilled water.

APPENDIX B

Details of methodology

B1. Extraction

Stem bark of *Cassia abbreviata* was air-dried and ground to powder (2mm mesh). The powdered plant material (200g) was extracted with methanol in a platform shaker machine for 24 h, at room temperature (157 rpm). The ratio of the plant material to the solvent was 1:5. The extract was separately filtered through Whatman no.1 filter paper.

B2. Phytochemical screening

B2.1 Test for saponins

The plant material (2.5 g) was extracted with boiling water and was allowed to cool. The extract was shaken vigorously to froth and then allowed to stand for 15-20min. The extract was then classified for saponin content as follows: no froth = negative (no saponins) and froth less than 1 cm = weakly positive (saponins present); froth 1.2 cm high = positive; and froth greater than 2 cm high = strongly positive.

B2.2 Test for tannins

The plant material (0.5 g) was boiled with 10 ml of water for 15 min, filtered and made up to 10 ml with distilled water. Two millilitres of the filtrate was put into another test tube and a few drops of 0.1 % $FeCl_3$ solution were added to the 2 ml of the filtrate. Black-blue, green or blue-green precipitate was taken as preliminary evidence of the presence of tannins.

B2.3 Test for steroids

Acetic anhydride (2 ml) and concentrated sulphuric acid (2 ml) were added to 0.5 g of the plant material and mixed. A colour change from violet to blue or green was taken as evidence of the presence of steroids.

B2.4 Test for terpenoids (Salkwoski test)

The plant material (0.5 g) was mixed with 2 ml of chloroform, and 3 ml of concentrated sulphuric acid was carefully added to form a layer. A reddish-brown colouration of the interface was taken as evidence of the presence of terpenoids.

B2.5 Test for alkaloids

The plant material (0.5 g) was dissolved in 5 ml 1 % HCl (aq). The solution was stirred over a steam bath and filtered. One millilitre of the filtrate was treated with Mayer's reagent. A precipitate was taken as preliminary evidence of the presence of alkaloids. Another 1 ml of the filtrate was treated with Dragendorff's reagent and turbidity or precipitate was also taken as evidence of the presence of alkaloids.

B2.6 Test for flavonoids

The flavonoids content was determined using the ferric chloride test. The plant material (1 g) was mixed with 1 ml of FeCl3. A dark brown or dirty brown precipitate was taken as evidence of the presence of flavonoids.

B3 Total phenolic content (gallic acid equivalent - GAE)

Gallic acid was prepared at different concentrations (0.01, 0.02, 0.04, 0.08 and 0.1 mg/ml diethylether). The plant extract (0.5 ml) was dissolve in 1 ml of diethylether and mixed. The diethyl ether was left to evaporate off, leaving the residue behind. Sodium carbonate (7.5 g/100ml) was prepared and Folin-Ciocalteu's Phenol reagent (FC) was diluted with distilled water (1:10).

Test tubes were set up in duplicates. To each test tube containing the residue, 1.5 ml FC and 1.2 ml Na_2CO_3 were added and they were well mixed to obtain solutions. The solutions were kept in the dark for 30 min. Absorbance of the blue coloured mixtures was read at 765 nm with the mixture of FC and Na_2CO_3 used as blank. The results were recorded and they were translated into a standard curve of absorbance (nm) versus concentration of gallic acid (mg/ml). See Figure B1.1. The total phenolic content of the extracts was calculated as gallic acid equivalent from the calibration curve of gallic acid and expressed as mg/g dry plant material.

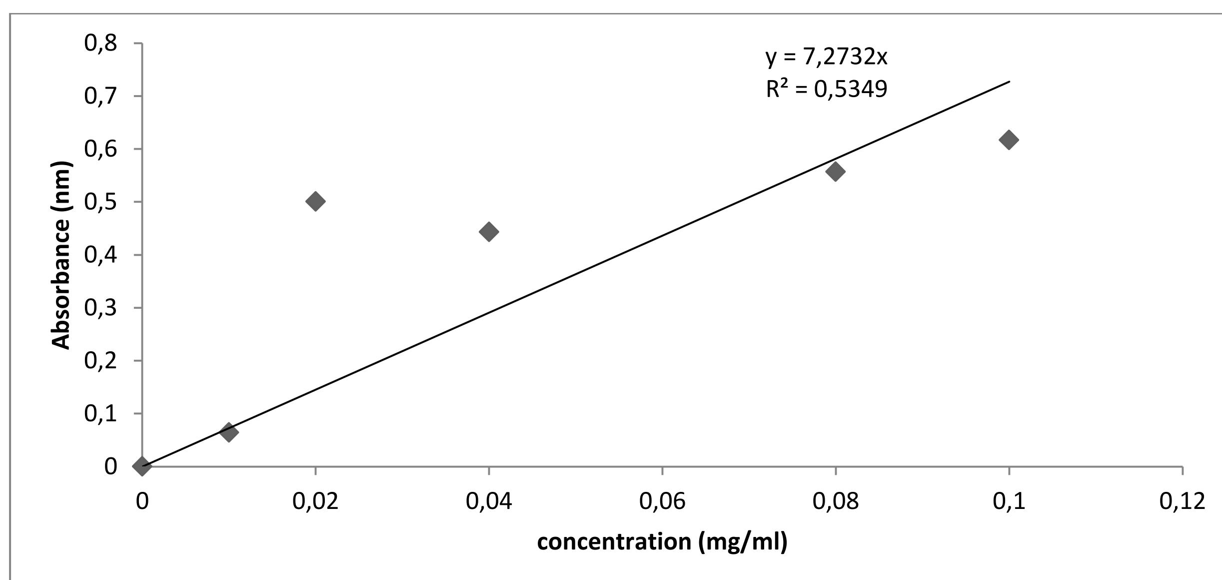

Figure B3.1. Calibration curve of gallic acid concentration (mg/ml) against absorbance (nm). The curve was used to determine the total phenolic content of the extract of *C abbreviate* as gallic acid equivalent.

B4 Flavonoid content (quercetin equivalent, QE)

Quercetin different concentrations (0.01, 0.02, 0.04, 0.08 and 0.1 mg/ml diethylether) were prepared. *C abbreviata* extract (0.5 ml) was dissolve in 1 ml of diethyl ether and mixed. The diethyl ether was evaporated off to leave behind the residues. Two

percent of AlCl3 ethanol solution was prepared. Test tubes were set up in duplicates. The residues were dissolved in 0.5 ml 2% AlCl3 ethanol solution. The solutions were allowed to stand for 1h at room temperature (a yellow colour indicated presence of flavonoids). Absorbance was read at 420 nm against a reagent blank (2% $AlCl_3$ ethanol solution). The flavonoid content of the extracts was determined as quercetin equivalent from the calibration curve of quercetin (figure B4.1 and expressed as mg/g dry plant material.

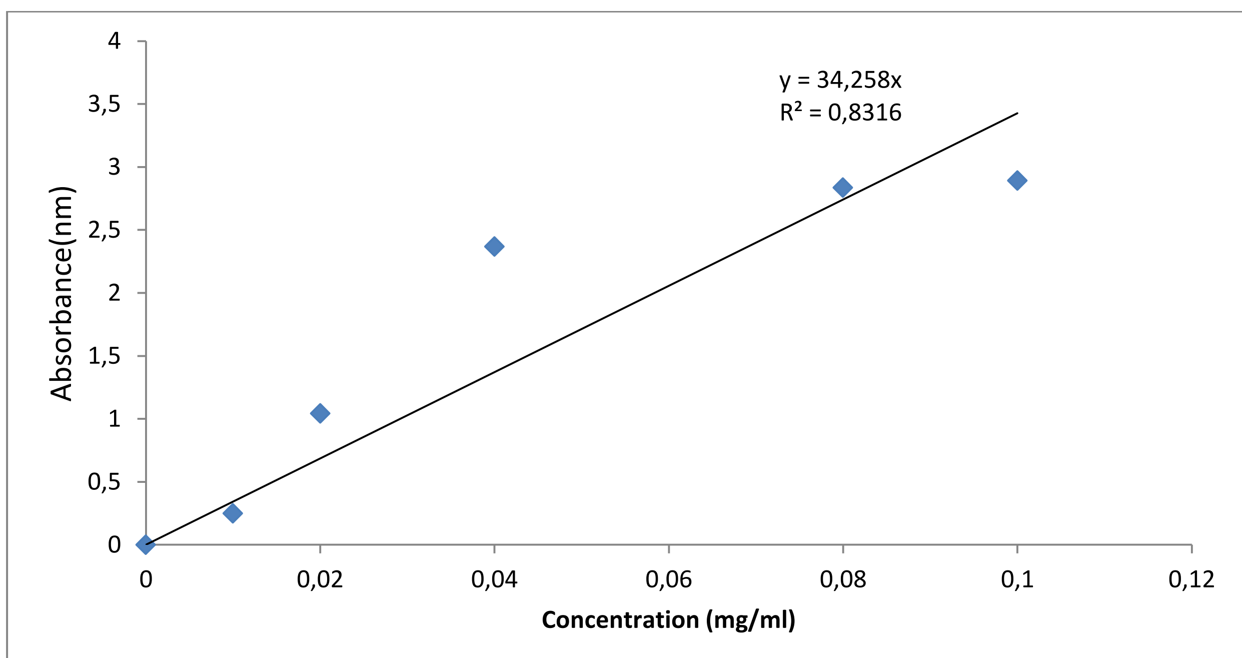

Figure B4.1Calibration curve of quercetin concentration (mg/ml) against absorbance (nm). The curve was used to determine the flavonoid content of the extracts of *C abbreviata* as quercetin equivalent.

Appendix C

UNIVERSITY OF ZULULAND
RESEARCH ETHICS COMMITTEE
(Reg No: UZREC 171110-30- RA Level 02)

RESEARCH & INNOVATION
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Fax: 035 902 6222
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ETHICAL CLEARANCE CERTIFICATE

<table>
<tr><td>Certificate Number</td><td colspan="8">UZREC 171110-030-RA Level 02 PGM 2014/124</td></tr>
<tr><td>Project Title</td><td colspan="8">Effects of Cassia abbreviate Oliv stem extracts on the oxidative stress caused by overcrowding in indigenous chicken of South Africa</td></tr>
<tr><td>Principal Researcher/ Investigator</td><td colspan="8">C Jobe</td></tr>
<tr><td>Supervisor and Co-supervisor</td><td colspan="4">Prof NW Kunene</td><td colspan="4">Prof A Opoku</td></tr>
<tr><td>Department</td><td colspan="8">Agriculture</td></tr>
<tr><td>Nature of Project</td><td>Honours/4th Year</td><td></td><td>Master's</td><td>x</td><td>Doctoral</td><td></td><td>Departmental</td><td></td></tr>
</table>

The University of Zululand's Research Ethics Committee (UZREC) hereby gives ethical approval in respect of the undertakings contained in the above-mentioned project proposal and the documents listed on page 2 of this Certificate.

Special conditions:

(1) The Principal Researcher must report to the UZREC in the prescribed format, where applicable, annually and at the end of the project, in respect of ethical compliance.

(2) Documents marked "To be submitted" (see page 2) must be presented for ethical clearance before any data collection can commence.

(3) The slaughtering of chicken must be done under supervision by a VETINERARIAN at the University of Zululand before transportation

The Researcher may therefore commence with the research as from the date of this Certificate, using the reference number indicated above, but may not conduct any data collection using research instruments that are yet to be approved.

Please note that the UZREC must be informed immediately of

- Any material change in the conditions or undertakings mentioned in the documents that were presented to the UZREC
- Any material breaches of ethical undertakings or events that impact upon the ethical

conduct of the research

Classification:

Data collection	Animals	Human Health	Children	Vulnerable pp.	Other
	X				

Low Risk	Medium Risk	High Risk
		X

The table below indicates which documents the UZREC considered in granting this Certificate and which documents, if any, still require ethical clearance. (Please note that this is not a closed list and should new instruments be developed, these would require approval.)

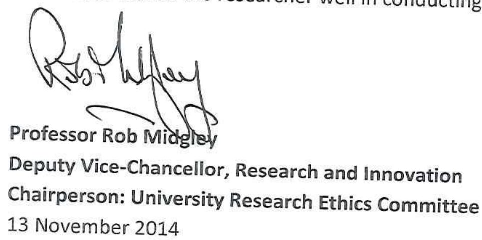
Documents	**Considered**	**To be submitted**	**Not required**
Faculty Research Ethics Committee recommendation	X		
Animal Research Ethics Committee recommendation	X		
Health Research Ethics Committee recommendation			X
Ethical clearance application form	X		
Project registration proposal	X		
Informed consent from participants			X
Informed consent from parent/guardian			X
Permission for access to sites/information/participants			X
Permission to use documents/copyright clearance			X
Data collection/survey instrument/questionnaire			X
Data collection instrument in appropriate language		Only if necessary	
Other data collection instruments		Only if used	

The UZREC retains the right to

- Withdraw or amend this Certificate if
  - Any unethical principles or practices are revealed or suspected
  - Relevant information has been withheld or misrepresented
  - Regulatory changes of whatsoever nature so require
  - The conditions contained in this Certificate have not been adhered to

- Request access to any information or data at any time during the course or after completion of the project

The UZREC wishes the researcher well in conducting the research.



Professor Rob Midgley
Deputy Vice-Chancellor, Research and Innovation
Chairperson: University Research Ethics Committee
13 November 2014

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