ANTHELMINTIC, ANTICANCER AND PHYTOCHEMICAL SCREENING OF *COTYLEDON ORBICULATA; HERMANNIA DEPRESSA; NICOTIANA GLAUCA* AND POTASSIUM PERMANGANATE

By

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DECLARATION

I, the undersigned, hereby declare that the work contained in this dissertation is my original work and that I have not previously in its entirety or in part submitted at any university for a degree. I furthermore cede copyright of the dissertation in favour of the University of the Free State.

Signature :……..

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DEDICATION

‘To the pillar of my strength, my best friend, my everything, my mother, Dieketseng Josephine Molefe for everything she has gone through in order to provide a better future for me and my little brother.’

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ABBREVIATIONS AND SYMBOLS

-	Negative
+	Positive
<	Less than
>	Greater than
±	More or less
≤	Less or equal to
®	Registered trade mark
µg/ml	Microgram per millilitre
µl	Microlitre
µm	Micrometer
ATCC	American Type Culture Collection
ATP	Adenosine Triphosphate
cell/ml	Cell per millilitre
cm	Centimetre
CO_2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid

ABBREVIATIONS AND SYMBOLS

EDTA	Ethylenediaminetetraacetic acid
FBS	Foetal Bovine Serum
g	Grams
g/l	Grams per litre
h	Hours
HeLa	Henrietta Lacks (uterine cell variety; named after deceased patient)
HPV	Human papillomavirus
In vitro	Laboratory experiment
In vivo	Experiments in a live specimen
L_1	First stage of nematode larvae, free living stage
L_2	Second stage of nematode larvae, free living stage
L_3	Third stage of nematode larvae, the infective stage
L_4	Fourth stage of nematode larvae, first moult to an adult stage in a host
LC_{50}	Lethal concentration that kills 50% of experimental specimen
LDH	Lactate dehydrogenase
MCF-7	Michigan Cancer Foundation-7
MDBK	Madin-Darby Canine Kidney
mg/ml	Milligram per millilitre

ABBREVIATIONS AND SYMBOLS

ml	Millilitre
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NAD+	Nicotinamide Adenine Dinucleotide
nm	Nanometer
PBS	Phosphate buffered saline
RNA	Ribonucleic acid

ABSTRACT

Cotyledon orbiculata, Hermannia depressa and *Nicotiana glauca* are widely used plants in traditional medicine for treatment of various infections and diseases. *C. orbiculata* is mostly used in treatment of epilepsy, earache and the removal of warts while *H. depressa* is used to relieve toothache, nerves and diarrhoea. Lastly, *N. glauca* has been mostly used as pain killer in relieving earache, toothache and seldom applied on body in treatment of soreness and inflammation. Traditional usages of these plants by indigenous people and local traditional healers have not yet been scientifically reported, that is why the current study was aimed at determining the anthelmintic and anticancer activity of these plants.

The anthelmintic activity of acetone and water extracts from the shoots of *Cotyledon orbiculata, Hermannia depressa* and *Nicotiana glauca* were investigated using the egg hatch, larval development and larval mortality assays. In all extracts tested, *C. orbiculata* water extract at 7.5 mg/ml prevented nematode eggs from hatching with 82.63% success rate. Other extracts exhibited egg hatch inhibition in a degree of less than 50%. Similarly, *C. orbiculata* water extracts suppressed nematode larval development with 85.32% at the concentration of 2.5 mg/ml followed by 66.69% of *H. depressa* extract at 7.5 mg/ml concentration. However, *N. glauca* water and all acetone extracts induced the 100% larval development inhibition. The *in vitro* larval mortality rate revealed that the water extracts from all the plants were able to kill all larvae at 2.5 mg/ml within 48 to 96 h. The results from this study have shown that the extracts from the three plants have the potential to prevent and ameliorate diseases associated with gastrointestinal nematodes.

The anticancer activity of the above mentioned plants was tested on two cancer cell lines, the MCF-7 and HeLa cells. All plants possessed anticancer activity at different durations and concentrations. The *N. glauca* demonstrated an activity against both cell lines, however, the plant acetone extracts were much effective on the MCF-7 line at 48 hours with inhibition percentages > 80% at all concentrations. The *N. glauca* acetone extracts were effective at 24 hours. The *H. depressa* acetone extracts also possessed much activity than water extracts at 24 hours, whereas *C. orbiculata* showed no activity at all on this cell line. The *C. orbiculata* acetone extracts followed by the water extracts were the most effective on the HeLa line ranging from 12 to

84% and 0 to 77% inhibition. *H. depressa* activity was observed at 48 hours of experimentation at all concentrations in both extracts. *N. glauca* exhibited significant inhibition percentages at 24 hours of water extracts and 48 hours of acetone extracts. This study has showed that the three plants are potential candidates for cancer treatment.

Brine shrimp lethality test was conducted on the nauplii stage of the shrimps. Furthermore, two assays, MTT and LDH cytotoxicity assays were conducted on the MDBK cells. None of the plants was toxic on the brine shrimps as most of them survived through 24 and 48 hours. *C. orbiculata* and *H. depressa* brine shrimp larval mortality was observed at 96, 72, 72, 96 and 96 h at 0.2, 0.4, 0.6, 0.8, and 1.0 mg/ml of water extracts respectively and 72 h at all concentrations of acetone extracts while *N. glauca* larval mortality was observed at 120 and 96 hours at all concentration of both extracts. There was a cell survival decrease of 50% at 0.65 mg/ml and 50-60% in *C. orbiculata* water and acetone extracts treated wells, respectively. No significant cell decrease was observed from both *H. depressa* and *N. glauca* water and acetone extracts except for *N. glauca* acetone extract at 2.50 mg/ml. None of the plants induced a significant toxicity on both the brine shrimps and the bovine cells.

Alkaloids, saponins, tannins and phenols were the constituents tested for in order to identify constituents responsible for observed activity of the whole study. No alkaloids were detected from all plants for both extracts, only saponins, tannins and phenols were present even though *N. glauca* acetone extracts possessed none of the tested constituents. Tested plants are therefore good candidates in treating parasitic gastrointestinal nematodes and cancer disease.

Potassium permanganate ($KMnO_4$) is a widely used chemical substance in the rural communities as prophylaxis or in treatment of various infections and diseases; however, there is no scientific validation of its usage. Anthelmintic activity of potassium permanganate was tested against parasitic gastrointestinal nematodes in comparison to anthelmintic commercial drugs, Tramisol®, Noromectin® and Valbazen®. There was no egg hatch inhibition induced (<10%) by potassium permanganate at all concentrations as compared to >80% inhibition of commercial drugs, however, 85.24, 98.10 and 90.91% of larval development was induced at 0.5, 1.0 and 1.5 mg/ml, respectively. Larval mortality was observed at 216, 144 and 144

hours at 0.5, 1.0 and 1.5 mg/ml, respectively. The cytotoxicity of this chemical was tested on the brine shrimps and MBDK cells as well. Within 24 hours all the brine shrimps larvae treated with $KMnO_4$ died. A significant decrease in MDBK cell survival was observed at 0.65 mg/ml at 24, 48 and 72 hours. This study has therefore showed that potassium permanganate have anthelmintic activity and concentration dependent cytotoxicity on the mammalian cell lines. In conclusion *Cotyledon orbiculata, Hermannia depressa, Nicotiana glauca* and potassium permanganate have the potential to supress the aggressiveness of parasitic gastrointestinal nematode and cancer diseases. These substances are therefore potential candidates in treatment of tested pathogens.

Keywords: Anthelmintic activity, anticancer activity, *Cotyledon orbiculata*, cell lines, cytotoxicity, *Hermannia depressa*, medicinal plants, *Nicotiana glauca*, parasitic gastrointestinal nematodes, Potassium permanganate.

Chapter 1

Introduction and literature review

1.1 Medicinal plants and their uses

Medicinal plants are plants which in one or more organs contain substances that can be used for therapeutic purposes and/or contain ingredients useful in medicinal preparation or discovery. They are used to cure, heal or reduce the aggressiveness of the disease (Kumar *et al.* 1997). They also provide potential for development of new pharmaceutical substances (Diehl *et al.* 2004). Many plants use secondary metabolites to defend themselves from pathogens, microbes and herbivores. When the plant is under any kind of stress may it be coldness, hotness, droughts, flooding or even being cut down, the secondary metabolites are released accordingly. Some of these metabolites such as the alkaloids and cardiac glycosides may be highly toxic, some are toxic in high concentrations. Other secondary metabolites are secreted at all times due to metabolic activities such as aging of the plant, these are not in most cases toxic or poisonous (Dearing *et al.* 2005). Other plants lose their toxic effects if exposed to heat or boiling than being consumed in a fresh state as it was stated by Asekum *et al.* (2007). It is believed that every component has its own specific function in a plant. During the experiment medicinal plant usage might mean toxic and/or poisonous substances are involved, the most certain way to determine the cytotoxicity of the plants is to conduct valid assays. There are two main toxicology studies that can be implemented in the determination of the safety measures of using any plant. Cytotoxicity is a process of detecting the quality of toxin of any substance on the cells and/or live specimen such as *Artemia salina*, the brine shrimps (Eloff *et al.* 1998).

According to Eguale *et al.* (2011), farmers started using the ethnoveterinary medicine as an alternative and a suitable way of treating gastrointestinal nematodes when they could not access the anthelmintic drugs due to their high costs. Medicinal plants are not synthetic, they are accessible, affordable and environmentally friendly (Pessoa *et al.* 2002). Furthermore, medicinal plants have not yet been documented with any resistance development from the gastrointestinal nematodes. Resistance

develop in most cases due to the synthetic chemicals used in drug manufacture. Naturally produced substances offer an alternative mode of treatment of many diseases and infections (Pessoa *et al.* 2002; Diehl *et al.* 2004). Numerous plants have been tested for anthelmintic activity in South Africa which includes *Harpephyllum caffrum, Tulbaghia violacea* (McGaw *et al*. 2000), *Felicia erigeroides, Hypoxis colchicifolia* (Aremu *et al.* 2010), *Pterocarpus angolensis, Schotia brachypetala* (McGaw *et al.* 2007), *Artemisia afra and Mentha longifolia* (Molefe *et al.* 2012).

Other alternative ways of treating parasitic nematode infections have been taken into consideration as well, however, not much has been reported. Diehl *et al.* (2004) suggested other possible ways such as breeding the resistant livestock against nematodes or improving food quality, whilst Adeomola & Eloff (2011) suggested prevention, control and treatment. However, there are obstacles to these suggestions; nematodes have their way of adapting to a new environment. It is quite hard to predict the infected grazing areas and treating hectares of land with anthelmintic drugs would be a huge waste of time and money.

Amongst other infections cancer infection is one of the most complex, fatal and difficult to treat (Merina *et al.* 2012). However, plant species have been documented to possess anticancer activity against certain cancer lines more especially plants containing flavonoids, diterpenes and stigmasterol (Kumar *et al.* 2004). In most cases plants tested for cytotoxicity are also tested for anticancer activity, using both mammalian cells, normal cells as well as cancer cell lines. Toxic plants may contain anticancer cells in certain percentages according to the chemical constituents of the plant. According to Steenkamp & Gouws (2006) reports on plants used for anticancer activities are rare in South Africa. Scientific reports of South African medicinal plants with anticancer activity includes *Cnicus benedictus, Dicoma capensis* (Steenkamp & Gouws 2006), *Solanum acanthoideum, Pelargonium acraeum* (Fouche *et al*. 2008), *Artemisia annua, Salvia albicaulis* (Kamatou *et al*. 2008). Research for traditional medicinal plants in treatment of parasitic gastrointestinal nematodes and cancer is still in progress.

A large South African population is depending on the usage of medicinal plants as primary health needs. Due to the lack of scientific validation of most traditionally used plants, the major problem remains, which is lack of regulation in the prescription and inadequate toxicological evidence of safety of medicinal plant usage (Fennell *et al.* 2004; Aremu *et al.* 2010). There is therefore a need of National government to encourage more research into traditional medicinal plants and emphasise on developing good policies and regulations for medicinal plants use. The South African government has proposed the integration of traditional medicinal plants into the national healthcare system; however, so far there are only few research groups which are actively involved in anthelmintic and anticancer research in South Africa aimed at the screening of plant extracts and the identification of potentially new anthelmintic and anticancer drug leads. Anthelmintic research groups include the Research Centre for Plant Growth and Development, University of KwaZulu-Natal and the Phytomedicine Programme, Department of Paraclinical Sciences, University of Pretoria (Aremu *et al.* 2012), while anticancer groups are Council for Scientific and Industrial Research (CSIR) and the NCI in South Africa (Fouche *et al.* 2008).

1.2 Parasitic gastrointestinal nematodes

Gastrointestinal nematodes are mostly known to invade the digestive tract, the abomasums or even the small intestines (Maphosa & Masika 2009). They are the worst parasites for livestock production worldwide (Diehl *et al.* 2004). They totally depend on their host for nourishment, nutrition and protection. Nematode infections are therefore of economic importance for they are a major cause of disease and results in decreased productivity. All types of livestock are at risk to gastrointestinal nematode infection which varies in their host range, life cycle and severity. However, small stock is the most susceptible to gastrointestinal nematode infections (Tsotetsi & Mbati 2003).

Nematode infection results in several clinical symptoms, however, in most cases no symptoms develop under high quantity of gastrointestinal nematodes in a single host. Some species of nematodes such as the *Haemonchus* and *Trichostrongylus* species are blood sucking parasites whose activity usually results in anemia. Most

common symptoms of nematode infection are irritation, loss of appetite, inflammation of the mucous membranes, diarrhea, loss of body mass, and death (Hendrix 1998; Hunter 1996). High rate of infection can cause reduction in feed intake, loss of condition, tissue damage, weight loss, skin pealing and eventually death (Bogish & Cheng 1990). Even though there are different types of nematodes, clinical symptoms might not differ that much, the only difference is their predilection sites (Hendrix 1998). Another major effect of high infection level is mortality and the survivors are less fertile and produce less milk, less quality of meat, less wool and lower calf birth percentage (Hunter 1996; Diehl *et al.* 2004).

Commercially available anthelmintics such piperazine, benzimidazoles, morantel and pyrantel have widely been used for a long time throughout the world to minimize the loss due to helminth infection. From the usage of the anthelmintic drugs, livestock production obtained considerable benefits. These drugs may not have been effective enough to totally cure the infection but at least they reduced the aggressiveness of the disease (Eguale *et al.* 2011). These treatments use synthetic chemicals (Pessoa *et al.* 2002). Apart from these synthetic anthelmintics, there are no other known treatments. However, anthelmintic drugs are expensive; it therefore takes a huge amount of money to combat helminth infections in livestock. Furthermore, there are specific stores where anthelmintic drugs are available, not every farmer particularly those in villages of resource poor countries could have access to them since they are mostly sold at the agriculture or veterinary services in bigger towns or cities (Tsotetsi & Mbati, 2003, Eguale *et al.* 2011). Otherwise, repeated administration of anthelmintic drugs is required in treating the internal parasites (Kamaraj & Rahuman 2010; Adeomola & Eloff 2011).

Nematodes are well documented for their good adaptability to various environments. Repeated administration of anthelmintic drugs leads to the development of nematode resistance against the drugs (Kotze *et al.* 2005). Nematode infections on livestock is increasing due to the built up resistance against anthelmintics and it only means that livestock is getting much susceptible to the infections (Hunter 1996). To date no vaccine has successfully been developed to combat gastrointestinal nematodes. Application of chemicals, sanitary to the environment, regular cleansing of the environment can reduce infection rates. This might leave an environment less original; however, it will be the best way of dealing with infectious diseases infecting

livestock. A possible way of dealing with nematode infections in an already highly infected area is to utilize the evasive grazing method. This is whereby the animals from that infected area will be moved from their natural environment or natural habitat into a more regulated pasture before dangerous pasture infectivity levels with infective larvae development are met. It takes about three months for contaminated pastures with eggs to decrease the infectivity level in summer periods hence the animals should be moved to a better place until their pastures are well treated (Githigia *et al.* 2001). After pasture treatment, the animals shall be brought back to their normal environment. Environmental management is still the better way of controlling the infection. Although pasture treatment does not guarantee 100% disinfection of an infected area since the nematode eggs can survive for more than a year. This method has already been practised in most countries which include Australia, Netherland, Georgia and Kenya, however, there is dearth of such studies on South African farms (Maingi *et al.* 1996; Eysker *et al.* 2005).

1.2.1 Parasitic gastrointestinal nematode life cycle

Generally gastrointestinal nematodes have a direct life cycle which consists of the free living and parasitic stages or phases. The host is infected *per os* by the infective staged larvae (L_3) (Fig. 1.1). The third staged larvae moults and develop into an adult worm in the gastrointestinal tract of the host. Within 3 to 4 weeks of infection, eggs are shed with faeces into the environment, which is why faecal samples are used in the diagnosis of helminth infections. Gastrointestinal nematode eggs transform into the first pre-infective staged larvae (L_1), and then moult into the second staged larvae (L_2) on the environment. With prevailing optimum environmental conditions necessary for nematode survival, the larvae will moult into the infective staged larvae (L_3) and continues in such an alternation (Roeber et al. 2013).

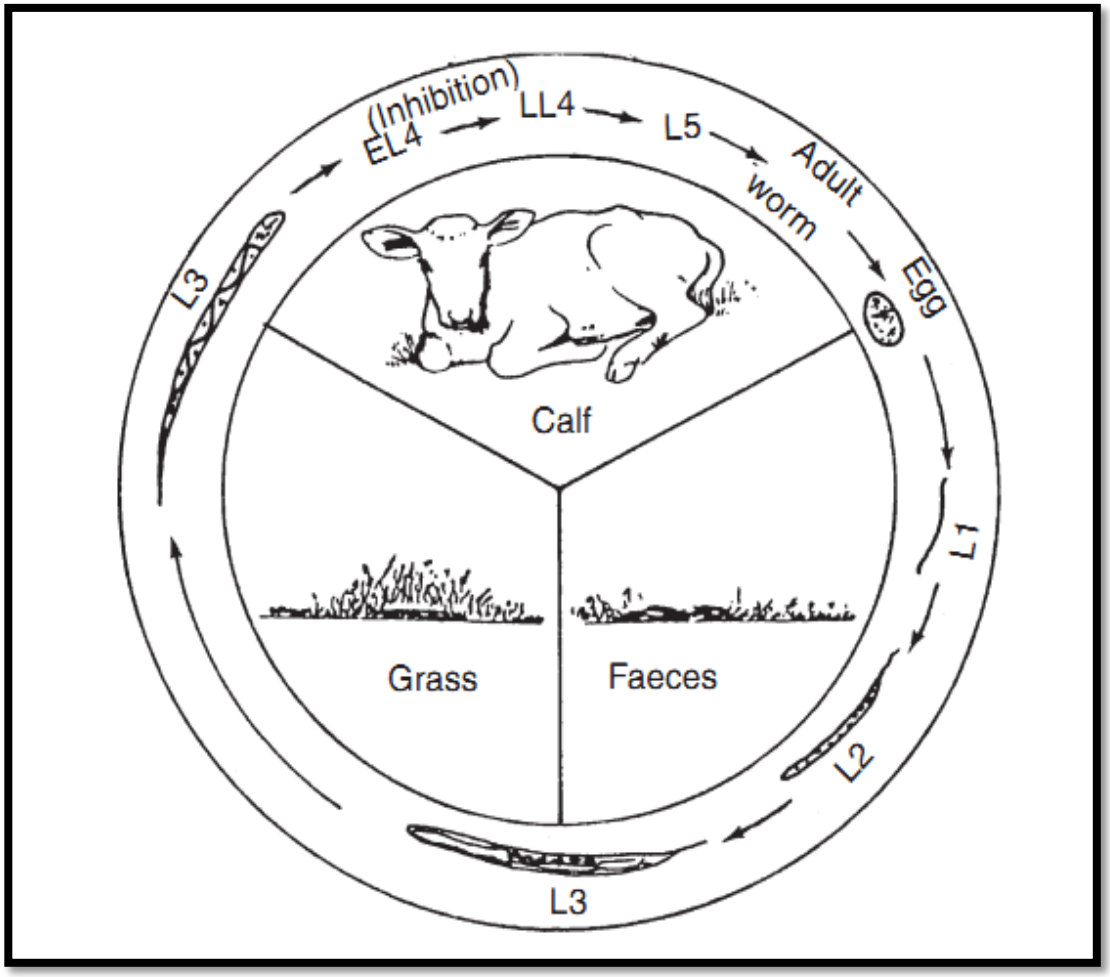

Fig. 1.1. Life cycle of parasitic gastrointestinal nematodes in cattle. EL: early larval stage; L: larval stage; LL, late larval stage (Charlier *et al.* 2011).

1.2.2 Economic importance of parasitic gastrointestinal nematodes

Parasitic gastrointestinal nematode infections are of major economic importance resulting in heavy production losses to the small ruminants than the big livestock. As stated by Tsotetsi & Mbati (2003) that the small stock including sheep and goats show high infection rates than cattle. Infected animals produce less quality of wool, meat and milk due to the observed clinical symptoms (Iqbal *et al.* 2010). According to Eguale *et al.* (2007), gastrointestinal nematode infections accounts for 28% mortality and 8% of weight loss in sheep mostly in developing countries with poor socio economic status and poor management practices. Rate of production is also hampered because reproduction performances of infected animals are affected (Bizimenyera *et al.* 2006; Botura *et al.* 2011; Nchu *et al.* 2011).

Anthelmintic resistance affects negatively the economic due to the fact that the anthelmintic drugs are much expensive to be afforded by countries with poor socio-economic status. Development of anthelmintic resistance begins with the loss of effectivity of the drug on the nematodes which on later stages rendours the expensive drug useless (Roeber *et al.* 2013). In a community where anthelmintic resistanve has occurred, it is difficult to treat or eradicate resistance, however, according to Sangster & Dobson (2002) loss of resistance is never observed if it has occured. Failure of anthelmintic drugs to kill gastrointestinal nematodes will lead to a widespread resistance to once susptible pathogens. Anthelmintic resistance is a threat to agricultural incomes because with resistance parasitic gastrointestinal infection rates only escalate and as a result heavy production losses will remain a major problem to livestock (Wolstenholme 2004).

1.3 Cancer disease

Like parasitic gastrointestinal nematodes infections and any other infections and/or disease, cancer is most prevalent in low income countries than developed countries. In developed countries it is much easier to treat and eradicate a disease than in under or developing countries. According to the American Cancer Society (2010) highest populated countries are the developing and the under developed where people do not have enough facilities and income to travel to the pharmacies and buy expensive drugs (Fig. 1.2). Therefore, the effects of cancer are felt more in mostly rural communities of most regions of the world. Since the inhabitants of such places are poor without adequate health care facility to initiate early diagnosis and treatment.

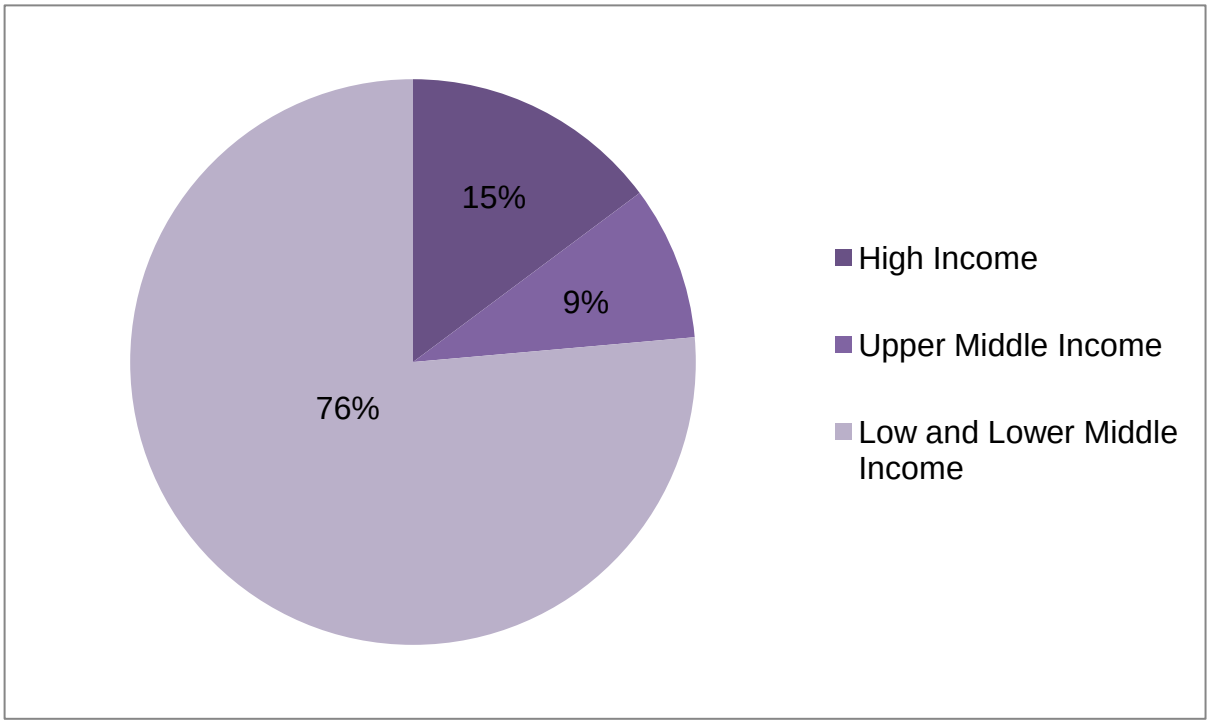

Fig. 1.2. Distribution of world population by country income group (American Cancer Society 2010).

Cancer is an irregular uncontrolled proliferation of abnormal cells. These cells divide rapidly, uncontrollably and independent from the tissue where they started. These cells can invade and damage tissues and organs near the tumor and can break away from the tumor, massive tissue forming from a bunch of unnecessary cells, and enter lymphatic system and/or blood streams by a process termed metastasis (ASTDR 2013). This is a potential fatal disease being one of the leading causes of death worldwide which is expected to increase with time because of the population growth as well as the increasing life expectancy (Merina *et al.* 2012). Cancer is of importance due to animal and human loss associated with the disease in under developed and developing countries from the lack of control measures.

In most cases cancer is caused by environmental factors that mutates or bring about changes in gene encoding critical cell-regulatory genes (Alison 2001; Shoeb 2006). These cancer causing agents (carcinogens) can be present in food, water and in the air, chemical and sunlight that people are exposed to. Cancer causes are complex; however, possible identified causes include exposure to chemicals and other substances, tobacco, ionizing radiation, unhealthy lifestyles and heredity. Exposure to chemicals that include asbestos, nickel, cadmium, uranium, radon and benzene

put people at a risk of contracting cancer. Cigarette smoke is documented to contain about 60 carcinogens and 6 developmental toxicants which are responsible for 80 to 90% of the lung cancer. Smoking is associated with mouth, pharynx, larynx, oesophagus, pancreas, and kidney and bladder cancer (van Leeuwen & Zonneveld 2001; Axelsson *et al.* 2010; Khlifi & Hamza-Chaffai 2010). Radioactive rays utilized during surgical processes like x-rays and ultraviolet rays from the sun can lead to cancer development by altering the DNA. Lastly, cancer can be passed from generation to generation in form of hereditary processes; this has been reported by Albano *et al.* (1982) since it has been observed that cancer types occur more frequently in some families than others.

Symptoms may mediate between different type of cancer, treatment and diminishing factor, however, pain and fatigue marks the first significant symptoms. In rural areas most common symptoms include pain and depression. Cancer is diagnosed more by clinical symptoms such as fatigue, pain, sleep concerns, memory loss, weight loss, unusual bleeding and/or discharge, persistent indigestion and presence of white patches on the mouth or white spots on the tongue, cognitive impairment, irritable mood, demoralisation depression and interpersonal problems. Symptom screening are therefore conducted to provide knowledge to health facilities as to what medication and which are the necessary and/or appropriate services to treat provide at that stage of the disease which is why symptoms management is complex and overwhelming to patients and caregivers (Berry 2011; Baili *et al.* 2013).

1.3.1 Development of cancer

When cancer develops due to exposure to toxic chemicals (Fig. 1.3), a toxic chemical is introduced to the cells of the body and causes mutation which is a reversible stage by either immune system attack to the abnormal cell and/or DNA repair process. The second step is the promotion and cell division of mutated cells. Continuation of the cell division leads to more cells which will end up forming a non-cancerous tumor in early stages, if the tumor is not treated in time it becomes a cancerous tumor with mutated cell proliferation. Cancerous tumor initially cannot be transported to other areas of the body but with the development of stem cells, cancer cells will start by infecting neighbouring organs and spread even further to far cells (Oliveira *et al.* 2007). According to the cancer hypothesis, a tumor needs the stem

cells which are responsible in giving rise to new cells, growth of those cells, cell proliferation and transport of the cells to various organs (O'Flaherty *et al.* 2012; Gou *et al.* 2013).

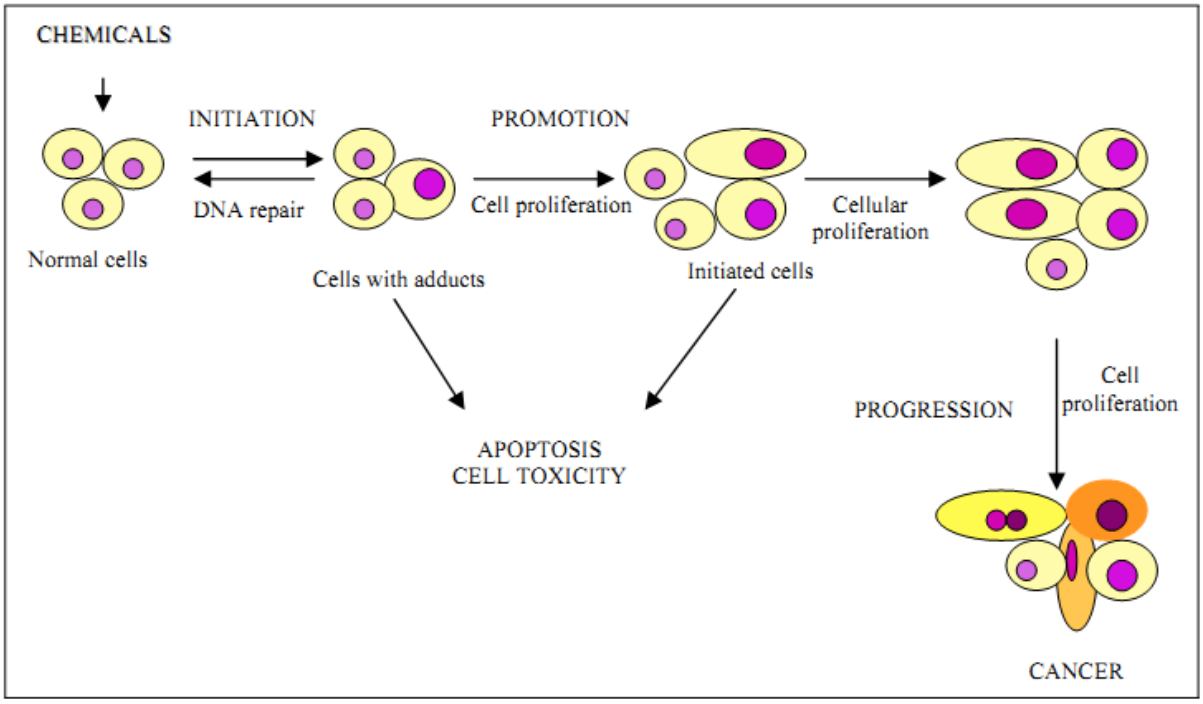

Fig. 1.3. Illustration of chemical carcinogenesis (Oliveira *et al.* 2007).

1.3.2 Diagnosis and treatment of cancer

Cancer leads to more deaths as compared to other diseases due to delayed diagnosis of the disease. It can take several years developing without any noticeable cases, a delayed diagnosis affect the chances of cure in patients and survival thereof (Brocken *et al.* 2012). Complicated diagnosis methods have been documented to lead to early cancer diagnosis, these techniques include 18-flourodeoxyglucose position emission (FDG-PET), contrast enhanced computerized tomography scan (CT), and mediastinoscopy. Disadvantages of most diagnostic method are the fact that they need a classifier system of diagnosis, organized, timely health care and skilled labour (Brocken *et al.* 2012). Quality of life of an individual and symptom profile are important factors when making decision of treatment in any cancer type and therefore an early screening is recommended as a manner to enable clinicians to identify patients at higher risks and therefore intervene at the perfect time and prevent development of crisis events. Androgen deprivation therapy improves even though it is associated with deleterious effects on the quality of life and bone health

(Sonpavde *et al.* 2007). There are surgeries developed for cancer treatment which require at all cost special training and expertise in cancer therapy (Cain *et al.* 2009). Chemotherapy is the usage of cytotoxic drugs or treatment to destroy or minimize cancer cell growth. Docetaxel chemotherapy is currently accepted as a standard care as it is modesty and extends life span than any other chemotherapy treatment offered (Sonpavde *et al.* 2007).

The main route chemotherapy drugs take to reach cancer cells all over the body is the circulation through the bloodstream; however, these drugs cannot differentiate between non-cancerous and cancerous cells and therefore target all cell divisions. Usually chemotherapy is given in a period of 3 to 6 months and administered orally through a pill, capsule of liquid format or as an intravenous tube. Chemotherapy studies show that it provides long term benefits including reducing the likelihood of reoccurrence of breast cancer among women under the age of 50. Despite the positive effects, chemotherapy is also associated with negative effects such as hair thinning and loss, dry, discoloured skin that is extra sensitive to the sun, changes of enamel, loss of appetite and change in taste of food and chronic fatigue and nausea. These side effects differ; depending on dose as well as the type of drug combination administered. It is recommended to attend to treatment related side effects as it promotes safe delivery of cancer therapies (Alison 2001; Cain *et al.* 2009; Berry 2011; ASTDR 2013).

1.4 Choice of plants

The overall aim of the present study was to investigate the anthelmintic and anticancer activity of *Cotyledon orbiculata, Hermannia depressa, Nicotiana glauca* and a potassium permanganate in order to validate their use in ethnoveterinary medicine of the Eastern Free State.

1.4.1 Cotyledon orbiculata

Plate 1.1. *Cotyledon orbiculata* plant commonly referred to as a Pig's ear (Molefe 2012).

Cotyledon orbiculata classification (NRCS 2013; USDA 2013):

Kingdom : Plantae
Phylum : Magnoliophyta
Class : Magnoliopsida
Order : Rosales
Family : Crassulaceae
Genus : *Cotyledon*
Species : *C. orbiculata*

This is a fast growing succulent shrub plant with thick, ovate leaves varying from grey to green in colour, often with a red line around the margin (Plate 1.1). *Cotyledon orbiculata* has varieties based on differences in leaf size, shape, colour and the shape of the flower influenced by the environment. It is widespread throughout the

whole of South Africa, Namibia and Angola, however, it usually confined to rocky outcrops in grassland fynbos and karoo regions. This plant is most tolerant to climatic conditions like moderate frosts and drought and that is how it survive in many regions. Even though *C. orbiculata* is often cultivated as a garden plant, it is actually a known medicinal plant. The fleshy part of the leaf is traditionally applied to soften and remove hard corns and to treat skin problems such as warts. The heated leaf is used as poultice for boils and inflammation. Warmed plant juice can be used as drops to relieve tooth and earache and treat epilepsy (van Wyk *et al.* 1995).

1.4.2 Hermannia depressa

Plate 1.2. *Hermannia depressa* plant commonly referred to as Doll's rose (Molefe 2012).

Hermannia depressa classification (NRCS 2013; USDA 2013):

Kingdom : Plantae
Phylum : Magnoliophyta
Class : Magnoliopsida

Order : Malvales

Family : Malvaceae

Genus : *Hermannia*

Species : *H. depressa*

This is a small shrub plant with stems and leaves flat on the ground which usually grow clumped in patches (Plate 1.2). The leaves are carried on short stalks which are generally large with purplish to reddish brown colour. The flowers vary from orange, pinkish to yellow cream in colour. The *H. depressa* possess a thick woody stem and root forming an underground stem which enables the plant to survive various conditions including dry periods and fires. It is mainly distributed across the flora of southern Africa which includes South Africa, Zimbabwe and Namibia, as inhabitants of open grassland, often at the margins of seasonal swamps in the Highveld regions. In seldom cases this plant can occur in Madagascar and extend through to the East Africa. The *H. depressa* is traditionally used by indigenous people in treatment of toothache and relieve of nerves, however others claim the usage of the plant ranging from respiratory diseases, coughs and internal aches, as stimulants or purgatives, to soothing wounds and cuts (Sobiecki 2008).

1.4.3 Nicotiana glauca

Nicotiana glauca is a perennial tree plant with loosely clustered flowers at the branch tips (Plate 1.3). The leaves are alternate, petioled, up to 7 inches long, egg-shaped, and a glaucous blue-green color. These fast-growing plants are slender, arching, and often lopsided with a leaning trunk. The plant is native to South America but has widely been introduced to many parts of the world including South Africa, Kenya, Tanzania, Uganda, Asia and U.S.A. This plant however occurs mostly in warm areas because of its sensitivity to frost (Henderson 2001). The N. glauca is commonly known as a toxic plant, however, it has been used medicinally where warmed leaves are applied to the head to relieve headache, on the throat to relieve sore throat and put in shoes for painful feet, lastly the plant has been used as an insecticide (GIS 2011).

Plate 1.3. *Nicotiana glauca* plant commonly referred as the Tree tobacco (http://writepass.co.uk 2013).

Nicotiana glauca classification (NRCS 2013; USDA 2013):

Kingdom	: Plantae
Phylum	: Magnoliophyta
Class	: Magnoliopsida
Order	: Solanales
Family	: Solanaceae
Genus	: *Nicotiana*
Species	: *N. glauca*

1.5 Potassium permanganate

Plate 1.4. Potassium permanganate (Molefe 2013).

Some farmers in the Eastern Free State use potassium permanganate to treat nematode infections but no documented report was found in literature to support the ethnoveterinary importance of this substance. A chemical of interest, potassium permanganate which is a chemical substance containing potassium, manganese as well as oxygen, with the chemical formula is $KMnO_4$. It is an oxidising agent widely used in aquaculture (Chanratchakool 1994). Potassium permanganate has been used for various purposes mainly for control of pathogens in aquaculture such as fungi, bacteria, algae and parasites (Franca *et al.* 2011). Potassium permanganate was included in the current study due to the folkloric claims of its usage by indigenous people including the local farmers as prophylaxis, treatment of infections as well as cleaning water. They use it to treat external parasites without any scientific report of the usage of the substance, however scientifically, potassium permanganate has been documented with the potential to control fungi, bacteria, algae and parasites (Chen & Yeh 2005; Franca *et al.* 2011).

1.6 OBJECTIVES OF THE STUDY

1.6.1 Statement of the problem

Parasitic gastrointestinal nematodes induce clinical symptoms such as loss of condition, diarrhea, anemia and death is likely to result if not treated in time. A single host can possess all the mentioned symptoms because each symptom results from various conditions. Loss of condition occurs in an animal infected with many species of nematodes competing for resources which may lead to tissue damage if infection site is not the specific site to nematode attachment (Molefe *et al.* 2012). *Haemonchus* and *Trichostrongylu*s species, for instance are blood sucking parasites and anemia is therefore likely to result in such infected host (Hepworth 2013). Clinical symptoms in overburdened hosts are in most cases irreversible and lead to a decrease in productivity due to reduced performance of the stock itself and there will therefore be a hindrance in production of growth rates, fiber, wool, meat and milk. On the other hand, cancer disease results from simple and everyday life such as cells as a leading global cause of human death. Breast and cervical cancer lead to more women death at reproductive stages of their lives. Cancer can only be reversed at an initial stage by a DNA repair process before any major tumor formation from abnormal cell proliferation and/or transportation of abnormal cells to affect neighboring areas (Oliveira *et al.* 2007).

Both parasitic infections and cancer have widely been treated with commercial drugs, however, their effectivity is now failing due to the emergence of resistant strains of the nematodes and cancer cell mutates into resistant cells (Wanyangu et al. 1996; Oliveira 2007). Resistance results from repeated administration of the drugs with a similar mode of action for a prolonged period as well as the lack of skills of farmers since source of the drugs need to be diluted accordingly before administration (Tsotetsi *et al.* 2013). Failure to prepare correct dosages of commercial drugs from lacking necessary skills results in over dosing and/or under dosing to the pathogens which surely leads to resistance development by the pathogens (Maingi *et al.* 1996). If resistance has occurred in a population regardless of the manner it was introduced, it is hard if not impossible to eradicate it due to lack of reversion of resistant isolates (Sangster & Dobson 2002). According to Coles (1999) and Sangster (2001) resistant isolates have a little tendency to convert back

to susceptible alleles of the parasites, however, resistant alleles have a strong tendency to remain dominant in the worm population until all susceptible allele have totally been diluted, developing into more resistant strains passed onto the next generations.

Development of new drugs is a costly and lengthy process, hence commercial anthelmintic and anticancer drugs are so expensive. Developing countries have always been at risk of both diseases due to poor socio-economic status where not all people or farmers could afford these drugs which are also inaccessible since they are only sold at agricultural offices (McGaw *et al.* 2000). Lastly, commercial drugs development consists of synthetic chemicals with various effects to the hosts, the drugs are therefore associated with severe side effects after administration (Pessoa *et al.* 2002). Chemotherapy has severe effects on the patients because it is developed using cytotoxic drugs or treatment to destroy or minimize cancer cell growth (Sonpavde *et al.* 2007).

These problems call for an urgent development or discovery of new inexpensive, accessible and environmental friendly treatment regimes that will act longer before resistance is built up. The current study was formulated for investigation of medicinal plant extracts as possible solution to both nematode infections and cancer as the case may be. According to Herdegen *et al.* (2003), Nassr-Allah *et al.* (2009), Iqbal *et al.* (2010) and Prakash *et al.* (2011) medicinal plants have almost always been primary health care substances in treatment of various infection and diseases. Developing countries show much interest now than ever in the usage of the indigenous plants than usage of modern drugs, however, there are factors to be taken into consideration when using medicinal plants such as toxicity effects of their constituents as some might be very toxic to both animals and humans if consumed in a fresh state or in excess (Botha & Penrith 2008).

1.6.2 Research hypothesis

1.6.2.1 Extracts of plants *Cotyledon orbiculata, Hermannia depressa* and *Nicotiana glauca* and a chemical potassium permanganate have anthelmintic and anticancer activities.

1.6.3 Specific objectives

1.6.3.1 To determine the *in vitro* anthelmintic activity of medicinal *C. orbiculata, H. depressa* and *N. glauca* on parasitic gastrointestinal nematodes.

1.6.3.2 To determine anticancer and/or antitumor activity of *C. orbiculata, H. depressa* and *N. glauca* on breast (MCF-7) and cervical (HeLa) cancer cell lines.

1.6.3.3 To determine the cytotoxicity effects of the medicinal plants on the mammalian (MDBK) cell lines (*in vitro*) and the brine shrimps (*in vivo*).

1.6.3.4 To document phytochemical constituents in the studied plants which might be responsible for any observed medicinal plant activities.

1.6.3.5 To determine the *in vitro* anthelmintic activity of potassium permanganate on parasitic gastrointestinal nematodes and its cytotoxicity effects on the mammalian cell line and the brine shrimps.

Chapter 2

In vitro anthelmintic activity of *Cotyledon orbiculata, Hermannia depressa* and *Nicotiana glauca* extracts against parasitic gastrointestinal nematodes of livestock

2.1 INTRODUCTION

Gastrointestinal nematodes are a major factor that limits small ruminant production worldwide (Cala *et al.* 2012) due to large economic losses resulting from reduced feed intake, impaired fertility, reduced immunity, damaged gastric function and high mortality rate (Carvalho *et al.* 2012). Nematosis is responsible for 28% of small stock mortality and between 3% and 8% of livestock weight loss in many countries, costing an affected country about US$2 billion per year; South Africa included (van Wyk *et al.* 1999, Zarlenga *et al.* 2001, Eguale *et al.* 2007).

Tembely *et al.* (1997) stated that in the determination of the degree of nematode infection several factors need to be taken into consideration such as the age of the host animal, breed type of the host, parasite species, and epidemiological patterns including husbandry practices, physiological status, as well as the environmental conditions including rainfall, temperature and humidity. Commercial drugs have been used effectively to control the infection by curing clinical symptoms and/or diseases and basically to reduce mortality rates (Molefe *et al.* 2012). However, repeated or indiscriminate administration of the drug on the host provides a suitable medium for nematodes to build up a wide range of resistance (Carvalho *et al.* 2012; Hernandez-Villegas *et al.* 2012). According to Carvalho *et al.* (2012) the first case of resistance against the commercial anthelmintic drugs was described accurately by Drudge *et al.* (1964) and afterwards many reports followed.

Commercial drugs are however, unaffordable and unavailable to farmers with poor socio-economic status in most of the developing countries since, in many cases, drugs are imported (Amin *et al.* 2009), hence are expensive and inaccessible as they are sold at the agricultural offices for safety purposes (Tsotetsi & Mbati 2003; Cala *et al.* 2012). Because of the un-affordability and unavailability farmers tend to rely on the ethnoveterinary medicine (Eguale *et al.* 2011). It has already been documented that more than 80% of the population in developing countries depend on plants for

medicinal needs (Fyhrquist *et al.* 2002). Numerous plants are capable of producing bioactive compounds such as the secondary metabolites, as a chemical defence mechanism against pathogens and predation (Sathiyamoorthy *et al.* 1997), and are friendlier to the environment than the synthetic chemicals used when producing anthelmintic drugs (Nwosu *et al.* 2006). The search is therefore ongoing at the direction of an alternative way of treating the infection using medicinal plants. The objective of this study was to determine the *in vitro* anthelmintic activity of *Cotyledon orbiculata*, *Hermannia depressa* and *Nicotiana glauca* extracts on gastrointestinal nematodes using the egg hatch, larval development and larval mortality assays.

2.2 MATERIALS AND METHODS

2.2.1 *Plant collection sites*

Plant materials were collected in May 2012 from multiple populations of *C. orbiculata, H. depressa* around Qwaqwa area in Maluti-A-Phofung Municipality of the eastern Free State Province (28°32'0"S and 28°49'0" E; altitude 1, 673 m). The mean annual rainfall of the Maluti-A-Phofung Municipality is about 653 mm per annum with temperatures ranging from as low as 0.1ºC during July nights, 14.2°C during the day in June, and maximum of 24.7ºC during the day in January (Wolfram 2012). The *N. glauca* was also collected in May 2012 from Wolmaranstad in Maquassi Hills Local Municipality of the North West Province (27°12'0"S and 25°58'0"E; altitude of 1170 m) of South Africa. The mean annual rainfall of the Maquassi Hills Local Municipality is about 391 mm per annum with temperatures ranging from as low as 0ºC during July nights, 17.6°C during the day in June, and maximum of 30ºC during the day in January (Wolfram 2012). The plants were identified by Dr. AOT Ashafa of the Department of Plant Sciences, University of the Free State (UFS) - Qwaqwa Campus and voucher specimens (MolMed/02/2012) were prepared and deposited at the UFS Qwaqwa Campus herbarium.

2.2.2 Plant material and preparation of extracts

The shoots of the plants were separated and dried in an Ecotherm oven (Laboratory Consumables Pty, South Africa) at a temperature of 40°C to a constant weight

before it was pulverized. Ten grams each of powdered material was extracted in acetone and distilled water. Acetone was of high analytical grade (Merck Chemicals Pty, Wadeville, South Africa). All extracts were filtered using Whatman No-1 filter paper (Whatman, United Kingdom) except *H. depressa* water extract which were filtered through a cotton wool and centrifuged. The crude extracts from acetone were concentrated under reduced pressure 40ºC using rotary evaporator (Cole-Parmer, Laboratory Consumables and Chemical Supplies Co.Ltd, China). The water extract was freeze dried using freeze dryer (Virtis SP Scientific, United States of America). Individual extracts were reconstituted in their respective solvents to give a stock solution of 50 mg/ml (Ashafa & Afolayan 2009). This was diluted to the required concentrations of 2.5 and 5.0 and 7.5 mg/ml for the bioassay analysis.

2.2.3 Faecal sample collection

Faecal samples were collected directly from the rectum of the sheep. The sheep were placed in a crash pan to stand upright. Faecal samples were collected by inserting two fingers in the rectum and tickled the upper part of the rectum. Faecal pellets were released into the palm of the hand then placed into a sealed collection plastic. Samples were transported to the Parasitology Research Program laboratory, University of the Free State, Qwaqwa Campus, in a cooler box and analysed within 24 hours of collection as recommended by Reinecke 1983.

Fig. 2.1. Collection of faecal samples from sheep (Molefe 2011).

2.2.4 *Diagnostic methods*

The McMaster technique (Soulby 1982; Reinecke 1983) was used to determine nematode egg presence in the collected faecal samples. A 40% sugar floatation medium was prepared by dissolving 400 g of sugar in a liter of distilled water. Two grams of pooled faecal samples were mixed with 58 ml of 40% sugar solution as a floatation medium. Samples were thoroughly crushed and mixed using a blender. Two chambers of a McMaster slide were filled with a Pasteur pipette. The slides were allowed to stand for about 4 minutes so that the eggs can float on the surface of the flotation medium and lie in contact with the upper glass of the chamber. A light microscope (Nikon Eclipse E100, Japan) was used for egg detection. Indistinguishable eggs coming from different genera such as the *Haemonchus, Trichostronglylus, Oesophagostomum* and *Chabertia* were grouped together, and recorded separately as strongyle eggs, whilst those that were easily distinguished such as the *Nematodirus, Strongyloides* and *Trichuris* were reported as such. The *Nematodirus* eggs are distinguished by a thin and colorless shell with a length of 150 µm and a width of 75 µm, *Strongyloides* eggs are broad eclipsed, slightly flattened and embryonated with the presence of L_1 larvae and *Trichuris* eggs have typical polar plugs on both ends. Egg identification was conducted according to the Atlas of Ovine Parasitology (http://issuu.com 2013).

2.2.5 *In vitro* assays

Three *in vitro* assays (egg hatch, larval development and larval mortality assays) were used to determine the anthelmintic activity of *C. orbiculata, H. depressa* and *N. glauca.* However, before the assays could be conducted, nematode eggs had to be recovered from faecal samples hence the egg recovery assay.

2.2.5.1 Egg recovery assay

Egg recovery was conducted according to Maphosa *et al.* (2010) protocol with some modifications. Four grams of collected faecal sheep pellets were weighed, then water was slowly added to them and the pellets were smashed until a relatively liquid suspension (slurry) was obtained. The slurry was then filtered through sieves of 117, 70 and 25 µm. The contents of 25 µm sieve were backwashed with distilled water

and transferred into 60 ml centrifuge tubes. The suspension was allowed to stand for 30 minutes and the supernatant was decanted while sediments were suspended in 40% sugar solution. The suspension was transferred into another set of tubes, allowed to stand for another 30 minutes and the supernatant was washed through a 25 µm pore mesh sieve using distilled water. The eggs were then washed off from the 25 µm sieve with distilled water into a 1 litre conical flask where they were allowed to sediment for 2 hours. The concentration of eggs was estimated by counting the number of eggs in 2 aliquots of 0.5 ml of the suspension in a microscope slide repeatedly, and the mean number of eggs per 0.5 ml was determined.

2.2.5.2 Egg hatch assay

The egg hatch assay was conducted as published by McGaw *et al.* (2007) and Bizimenyera *et al.* (2006). The number of eggs that were contained in the egg suspension of 0.5 ml was counted and afterwards they were pipetted into a 96-well microtitre plate. The numbered wells from 1 to 3 were used for the *C. orbiculata* experiments, from 5 to 7 for the *H. depressa* experiments and from 9 to 12 for the *N. glauca*. In addition, 0.5 ml of each plant extract at different concentrations of 7.5, 5 and 2.5 mg/ml were added. A commercial anthelmintic drug Tramisol® (Afrivet, South Africa) was used as the positive control at the same concentrations while distilled water was used as a negative control. All tests were repeated 3 times. The plate was incubated under humidified temperature of 25ºC temperature for 48 hours thereafter a drop of Lugol's iodine solution was added to each well so as to stop further hatching. All unhatched eggs and first-stage larvae (L_1) were then counted.

Inhibition percentages were calculated using a formula by Cala *et al.* (2012).

$$E = \frac{(Eggs + L_1) - L_1}{Eggs + L_1} \text{ X } 100$$

2.2.5.3 Larval development assay

The larval development assay was conducted as described by Bizimenyera *et al.* (2006). The counted number of eggs in a 0.5 ml of the egg suspension was put into

each well in a 96 well microtitre plate with a 100 µl of lyophilized penicillin-streptomycin in order to combat fungal growth. The contents of the wells were then mixed, and the plates were placed in an incubator under humidified conditions at ambient temperature for 48 hours. Forty eight hours later, 0.5 ml of the water and acetone extracts of *C. orbiculata, H. depressa* and *N. glauca* as well as Tramisol® (Afrivet, South Africa) as a positive control at 7.5, 5 and 2.5 mg/ml were added to respective plates. The negative control plates received 0.5 ml of distilled water. All experiments were repeated 3 times. Incubation of the plates was continued for 5 days, after which all the plates were examined to determine the survival of larvae at different concentrations. All the third-stage larvae (L_3) in each well were counted and a percentage inhibition of larval development was calculated using the formula described by Cala *et al.* (2012):

$$E = \frac{(L_1 + L_2 + L_3) - L_3}{L_1 + L_2 + L_3} \text{ X } 100$$

2.2.5.4 Larval mortality assay

The larval mortality assay was conducted according to the method described by McGaw *et al.* (2000) and Zafar *et al.* (2006) with minor modifications (Molefe *et al.* 2012). *In vitro* cultures from nematode eggs were prepared after collection from microscopically positive sheep faecal samples. After seven days of incubation, the eggs hatched into larvae. The L_3 larvae were harvested from the *in vitro* cultures prepared and was transferred into a single petri dish. The 150 µl of L_3 larvae solution were placed in microtitre plate and crude extracts of the same volume were added at different concentrations, Tramisol® (Afrivet, South Africa) was used as positive control and distilled water as a negative control. After the addition of extracts, larval counts were conducted on daily basis and the larval mortality rates were recorded until all the larvae had died. All tests were repeated three times. All live and motile L_3 stage larvae in each well were counted and a percentage inhibition of larval development was calculated using the formula described by Coles *et al.* (1992) and Bizimenyera *et al.* (2006) with slight changes:

$$\text{Inhibition percentage (\%)} = 100(1-X_1/X_2)$$

Where X_1 is the initial number of larvae in test extracts pre-treatment, and X_2 is the number of larvae obtained post-treatment.

2.2.5.5 Larval identification

Infective larvae were prepared for examination by adding a drop of diluted Lugol's iodine solution to a drop of larval suspension on a glass microscope slide. The iodine solution was diluted to a level where L_3 do not stain darkly before a few minutes have elapsed. The caudal and cranial extremities were taken into much consideration since they are most important for differentiating L_3 of most of the parasitic nematode genera, other features such as the length or shape of the oesophagus are also important in some genera. To facilitate identification, the lengths of the sheath tails of the various species were related according the larval identification key using the total larvae length, the tip, sheath tail length and the larval filament as illustrated in Fig. 2.2 (van Wyk *et al.* 2004).

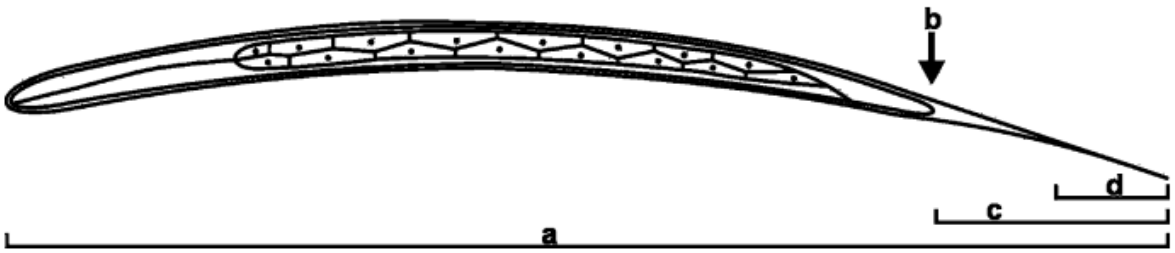

Fig. 2.2. Diagram of nematode infective larvae (van Wyk *et al.* 2004) a: total length; b: tip of larvae tail; c: sheath tail and d: filament.

2.2.6 Statistical analysis

Kruskal-Wallis test was used to analyse the egg hatching and larval mortality assays in order to determine if there was significant difference in the hatched eggs and larval mortality from different treatments with different concentrations (Kruskal & Wallis 1952). P values ≤ 0.05 were considered significant while $p < 0.001$ and $p > 0.05$ not significant.

2.3 RESULTS

In the current study, nematode genera including *Haemonchus*, *Oesophagostomum*, *Trichostrongylus* and/or *Teladorsagia* were determined by L_3 larval identification.

Water extracts inhibited a higher percentage of nematode eggs from hatching when compared to the acetone extracts (Table 1). The *H. depressa* water extract inhibited the highest egg percentages of 39.17 followed by *C. orbiculata* with 38.96% and lastly *N. glauca* with 15.46% at the lowest concentration of 2.5 mg/ml. On the other hand, Acetone extracts of all extracts at the same concentration inhibited less than 20% eggs from hatching. At a concentration of 5.0 mg/ml both water and acetone extracts of all three tested plants inhibited no greater than 25% of eggs from hatching. The *C. orbiculata* water extract induced the highest egg inhibition percentage of 82.62 at 7.5 mg/ml, whilst the acetone extracts exhibited less or equal to 10.00% egg hatching inhibition. The controls gave 100% of egg inhibition for the positive and 0% for the negative. There was a statistically significant difference ($p = 0.0049$) in the activity of treatments on the egg hatchability.

Table 2.1. Inhibition percentages on egg hatching of gastrointestinal nematodes of livestock with different water and acetone extracts concentrations of *C. orbiculata, H. depressa* and *Nicotiana glauca.*

Concentration (mg/ml)	***Cotyledon orbiculata***		***Hermannia depressa***		***Nicotiana glauca***		**Controls**	
Extracts	Water	Acetone	Water	Acetone	Water	Acetone	Positive	Negative
2.5	38.96	7.60	39.17	22.47	15.46	15.91	100.00	0.00
5.0	19.84	17.84	22.73	7.06	10.68	4.11	100.00	0.00
7.5	82.62	10.00	40.14	7.40	19.12	7.03	100.00	0.00

Acetone extracts inhibited a total larval development from pre-infective (L_1) to the infective staged larvae (L_3), whilst water extracts inhibited various percentages at different concentrations (Table 2.2). *Nicotiana glauca* water extracts inhibited 100% larvae from developing followed by *C. orbiculata* with 85.32% and *H. depressa* with 44.12 at 2.5 mg/ml. Water extracts of all 3 plants at 5.0 mg/ml inhibited more than 50% with a 100% inhibition by *N. glauca*, 81.80% by *C. orbiculata* and 58.16% by *H. depressa.* At the concentration of 7.5 mg/ml *N. glauca* water extracts inhibited once again a total larval development with 100% inhibition while *C. orbiculata* inhibited 78.88 and lastly *H. depressa* inhibited 66.69% of larvae from developing. Positive control induced a 100% larval development inhibition whereas the negative control inhibited 0% of pre-infective larvae from developing to the infective stage.

Table 2.2. Inhibition percentages on larval development of gastrointestinal nematodes of livestock at different concentrations of water and acetone extracts of *C. orbiculata, H. depressa* and *Nicotiana glauca.*

Concentration (mg/ml)	***Cotyledon orbiculata***		***Hermannia depressa***		***Nicotiana glauca***		**Controls**	
Extracts	Water	Acetone	Water	Acetone	Water	Acetone	Positive	Negative
2.5	85.32	100.00	44.12	100.00	100.00	100.00	100.00	0.00
5.0	81.80	100.00	58.16	100.00	100.00	100.00	100.00	0.00
7.5	78.88	100.00	66.69	100.00	100.00	100.00	100.00	0.00

The water extracts showed to be much effective on the infective stage larvae than on the pre-infective stage larvae. All experimental larvae mortality was observed within 48 hours, 96 hours and 72 hours of experimentation in water extracts of *C. orbiculata, H. depressa and N. glauca*, respectively ($p= 0.0695$). Acetone extracts of all plants were less effective, as more time than expected was necessary to totally kill the experimental larvae ($p < 0.0001$). The *C. orbiculata* acetone extracts duration differed with concentrations, however, the results were not concentration dependent whereby all the larvae died within 96, 120 and 96 hours at 2.5, 5.0 and 7.5 mg/ml, respectively (Table 2.3). The *H. depressa* acetone extracts duration was consistent for all concentrations with all larval mortality been observed within 120 hours (Table 2.4). About 192 hours was needed to result in a total larval mortality from *N. glauca* acetone extract with 5.0 mg/ml concentration which was the longest experimental time. The 2.5 mg/ml and 5.0 mg/ml took 120 and 144 hours for total mortality of experimental larvae, however, it took longer with the 5.0 mg/ml concentration (Table 2.5).

Table 2.3. Effects of *C. orbiculata* extracts on larval mortality of gastrointestinal nematode of livestock.

Time	Water extracts (mg/ml)			Acetone extracts (mg/ml)			Controls	
(Hours)	2.5	5.0	7.5	2.5	5.0	7.5	Positive	Negative
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	42.29	29.76	48.88	56.39	64.36	55.63	100.00	0.00
24	98.60	96.13	98.75	81.869	80.1896	87.39	100.00	0.00
48	100.00	100.00	100.00	92.3	83.02	95.04	100.00	0.00
72	*	*	*	95.599	90.56	99.55	100.00	0.00
96	*	*	*	100.00	96.22	100.00	100.00	0.00
120	*	*	*	**	100.00	**	100.00	0.00

* (All larvae died within 48 hours)

** (All larvae died within 96 hours)

Table 2.4. Effects of *H. depressa* extracts on larval mortality of gastrointestinal nematode of livestock.

Time	**Water extracts (mg/ml)**			**Acetone extracts (mg/ml)**			**Control**	
(Hours)	2.5	5.0	7.5	2.5	5.0	7.5	Positive	Negative
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	51.59	54.51	49.64	36.21	40.69	25.44	100.00	0.00
24	98.05	99.76	100.00	53.61	53.68	31.84	100.00	0.00
48	97.08	100.00	*	92.77	87.88	86.91	100.00	0.00
72	98.30	**	*	90.50	98.49	91.96	100.00	0.00
96	100.00	**	*	99.24	99.79	99.41	100.00	0.00
120	***	**	*	100.00	100.00	100.00	100.00	0.00

* (All larvae died within 24 hours)

** (All larvae died within 48 hours)

*** (All larvae died within 96 hours)

Table 2.5. Effects of *N. glauca* extracts on larval mortality of gastrointestinal nematode of livestock.

Time (Hours)	Water extracts (mg/ml)			Acetone extracts (mg/ml)			Controls	
	2.5	5.0	7.5	2.5	5.0	7.5	Positive	Negative
0	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
6	41.09	31.45	66.06	30.56	36.97	42.13	100.00	0.00
24	80.30	84.03	76.08	46.25	53.04	62.93	100.00	0.00
48	98.74	91.89	99.32	56.67	64.70	62.62	100.00	0.00
72	100.00	100.00	100.00	62.92	82.43	78.81	100.00	0.00
96	*	*	*	86.25	91.22	97.20	100.00	0.00
120	*	*	*	100	98.56	96.20	100.00	0.00
144	*	*	*	**	99.52	100.00	100.00	0.00
168	*	*	*	**	99.86	***	100.00	5.00
192	*	*	*	**	100.00	***	100.00	5.63

* (All larvae died within 96 hours)

** (All larvae died within 120 hours)

*** (All larvae died within 144 hours)

2.4 DISCUSSION

In this study, microscopic diagnosis from sheep faecal samples revealed the presence of *Haemonchus, Oesophagostomum, Trichostrongylus* and/or *Telardosagia* which have been documented as medically important species of gastrointestinal nematodes worldwide (Coles *et al.* 2006). The anthelmintic activity of three medicinal plants namely *C. orbiculata, H. depressa* and *N. glauca* was therefore determined on the eggs and the larvae of these gastrointestinal nematodes of livestock.

The major finding on the present study was the high effectiveness of water extracts compared to acetone extracts on the egg hatching and larval mortality assay. According to Maphosa *et al.* (2010) anthelmintic activity of medicinal plants in most cases result in unexpected manner because of different conditions in gastrointestinal tract and the *in vitro* provided conditions. In the egg hatch assay, water extracts proved to be much more ovicidal than the acetone extracts, in all concentrations of all tested plants. Similar results have been reported by Maphosa *et al.* (2010) where water extracts of *Leonotis leonorus, Aloe ferox* and *Elephantorrhiza elephantine* were effective on the known problematic helminths such as *Haemonchus contortus* and inhibited 11.5% of eggs from hatching.

From the larval development assay, *N. glauca* extracts induced 100% larval development inhibition in both water and acetone extracts. The *C. orbiculata* and *H. depressa* plants resulted in 85.32 and 44.12% larval development inhibition at 2.5 mg/ml concentration. However, acetone extracts of these two plants induced a 100% larval development inhibition. The inhibition of larval development was most probably due to the mortality of the pre-infective larvae.

In the larval mortality assay all larvae died and the only difference was found to be the duration it took for the larvae to die. Water extracts proved to be more effective than the acetone extracts. Despite the difference in concentration, water extracts of all the plants resulted in a total death of all the larvae within 48, 96 and 72 hours for *C. orbiculata, H. depressa* and *N. glauca*. However, acetone extract's total larval mortality was observed within 120 (*C. orbiculata*), 120 (*H. depressa*) and 192 (*N. glauca*) hours of experimentation. The first larval mortality monitoring was performed

within six hours post-treatment, within which all the larvae in the positive control had died.

Maphosa *et al.* (2010) stated that the efficacy of any plant extracts at the lowest concentrations against the gastrointestinal nematodes proves the anthelmintic activity of that plant. It is therefore concluded that *C. orbiculata, H. depressa* and *N. glauca* have an anthelmintic activity. Overall, the study revealed that the aqueous extracts of the shoots of all tested plants were more effective than acetone extracts with respect to the egg hatch and larval mortality assays.

The ability of eggs and larvae of the gastrointestinal nematodes to withstand unfavourable environmental conditions depend on the species and developmental stage of the parasite as well as the geo-ecological regions (Tembley 1998). Egg inhibition was observed in the current study, however, not all eggs were inhibited from hatching and most managed to hatch, this might be because the egg is the stage disseminated into the environment and protected with a thick wall making it resistant to various environmental conditions (Hounzangbe-Adote *et al.* 2005). According to Cheah & Rajamanickan (1997) the most favourable condition for the development of L_1 larvae to L_3 larvae are fairly high temperature in order to facilitate a rapid development rate and adequate humidity. The most susceptible stage of the larvae is the pre-infective one, the feeding stage in order to develop to the infective stage, however, it is the much exposed to medicinal components than the eggs hence 100% larval development inhibition rate (Molan *et al.* 2003). The plants that took part in the current study led to a total larval mortality at different durations and concentrations. Significant anthelmintic effects of both acetone and water extracts of the three plants on the L_3 larvae were observed in terms of paralysis and death in different hours post treatment with greater activity in crude acetone and crude aqueous extracts as confirmed previously by Tariq *et al.* (2009). The *N. glauca* at 5.0 mg/ml killed larvae with a reduction of larvae in the negative control, which might be due to lack of nutrients or exhaustion in the negative control (Adamu *et al.* 2010).

Chemical constituents can vary considerably between individual plant species due to genetic or environmental differences, age or developmental stage at harvesting, method of plant material drying, the storage technique and the type of solvent (Hördegen *et al.* 2003; Ononuju & Nzenwa, 2011). Acetone and water extracts were

selected for experiments in the current study because not every medicinal component is water soluble. Acetone extract has been documented as suitable extractant in most cases because it has the ability of extracting compounds from a wide variety of polarity range (Bizimenyera *et al.* 2006). Chemical constituents play a major role in the plant activities such as the anthelmintic activity, antimicrobial and so forth. The *C. orbiculata* has been documented to carry saponins, cardiac gylcosides, tannins, reducing sugars and triterpene steroids (Amabeoku *et al.* 2007) whereas *H. depressa* contains tannins and about less than 1% of cardiac glycosides (Reid *et al.* 2005) and *N. glauca* with nicotine, anabasine (Mhinana *et al.* 2010). Each of the chemical components has its own function. Tannins are rich in glycoproteins which have the ability of binding onto the free proteins of the larval cuticle and therefore reducing the availability of nutrients to the larvae resulting in the death due to starvation (Cala *et al.* 2012). The *N. glauca* activity might be due to the presence of nicotine, the piperidine alkaloid from tobacco plant which can be toxic in high doses to both animals and humans and is also soluble in water therefore explaining the high effectivity of water extracts (Webb & Dalzell, 1997), and anabasine as a highly toxic piperidine like alkaloid constituting about 70% of the plant as a whole (Mizrachi *et al.* 2000).

Conclusions

The *C. orbiculata, H. depressa* and *N. glauca* are therefore good candidates for treatment of gastrointestinal larval infections. However, the mechanisms of their effectiveness still remain to be tested *in vivo.* Furthermore, safety and toxicity studies must be conducted *in vivo* to determine the minimum non-lethal concentrations needed for the treatment of nematode infections.

Chapter 3

In vitro anticancer activity of medicinal plants *Cotyledon orbiculata; Hermannia depressa* and *Nicotiana glauca* on breast and cervical cancer cell lines

3.1 INTRODUCTION

A body consists of numerous cells, normal cells to be specific, which divide at a standard or a normal rate. These cells have various functions in the body; they play a role in growth of young and as well as fully grown, the very same cells change their function into repairing the damaged and replacing old dead cells. However, in some cases normal cells undergo mutation which is a process whereby DNA will be damaged and/or changed resulting in an altered functioning of DNA or cells. The mutated DNA strand can be transmitted from generation to generation through heredity (Merina *et al.* 2012). It is not yet clear what brings about mutation in cells, however, reports claim exposure to heavy metallic chemicals, environmental conditions, tobacco, radiation and ultraviolet exposure. Mutation has various types, such as introduction of a stop codon in proteins. Nonetheless, mutation is a two way processes resulting in both positive and negative effects, other cases of mutations have led to the known process of evolution and adaptations but negatively leads to cases such as cancer. As time goes by more cells including genes which are used to repair damaged DNA will mutate and end up being dysfunctional (Giraud *et al.* 2001).

Cancer develops as more and more cells become mutated with an alteration of function with their ability to divide quicker and at high rates than normal cells. Cancer cells act independently from any other cells, have the ability to outgrow normal cells and travel to other body parts to form tumors. Their abilities are orientated by a damaged DNA in them hence they do not behave like normal cells. Even though the cells can travel and affect other body areas but all cancers are named from their place of origin e.g. brain cancer, pancreatic cancer and eye cancer, colon cancer, breast cancer and cervical cancer (http://www.exploratorium.edu 2013).

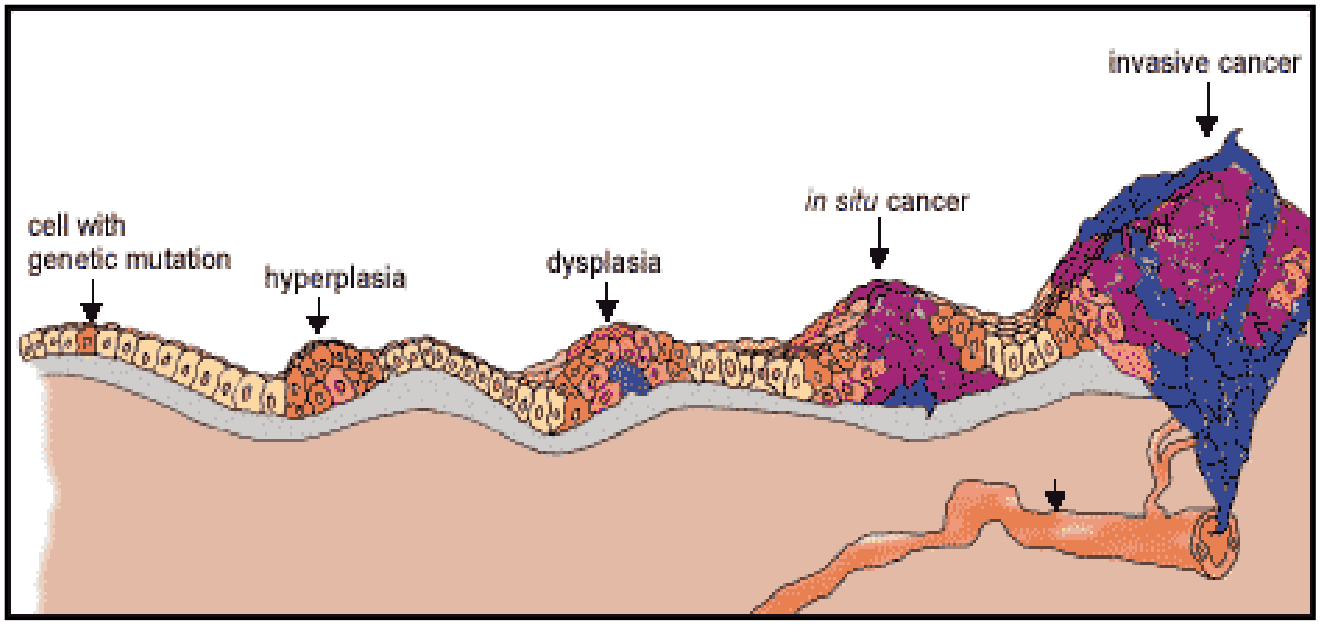

Fig. 3.1. Cancer developmental stages (http://science.education.nih.go 2013).

Cancer develops from a sequence of stages which finally results in malignant tumor (Gurney & Kadan-Lottick 2013). It is not clear how many mutations should a normal cell undergo before it is a cancer cell but steps of mutations definitely take place. The process begins with a single cell mutation which will enable the cell to develop more than normal cells do. Hyperplasia is a condition developing from a division and growth of a mutated cell which becomes a second stage (American Urological Association 2013). Thirdly, it is development of dysplasia, which is when the descendants' cells divide excessively and look abnormal. It is in this very same stage that another mutation might occur from one or more cells. It takes growth and division of descendant cells for one to experience an abnormal appearance. Cells at this stage can either migrate to other organs or remain contained in the tissue of origin (CAP 2011). Disadvantages of migrating cells is that they have the ability of spreading the disease to other body parts and eventually the whole body from a single cell that undergone mutation. The most important stage of cancer, the last stage, is the malignant tumor. Most cancer cases are realized in this stage which is too late to treat. In this stage cancer cells undergo excessive mutations which further assist in spreading the disease to neighbouring tissues through blood or lymph. These cells may establish a formation of another one or more tumors wherever they land (Izrailit & Reedijk 2012).

Cancer types of interest were breast and cervical cancer, which are types affecting women worldwide but severe cases have been documented in under developed

and/or developing countries (Barr & Sings 2008). Both types are leading high mortality rate factors of women already, even though the numbers are still expected to increase in the coming decades (Hasima & Aggarwal 2012). There are many various causes of cancer, breast cancer results from more of environmental based factors such as radiation exposure, obesity, consumption of oral contraceptive, family history and personal history whereas cervical cancer is likely to develop from human papillomavirus (HPV). The HPV infection is widespread with limited methods of preventing transmission, however, according to Barr & Sings (2008), the most effective way of preventing HPV transmission is a total abstinence from all genital contact while lifetime monogamy is also advised. There has been a dearth of information about breast cancer for many years until a group by the name of Breast Cancer Group was introduced (Cufer *et al.* 2012). The main function of this group was to conduct high quality international clinic trials, investigate new anticancer agents as well as applying the new diagnostic techniques of the disease.

So far, different vaccines are being developed every-now and then in prevention of both cancer types. Although there are yet changes, it is difficult to know which vaccine to use. Decline in cancer incidences have been and are still observed due to improving socio economic status of a country which makes it easier to diagnose the disease at an early stage than in countries with poor socio economic status. Vaccines are complicated to administer, body takes time before adapting to changes of antibodies introduced to the system and a multi-step process to develop (Mackiewics & Mackiewics 2009). Diagnosis of cancer plays a major role in deciding the necessary treatment (Colantonio *et al.* 2009; Berry 2011; Baili *et al.* 2013). Chemotherapy is the most applied treatment to most cancer types, numbers increased at the beginning of the usage of chemotherapy and many are still surviving the disease, however, there are severe effects accompanying the treatment that patients feel is better to quit treatment. Failure in development of safe treatment calls for environmental friendly substances in cancer treatment hence the current study is proposing the usage of medicinal plants.

Natural products from plants have widely been used in drug discovery of many infections and diseases (Nassr-Allah *et al.* 2009; Prakash *et al.* 2011). Plant bioactive compounds, among 40 000 plant species have been tested, however, only a few percentage possess the anticancer activity and the studies are still continuing

as an important source of anticancer agents (Shoemaker *et al.* 2005). Secondary metabolites documented with the anticancer are vincristine, vinblastine, camptothecin and taxol which are effective against various types of cancer and cancer stages such as the primary stages of breast, lung and advanced testicular cancer, these metabolites have the ability to treat leukaemia and lymphomas as well (Shoeb 2006; Merina *et al.* 2012). Extracts of *Cotyledon orbiculata, Hermannia depressa* and *Nicotiana glauca* plants were then used to test their anticancer activity against MCF-7 and HeLa cell lines.

3.2 MATERIALS AND METHODS

3.2.1 Plant collection sites

Plant materials were collected in May 2012 from multiple populations of *C. orbiculata, H. depressa* around Qwaqwa area in Maluti-A-Phofung Municipality of the eastern Free State Province (28°32'0"S and 28°49'0" E; altitude 1, 673 m). The mean annual rainfall of the Maluti-A-Phofung Municipality is about 653 mm per annum with temperatures ranging from as low as 0.1ºC during July nights, 14.2°C during the day in June, and maximum of 24.7ºC during the day in January. The *N. glauca* was also collected in May 2012 from Wolmaranstad in Maquassi Hills Local Municipality of the North West Province (27°12'0"S and 25°58'0"E; altitude of 1170 m) of South Africa (Wolfram 2012). The mean annual rainfall of the Maquassi Hills Local Municipality is about 391 mm per annum with temperatures ranging from as low as 0ºC during July nights, 17.6°C during the day in June, and maximum of 30ºC during the day in January (Wolfram 2012). The plants were identified by Dr. AOT Ashafa of the Department of Plant Sciences, University of the Free State (UFS) - Qwaqwa Campus and voucher specimens (MolMed/02/2012) were prepared and deposited at the UFS Qwaqwa Campus herbarium.

3.2.2 Plant material and preparation of extracts

The shoots of the plants were separated and dried in an Ecortherm oven (Laboratory Consumables Pty, South Africa) at a temperature of 40°C to a constant weight before it was pulverized. Ten grams each of powdered material was extracted in acetone and distilled water. Acetone was of high analytical grade (Merck Chemicals Pty, Wadeville, South Africa). All extracts were filtered using Whatman No-1 filter

paper (Whatman, United Kingdom). The filtrates from acetone were concentrated under reduced pressure 40ºC using rotary evaporator (Cole-Parmer, Laboratory Consumables and Chemical Supplies Co.Ltd, China). The water extract was freeze dried using freeze dryer (Virtis SP Scientific, United States of America). Individual extracts were reconstituted in their respective solvents to give a stock solution of 50 mg/ml (Ashafa and Afolayan 2009). This was diluted to the required concentrations of 1, 0.5, 0.25, 0.125, 0.075, 0.025, 0.005 mg/ml for the bioassay analysis. The crude extracts were diluted to required concentrations in 50% DMSO (500 µl + 500 µl H_2O).

3.2.3 *Cell lines*

Human cancer cell lines MCF-7 and HeLa were purchased from American Type Culture Collection, Manassas, USA. All cells were maintained with RPMI 1640 or (DMEM/High glucose) medium supplemented with 10% foetal bovine serum. A 200 µl of medium containing cells was added into a 96 well plate in replicates and incubated at 37°C in a humidified incubator with 5% CO_2 for 24 hours.

3.2.4 *Cell seeding*

The method was conducted as described by Tanih & Ndip (2013). Purchased cells were reconstituted in the laboratory in a flask incubated at 37°C in a humidified incubator (Heal Force Incubator) with 5% CO_2. Growth performance was monitored before experimental usage. The cells were washed with 10 ml PBS-EDTA and then 1 ml trypsin-EDTA was added to detach the cells from the culture flask. Interface staged cells were selected for experimental studies and the medium in which they were cultured was discarded into sodium hydrochloride. The cells were incubated for 10 minutes after ensuring that they are detached from the surface of the flask under Olympix CK 316 microscope. Twenty microlitres cells were then stained in 20 µl trypan blue for counting and 10 µl was pipetted in a haemocytometer slide and counted under a light microscope (CK × 31). Cell counts estimated under a million in a flask were not used.

Standard formula of counting the cells

= number of cells × 2 × 10 000

The cells were then added into a medium (DMEM/High glucose). A 200 µl of a medium containing cells was added into a 96 well plate with repetitions and incubated at 37°C in a humidified incubator with 5% CO_2 for 24 hours.

3.2.5 Cell growth assay

After 24 hours of seeding of the cells, medium was pipetted out and the plates were labelled 24 hours and 48 hours leaving the cells attached. The cells were treated with 200 µl of different crude plant extracts (*C. orbiculata, H. depressa* and *N. glauca*) dissolved in 50% DMSO and were incubated for required time. Curcumin C was used as a positive control while DMEM/High glucose medium was used as a negative control. After 24 and 48 hours of incubation a 40 µl cell titre blue dye was added into each well which provides a perfect medium for cell growth detection and cell counting. The plate was left for 4 hours before the actual counting. The cells death was determined using ELISA microplate reader (BioTek, Tokyo, Japan) at an excitation wavelength of 620nm and an emission wavelength of 573nm (Tanih & Ndip 2013).

3.2.6 Statistical analysis

Mann-Whitney U test was used in determining the significant difference the extracts and the cancer cell line used. P values ≤ 0.05 were considered significant while $p < 0.001$ and $p > 0.05$ not significant (Milenkovic 2011).

3.3 Results

The highest cancer growth inhibition percentages of the MCF-7 cells were observed with the treatments of *N. glauca* water extracts at 48 hours whereby there was a recorded 88.89, 86.67, 87.76, 92.01, 85.30, 96.21, 94.35% inhibition at 5, 25, 75, 125, 250, 500, 1000 µg/ml concentrations respectively and 81.03% inhibition with the positive control tested, respectively, followed by *H. depressa* acetone extracts at 24 hours with inhibition percentages of 64.12, 54.78, -6.33, 61.39, 50.06, 64.06, 33.44 and 14.07% at concentrations from 5 to 1000 µg/ml and positive control. The *N. glauca* acetone extracts at 24 hours inhibited 18.51, 63.87, 4.30, 49.81, -18.28, 5.89, and 23.08% at 5, 25, 75, 125, 250, 500 and 1000 µg/ml concentrations respectively with no inhibition percentage observed with the positive control. There was in another case no inhibition observed with the positive control under *H. depressa* water extract at 24 hours. The rest of the extracts inhibited cancer cell growth at lower concentrations whereby *C. orbiculata* possessed the least anticancer activity in both extracts at 24 and 48 hours. *H. depressa* showed a better activity at 24 hours of both water and acetone extracts whereas *N. glauca* activity was observed at 48 hours with water extracts and 24 hours with the acetone extracts. The *N. glauca* water extracts were most effective at 48 hours as compared to acetone extracts no inhibition observed at 24 hours while *H. depressa* activity was observed at 24 hours with both extracts and no activity at all was observed with *C. orbiculata* (Table 3.1). There statistical difference between acetone and water extracts treated wells was not significant ($p \leq 0.01$).

There was remarkable growth inhibitions of the HeLa line cells from all experimental plant extracts. The *C. orbiculata* acetone extracts at 24 hours showed the best inhibition percentages of 60.90, 60.48, 80.83, 83.15, 68.70, 38.86 and 11.93 at 5, 25, 75, 125, 250, 500 and 1000 µg/ml, respectively, which are good percentages as compared to the positive control with 69% inhibition. The second best inhibition percentages was observed in treatments of *C. orbiculata* water extracts at 24 hours with inhibition percentages of 57.17, 64.92, 77.51, 66.37, 19.69, -4.18 and -15.19 at 5, 25, 75, 125, 250, 500 and 1000 µg/ml concenrations, respectively. These extracts showed to be much effective at lower concentrations than higher concentrations. The *N. glauca* water extracts also proved to contain some anticancer agents with higher effectivity at 75, 125 and 250 µg/ml with 55.49, 85.20 and 76.07% growth inhibition,

respectively. Both *C. orbiculata* and *N. glauca* extracts were the most effective at 24 hours of experimentation period as compared to *H. depressa* water and acetone extracts which were much effective at 48 hours of experimentation periods, however, *N. glauca* acetone extracts did not show any anticancer activity with the highest inhibition percentage of 30.90% at 25 µg/ml (Table 3.2). There was no statistical significant difference observed on the survival and inhibition of the cancer cell exposed to water and acetone extracts used for HeLa cancer cell line was not significant; however, there was a significant difference ($p < 0.05$) of cell survival in the MCF-7 and HeLa cancer line used in the study. All inhibition percentages were compared to the negative control inhibition percentages which were at all tests 0.00%. Most of the extracts possessed moderate to strong anticancer activity, however, others did not show any effect while others with negative marks induced cancer cell growth.

Table 3.1. Inhibition percentages of *C. orbiculata, H. depressa* and *N. glauca* on the MCF-7 cell line.

	Water extracts (%)						**Acetone extracts (%)**					
	C. orbiculata		***H. depressa***		***N. glauca***		***C. orbiculata***		***H. depressa***		***N. glauca***	
Concentrations	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5 µg/ml	30.63	38.13	37.87	-28.38	-541.23	88.89	23.01	-10.53	64.12	5.11	18.51	-42.67
25 µg/ml	-137.01	28.94	49.95	-38.07	-589.64	86.67	-137.12	-37.53	54.78	-112.46	63.87	-47.89
75 µg/ml	-117.56	-71.67	8.33	-30.78	-522.01	87.76	-302.65	-32.04	-6.33	-42.19	4.3	-388.87
125 µg/ml	-187.63	-141.07	40.57	-8.21	-347.76	92.01	-453.91	-46.62	61.39	-20.32	49.81	-146.57
250 µg/ml	-316.45	-124.12	14.23	-16.84	-193.52	85.3	-213.16	-20.71	50.06	11.47	-18.28	14.27
500 µg/ml	-490.24	-108.49	42.31	-238.79	-175.4	96.21	-103.22	-33.99	64.06	-42.62	5.89	-226.4
1000 µg/ml	-441.28	-159.8	-249.5	-456.4	-173.12	94.35	-79.07	-43.59	33.44	-18.53	23.08	-78.82
Positive control	-32.44	6.9	41.32	-101.31	-4.56	81.03	-39.3	43.36	14.07	-5.85	-3.37	-80.89

Values with a negative (-) sign indicates no inhibition of the cancer cells but cell growth stimulatation.

Table 3.2. Inhibition percentages of *C. orbiculata, H. depressa* and *N. glauca* on the HeLa cell line.

	Water extracts (%)						**Acetone extracts (%)**					
	C. orbiculata		***H. depressa***		***N. glauca***		***C. orbiculata***		***H. depressa***		***N. glauca***	
Concentrations	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**	**24 h**	**48 h**
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
5 µg/ml	57.17	11.28	-25.67	41.18	32.29	-50.5	60.9	18.03	8.2	38.11	-35.86	23.17
25 µg/ml	64.92	28.46	-15.89	24.91	42.97	-41.64	60.48	22.12	21.21	37.3	-68.8	30.9
75 µg/ml	77.51	19.43	4.63	24.37	55.49	6.29	80.83	17.1	13.12	30.07	-20.95	20.3
125 µg/ml	66.37	18.62	37.97	17.2	85.2	31	83.15	12.51	16.66	23.4	-7.2	17.73
250 µg/ml	19.69	14.65	29.28	19.5	76.07	29.68	68.71	6.64	22.94	15.3	6.78	9.92
500 µg/ml	-4.18	5.57	38.26	15.94	36.15	37.62	38.86	3.43	30.76	11.74	24.83	1.29
1000 µg/ml	-15.19	1.25	50.01	5.58	16.73	45.36	11.93	-23.56	45.56	2.71	54.88	-1.92
Positive control	60.81	27.67	27.25	37.33	21.62	72.09	69.51	21.85	33.38	30.43	75.34	19.73

Values with a negative (-) sign indicates no inhibition of the cancer cells but cell growth stimulatation.

3.4 DISCUSSION

In the current study MCF-7 and HeLa cancer cell lines were used in the investigation of the anticancer and/or antitumor effects of *C. orbiculata, H. depressa* and *N. glauca.* According to Prakash *et al.* (2011) the most reliable criteria of concluding on the influence brought by the plant extracts on the cancer cells is judging according to the prolongation of the life span and by body weight of animals bearing tumors. The duration of 24 hours was utilized in treatment of the cells, data was collected firstly at 24 hours then at 48 hours. Each extract possessed a unique activity on different cancer line. There was no anticancer activity observed from both water and acetone extracts of *C. orbiculata* on the breast cancer. *H. depressa* water extracts evaluated anticancer activity against the breast cancer at the lowest concentrations in the 24 hours duration, however, no activity was observed at 48 hours which was a similar case even with the positive control. The acetone extracts also showed an activity of ≤ 50% at 24 hours at all concentrations as compared to the activity obtained only at 5; 125 and 250 µg/ml at 48 hours. The highest inhibition percentages of the breast cancer were obtained at 48 hours by *N. glauca* water extracts at all concentrations as compared to the negative control and no activity at 24 hours of the same extracts. Acetone extracts at 24 hours possessed anticancer activity only at selected concentrations but none at 48 hours.

For cervical cancer cell lines, the *C. orbiculata* water extracts exhibited anticancer activity at all concentrations except at 500 and 1000 µg/ml at 24 hours and only less than 50% activity was observed at 48 hours of the same extracts. The highest anticancer activity of all extracts was exhibited in *C. orbiculata* at 24 hours and less than significant activity was observed at 48 hours with the acetone extracts. The *H. depressa* water extracts activity was observed from 75 to 1000 µg/ml at 24 hours with less than significant inhibition percentages at 48 hours and less than 50% inhibition at all concentrations of acetone extracts. The *N. glauca* extracts have shown to be the better candidate with an anticancer activity on both cell lines. Water extracts at 24 hours inhibited the cell growth at all concentrations while inhibition was observed from 75 to 1000 µg/ml in acetone extracts whereas 24 hours of acetone extracts exhibited anticancer activities from 250 to 1000 µg/ml, with less than 50% inhibition at 48 hours. Curcumin C was used as a positive control and 10% PBS-EDTA medium as negative control. Curcumin C has been stated by Hasima &

Aggarwal (2012) as the best candidate for this sort of tests due to the ability of targeting potent growth factors and might therefore be an antigrowth agent, it has been documented to block breast cancer proliferation by down regulating endoplasmic reticulum and reducing the toxic effects of the drugs and lastly it has the potential of directly binding to the DNA and RNA therefore altering their functions to anti-DNA and anti-RNA which in most cases retard cellular growth and migration.

From the current study some of the extracts exhibited dose and time-dependent activities as compared to the others. The effectivity of *H. depressa* water extract and *N. glauca* water extracts on the cervical cancer cells were dose dependent. Dose plays an important role in treatment of every disease or infection. The ability of anticancer drugs depends on the drug delivery through the vascular system to the tumor and penetration of drug to tumor cells that are distant from the point of injection or the blood vessels (Patel *et al.* 2013). Cancer hypothesis suggests that only a small subpopulation of cells known as stem cells which have the ability to give rise to new cells, are responsible for growth, invasion, metastasis, recurrence and resistance of the cells to drugs provided for cancer treatment. It is therefore stated that not much concentration is needed in treatment of cancer, only enough to target and attack the stem cells, which might have been the case in situation of low concentration inhibiting cancer cells than high concentrations in treatment of cervical cancer with *C. orbiculata* water and acetone extracts at 24 hours (O'Flaherty *et al.* 2012; Gou *et al.* 2013). Higher concentrations of *N. glauca* water extracts in both breasts and cervical cancer tests were observed to inhibit cell growth as well, in a well with many cells, higher concentrations and a prolonged time are needed in order to attack and inhibit growth of normal cancerous cells before actually reaching the stem cells. Time is also a major factor influencing the effectivity of the drug; however, there are many factors to be taken into consideration such as the blood flow to and from different organs, diffusion, tissue binding and lipid solubility which influence time a drug will take to reach all tumors. No prolonged time will be needed by any extracts should all the influencing factors happen quicker, the inverse is also correct (Patel *et al.* 2013). Time dependent effectivity in the current study was observed on *C. orbiculata* and *N. glauca* water extracts in treatment of breast cancer cells.

Chemical constituents of plants have always proved to contain different functions. Plants are the largest reservoir of secondary metabolites playing a major role in

combating many different diseases and infections from early times (Shoeb 2006; Merina *et al.* 2012). As documented by Amabeoku *et al.* (2007); Reid *et al.* (2005) and Mhinana *et al.* (2010) *C. orbiculata* contains saponins, cardiac glycosides, tannins, reducing sugars and triterpene steroids while *H. depressa* contains tannins and less than 1% cardiac glycosides and *N. glauca* contains the most toxic constituents as nicotine and anabasine. Most of these constituents have been documented with anticancer activity (Tiwari *et al.* 2011). Saponins discovery has contributed much into the pharmacological sector of cancer treatment since various identified saponins are used in clinical practices. Saponins possess antitumor effects on various cancer cells by a process known as cell cycle arrest and apoptosis of cancer cells as well as suppressing tumor invasion and migration (Man *et al.* 2010). Treatment of tumors is not only by cell inhibition but mostly by apoptosis of cancer cells leading to better results of relieving the body of an animal containing tumor. Apoptosis permits the removal of damaged and/or unwanted cells in an organism without any major damage to the microenvironment. The main potential of saponins in combating tumor growth or development is due to the ability to bind onto the membrane and therefore changing membrane permeability and forming pores (Kaskiw *et al.* 2009; Yan *et al.* 2009). Phenolic compounds including flavonoids, glycosides and tannins are promising candidates for cancer prevention for most of them can dissolve in numerous solvents and contain therefore major components in destruction of unwanted cells, however, in rare cases these components should be combined together into delivering best treatments (Nassr-Allah 2009; Sharma *et al.* 2009).

Most anticancer therapies are focused on the induction of apoptosis even though cancer cell have the ability to evade apoptosis, however, researchers are making sure that apoptosis is re-introduced directly into the tumor cells which will decrease the rate of resistance as it has escalated as detected by the apoptosis program (Do & Lim 2008). Consumption of the tested concentrations of these plants may be of help in induction of apoptosis although this requires further experimental confirmation.

Conclusions

Results of the current study showed that the three tested plants (*C. orbiculata, H. depressa* and *N. glauca*) possess anticancer activity although every extract had a specific activity at various concentrations on different cells. The study has proven that *N. glauca* was the most effective plant on both breast and cervical cancer cell lines while *C. orbiculata* and *H. depressa* were effective on cervical and breast cancer cells, respectively.

Chapter 4

In vitro and *in vivo* cytotoxicity analysis of *Cotyledon orbiculata, Hermannia depressa* and *Nicotiana glauca* on the bovine kidney cells and brine shrimps

4.1 INTRODUCTION

There is a continuous search is for new sources of biologically active compounds for the discovery of new drugs for the treatment of infectious pathogens including parasites, bacteria, viral infections and immunological disorders (Steenkamp & Gouwns 2006; Prasad *et al.* 2012; Prema *et al.* 2012; Sini *et al.* 2012). According to Prema *et al.* (2012) natural and related product are successfully used globally in treatment of up to 80% of most categorised diseases, 25% of prescribed drugs worldwide naturally originate from medicinal plants and 80% of people in the whole world depend solely on traditional based medicine for primary health care needs. Medicinal plants have and are still playing an important role in the discovery of modern novel drugs, they can be in twofold of drug development either as a base for drug development or as the main phytomedicine for treatment of the certain diseases (Prasad *et al.* 2012). Medicinal plants are used to treat and cure diseases, however, in other cases they cannot totally cure the disease but they reduce the aggressiveness of the disease (Leto *et al.* 2012).

Medicinal plants are used in treatment of pathogens due to the fact that they contain active chemical components of therapeutic value. Medicinal values depend on the chemical composition of the plants mostly known as the secondary metabolites which include alkaloids, flavonoids, tannins, glycosides and phenolics (Sini *et al.* 2012). These active compounds present in a plant provide large quantity resources for pharmaceutical, cosmetics and food industry purposes. Furthermore, it has recently been documented that these compounds can be of agricultural purposes and can possibly be used in pest control (Prasad *et al.* 2012). Secondary metabolites are secreted by plants for various functions in various manners. Some metabolites which are constitutively produced compounds meaning they are secreted by different parts of plants at different stages, biochemically activated are always present in a plant, however, they are of no use until they are stimulated by other chemicals to protect plants. Compounds such as morhine and digitoxin are mostly known to treat and control pain and congestive heart in animals, respectively. These metabolites

can be used as pure compounds extracted from the medicinal plants with others such as atropine, quinine whereas others such as asprin and anasthetics are modifications (Verpoorte 1998). Plant poisoning of livestock due to toxic plant consumption can be fatal and also affect economic status of farmers negatively. In most cases toxic plant consumption by animals, much results from poor pasture periods or condition meaning the medicinal plants will then be the better source of green feed.

Plants used for ethnobotanical need or purposes should thoroughly be tested for cytotoxicity since some of the secondary metabolites secreted can be toxic. According to Botha & Penrith (2008), poisonous plants as well may contain effective biological components that can treat infectious diseases and illnesses. Most of the secondary metabolites are only present in the plants as the mechanism of defence, however, other amongst all are toxic and death may results with their consumption. Toxic secondary metabolites include cardiac glycosides, pavetamine, pyrrolizidine alkaloids, pentacyclic triperpene acids and sesquiterpene lactones (Botha & Penrith 2008). Cardiac glycosides found in plants result in livestock poisoning in sheep and goats if consumed (Leto *et al.* 2012). Cardiac glycosides are divided into two categories namely cardenolides and bufadienolides. The cardenolides are much unpalatable to livestock and bufadienolides result in an acute and chronic poisoning and death may result due to cardiotoxic effect (Schultz *et al.* 2005). Even though cardiotoxic glycosides are known to affect the heart but they can also affect gastro-intestinal sides. Pavetamine results in a disease called gousikte which is a heart failure disease in ruminants after ingesting toxic rubiaceous plant. In this case affected cattle and small stock will reveal histophathologically typical lesions on the heart (Botha & Penrith 2008). Pyrrolidizidine alkaloids can result in hepatotoxicosis and cyanogenic glycosides in combination with glucosidases have the potential of yielding a highly toxic hydrogen cyanide (Verpoorte 1998).

It is due to these components that cytotoxicity studies were conducted in both *in vitro* and *in vivo* environments. In the current study cytotoxicity of three medicinal plants: *Cotyledon orbiculata, Hermannia depressa* and *Nicotiana glauca* were tested *in vitro* on mammalian cell lines and also *in vivo* on brine shrimps.

4.2 MATERIALS AND METHODS

4.2.1 *Plant collection sites*

Plant materials were collected in February 2013 from multiple populations of *C. orbiculata* (Plate 1.1), *H. depressa* (Plate 1.2) around Qwaqwa area in Maluti-A-Phofung Municipality of the eastern Free State Province (28°32'0"S and 28°49'0" E; altitude 1, 673 m). The mean annual rainfall of the Maluti-A-Phofung Municipality is about 653 mm per annum with temperatures ranging from as low as 0.1ºC during July nights, 14.2°C during the day in June, and maximum of 24.7ºC during the day in January (Wolfram 2012). *N. glauca* (Plate 1.3) was also collected in February 2013 from Wolmaranstad in Maquassi Hills Local Municipality of the North West Province (27°12'0"S and 25°58'0"E; altitude of 1170 m) of South Africa. The mean annual rainfall of the Maquassi Hills Local Municipality is about 391 mm per annum with temperatures ranging from as low as 0ºC during July nights, 17.6°C during the day in June, and maximum of 30ºC during the day in January (Wolfram 2012). The plants were identified by Dr. AOT Ashafa of the Department of Plant Sciences, University of the Free State (UFS) - Qwaqwa Campus and voucher specimens (MolMed/02/2013) were prepared and deposited at the UFS Qwaqwa Campus herbarium.

4.2.2 *Preparation of crude extracts*

The shoots of the plants were separated and dried in an Ecortherm oven (Laboratory Consumables Pty, South Africa) at a temperature of 40°C to a constant weight before it was pulverized. Ten grams each of powdered material was extracted in acetone and distilled water. Acetone was of high analytical grade (Merck Chemicals Pty, Wadeville, South Africa). All extracts were filtered using Whatman No-1 filter paper (Whatman, United Kingdom). The filtrates from acetone were concentrated under reduced pressure 40ºC using rotary evaporator (Cole-Parmer, Laboratory Consumables and Chemical Supplies Co.Ltd, China). The water extract was freeze dried using freeze dryer (Virtis SP Scientific, United States of America). Individual extracts were reconstituted in their respective solvents to give a stock solution of 50 mg/ml (Ashafa & Afolayan 2009). This was diluted to the required concentrations of 2.5 and 5.0 and 7.5 mg/ml for the bioassay analysis.

4.2.3 *In vitro* cytotoxicity tests

4.2.3.1 Cell maintenance and preparation

The MDBK cells were obtained from the American Type Culture Collection (ATCC). The cells were removed from the incubator and the adherence of the cells was observed microscopically. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) and Hams F-12 Nutrient Mixture supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Fungizone) in a humidified atmosphere of 5% CO_2 at 37°C. The cells were cultured in 75 cm^2 cell culture flasks.

The flask was shaken gently on the platform in order to assess adherence and thoroughly sprayed with ethanol before introduced into hood; the medium was discarded into the waste beaker. Cells were rinsed 3 times with PBS to remove all debris and leave cells attached to the surface of the flask. Trypsin/EDTA solution was introduced to the flask to detach the cells and the solution was allowed 3 minutes to work. Carefully the supernatant was discarded into the waste beaker. A 500 µl of FBS was placed into a tube with 50 ml Dulbecco's modified eagle's medium in preparation of 1% FBS medium to suspend the cells for cell counts.

4.2.3.2 Cell counts

A 200 µl trypan blue was added into a 2 ml eppendorf tube containing 800 µl 1% FBS medium. From the solution a 500 µl was pipette into an empty tube with an addition of 500 µl of the cell suspension. Thereafter 10 µl of the solution was pipetted into two sites of the haemacytometer slide and counted to 20 000 cell/ml.

A 200 µl volume of the cells was added into a 96 well plate and incubated for 24 hours. Plant extracts of concentrations 0.65, 1.25 and 2.50 mg/ml, negative control (50% DMSO: H_2O) except the positive control (Trixton X100 was added into 24, 48 and 72 hours labelled plates. The LDH and MTT assays were conducted on the same plate, beginning with an LDH assay in 24 hour duration.

4.2.3.3 MTT assay

Assessment of cell survival was carried out using the method of Mosmann (1983). At the end of the exposure period, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazolium bromide (MTT) (5 mg/ml in PBS) was added into each well and the cells were further incubated for 4 h. Thereafter, cells were lysed with DMSO for 30 min in order to dissolve the formazan crystals. The formation of colour (formazan) was measured with a microtitre plate spectrophotometer (Bio-Tek µQuant) at 570 nm. Results were analysed with the GraphPad Prism software (version 4.0). Cell viability was estimated as the percentage absorbance of sample relative to control. Two independent experiments were carried out with three replicate wells for each toxin concentration.

4.2.3.4 *LDH assay*

Cell membrane integrity was evaluated by measuring the lactate dehydrogenase (LDH) enzyme activity using the commercial LDH cytotoxicity detection kit (Roche Diagnostics GmbH). This is a colorimetric assay for quantification of cell death and cell lysis, based on the determination of LDH activity released from the cytoplasm of damaged cells into the medium, thus indicating cell membrane damage. At the end of the exposure period, 100 µL of cell culture medium was removed and transferred into a 96-well plate. An equal volume (100 µL) of the reaction mixture (250 µL of diaphorase/NAD^+ mixture premixed with 11.25 ml of iodotetrazolium chloride/sodium lactate) was added to each well and the plate was incubated for 30 min at room temperature. The absorbance was then measured with a microtitre plate spectrophotometer (Bio-Tek µQuant) at 490 nm. LDH release was expressed as percentage of the compounds tested relative to control cells. Two independent experiments were carried out with three replicate wells for each toxin concentration.

Total LDH release was calculated using a formula described by Konjevic *et al.* (1997):

$$\frac{LDH_{experimental} - LDH_{effector\ cells} - LDH_{spontaneous}}{LDH_{maximal} - LDH_{spontaneous}} \text{ X } 100$$

Where $LDH_{experimental}$ represents LDH release activity resulting from co-cultures of effector and target cells; $LDH_{effector\ cells}$ represents released LDH activity from separately cultured effector cells, $LDH_{spontaneous}$ represents activity released from cultures of MDBK cells and LDH maximal represents LDH activity released from MDBK cells after lysis.

4.2.3.5 *Data analysis*

Data was analyzed using the Manh-Whitney U test for comparison between the MTT and the LDH assays (Milenkovic 2011).

4.2.4 *In vivo* cytotoxicity assay

4.2.4.1 Hatching of the brine shrimp eggs

The method was conducted as described by Padmaja *et al.* (2002) and Manilal *et al.* (2006). *In vitro* lethality assay of *Artemia salina* (brine shrimps) was used to detect cell toxicity. A spatula tip was filled with the eggs and placed into 100 ml of sea water and left at 24-28^{o}C in front of a contact light. Eggs hatched within 48 hours providing large number of larvae also known as nauplii. Ten to fifteen nauplii were placed in vials containing 5 ml of seawater and increasing concentrations of *Cotyledon orbiculata, Hermannia depressa* and *Nicotiana glauca* shoot extract. Alive nauplii were counted in duration of 24 hours until all died and the lethal concentration (LC_{50}) was calculated. Concentrations prepared were 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml for all materials experimental plants, pure saline water was used as negative control.

Equation published by the Laboratory of Ecotoxicity and LAC (2013) was used to determine mortality rate. The equation is:

$$Mm_{ct} = \frac{N_{Mm}}{N_o} X\ 100$$

Where:

Mm_{ct} is mortality of individuals in time t (%)

N_{Mm} is average number of dead individuals

N_o is the initial number of living individuals put into every concentration at the start

Lethal concentration (LC_{50})

4.2.4.2 Data analysis

Significant differences between extracts and the assays used were assessed by the Student's T-test. A probability level $P < 0.05$ was considered to indicate statistical significance (Saghal *et al*. 2010).

4.3 RESULTS

4.3.1 In vitro cytotoxicity

There was a significant difference ($p < 0.05$) in cytotoxicity observed on MDBK cells exposed to both water and acetone extracts of *C. orbiculata, H. depressa and N. glauca* assessed by MTT and LDH assays. The water extracts at 72 h exposure induced a significant decrease in the cell survival to a range of 50-60%. In general, prolonged exposure to this extract resulted in increased cytotoxicity (Fig. 4.1a). Significant cytotoxic effects were observed when MDBK cells were exposed to lower dilutions (0.56 and 1.25 mg/ml) of *C. orbiculata* acetone extracts at the three exposure periods (Fig. 4.1b). Recovery of the cells from the cytotoxic effects of the acetone extracts was recorded as the extract dilutions were increased.

A significant release in LDH enzyme was only observed when MDBK cells were exposed to the highest *C. orbiculata* water extract concenration (2.5 mg/ml) at 24 and 48 h exposure periods (Table 4.1). No release in LDH enzyme was recorded at 72 h exposure periods for the different water extract dilutions when compared to the control (100%). There was a significant release of cytotplasmic LDH enzyme at 24 and 48 h exposure periods demonstrated for the different *C. orbiculata* acetone extract dilutions (Table 4.1). The release of LDH enzyme was slightly more than that of the control (100%) after 72 h of exposure to the three extract dilutions.

There was no difference in the cytotoxic response of MDBK cells following exposure to the three dilutions of *H. depressa* water extract at the different exposure periods (Fig. 4.2a). An increase in the cytotoxicity induced by this extract was observed as the exposure period was extended. Hence, a significant cytotoxicity characterized by ±40% of cell death was recorded at 72 h exposure period. No cytotoxicity was observed with *H. depressa* acetone extracts at the three dilutions recorded for the different exposure periods (Fig. 4.2b). This extract stimulated the growth of MDBK cells. A dose-dependent stimulation in cell growth characterized by cell survival of more than 350% was observed at the three exposure periods.

There was a significant release of cytoplasmic LDH enzyme following exposure to the three dilutions of *H. depressa* water extracts at the different exposure periods (Table 4.2). The release of LDH was dose-dependent at 48 and 72 h exposure

periods, however, at 24 h exposure period the release in LDH decreased from 228 to 157% as the extract dilutions increased. An increase in release of cytoplasmic LDH was recorded as the dilutions of *H. depressa* acetone extracts were increased (Table 4.2). This release in LDH enzyme was more significant (351-1159%) at 24 and 48 h exposure periods than at 72 h (188-376%).

No cytotoxicity was observed on MDBK cells following exposure to the three dilutions of *N. glauca* water extract at 24 and 48 h exposure periods (Fig. 4.3a). Cytotoxicity characterized by ±25% of cell death was demonstrated at 72 h exposure period, implying that prolonged exposure to this extract results in cytotoxicity. A dose-dependent cytotoxicity was recorded on MDBK cells after exposure to the three dilutions of *N. glauca* acetone extract (Fig. 4.3b). About 50% of the cells died following exposure to the highest dilution (2.5 mg/ml) of *N. glauca* acetone extract.

There was a significant release of LDH enzyme from the cytoplasm of MDBK cells after exposure to *N. glauca* water extracts (Table 4.3). A dose-dependent release in LDH enzyme (229-374%) was observed only at the 24 h exposure period. However, at 48 and 72 h exposure periods the LDH release remained approximately the same. Small amounts of LDH enzyme were released from the cytoplasm of MDBK cells following exposure to the *N. glauca* acetone extract at the different exposure periods (Table 4.3).

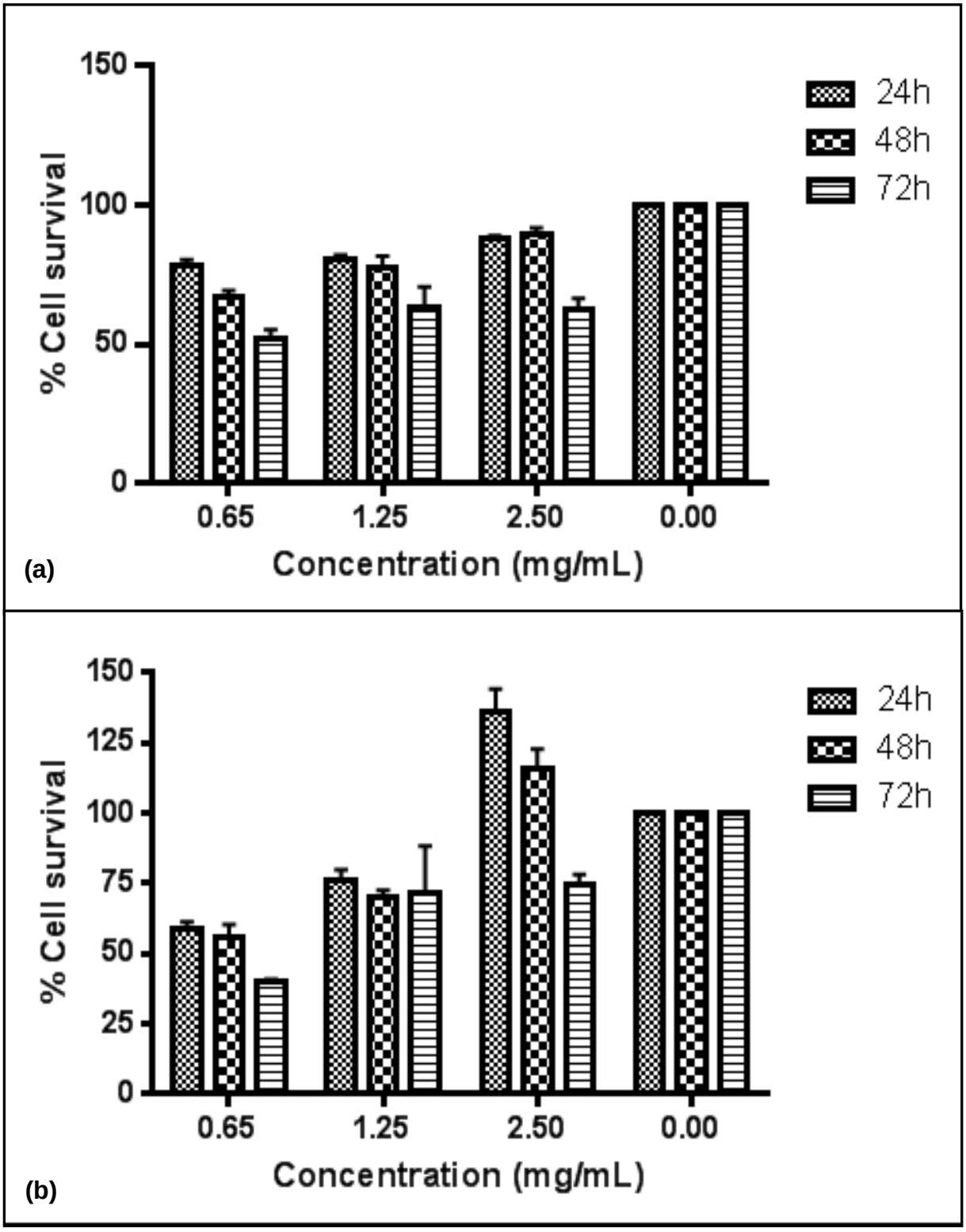

Fig. 4.1. Cytotoxic response of the MDBK cells measured using the MTT assay after exposure for 24, 48 and 72h to *C. orbiculata* (**a**) water extract, and (**b**) acetone extract. Results are presented as percentage of control.

Table 4.1. LDH release assay after exposure of MDBK cells to *C. orbiculata* water and acetone extracts for 24, 48 and 72 h.

Concentration (mg/ml)	***C. orbiculata* water extracts**			***C. orbiculata* acetone extracts**		
	24h	48h	72h	24h	48h	72h
0.65	120±16	111±70	<QC	222±26	200±70	131±70
1.25	117±50	128±90	<QC	237±35	211±70	131±90
2.50	167±25	177±10	<QC	295±16	212±14	142±15

Data presented as percentage of control ±SEM; SEM: standard error mean. LDH values less than that of the control cells (control = 100%) are designated as <QC (Statistical Quality Control).

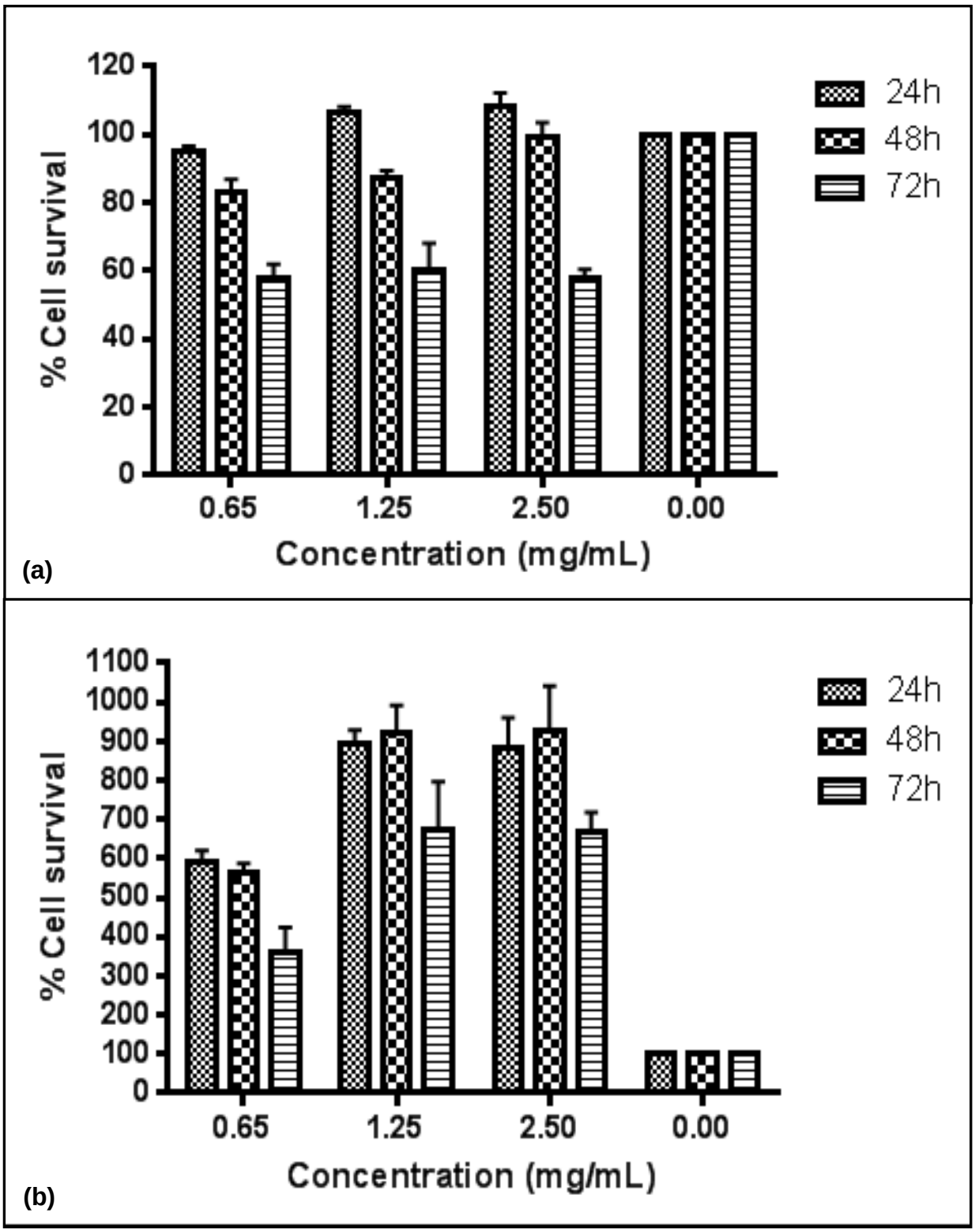

Fig. 4.2. Cytotoxic response of the MDBK cells measured using the MTT assay after exposure for 24, 48 and 72h to *H. depressa* (**a**) water extract, and (**b**) acetone extract. Results are presented as percentage of control.

Table 4.2. LDH release assay after exposure of MDBK cells to *H. depressa* water and acetone extracts for 24, 48 and 72 h.

Concentration (mg/ml)	*H. depressa* water extracts			*H. depressa* acetone extracts		
	24h	48h	72h	24h	48h	72h
0.65	228±33	188±12	141±60	573±25	351±12	188±10
1.25	185±16	209±90	148±11	835±56	485±12	284±24
2.50	157±15	242±39	181±23	1159±82	653±58	376±50

Data presented as percentage of control ±SEM; SEM: standard error mean. LDH values less than that of the control cells (control = 100%) are designated as <QC (Statistical Quality Control).

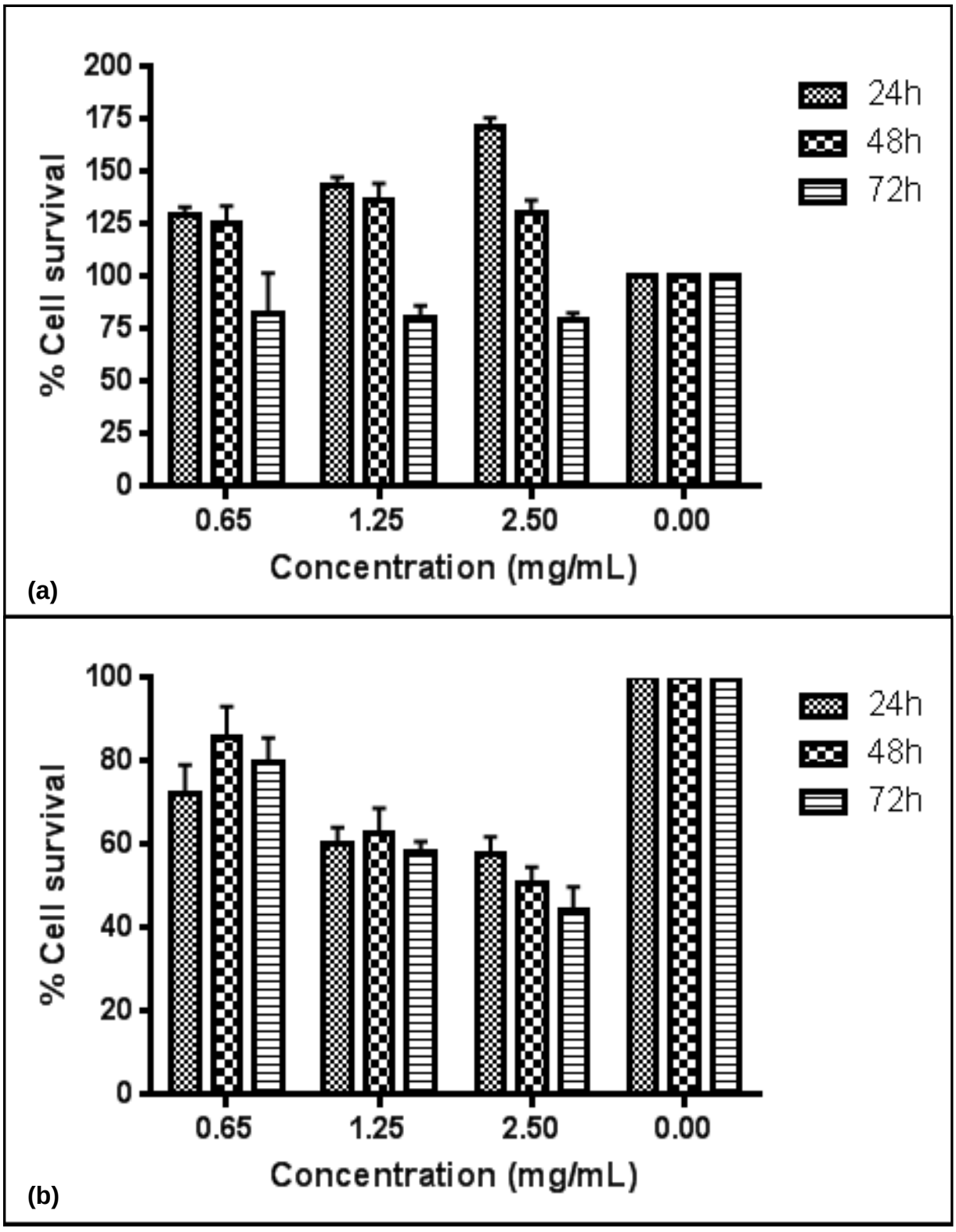

Fig. 4.3. Cytotoxic response of the MDBK cells measured using the MTT assay after exposure for 24, 48 and 72h to *N. glauca* (**a**) water extract, and (**b**) acetone extract. Results are presented as percentage of control.

Table 4.3. LDH release assay after exposure of MDBK cells to *N. glauca* water and acetone extracts for 24, 48 and 72 h.

Concentration (mg/ml)	*N. glauca* water extracts			*N. glauca* acetone extracts		
	24h	48h	72h	24h	48h	72h
0.65	229±12	255±10	180±10	136±31	97±10	122±19
1.25	322±48	222±18	213±33	<QC	126±18	109±11
2.50	374±28	235±10	237±25	137±20	123±8	133±13

Data presented as percentage of control ±SEM; SEM: standard error mean. LDH values less than that of the control cells (control = 100%) are designated as <QC (Statistical Quality Control).

4.3.2 In vivo cytotoxicity

Cytotoxicity rate of the tested material was determined by mortality rate of the brine shrimps. Percentage mortality was recorded in each case. Durations were recorded until all the brine shrimps died as the highest toxicity level. For *Cotyledon orbiculata* water extract, all the larvae in 0.2, 0.4 and 0.6 mg/ml died within 72 hours of experimentation whereas those in 0.8 and 1.0 mg/ml died in 92 hours of experimentation. At 0.2 mg/ml concentration there was less toxicity rate as no larval mortalities were observed at the initial 24 hour duration, 30% mortality rate in 48 hours and 100% mortality in 72 hours. The highest mortality rate in the current experiment was at 24 hours with 0.6 mg/ml where 40% larval mortality was recorded, whereas only 20% mortality was observed at 0.8 and 1.0 mg/ml at the same time (Fig. 4.4a). Total toxicity of *C. orbiculata* acetone extracts total toxicity was at 72 hours at all concentrations. No larval mortality was observed at 24 hours with 0.4, 0.6, 0.8 and 1.0 mg/ml concentrations, however, 30% mortality was observed at 0.2 mg/ml and 10% with negative control. Mortalities resulting in larval reductions were however observed to be 60, 43, 64, 60, 60 and 90% at 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml and negative control at 48 hours, respectively (Fig. 4.4b).

Hermannia depressa water extract total toxicity was observed within 72 hours of experimentation from the lowest concentration of 0.2 mg/ml to 0.8 mg/ml. Mortality rate was concentration dependent and the highest larval mortality was observed 0.6 mg/ml, 48 hours with 70% reduction as compared to the negative control (Fig. 4.5a). Acetone extracts total mortality was observed within 72 hours as well, with the highest reduction percentage of 80% at 0.6 mg/ml. Surprisingly, the range of negative control was more/less at the same percentages as the treated experiments with mortality rate of 27, 55 and 100% at 24, 48 and 72 hours, respectively (Fig. 4.5b).

Both *Nicotiana glauca* water and acetone extracts needed a prolonged time to lead to a total mortality of the larvae, 120 hours as compared to the other two plants. No concentration dependent results were obtained in this plant's water extract. Total larval mortality at 0.2, 0.6 and 0.8 mg/ml as well as the negative control was observed within 96 hours whereas it was 48 hours at 0.6 mg/ml and 120 hours at 0.4 and 1.0 mg/ml (Fig. 4.6a). The acetone extracts toxicity rate were concentration

dependent whereby an escalation of mortality rate was observed with an increase in concentration. At 24 hours mortality rates were observed as 70, 80, 90, 100 and 100% at 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml and 30% in the negative control. All larvae died within the initial 24 hour duration at the highest concentrations tested which are 0.8 and 1.0 mg/ml followed by 0.4 mg/ml at 72 hours then 0.2 and 0.8 mg/ml at 96 hours and lastly 0.6 mg/ml at 120 hours (Fig. 4.6b). There was a significant difference ($p \leq 0.05$) to the survival of the brine shrimps observed from water and acetone extracts of *C. orbiculata, H. depressa* and *N. glauca*. Although different plant extracts executed either dose or time dependent toxicity on brine shrimp nauplii, yet, the LC_{50} of all tested extracts was greater than 100 µg/ml, therefore rendering the extracts non-toxic to brine shrimp larvae.

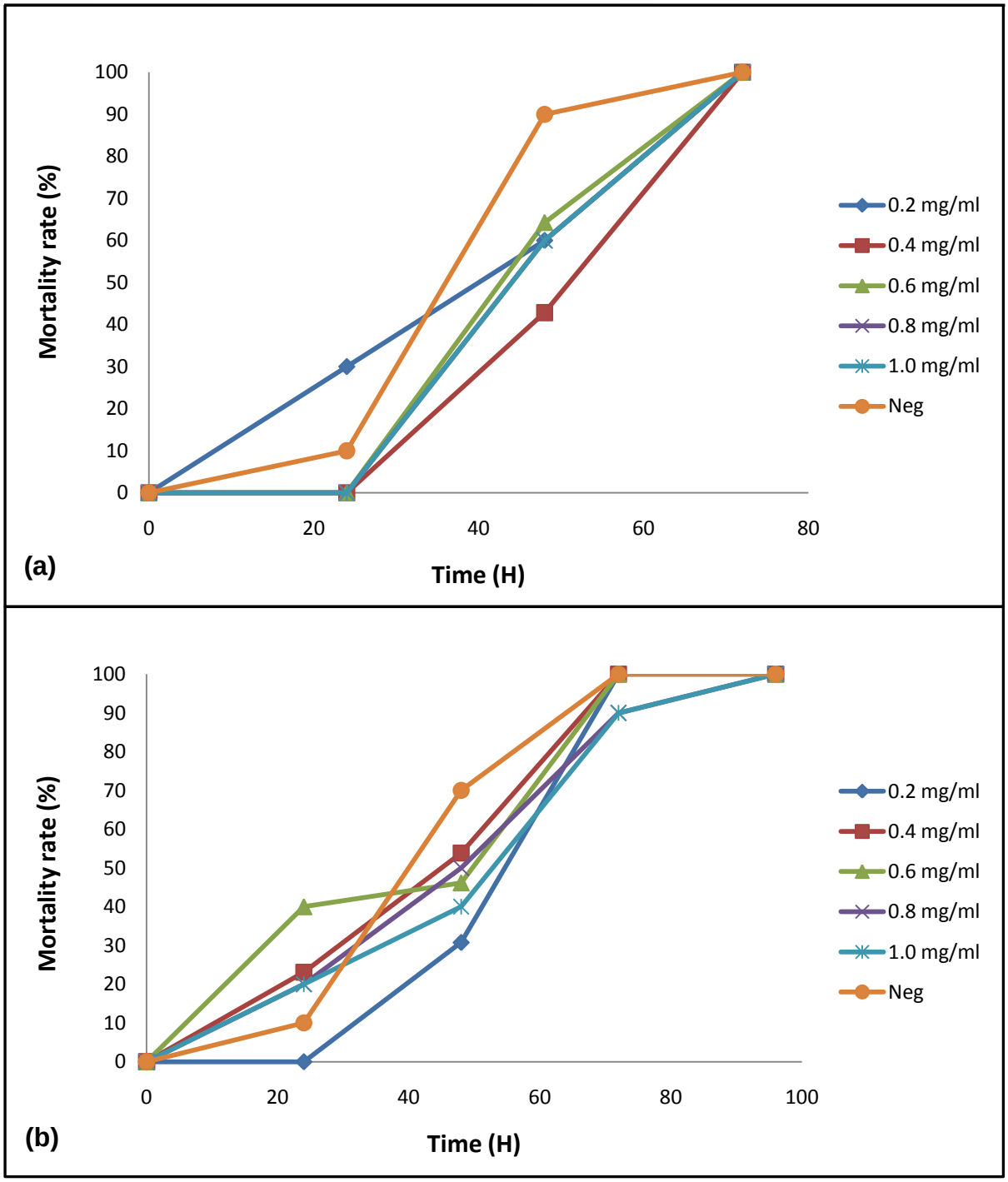

Fig. 4.4. Effects of *Cotyledon orbiculata* (**a**) water and (**b**) acetone extracts on the brine shrimp motility.

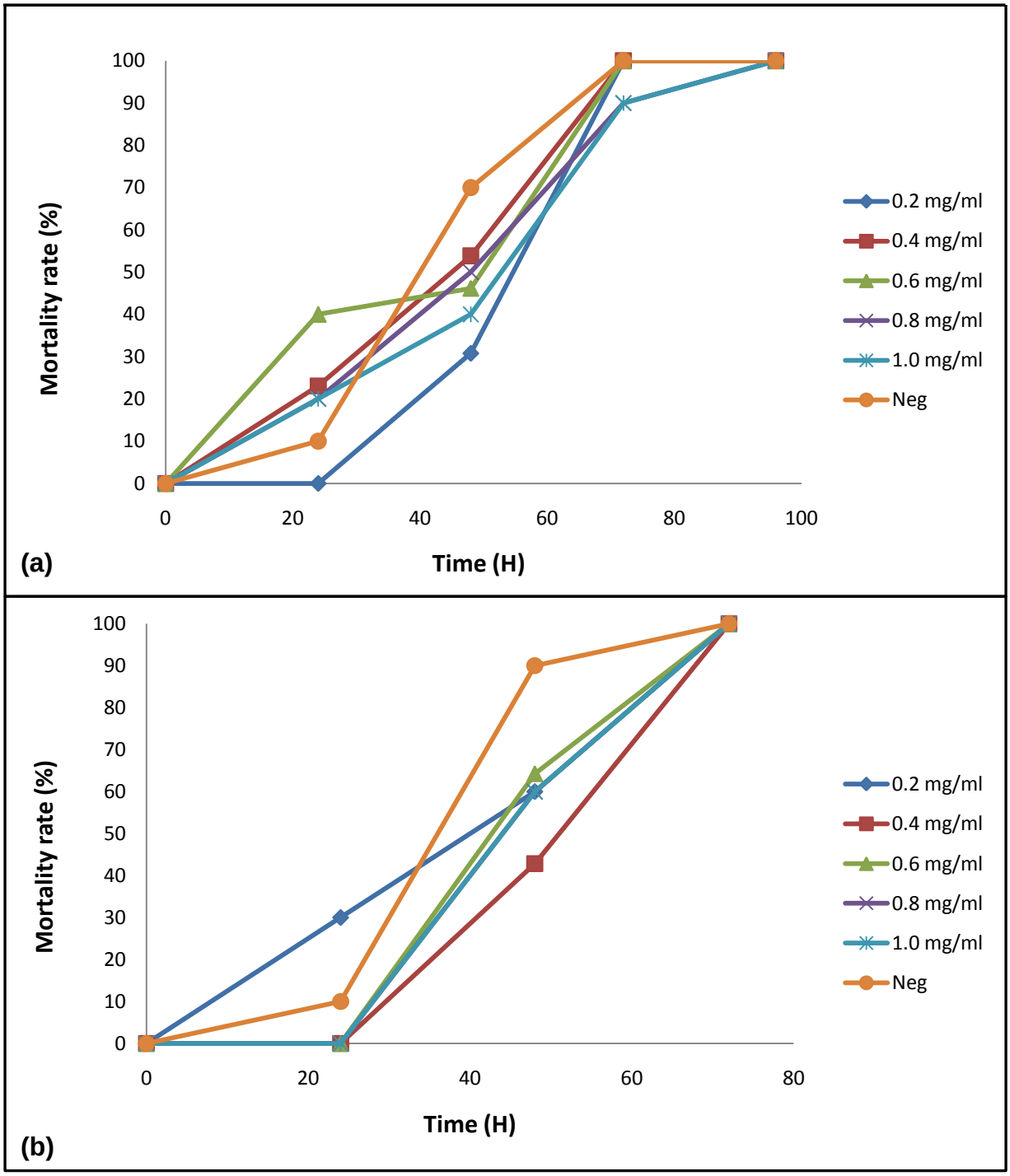

Fig. 4.5. Effects of *Hermannia depressa* (**a**) water and (**b**) acetone extracts on the brine shrimp motility.

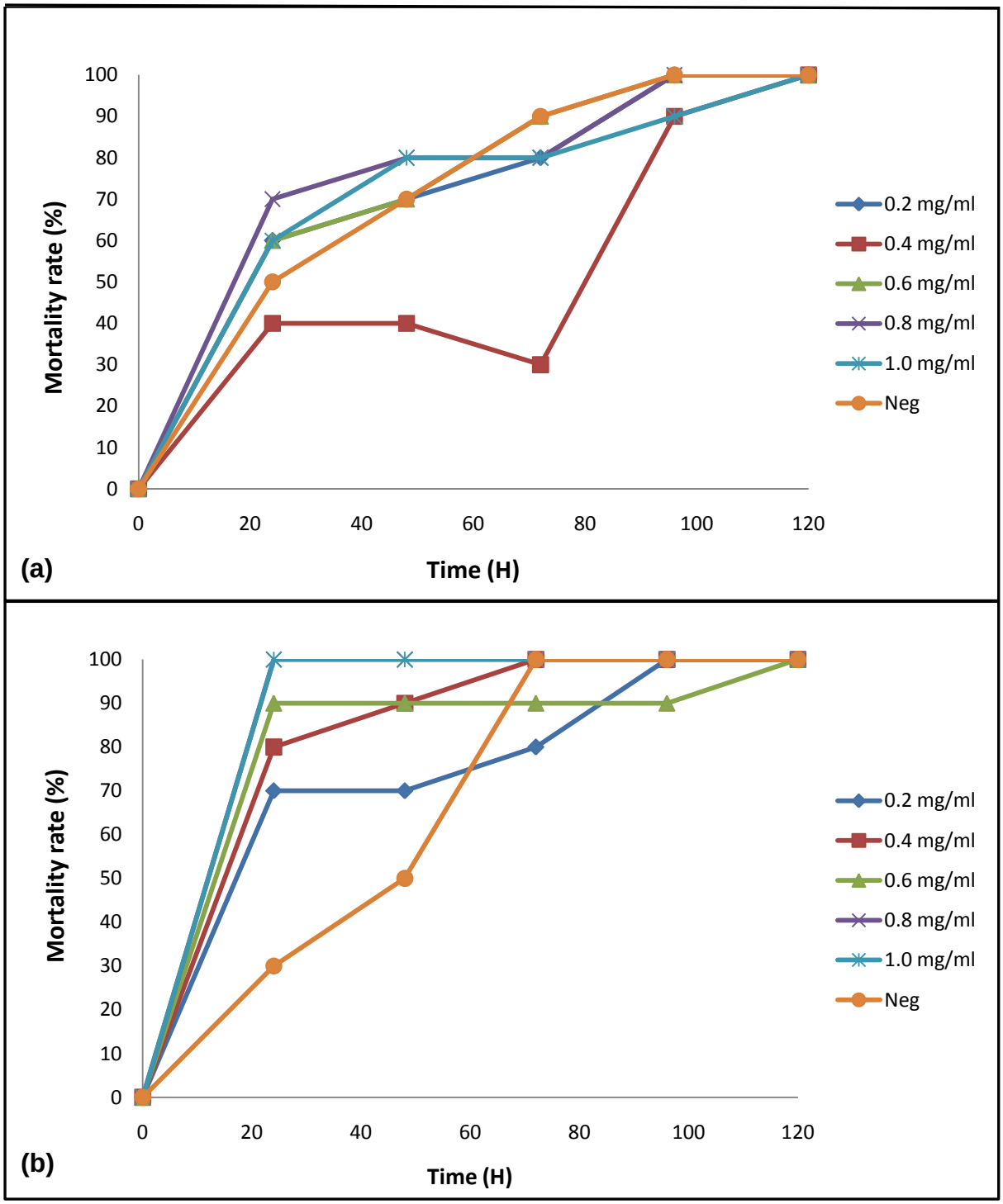

Fig. 4.6. Effects of *Nicotiana glauca* (**a**) water and (**b**) acetone extracts on the brine shrimp motility.

4.4 DISCUSSION

In the current study brine shrimps were used to test preliminary toxicity effects of *C. orbiculata, H. depressa* and *N. glauca.* In all tests the organisms survived for few days and died off. The dying off process began with a daily decrease in motility of the brine shrimps. Hlywka *et al.* (1997) stated that the age of the brine shrimps also influence the organisms' sensitivity towards any toxic substance. Age might have therefore been a reason of the gradual decrease of the number and motility of the organisms in each test hence freshly hatched nauplii should always be used in toxicity studies. Total motility (brine shrimp movement) inhibition was observed within 72 and 96 hours in *C. orbiculata* and *H. depressa* water and acetone extracts, respectively. Both extracts of *N. glauca* resulted in total mortality at 120 hours indicating that extracts of this plant was far less toxic than any of the other tested plants. This plant is known to possess nicotine which is known to be highly toxic (Mhinana *et al.* 2010), however in this study the plant did not prove to be any toxic since the extracts at the tested concentrations could not kill brine shrimps larvae at the expected time of 24 hours. It might mean that the plant is not toxic to the organism since nicotine has been documented with other bioactivities such as anthelmintic and antimicrobial (Iqbal *et al.* 2006; Hadaruga *et al.* 2010) or that brine shrimps managed to acclimatize in the extracts quicker than in the other extracts since they can withstand various conditions (Turker & Usta, 2008).

According to Moshi *et al.* (2010) a substance or a material is considered toxic if the LC_{50} counts ranges less than 100 µg/ml and counts greater are non-toxic. In the current study LC_{50} values were calculated in mg/ml as in the study by McGaw *et al.* (2007) the reason being during the anthelmintic activity study of the plants tested concentrations were in mg/ml therefore even toxicity studies had to be in mg/ml so that consistency is kept between the studies. The lowest concentration tested was 0.2 mg/ml which is over a 100 µg/ml already. The plants are therefore unlikely to possess any acute or chronic toxic substances to the brine shrimps and there was no concentration dependency observed in the study as well.

The second aim of the study was to evaluate the toxicity effects of *C. orbiculata, H. depressa, N. glauca* on the MDBK cell lines for 24, 48 and 72 hours at 0.65, 1.25 and 2.50 mg/ml using the LDH and MTT assays. A number of naturally derived

agents have been entered into the clinical trials and terminated due to lack of toxicity tests. Plants with variable activities such as anthelmintic or anticancer should further be tested for cytotoxicity effects on normal cells as a way of combating unnecessary deaths or effects from toxic plants (Craig & Newman 2005). The LDH assay is associated with the leakage of an enzyme into the culture medium due to membrane disruption and damage from the effects of the extracts whereas the MTT assay is mainly based on enzymatic conversion of MTT in the mitochondrial functioning. The LDH release plays a vital role in assessing the cytotoxicity of the extracts on the cells whereby the high LDH release is associated with toxicity of the extracts therefore fewer cells are expected to survive (Agiga & Jagetia 1999; Fotakis & Timbrell 2006).

C. orbiculata and *H. depressa* water extracts toxicity were time dependent. According Agarwala *et al.* (2009) cells would usually undergo the necrosis stage when they can no longer produce adequate ATP for sustainability measures. Cells need energy for metabolism process in the form of ATP, should cells lack energy they are therefore likely to fail maintaining homeostasis which should be the balance between tumor growth and the ability to supply necessary nutrients including oxygen, cell proliferation and apoptosis and therefore result in LDH spillage.

The results from *C. orbiculata* acetone extracts were in agreement with the study by Adiga & Jagetia (1999) and Agarwala *et al.* (2009) which stated that elevated LDH levels were observed as the cell survival worsen even at optimum concentrations. Cell viability decrease demonstrated the dying of the cells which precedes the plasma membrane breakdown because as stated by Fotakis & Timbrell (2006), mitochondrial respiration inhibition leads to a decrease in ATP produced to maintain metabolism therefore oxygen related death of the cell will result before the main membrane breaks down.

H. depressa acetone extracts did not show cytotoxic effects on the cells since there was no cell growth inhibition observed but the cells were stimulated to grow more than the negative control. Biological membranes are composed of various protective structures such as the phospholipids bi-layers of hydrophilic and hydrophobic characters, which consist of cholesterols and proteins. The main aim of the cell membrane is to regulate the movement of particles in and out of the cell. Agarwala *et al.* (2009) reported resistance of the cell membranes to detergent which might have

been the case in the current study, resistance of the cell membrane to the plant extract. In both extracts of *H. depressa* there were low cytotoxic effects associated with high leakages of LDH, normal cells do release LDH, there were high stimuli of the cell growth in this study hence the observed high LDH percentages than those observed in the negative control.

N. glauca water extracts showed little toxicity at higher concentration while acetone extracts were toxic at lower to higher concentrations with much LDH release at all concentrations. *Nicotiana* species has been documented with the presence of pyridine alkaloid of nicotine and anabasine which are very toxic to herbivores and humans (Baldivin & Callahan 1993). Plants usually accumulate chemical constituents as defence mechanisms and therefore exhibit numerous biological activities. Preparations of extracts begin with cutting of the plant and grindings which might influence the secretion of these metabolites (Baldwin & Huh 1994). Data obtained from the current study was similar to that reported by Tadmor-Melamed *et al.* (2004) where they reported that toxicity exhibited by *Nicotiana* species is dose dependent.

In vitro cytotoxicity studies were conducted using the LDH and the MTT assays on the MDBK which are the normal bovine kidney cells. The LDH and MTT assays are the most commonly employed cytotoxicity tests. LDH assay is used to assess the loss of membrane integrity by measuring release into the cytosol enzyme lactate dehydrogenase. The assay determines the neuronal apoptosis in cortical cultures (Lobner 2000). MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) assay quantifies mitochondrial activity by measuring the formation of blue formazan product formed by the reduction of tetrazolium ring of MTT. The MTT reduction is thought to happen in the mitochondria through the action of succinate dehydrogenase and therefore providing a measure of mitochondrial function by determining the neuronal injury (Fotakis & Timbrell 2006).

Cytotoxicity studies can be conducted in two ways either *in vitro* or *in vivo*. In the current study *in vivo* cytotoxicity study was conducted using the brine shrimps. According to Manial *et al.* 2009 the usage of brine shrimps for cytotoxicity tests may be used as a preliminary assessment of toxicity that is very useful in isolation of biogeric compounds from the plant extracts. These organisms have the ability to

withstand various conditions including salinity at the range of 5 to 250 g/L and temperature range of 6 to 35°C and have been documented with an extreme adaptability rate to survive environments where no life forms are found. They obtain this ability from characters such as having short life cycle, high fecundity, parthogenetic strategy of reproduction, their small body size as well as a wide nutrient range (Nunes *et al.* 2006).

Overall observation from the study is that acetone extracts were more toxic than water extracts. Acetone as a solvent has the ability to dissolve and extracts numerous compounds and secondary metabolites than water. Water can dissolve and therefore extract polar compounds only whereas acetone can dissolve a wide range of polarity and can form a homogenous mixture with different solvents (Eguale *et al.* 2011). Acetone is an organic extractant which might have extracted most of the toxic compounds hence acetone extracts were much toxic than any other (Ademu *et al.* 2013). Water extracts have in most cases been documented with few compounds extracted which contains less biological activities against most infections or organisms. The main difference between the results obtained from solvents used in the current study is polarity and compounds solubility hence different cytotoxicity levels observed (Wang *et al.* 2011). Generally, the release of LDH plays a major role in determining the tumor burden dealt with at all cytotoxicity studies. LDH release shows the occurrence of anaerobic respiration by tumor cells since it is associated with the process known as the glycolysis in the absence of oxygen. According to Lobner (2000) prevention of cell death by any substance as assayed by LDH can only be maintained for 48 hours of experimentation. There are other factors associated with the cytotoxicity results of any plant that might be tested such as temperature, solvent and particle size.

4.5 Conclusions

In this study, *C. orbiculata, H. depressa* and *N. glauca* have appeared to be non-toxic, and can therefore be good candidates for development of alternative anthelmintic and anticancer treatment regimes for livestock and humans, however, it is advisable that the plant extracts are taken through further cytotoxicity studies of other cell lines as well as *in vivo* tests using mammalian models**.**

Chapter 5

Qualitative phytochemical analysis of *Cotyledon orbiculata, Hermannia depressa* and *Nicotiana glauca*

5.1 INTRODUCTION

From early ages, various plants have been used for different folkloric purposes and are known to relieve and heal some diseases including cough, headache, fever and stomach gramps, however, in other cases a total relief might not be observed but at least reduction of aggressiveness. Medicinal plants are used because of their biologically active substances also termed the biological constituents (Hornok 1992). Biological constituents include secondary metabolites which are compounds produced, triggered or originally synthesized by plants. Unlike primary metabolites which are responsible for cell division and growth of the cells, secondary metabolites are most associated with defence mechanism by plants against adaptation to severe climatic conditions, stressful situations and protection against predation by either snail, insects or even herbivores due to the fact that plants are sessile. Phenolics, steroids and alkaloids are considered the large molecule families with phenolics being involved in lignin synthesis (Bourgaud *et al.* 2001; Wink 2003).

Medicinal plants are natural and can be cultivated wherever possible, accessible, affordable, not synthetic therefore they are environmentally friendly and are also used for cultural importance (Pessoa *et al.* 2002; Umadevi *et al.* 2013). Medicinal plants also provide a potential of manufacture of important new pharmaceutical substances. According to Eguale *et al.* (2011) farmers started using the ethnoveterinary medicine as an alternative and a suitable way of treating various infections when they could not get access to the commercial drugs due to their high cost. There are other alternative way of treating and reducing resistance of the parasitic gastrointestinal nematodes which includes breeding the resistant livestock and improving livestock feed quality (Diehl *et al.* 2004). While, Adeomola & Eloff (2011) suggested prevention, control and treatment. However, there are obstacles to these suggestions, nematodes have their way of adapting to a new environment and then actually infect the resistant breed livestock, which might have cost a fortune. It is quite hard to predict the infected grazing areas in order to prevent the infection for

these are microscopic parasites. Similarly treating hectares of land with anthelmintic drugs would be a huge waste of time and money.

Discovery of new pharmaceutical agents from medicinal plants can combat many drastic diseases more especially in rural areas or even developing countries as South Africa (Aremu *et al.* 2010). The potential activities of plants are associated with the presence of secondary metabolites but small amounts of medicinal plants have been investigated of biological and pharmacological properties since most are used by non-scientific herbalists. Due to escalating dependency on plant derived drugs factors such as biological constituents or phytochemical analysis should be taken into account (Lachumy *et al.* 2010; Tiwari *et al.* 2011). Behind every medicinal activity proclaimed by a single plant there is always one or more biological constituents responsible for the potential, may it be antibiotic, antifungal, antiviral or even anti-germinative.

Three plants tested in this study are *Cotyledon orbiculata, Hermannia depressa* and *Nicotiana glauca.* These plants were earlier evaluated for anthelmintic and anticancer activities as reported in chapters 2 and 3, respectively. At different concentrations the three plant extracts displayed the tested activity. *Cotyledon orbiculata* from family crassulaceae has traditionally been used to treat and remove warts and corns as well as local treatment of ruptured skin. Warmed liquid extracted from the plant can be applied in infected ears or painful tooth, treatment of epilepsy and also applied as an ornament. Documented biological constituents of this species are novel bufadienolide glucosides and tyledoside D (van Wyk & Winter 1995). *Hermannia depressa,* family malvaceae is a highly ornamental species. According to Mizrachi *et al.* (2000) these plants have the horticultural potential and medicinally range from respiratory diseases, coughs, and internal aches to soothing of wounds but are undervalued by being classified by people as garden plants. *Nicotiana glauca,* family solanaceae are highly insecticidal plants and ornaments used as treatment to ameliorate prehepatic jaundice, burns and inflammatory diseases. However due to the presence of anabasine *N. glauca* are known to be toxic (Janakat & Al-Merie 2002; Furer *et al.* 2011). The main aim of this study was to determine the presence of documented anthelmintic and anticancer constituents of *C. orbiculata, H. depressa* and *N. glauca* which might be responsible for the anthelmintic and anticancer activities observed in chapter 2 and 3.

5.2 MATERIALS AND METHODS

Biological constituents tested in this study were documented by Tiwari *et al.* (2011) as possessing anthelmintic and anticancer activities, namely tannins, saponins, phenol and polyphenol as well as the alkaloids. Phytochemical analysis examination was performed on water and acetone extracts because during the anthelmintic and anticancer studies in chapter 2 only extracts from these two solvents were tested against parasitic gastrointestinal nematodes.

5.2.1 *Plant collection sites*

Plant materials were collected in February 2013 from multiple populations of *C. orbiculata* (Plate 1.1)*, H. depressa* (Plate 1.2) around Qwaqwa area in Maluti-A-Phofung Municipality of the eastern Free State Province (28°32'0"S and 28°49'0" E; altitude 1, 673 m). The mean annual rainfall of the Maluti-A-Phofung Municipality is about 653 mm per annum with temperatures ranging from as low as 0.1ºC during July nights, 14.2°C during the day in June, and maximum of 24.7ºC during the day in January (Wolfram 2012). *N. glauca* (Plate 1.3) was also collected in February 2013 from Wolmaranstad in Maquassi Hills Local Municipality of the North West Province (27°12'0"S and 25°58'0"E; altitude of 1170 m) and Kroonstad (27.6500° S and 27.2333° E; attitude 1399 m) of South Africa. The mean annual rainfall of the Maquassi Hills Local Municipality is about 391 mm per annum with temperatures ranging from as low as 0ºC during July nights, 17.6°C during the day in June, and maximum of 30ºC during the day in January (Wolfram 2012).

5.2.2 *Plant extract preparations*

The shoots of the plants were separated and dried in an Ecortherm oven (Laboratory Consumables Pty, South Africa) at a temperature of 42°C to a constant weight before it was pulverized. Ten grams each of powdered material was extracted in acetone and distilled water. Acetone was of high analytical grade (Merck Chemicals Pty, Wadeville, South Africa). All extracts were filtered using Whatman No-1 filter paper (Whatman, United Kingdom). The filtrates from acetone were concentrated under reduced pressure 40ºC using rotary evaporator (Cole-Parmer, Laboratory

Consumables and Chemical Supplies Co.Ltd, China). The water extract was freeze dried using freeze dryer (Virtis SP Scientific, United States of America).

Different methods were carried out as documented by Tiwari *et al.* (2011) and Govindasamy & Srinivasan (2012).

5.2.2.1 *Detection of alkaloids*

Extracts were dissolved individually in dilute hydrochloric acid and filtered then followed by the Wagner's test. Filtrates were treated with iodine on potassium iodide, a formation of brown/reddish precipitate indicated the presence of alkaloids.

5.2.2.2 *Detection of saponins*

Froth test: extracts were diluted with distilled water to 20 ml and shaken in a graduated cylinder for 15 minutes. Formation of 1 cm layer of foam indicated the presence of saponins.

5.2.2.3 *Detection of tannins*

Gelatin test: 1% gelatin solution was prepared by adding 5 g of gelatine powder into 500 ml of endotoxin free (Milli-Q) water, autoclaved for 30 minutes and stored in a 4°C refrigerator until use. The 1% gelatine solution containing sodium chloride was added into the extract. Formation of white precipitate indicated the presence of tannins.

5.2.2.4 *Detection of phenols*

Ferric Chloride test: extracts were treated with 4 drops of ferric chloride solution. Formation of bluish black colour indicated the presence of phenols.

5.3 RESULTS

Table 5.1. Phytochemical compounds present in the water and acetone extracts of the shoots of *C. orbiculata.*

Compounds	Water extracts	Acetone extracts
Alkaloids	-	-
Saponins	+	+
Tannins	+	+
Phenols	+	-

+ : Presence of a tested constituent; – : absence of the constituent

Table 5.2. Phytochemical compounds present in the water and acetone extracts of the shoots of *H. depressa*.

Compounds	Water extracts	Acetone extracts
Alkaloids	-	-
Saponins	+	+
Tannins	+	+
Phenols	+	-

+ : Presence of a tested constituent; – : absence of the constituent

Table 5.3. Phytochemical compounds present in the water and acetone extracts of the shoots of *N. glauca.*

Compounds	Water extracts	Acetone extracts
Alkaloids	-	-
Saponins	+	-
Tannins	-	-
Phenols	+	-

+ : Presence of a tested constituent; – : absence of the constituent

Water extracts of all plants possessed more anthelmintic constituents than the acetone extracts. All water extracts had at least the saponins, tannins and phenols, except for *N. glauca* which did not contain tannins.

5.4 DISCUSSION

The objective of this study was to document anthelmintic and anticancer medicinal components in three plants namely *C. orbiculata, H. depressa* and *N. glauca*. Presence of anthelmintic medicinal components tested amongst others as stated by Govindasamy & Srinivasan (2012) were alkaloids, saponins, phenols and tannins. According to Tiwari *et al.* (2011) amongst the four tested components for anthelmintic activity, only saponins possess the anticancer activity. Other constituents such as phenolic compounds and tannins can dissolve in solvents of wide range polarity and therefore contain major components in destruction of unwanted cells, however, these compounds should be combined together in order to deliver best cancer treatment (Nassr-Allah 2009; Sharma *et al.* 2009). The evaluated plants have been documented with anthelmintic activity against parasitic gastrointestinal nematodes of sheep including *Haemonchus, Oesophagostomum, Strongyloides* and *Trichuris* species. Water and acetone were the only solvents used for extract preparations. Different solvents were utilised because constituents of different polarity cannot be extracted from a single solvent such as water (Eguale *et al.* 2011).

Components observed from the water extracts in this study were the saponins, phenolic components and tannins and no alkaloids for all plants. Reid *et al.* (2005); Amabeoku *et al.* (2007) and Mhinana *et al.* (2010) studies showed that *C. orbiculata* contains saponins, cardiac glycosides, tannins, reducing sugars and triterpene steroids while *H. depressa* contains tannins and less than 1% cardiac glycosides and *N. glauca* contains the most toxic constituents as nicotine and anabasine. The outcomes of this study suspecting the anthelmintic and anticancer activities from medicinal plants containing saponins, phenolic compounds and tannins is supported from literature (Wang *et al.* 2010; Ali *et al.* 2011; Hussain *et al.* 2011). Saponins, phenolic compounds and tannins have already been documented with antiflammatory, anthelmintic, analgesic, anticancer and antidiabetic activities. Saponins are a large family of structurally related heterosides compounds of steroid linked to one or more oligosaccharide moieties by glycosidic linkages. According to Ali *et al.* (2011) the parasiticidal activity observed on their study was comparable to the anthelmintic commercial drug albendazole at concentrations of 10 and 20 mg/ml, however at 40 mg/ml saponins showed anthelmintic activity better than that of the

commercial drug by 1.32 times and it was therefore stated that saponins possess excellent anthelmintic activity. The anticancer and/or antitumor activity of saponins results from the cell cycle arrest, apoptosis and the ability to bind onto either the cell or mitochondrial membrane resulting in membrane permeability and pore formation of the cell (Man *et al.* 2010; Kaskiw *et al.* 2009; Yan *et al.* 2009). From observed results in Chapter 2, *N. glauca* water extracts unlike *C. orbiculata* and *H. depressa* water extracts needed a prolonged time to lead to a total death of the experimental larvae. It has been observed from the current study that *N. glauca* water extracts possesses no saponins while *C. orbiculata* and *H. depressa* water extracts possesses no alkaloids. Wang *et al.* (2010) stated that saponins activity results from the ability of their membrane permiabilizing property and pore formation on the parasites which has also been observed from anthelmintic commercial drugs as praziquantel and toltrazuril.

Hossain *et al.* (2012) stated that phenolic compounds contain anthelmintic activity since phenols are shown to interfere with energy generation in parasitic worms by uncoupling the oxidative phosphorylation which is the process to generate energy in organisms, these processes have been documented from the phenolic synthentic anthelmintics including niclosamide, oxyclozanide and bithionol. Chemical tannins demonstrated to possess anthelmintic activity. Tannins are in most cases associated with plant defence mechanism against insects and herbivores preying on the plants, tannins at the far end result in detrimental effects on ruminants but with benefits as well such as wool growth, weight gain, milk secretion and decrease in parasitism (Ramirez-Restrepo *et al.* 2004). Literature has have demonstrated that tannins have a direct inhibitory activity against *in vitro* L_1 and L_3 larval stages of sheep gastrointestinal nematodes *Haemonchus* and *Trichostrongylus columbriformis*. The anthelmintic ability of tannins is brought about by the potential to bind to free protein and form complex with either the protein or any other structure of the parasite in the gastrointestinal, digestive or reproductive tract of host animal or even on the glycoprotein making up the cuticle and eventually cause death of parasites (Athanasiadou *et al.* 2001; Iqbal *et al.* 2010; Alonso-Diaz *et al.* 2011; Hussain *et al.* 2011). Tannins therefore can decrease female worm production by resulting in cuticular lesions and improving the immune response (Ramirez-Restrepo *et al.* 2004).

The type and level of biological activity exhibited by any plant or material depends on various factors including the tested plant part, geographical sources, soil conditions, harvest period, moisture content, drying methods implemented, storage conditions as well as the post-harvest processing. While on that aspect it has been documented that higher temperatures generated during tissue grinding can lead the denaturation of the chemical constituents, whereas, low temperature cannot activate certain constituents. Biological constituents are produced and function best at an optimum temperature and pH (Wendakoon et al. 2012). Solvents utilized to prepare crude extracts can as well affect the level and composition of the secondary metabolites extracted from tissues. Konate *et al.* (2011) reported that acetone is the best solvent in dissolving and extracting phenolic compounds whereas water has been reported by Malvankar (2012) as best in dissolving alkaloids, tannins, phenols, flavonoids, steroids, lignin and saponins. Due to the variation in composition in actively compound composition, different plant types may require different solvent concentration to achieve maximum extraction of bioactive components.

Acetone extracts from the current study showed the presence of saponins and tannins. The *C. orbiculata* and *H. depressa* acetone extracts showed no presence of phenols and alkaloids, whilst *N. glauca* had none of the tested constituents which might be the reason for a prolonged time it took to lead to a total death of all the larvae in larval mortality assay. In chapter 3, *N. glauca* was the only plant that possessed anticancer activity against both MCF-7 and HeLa cancer cell lines. Both water and acetone extracts of *N. glauca* were effective on the cancer cells even though water extracts possessed no tannins and acetone extract possessed none of the tested constituents. The general alkaloid test indicated the absence of alkaloid, however, *N. glauca* has been documented with toxic nicotine and anabasine which are currently still suspected for the anthelmintic and best anticancer activity observed from this plant (Mhinana *et al.* 2010). Nicotine is the main alkaloid of tobacco plants from genus *Nicotiana* and it is used in respiration assistance lower concentrations but can lead to respiratory problems in higher concentrations. The constituent is suspected to have provided the anthelmintic activity documented of *N. glauca* for it has earlier been documented with ectoparasiticidal and anthelmintic properties (Iqbal *et al.* 2006; Hadaruga *et al.* 2010). Anabasine is a pyridine alkaloid which might not have been detected since the test was not specifically on pyridine alkaloid.

Anabasine possess insecticidal and antimicrobial properties as stated by Baikenova *et al.* (2004). All in all water extracts possessed many constituents than did the acetone extracts which might explain the better activity of water extracts as compared to acetone extracts.

5.5 CONCLUSIONS

Medicinal plants have demonstrated to provide necessary metabolites or compounds in treatment of different numerous infections. In this study, saponins, tannins and phenols were present in *C. orbiculata, H. depressa* and *N. glauca*. These compounds are known worldwide for their effectiveness on infestations and infections. Clearly these compounds explain the anthelmintic and anticancer and/or antitumor effects of experimental plant extracts observed in chapters 2 and 3. Therefore, it is only reasonable to diverge onto the medicinal plant usages instead of expensive, inaccessible commercial drugs (Eguale *et al.* 2007; Hadaruga *et al.* 2010; Molefe *et al.* 2012).

Chapter 6

Anthelmintic activity of potassium permanganate on the parasitic gastrointestinal nematodes and cytotoxicity analysis on the MDBK cell lines and brine shrimps

6.1 INTRODUCTION

Gastrointestinal nematodes are a class of parasitic worms of economic importance due to resulting clinical symptoms on their host which include anaemia, loss of condition, tissue damage and diarrhoea. Genera such as *Haemonchus, Oesophagostomum, Trichostrongylus* and *Trichuris* are the most prevalent and the most problematic because they lead to a decreased productivity rate of all the affected countries because infected sheep will produce less meat and wool which negatively affect the economic status of the country. Re-infection is a common process with parasitic gastrointestinal nematodes whereby a single host shall be infected for the second, third and so forth by a single species of infection, leading to economic loss, hence most farmers in the *Haemonchus* endemic areas were forced to leave the industry (Waller 1997; Tsotetsi & Mbati 2003; Molefe *et al.* 2012).

Many industries rely more on commercial anthelmintic drugs which have effectively been used in treating the infections, however, their effectiveness is now failing due to the emergence of resistant strains of the nematodes (Wanyangu *et al.* 1996). According to Holden-Dye & Walker (2007) there are about seven classes of anthelmintic drugs namely Piperazine, Benzimidazole, Levamisole, Paraherquamide, Ivermectin, Emodepside and Nitazoxamide. Each discovery of anthelmintic group comes with a failure of the previously used drug, the first discovery of the group Piperazine was in 1950 which was followed by manufacture of Benzimidazole in 1961 and so forth until the recent manufacturing of the anthelmintic group monepantel (Epe & Kaminsky 2013) registered in 2009. This group is considered highly effective on the parasitic gastrointestinal nematodes, Kaminsky et al (2011) reported 100% egg hatch inhibition and 99.9% worm burden reduction of both *Haemonchus* and *Trichostrongylus* species of the group monepantel. Drug efficacy, effectivity, safety and cost have always been the driving force behind the usage of all the groups as most farmers with poor socio-economic status would do. Efficacy is the determination of the drug effectivity to reduce or kill parasitic pathogens in a

maintained or manipulated area such as *in vitro* environments (Albonico 2003; Holden-Dye & Walker 2007).

Drug resistance is a problem that is quite difficult to conquer on a daily basis due to the difficulty of introducing non-chemical mode of controlling the parasites (Sangster 1999). Warmer and sheep-producing countries are at a high risk of infections because the nematodes are well suited in warm and humid countries and therefore resistance has been much documented from such places due to frequent need of anthelmintic usage. According to Kaplan & Vidyashankar (2012) multiple-resistant parasites are highly prevalent in most parts of the world. Drug resistance has been documented to develop in various ways and sometimes inherited. Newly produced anthelmintics are initially much effective on the parasites, however, during the treatment few of the nematodes survive as the resistant portion which in time shall contribute and produce many resistant strains in the following generations and eventually the susceptible genes will gradually get diluted then lost through time (Coles 1999; Papadopoulos 2008). Many parasitic nematodes of veterinary importance have genetic features which in most cases have the potential to favour the development of resistance by responding successfully to chemical attacks from provided drugs and dissemination of resistant genes through a single host to the other (Sangster 1999; Papadopoulos 2008).

Potassium permanganate is a chemical substance containing potassium, manganese as well as oxygen with a chemical formula of $KMnO_4$. It is an oxidising agent widely used in aquaculture. Potassium permanganate has been used for various purposes mainly for control of pathogens in aquaculture such as fungi, bacteria, algae and parasites (Franca *et al.* 2011). This chemical is currently used in municipal water treatment in removing manganese, hydrogen sulphite as well as in control of algal growth. Algae in drinking water results in bad taste and odour. It has never been easy removing algae; however, potassium permanganate has proven to improve algae removal by coagulation and filtration process (Chen & Yeh 2005). Potassium permanganate is also used as a disinfectant in aquariums and tanks for removal and control of fish parasites such as *Ichthyophthirius multifiliis, Pseudodactylus anguillae* and *P. bini* (Franca *et al.* 2011). It is vital to control external pathogens to combat establishment of most internal or systematic infections as well as limiting antibiotic therapy which at the later stage result in resistant strains

of bacteria, fungi and other parasites (Franci-Floyd & Klinger 2005). According to Thekisoe *et al.* (2003) and Chernos *et al.* (2008) this chemical is already used as prophylaxis, treatment and as an antiseptic for internal and topical use in village poultry system. Other functions of potassium permanganate include chemical manufacture and processing of organic products for the chemical process, field of feeds, livestock breed, pharmaceutical and drinking water industries (Earnest 1974).

The main criteria used in determining the toxicity effects of any drug or chemical are either by *in vitro* or *in vivo* cytotoxicity analysis of the substance. In some instances a substance that contains many different activities such as potassium permanganate have effects on other sides of an organism. Even so, cytotoxicity studies should still be conducted in making sure that this chemical will not affect organisms in a negative way with its wide variety of utilization. So far there is dearth of information of an anthelmintic activity of potassium permanganate as well as its cytotoxicity on mammalian cells. The main aim of the current study was to evaluate the anthelmintic activity of potassium permanganate *in vitro* in comparison to the commonly used anthelmintic drugs namely; Tramisol®, Noromectin® and Valbazen® and the cytotoxicity effects of potassium permanganate on the bovine kidney cell lines in order to obtain scientific data on the potency of this chemical as well as its toxicity status on mammalian cells.

6.2 MATERIALS AND METHODS

6.2.1 Drug dilution preparations

Commercial drugs Tramisol®, Noromectin® and Valbazen® were purchased from Afrivet, South Africa whilst potassium permanganate (Kayo Fine Chemicals, South Africa) was obtained from the Chemistry Department of the University of the Free State, Qwaqwa Campus. Drug dilutions were prepared from the original mass/volume percentage of the drug to 0.5, 1.0 and 1.5 mg/ml for anthelmintic activity, 0.65, 1.25 and 2.50 mg/ml for *in vitro* cytotoxicity assay and lastly 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml for *in vivo* cytotoxicity assays of all the drugs used as positive control including potassium permanganate (Plate 1.4).

6.2.2 Faecal sample collection

Faecal samples were collected directly from the rectum of the sheep. The sheep were placed in a crash pan to stand upright. Faecal samples were collected by inserting two fingers in the rectum and tickled the upper part of the rectum. Faecal pellets were released into the palm of the hand then placed into a sealed collection plastic. Samples were transported to the Parasitology Research Program laboratory, University of the Free State, Qwaqwa Campus, in a cooler box and analysed within 24 hours of collection as recommended by Reinecke 1983.

6.2.3 *Diagnostic methods*

The McMaster technique (Soulby 1982; Reinecke 1983) was used to determine nematode egg presence in this study. A 40% sugar floatation medium was prepared by dissolving 400 g of sugar in a liter of distilled water. Two grams of pooled faecal samples were mixed with 58 ml of 40% sugar solution as a floatation medium. Samples were thoroughly crushed and mixed using a blender. Two chambers of a McMaster slide were filled with a Pasteur pipette. The slides were allowed to stand for about 4 minutes so that the eggs can float on the surface of the flotation medium and lie in contact with the upper glass of the chamber. A light microscope (Nikon Eclipse E100, Japan) was used for egg detection. Indistinguishable eggs coming from different genera such as the *Haemonchus, Trichostronglylus,*

Oesophagostomum and *Chabertia* were grouped together, and recorded separately as strongyle eggs, whilst those from those from those that were easily distinguished such as the *Nematodirus, Strongyloides* and *Trichuris* were reported as such. The *Nematodirus* eggs are distinguished by a thin and colorless shell with a length of 150 µm and a width of 75 µm, *Strongyloides* eggs are broad eclipsed, slightly flattened and embryonated with the presence of L_1 larvae and *Trichuris* eggs have typical polar plugs on both ends. Egg identification was conducted according to the Atlas of Ovine Parasitology (http://issuu.com 2013).

6.2.4 *In vitro* assays

Three in vitro assays (egg hatch, larval development and larval mortality assays) were used to determine the anthelmintic activity of *C. orbiculata, H. depressa* and *N. glauca.* However, before the assays nematode eggs had to be recovered from faecal samples hence the egg recovery assay.

6.2.4.1 Egg recovery assay

Egg recovery was conducted according to Maphosa *et al.* (2010) protocol with some modifications. Four grams of collected faecal sheep pellets were weighed, then water was slowly added to them and the pellets were smashed until a relatively liquid suspension (slurry) was obtained. The slurry was then filtered through sieves of 117, 70 and 25 µm. The contents of 25 µm sieve were backwashed with distilled water and transferred into 60 ml centrifuge tubes. The suspension was allowed to stand for 30 minutes and the supernatant was decanted while sediments suspended in 40% sugar solution. The suspension was transferred into another set of tubes, allowed to stand for another 30 minutes and the supernatant was washed through a 25 µm pore mesh sieve using distilled water. The eggs were then washed off from the 25 µm sieve with distilled water into a 1 litre conical flask where they were allowed to sediment for 2 hours. The concentration of eggs was estimated by counting the number of eggs in 2 aliquots of 200 µl of the suspension in a microscope slide repeatedly, and the mean number of eggs per 200 µl was determined.

6.2.4.2 Egg hatch assay

The egg hatch assay was conducted as published by Bizimenyera *et al.* (2006) and McGaw *et al.* (2007). The number of eggs that were contained in the egg suspension of 200 µl was counted and afterwards they were pipetted into a 96-well microtitre plate. The numbered wells from 1 to 3 were used for the Tramisol® experiments, from 4 to 6 for the Noromectin®, from 7 to 9 for Valbazen® experiments and from 9 to 12 for the potassium permanganate. A volume of 200 µl potassium permanganate was added onto each well at different concentrations of 0.5, 1.0 and 1.5 mg/ml. Distilled water used as a negative control. All tests were repeated 3 times. The plate was incubated at the temperature of 25ºC for 48 hours thereafter a drop of Lugol's iodine solution was added to each well so as to stop further hatching. All unhatched eggs and first-stage larvae (L_1) were then counted.

Inhibition percentages were calculated using a formula by Cala *et al.* (2012).

$$E = \frac{(Eggs + L_1) - L_1}{Eggs + L_1} \text{ X } 100$$

6.2.4.3 Larval development assay

The larval development assay was conducted as described by Bizimenyera *et al.* (2006). The counted number of eggs in a 200 µl of the egg suspension was put into each well in a 96-microtitre plate with a 50 µl of lyophilized penicillin-streptomycin in order to combat fungal growth. The contents of the wells were then mixed, and the plates were placed in an incubator under humidified conditions at ambient temperature for 48 hours. Forty eight hours later, 200 µl of the drugs (Tramisol®, Noromectin ®, Valbazen® and potassium permanganate) at 0.5, 1.0 and 1.5 mg/ml were added to respective plates. The negative control plates received 200 µl of distilled water. All experiments were repeated 3 times. Incubation of the plates was continued for 5 days, after which all the plates were examined to determine the survival of larvae at different concentrations. All the third-stage larvae (L_3) in each well were counted and a percentage inhibition of larval development was calculated using the formula described by Cala *et al.* (2012):

$$E = \frac{(L_1 + L_2 + L_3) - L_3}{L_1 + L_2 + L_3} \text{ X } 100$$

6.2.4.4 Larval mortality assay

The larval mortality assay was conducted according to the method described by McGaw *et al.* (2000) and Zafar *et al.* (2006) and modified by Molefe *et al.* (2012). Briefly, *in vitro* cultures from nematode eggs were prepared after collection from microscopically positive sheep faecal samples. After seven days of incubation, the eggs hatched into larvae. L_3 larvae were harvested from prepared *in vitro* cultures and were transferred into a single petri dish. The 200 µl of L_3 larvae solution were placed in microtitre plate and crude extracts of the same volume were added at different concentrations, with distilled water as a negative control. After the addition of drugs, larval counts were conducted on daily basis and the larval mortality rates were recorded until all the larvae had died. All tests were repeated three times. All live and motile L_3 stage larvae in each well were counted and a percentage inhibition of larval development was calculated using the formula described by Coles *et al.* (1992) and Bizimenyera *et al.* (2006) with slight changes:

$$\text{Inhibition percentage (\%)} = 100(1\text{-}X_2/X_1)$$

Where X_1 is the initial number of larvae in test extracts pre-treatment, and X_2 is the number of larvae obtained post-treatment.

6.2.4.5 Larval identification

Infective larvae were prepared for examination by adding a drop of diluted Lugol's iodine solution to a drop of larval suspension on a glass microscope slide. The iodine solution was diluted to a level where L_3 do not stain darkly before a few minutes have elapsed. The caudal and cranial extremities were taken into much consideration since they are most important for differentiating L_3 of most of the parasitic nematode genera, other features such as the length or shape of the oesophagus are also important in some genera. To facilitate identification, the lengths of the sheath tails of the various species were related according the larval

identification key using the total larvae length, the tip, sheath tail length and the larval filament as illustrated in Fig. 6.1 (van Wyk *et al.* 2004).

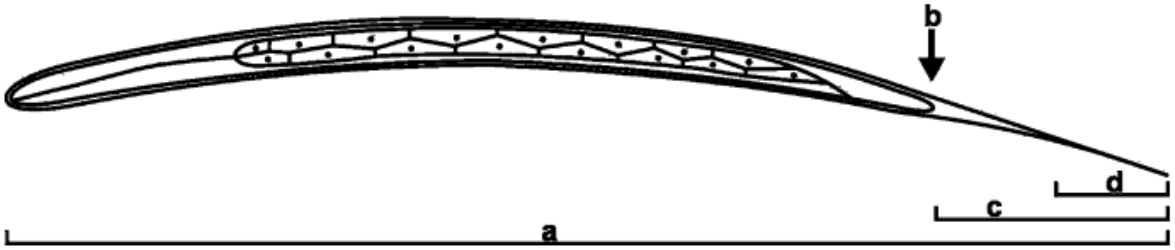

Fig. 6.1. Diagram of nematode infective larvae (van Wyk *et al.* 2004) a: total length; b: tip of larvae tail; c: sheath tail and d: filament.

6.2.5 *In vitro* cytotoxicity tests

6.2.5.1 Cell maintenance and preparation

The MDBK cells were obtained from the American Type Culture Collection (ATCC). The cells were removed from the incubator and the adherence of the cells was observed microscopically. The cells were grown in Dulbecco's modified Eagle's medium (DMEM) and Hams F-12 Nutrient Mixture supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B (Fungizone) in a humidified atmosphere of 5% CO2 at 37 °C. The cells were cultured in 75 cm² cell culture flasks.

The flask was shaken gently on the platform in order to assess adherence and thoroughly sprayed with ethanol before introduced into hood; the medium was discarded into the waste beaker. Cells were rinsed 3 times with PBS to remove all debris and leave cells attached to the surface of the flask. Trypsin/EDTA solution was introduced to the flask to detach the cells and the solution was allowed 3 minutes to work. Carefully the supernatant was discarded into the waste beaker. A 500 µl of FBS was placed into a tube with 50 ml Dulbecco's modified eagle's medium in preparation of 1% medium to suspend the cells for cell counts.

6.2.5.2 Cell counts

A 200 µl trypan blue was added into a 2 ml eppendorf tube containing 800 µl of 1% FBS medium. From the solution a 500 µl was pipetted into an empty tube with an addition of 500 µl of the cell suspension. Then 10 µl of the solution was pipette into two sites of the haemacytometer slide and counted to 20 000 cell/ml.

A 200 µl of the cells were added into a 96 well plate and incubated for 24 hours. Potassium permanganate of concentrations 0.65, 1.25 and 2.50 mg/ml, negative control, 50% DMSO: water except the positive control was added into 24, 48 and 72 hours labelled plates. LDH and MTT assays were conducted on the same plate, beginning with an LDH assay in 24 hour duration.

6.2.5.3 MTT assay

Assessment of cell survival was carried out using the method of Mosmann (1983). At the end of the exposure period, 10 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml in PBS) was added into each well and the cells were further incubated for 4 h. Thereafter, cells were lysed with DMSO for 30 min in order to dissolve the formazan crystals. The formation of colour (formazan) was measured with a microtitre plate spectrophotometer (Bio-Tek µQuant) at 570 nm. Results were analysed with the Graph Pad Prism software (version 4.0). Cell viability was estimated as the percentage absorbance of sample relative to control. Two independent experiments were carried out with three replicate wells for each toxin concentration.

6.2.5.4 *LDH assay*

Cell membrane integrity was evaluated by measuring the lactate dehydrogenase (LDH) enzyme activity using the commercial LDH cytotoxicity detection kit (Roche Diagnostics GmbH). This is a colorimetric assay for quantification of cell death and cell lysis, based on the determination of LDH activity released from the cytoplasm of damaged cells into the medium, thus indicating cell membrane damage. At the end of the exposure period, 100 µL of cell culture medium was removed and transferred into a 96-well plate. An equal volume (100 µL) of the reaction mixture (250 µL of diaphorase/NAD^+ mixture premixed with 11.25 ml of iodotetrazolium chloride/sodium lactate) was added to each well and the plate was incubated for 30 min at room

temperature. The absorbance was then measured with a microtitre plate spectrophotometer (Bio-Tek µQuant) at 490 nm. LDH release was expressed as percentage of the compounds tested relative to control cells. Two independent experiments were carried out with three replicate wells for each toxin concentration.

Total LDH release was calculated using a formula described by Konjevic *et al.* (1997):

$$\frac{LDH_{experimental} - LDH_{effector\ cells} - LDH_{spontaneous}}{LDH_{maximal} - LDH_{spontaneous}} \text{ X } 100$$

Where $LDH_{experimental}$ represents LDH release activity resulting from co-cultures of effector and target cells; $LDH_{effector\ cells}$ represents released LDH activity from separately cultured effector cells, $LDH_{spontaneous}$ represents activity released from cultures of MDBK cells and LDH maximal represents LDH activity released from MDBK cells after lysis.

6.2.5.5 Data analysis

Data was analyzed using the Manh-Whitney U test for comparison between the MTT and the LDH assays. A probability level $P < 0.05$ was considered to indicate statistical significance (Milenkovic 2011).

6.2.6 *In vivo* cytotoxicity tests

6.2.6.1 Hatching of the brine shrimp eggs

The method was conducted as prescribed by Padmaja *et al.* (2002) and Manilal *et al.* (2006). *In vitro* lethality assay of *Artemia salina* (brine shrimp) was used to detect cell toxicity. A spatula tip was filled with eggs and placed into a 100 ml of saline water and left at 24-28^{o}C in front of a contact light. Eggs hatched within 48 hours providing large number of larvae also known as nauplii. About 10 nauplii were counted into vials containing 5 ml of 0.2, 0.4, 0.6, 0.8 and 1.0 mg/ml of potassium permanganate. Pure saline water was used as negative control. Control was made with the same volume of saline water without addition of the any substance. Nauplii were counted in duration of 24 hours until all died and the lethal concentration (LC_{50}) was calculated.

Equation published by the Laboratory of Ecotoxicity and LAC (2013) was used to determine mortality rate. The equation is:

$$Mm_{ct} = \frac{N_{Mm}}{N_o} \text{ X } 100$$

Where:

Mm_{ct} is mortality of individuals in time t (%)

N_{Mm} is average number of dead individuals

N_o is the initial number of living individuals put into every concentration at the start

Lethal concentration (LC_{50})

6.2.6.2 Data analysis

Significant differences between control and experimental groups were assessed by the Student's T-test. A probability level $P < 0.05$ was considered to indicate statistical significance (Saghal *et al.* 2010).

6.3 RESULTS

6.3.1 *Anthelmintic activity*

Potassium permanganate inhibited less percentage of eggs from hatching resulting in 1.82, 6.52 and 4.98% at 0.5, 1.0 and 1.5 mg/ml, respectively (Fig. 6.2). The three commercial drugs showed a significant effect on the egg hatchability from the lowest concentration of 0.5 mg/ml whereby Tramisol® inhibited 93.74, 83.71 and 95.83% of eggs from hatching at 0.5, 1.0 and 1.5 mg/ml, respectively, whereas Noromectin® at similar concentrations inhibited 78.94, 74.77 and 66.27% of eggs from hatching and 94.27, 27.93 and 89.78% eggs were inhibited from hatching by Valbazen®.

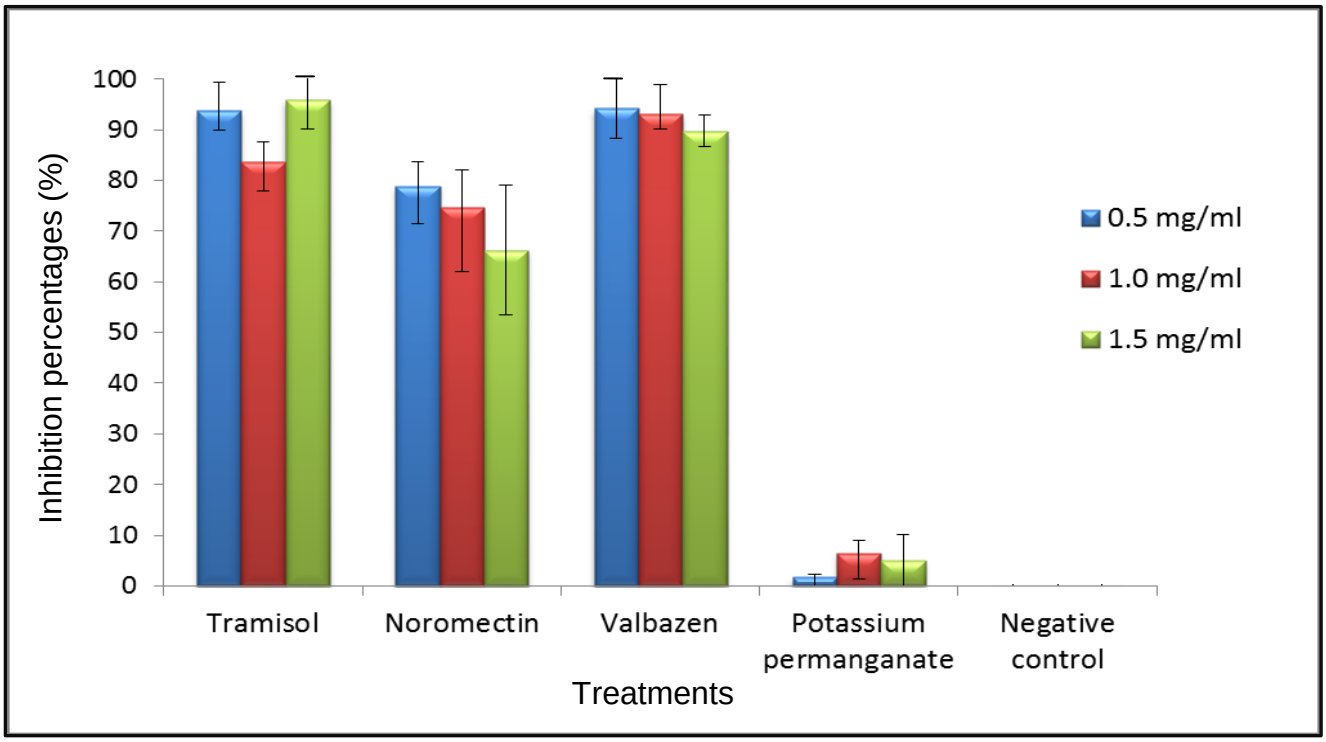

Fig. 6.2. Comparative egg hatch inhibition percentages of commercial anthelmintic drugs and potassium permanganate at various concentrations.

In contrast to egg hatch inhibition, a high percentage of larval development was inhibited by potassium permanganate at various concentrations, a 1.0 mg/ml showed the highest inhibition percentage (98.10%). Amongst the three tested commercial drugs, Tramisol® performed best in terms of larval development inhibition (Fig. 6.3).

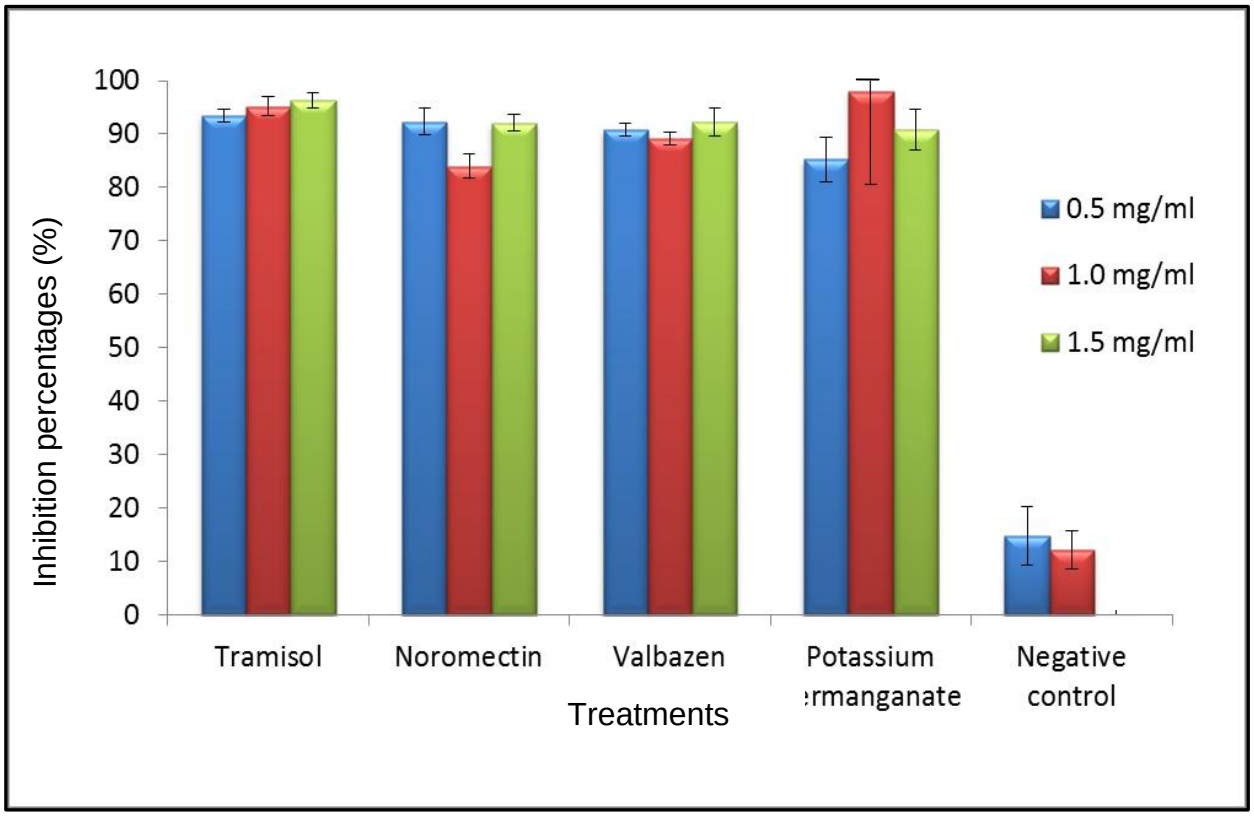

Fig. 6.3. Comparative larval development inhibition percentages of commercial anthelmintic drugs and potassium permanganate at various concentrations.

Time kill of the tested drugs as well as potassium permanganate on the infective nematode larvae was observed and plotted as shown in figure 6.4. Tramisol® was the most effective drug as compared to the other tested chemicals including potassium permanganate, where a total death of experimental larvae was observed within 72 hours at the concentration of 0.5 and 1.5 mg/ml and 144 hours at 1.0 mg/ml (Fig. 6.4a). Figure 6.4d is presenting the results of potassium permanganate, larval mortality as observed within 216, 144 and 144 hours at 0.5, 1.0 and 1.5 mg/ml of treatment concentration, respectively. However, Noromectin® and Valbazen® did not reach any total larval mortality (Fig. 6.4b and 6.4c) where a total death of larvae was observed only with Noromectin® within 216 hours of experimentation at 0.5 mg/ml (Fig. 6.4b). At other concentrations including 1.0 and 1.5 mg/ml of Noromectin® as well as all concentrations tested for Valbazen® did not lead to any total larval mortality until 216 hours was reached which concluded the experiment since all the larvae in potassium permanganate wells had already died off.

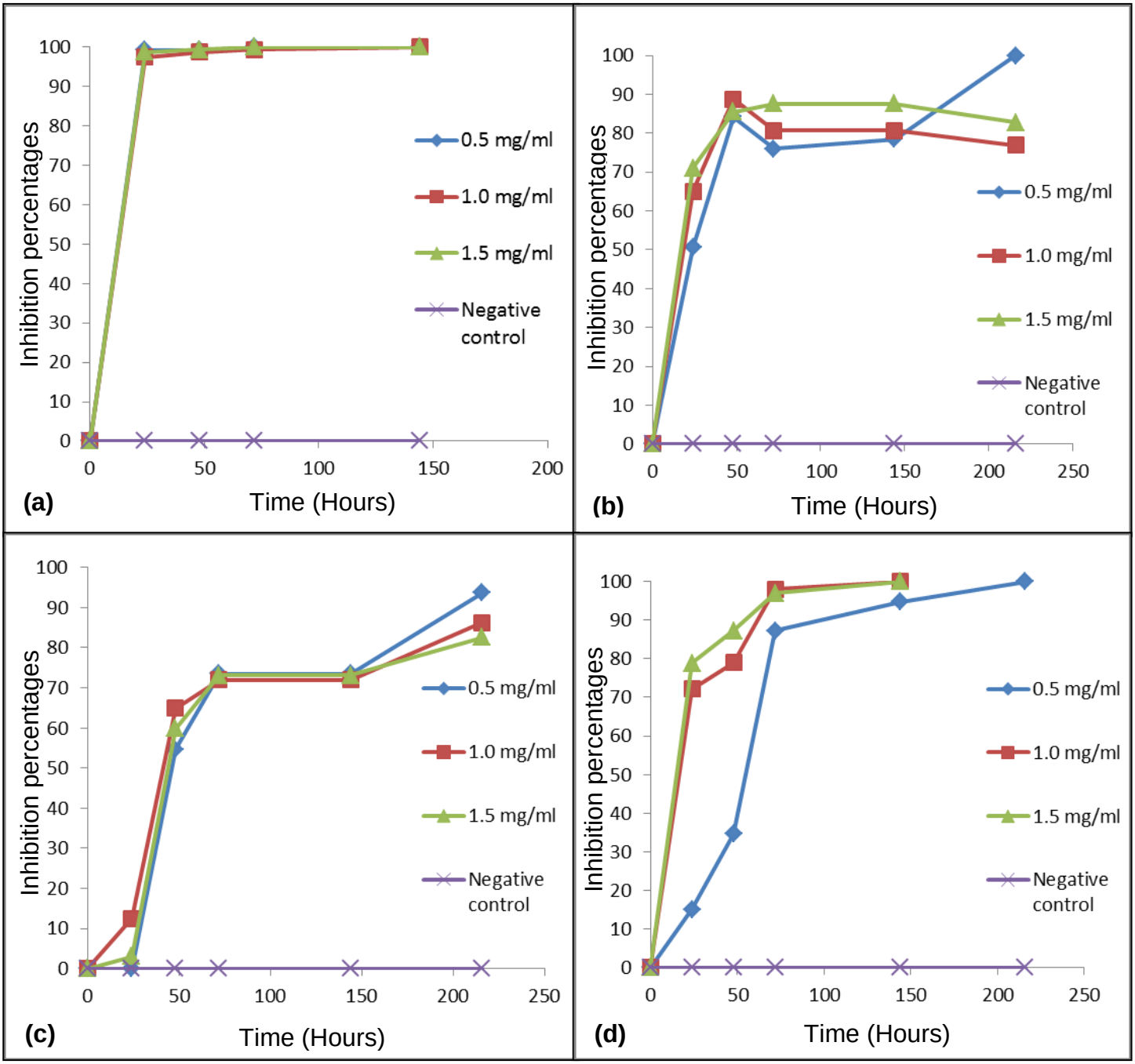

Fig. 6.4. Inhibition percentages of (**a**) Tramisol®, (**b**) Noromectin®, (**c**) Valbazen® and (**d**) Potassium permanganate on larval mortality of gastrointestinal nematodes.

6.3.2 *The in vitro* cytotoxicity *tests*

Cytotoxicity was observed on MDBK cells exposed only to the lowest dilutions of potassium permanganate (0.65 mg/ml) (Fig. 6.5). No statistically significant difference ($p \leq 0.05$) in brine shrimp survival was observed in comparison to the MTT and the LDH assay. A stimulating effect on the growth of MDBK cells, characterized by cell survival of up to 190%, was recorded when the cells were exposed to potassium permanganate at 1.25 and 2.5 mg/ml dilutions. There was no significant release of LDH enzyme (<100%) from MDBK cells when compared to the control (100%) exposed to the 1.25 and 2.50 mg/ml potassium permanganate dilutions (Table 6.1). However, cells exposed to the lowest dilution (0.65 mg/ml) at the 24 h exposure period showed a significant release in LDH enzyme (±143%).

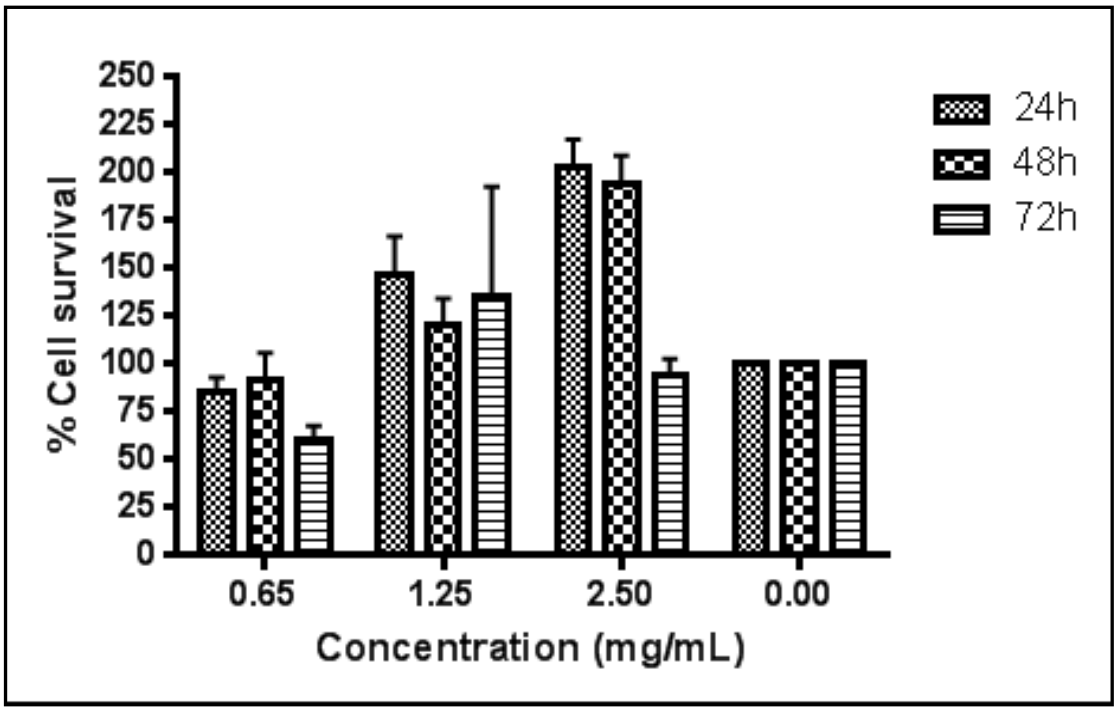

Fig. 6.5. Cytotoxic response of the MDBK cells measured using the MTT assay after exposure for 24, 48 and 72h to potassium permanganate. Results are presented as percentage of control.

Table 6.1. LDH release assay after exposure of MDBK cells to Potassium permanganate for 24, 48 and 72 h.

Concentration (mg/ml)	Potassium permanganate		
	24h	48h	72h
0.65	143±32	<QC	<QC
1.25	<QC	<QC	<QC
2.50	<QC	<QC	<QC

Data presented as percentage of control ±SEM; SEM: standard error mean. LDH values less than that of the control cells (control = 100%) are designated as <QC.

6.3.3 *In vivo cytotoxicity tests*

Potassium permanganate was extremely toxic to the brine shrimp larvae at all concentrations resulting in a total death of all the larvae within 24 hours of experiment as compared to negative control that initially led to 30% death of the larvae followed by 70% and 100 at 24, 48 and 72 hours.

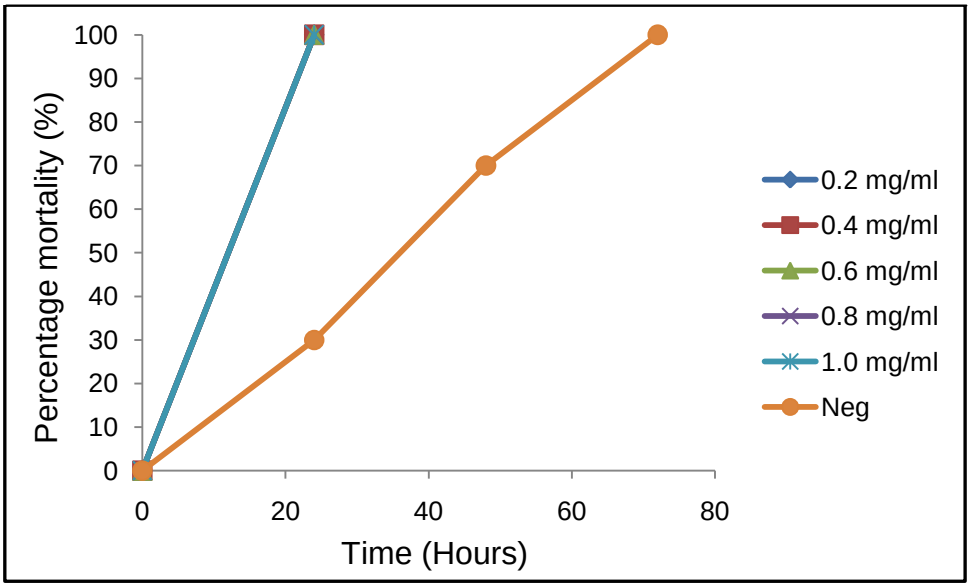

Fig. 6.6. Potassium permanganate lethality percentages on brine shrimp.

6.4 DISCUSSION

The aim of this study was to determine anthelmintic activity and cytotoxicity effects of potassium permanganate in comparison with Tramisol®, Noromectin® and Valbazen® representing anthelmintic groups namely levamisole, ivermectin and albendazole respectively on the parasitic gastrointestinal nematodes, brine shrimps and the bovine kidney cells. Parasitic gastrointestinal nematode egg is the stage disseminated into the environment and is therefore the most protected stage in the whole life cycle which can survive for decades regardless of variation in environmental conditions (Molefe *et al.* 2012). Less significant percentages were observed with potassium permanganate where the egg inhibition percentages ranged from 1-7% whereas the 3 tested anthelmintic drugs Tramisol®, Noromectin® and Valbazen® inhibited the egg hatchability at the range of 83-95%, 66-79% and 89-94%, respectively. Failure to inhibit egg hatchability by $KMnO_4$ might be due to the hydrophobic nature of the egg shell as well as the ability to withstand various climatic conditions (Gill *et al.*, 1995; Salih, 2003; Hounzangbe- Adote, 2005). Parasitic gastrointestinal nematode egg is the stage disseminated into the environment and is therefore the most protected stage in the whole life cycle which can survive for decades regardless of variation in environmental conditions (Salih, 2003).

The $KMnO_4$ inhibited 85-98% of larvae from developing at all concentrations as compared to distilled water which has 85% of larvae developing to the infective stage. In larval development assay the inhibition percentages ranged from 93-96%, 84-92% and 89-92% with Tramisol®, Noromectin® and Valbazen®, respectively. These are significant inhibition percentages for tested commercial anthelmintic drugs. The first (L_1) and the second (L_2) staged larvae are most sensitive to drugs because they are the feeding stages of the nematodes and are therefore easily affected with whatever components applied on them. During the experiments, extracts are applied on the larvae for a longer duration than on the eggs because it takes 5 days for the L_1 larvae to develop onto the L_3 while eggs take 2 days to hatch into L_1 larvae (Cala *et al.*, 2012; Molefe *et al.*, 2012) which explains the highest larval mortality percentages obtained in the current study.

Potassium permanganate treated larvae died off at 144, 144 and 216 hours of experimentation at 0.5, 1.0 and 1.5 mg/ml, respsctively. No larval mortality was observed using Noromectin® and Valbazen® at a given time of the experiment; 17-24% and 6-18% of experimental larvae did not die within the experimental duration, respectively. Despite the ability of nematode larvae to rapidly adapt and survive in various environment (Bizimenyera *et al.*, 2006), levamisole was the most effective drug in the study whereby all the larvae died at an anticipated duration of 24 hours at all concentrations. Wanyangu *et al.*, (1996) stated that due to levamisole effectivity sheep require less dosages of levamisole than any other species and levamisole was indeed the most effective group tested in the current study. In most cases drug effectivity is reduced if the animals which samples were collected from have previously been treated with the drug of the specific anthelmintic group. Ivermectin resistance is documented with an increasing frequency in sheep flock throughout the world (Eddi *et al.*, 1996). Albendazole has the ability to paralyze nematodes within 8.9 minutes and death usually occurred within 28.87 minutes at 20 mg/ml (Aleemuddin *et al.*, 2012) which is in contrast to the data obtained in the current study, this shows resistance at less concentrations of the drug. According to Franca *et al.*, (2011) potassium permanganate has the ability to remove parasites from the intestinal tract and detoxify poisons and assist in pharmaceutical productions. Potassium permanganate showed to possess larvicidal activity as compared to less than 10% ovicidal activity observed.

Infective (L_3) larval identification was conducted to identify the possible genera that were dealt with in the study. Genera obtained included *Haemonchus, Oesophastomum* and *Charbetia.* Literature states that the most problematic parasitic gastrointestinal nematodes are the trichostrongyloides nematodes including *Haemonchus contortus, Trichostrongylus* and *Ostertagia* species. The infective larvae have the ability to withstand and adapt to different conditions within no times which might result in resistance in most cases. A decrease in the effectiveness of the anthelmintic groups used in the study has widely been reported (van Wyk *et al.*, 2004). Levamisole and benzimidazole are the much widespread and have readily been available for three decades, this makes them the most frequently used anthelmintics which should not be of any surprise if they were not effective on the identified genus of nematodes (Wanyangu *et al.*, 1996; Sangster and Gill, 1999).

Ivermectin on the other hand has the potential of inhibiting the motility, development of the free living stages and inhibit the development of *T. colubriformis* L_4 staged larvae to the young adults but it does not cause any lethal or acute effects on the nematodes unless higher concentrations are tested (Gill *et al.*, 1995).

Potassium permanganate was observed to be toxic for MDBK cell lines at the lowest concentration of 0.65 mg/ml even though the toxicity was time dependent as observed using the MTT assay, however, there was high emission of LDH at 0.65 mg/ml, 24 hours which was the least toxic but less LDH was released at 48 and 72 hours which was the most toxic. At other concentrations both assays provided expected results, the higher the cell survival rate there lesser LDH release. According to Lobner (2000) and Fotakis & Timbrell (2006) the LDH release assay is used in assessing the loss of membrane integrity due to the release of enzyme LDH into the bathing medium while the MTT assay assesses the cell survival. Expectation is the lesser the cell survival percentages, the higher the LDH release, however, this was not the case whereby there less cells survived at 48 and 72 hours of 0.65 mg/ml with less than a 100% LDH release. It has been reported that different assays can provide different results depending on the test agents used and the assay employed that is why it is advisable to repeat the assays, use different concentrations at various durations to avoid underestimations and/or overestimations of cytotoxicity (Fotakis and Timbrell, 2006). According to Sahgal *et al.*, (2010) substances with high amount of bioactive components are likely to possess cytotoxicity effects. Potassium permanganate appeared to stimulate cell growth with an MTT assay at higher concentrations with lesser LDH released. $KMnO_4$ is a widely used chemical as prophylaxis and in treatment of some livestock infections by communal farmers (Thekisoe *et al.*, 2003). Potassium permanganate was highly toxic to the brine shrimps whereby all the nauplii died within 24 hours at all concentrations as compared to the negative control utilised. Potassium permanganate is used in purification of water and pharmaceutical industries which might be killing all microorganisms, brine shrimps included (Franca *et al.*, 2011). Previous study has shown that potassium permanganate prepared at lower concentrations can cause damage to delicate tissues of some organisms such as the skin and should be prepared along with formaldehyde (Chernos *et al.*, 2009; Francis-Floyd and Klinger,

2002). Interestingly, in this sudy has been demonstrated by cytotoxicity observed at the lowest concentration tested on the MDBK cell line.

6.5 CONCLUSIONS

The current study revealed that potassium permanganate is effective for treatment of parasitic gastrointestinal nematode larvae, the pre-infective as well as the infective stage of the larvae. The mechanisms of its effectiveness still remain to be elucidated. This study has further revealed that known commercial drugs (Tramisol®, Noromectin® and Valbazen®) are potent ovicidal agents against nematode eggs, while potassium permanganate was very effective against larval development and larval mortality. It is therefore recommended that potassium permanganate should be administered simultaneously with the standard drugs in order to effectively manage nematode infection on livestock.

CHAPTER 7

General discussion, conclusions and recommendations

The general objective of this research project was to determine the anthelmintic effects on parasitic gastrointestinal nematode and anticancer effects on MCF-7 and HeLa cancer cells of *C. orbiculata, H. depressa, N. glauca* and potassium permanganate. Gastrointestinal nematodes are parasitic worms infecting both humans and animals, however, in the current study the focus was on parasitic gastrointestinal nematode infecting small stock. Parasitic gastrointestinal nematodes totally depend on their hosts for nutrition, protection and nourishment (Tsotetsi & Mbati, 2003). Infections result in changing the host's health due to clinical symptoms posed on the hosts by the nematodes. These infections negatively affect the economic status of most countries more especially sheep producing countries (Kamaraj & Rahuman 2010, Eguale *et al.* 2011). Cancer is on the other hand an abnormal cell proliferation resulting from a single cell that undergone DNA mutation. Cancer develops as more and more cells become mutated which has the ability to divide quicker and at high rates than normal cells (Merina *et al.* 2012). There are many types of cancer; however the current study focused only on breast and cervical cancer. These cancer types are of economic importance due to the fact that they lead to high mortality rate factors of women, even though mortality rates are still expected rise in the coming decades (Barr & Sings 2008; Hasima & Aggarwal 2012).

Anthelmintic and anticancer commercial drugs have widely been used in treatment of the disease however, these drugs are accompanied by severe effects, they are expensive, inaccessible and above all nematode worms and cancer cells have developed resistance against them (Maphosa *et al.* 2010). There is therefore a call for an alternative mode of treatment of these particularly for resource-poor farmers and countries. Medicinal plants are plants used for medicinal purposes due to their active ingredient content. They are used to relieve, heal or reduce the aggressiveness of the disease (Hornok 1992). Medicinal plants have been effective on various infections by indigenous people. Potassium permanganate is a synthesised chemical used as an ethnoveterinary and aquaculture medicine and prophylaxis (Chanratchakool 1994). It is a widely used chemical for treatment of numerous diseases such as sores and stomach ache in livestock.

Anthelmintic activity of *C. orbiculata, H. depressa* and *N. glauca* was demonstrated on microscopically diagnosed nematode species including *Haemonchus, Oesophagostomum, Trichostrongylus* and/or *Telardosagia* species which were found infecting sheep in Maluti-A-Phofung municipality of the North eastern Free State province of South Africa. These species of nematodes are worms of economic importance due to their effects on infected hosts which result in loss of production (Coles *et al.* 2006). The efficacy of *C. orbiculata, H. depressa* and *N. glauca* can be judged from the inhibition of nematode eggs from hatching, loss of spontaneous movement of the larvae and lastly the complete destruction of the larvae (Tandon *et al.* 1997). Commercial drugs have successfully been used globally in treatment of the infections. However due to adaptation and concepts such as radiation, resistance has been observed in most cases resulting from the continuous use and administration of these drugs (Leto *et al.* 2012). The most disadvantageous effects of commercial drugs is the association with severe side effects on the host including amongst all liver, kidney damage, bone marrow suppression, carcinogenesis and disordered foetal development. It has been documented that it is difficult to discover and develop chemicals that can effectively treat the infections without any undesirable effects (El-Rahan *et al.* 1999). Most countries which are affected by infections are of poor socio-economic status to afford these expensive, unaffordable and inaccessible drugs, which are only sold at the agricultural offices (Prema *et al.* 2012). On the other hand medicinal plants are much cheaper and highly accessible to most of the populations all over the world. Herbal products extracted from medicinal plants are much preferred because less testing time is required, higher safety levels, efficiency, and cultural acceptability and are associated with lesser side effects. Medicinal plants are believed to have a better compatibility with the host body due to the presence of chemical composition which are believed to be a part of physiological functions of living organisms (Prasad *et al.* 2012).

In the egg hatching assay conducted in the current study, water extracts inhibited higher percentages of eggs from hatching at 7.5 mg/ml concentrations with 82.62, 40.14 and 19.12% of all plants respectively as compared to the acetone extracts.

From the larval development assay, acetone extracts were more effective than water extracts as it has been documented by the study of Adeomola & Eloff (2011). *N. glauca* extracts induced 100% larval development inhibition in both water and

acetone extracts. The *C. orbiculata* and *H. depressa* plants water extracts at 2.5 mg/ml resulted in 85.32 and 44.12% larval development inhibition, however, acetone extracts of both plants induced a 100% inhibition. The pre-infective staged larvae either L_1 and/or L_2 are the most susceptible stages of nematode development because they are the feeding stages (Ferhat *et al.* 2011). All larvae tested in the larval mortality assay died at different durations in various concentrations of tested plants. Water extracts were more effective on the timed mortality of the nematode larvae than the acetone extracts. Despite concentrations a total death of larvae of plants *C. orbiculata, H. depressa* and *N. glauca* water extracts were observed at 48, 96 and 72 hours of experimentation while acetone extracts larval time kill of the same plants were observed at 120, 120 and 192 hours. The first larval mortality monitoring was performed within six hours post-treatment, within which all the larvae in the positive control, Tramisol® had died.

The plant activity on both larval development and larval mortality assays were demonstrated to be larvicidal following the observed paralysis and a total death of the larvae resulting from treatment with the tested medicinal plants (Kosalge & Fursule 2009). Maphosa *et al.* (2010) stated that the efficacy of plant extracts at the lowest tested concentrations against the gastrointestinal nematodes proves the anthelmintic activity of that plant. The lowest concentration tested in the current study was 2.5 mg/ml. The *C. orbiculata, H. depressa* and *N. glauca* have the ability to inhibit eggs from hatching, larvae from developing and lead to mortality of infective staged larvae and therefore these plants possess anthelmintic activity. Overall, the study revealed that the aqueous extracts of the shoots of all tested plants were more effective than acetone extracts concerning the egg hatching and larval mortality assays.

Anticancer and/or antitumor effects of *C. orbiculata, H. depressa* and *N. glauca* were tested on women cancer cells of economic importance namely the breast (MCF-7) and cervical (HeLa) cancer cell lines. According to Prakash *et al.* (2011) the most reliable criteria of concluding on the influence brought by the plant extracts on the cancer cells is judging according to the prolongation of the life span and by body weight of animals bearing tumors. The experiments were run from 24, 48 to 72 hours at 0.65, 1.25 and 2.50 mg/ml concentrations. Every cell line has specific treatment; it is therefore possible that a single plant might be more effective on one cell line than

the other. In the current study, various extracts were effective on different cell lines as it was demonstrated by Kuete *et al.* (2011) whereby the MCF-7 and HeLa cell lines were tested on many different chemical compounds extracted from 6 Cameroonian medicinal plants, however, both of the cells were more sensitive to xanthone than any of the tested compounds. In the current study there was no anticancer activity observed from both water and acetone extracts of *C. orbiculata* on the breast cancer. The *H. depressa* water extracts anticancer activity against the breast cancer was at the lowest concentrations in the 24 hours duration, however, no activity was observed at 48 hours which was a similar case even with the positive control. Less than 50% activity was detected from acetone extracts at 24 hours at all concentrations as compared to the activity obtained only at 5, 125 and 250 µg/ml in the 48 hour tests. The highest inhibition percentages of the breast cancer were obtained at 48 hours of *N. glauca* water extracts at all concentrations as compared to the negative control and no activity at 24 hours of the same extracts. Acetone extracts at 24 hours possessed anticancer activity only at selected concentrations but none at 48 hours.

The *C. orbiculata* water extracts exhibited anticancer activity against cervical cancer cell line at all concentrations except at 500 and 1000 µg/ml at 24 hours and only less than 50% activity was observed at 48 hours of the same extracts. The highest anticancer activity of all extracts was exhibited in *C. orbiculata* at 24 hours and less than significant activity was observed at 48 hours with the acetone extracts. The *H. depressa* water extracts activity was observed from 75 to 1000 µg/ml at 24 hours with less than significant inhibition percentages at 48 hours and less than 50% inhibition at all concentrations of acetone extracts. The *N. glauca* has proven to be the one candidate with an anticancer activity on both cell lines, similarly, the *N. tabacum* leaves have also been documented with anticancer activity (Umadevi *et al.* 2013). Water extracts at 24 hours inhibited the cell growth at all concentrations while inhibition was observed from 75 to 1000 µg/ml in acetone extracts whereas 24 hours of acetone extracts exhibited anticancer activities from 250 to 1000 µg/ml, with less than 50% inhibition at 48 hours. Curcumin C was used as a positive control and 10% PBS-EDTA medium as negative control. An extract is concluded with anticancer activity due to the observed cell proliferation inhibition, induction of apoptosis and cell growth arrest in any of the cancer cell lines (Hu *et al.* 2013).

In vitro cytotoxicity effects of the plants were tested on the MDBK cells using the LDH and MTT cytotoxicity assays. Elevated LDH levels correspond with a decrease in the cell survival at optimum concentrations (Adiga & Jagetia 1999; Agarwala *et al.* 2009). The LDH release were dose and time dependent on *C. orbiculata* and *N. glauca* extracts therefore showing signs of toxicity at different concentrations. *H. depressa* did not demonstrate any toxic effects on the cells as according to the cell viability results, however, there was a high release of LDH. According to Maulik *et al.* 1997, other plants are high effective of protecting the cells which leads to an increase in cell viability. Other plant extracts need time to release toxic effects which are the reason all cytotoxicity studies should expose normal cells to a prolonged treatment than concluding after 24 hours (Val'ko *et al.* 2007). Brine shrimps were used for *in vivo* toxicity studies of *C. orbiculata, H. depressa* and *N. glauca.* The brine shrimp mortality assay is a widely accepted method of detecting toxicity effects of a plant following very simple and inexpensive criteria.

Plant extracts showing lethal effects on the brine shrimps are likely to possess toxic constituents at low concentrations because brine shrimps are well documented for their ability to withstand extreme conditions (McGaw *et al.* 2007). In all tests the organisms survived for more than 24 hours before dying off. The dying off process began with a decrease in motility of the larvae as they were on a daily basis becoming slower. Hlywka *et al.* (1997) stated that the age of the brine shrimps also influence the organisms' sensitivity towards any toxic substance hence freshly hatched brine shrimp larvae should always be used in such studies. Total larval mortality was observed within 72 and 96 hours in *C. orbiculata* and *H. depressa* both water and acetone extracts, respectively. Both extracts of *N. glauca* showed a total mortality at 120 hours as it was observed at the anthelmintic study of the plant which means that this plant either does not possess any toxicity or possess toxicity at lower levels. Genus *Nicotiana* is widely known to possess toxic effects, however, according to Maulik *et al.* 1997 a single or two species of a widely studied plant might not be toxic at other points. This plant is known to possess nicotine which is known to be highly toxic (Mhinana *et al.* 2010), however, in this study the experimental plant (*N. glauca*) did not prove to be toxic. It might mean that the plant is not toxic to the organism since nicotine has been documented with other bioactivities such as anthelmintic and antimicrobial (Iqbal *et al.* 2006; Hadaruga *et al.* 2010) or that brine

shrimps managed to acclimatize in the extracts quicker than in the other extracts since they can withstand various conditions (Turker & Usta, 2008).

Overall observation from the study is that acetone plant extracts were more toxic than water extracts on the parasitic gastrointestinal nematodes, cancer cells, normal mammalian cells brine shrimps and the. The difference in cytotoxicity of the tested plant extracts on any organisms is based on their chemical composition and relative biologically active substance (Val'ko *et al.* 2007). Acetone as a solvent has the ability to dissolve and extract numerous compounds and secondary metabolites of wide range of polarity and can form a homogenous mixture with different solvents than water. Water is the most common solvent used amongst indigenous people even though it can only dissolve and extract polar compounds therefore fewer compounds are extracted with water (Eguale *et al.* 2007; Wang *et al.* 2011; Ademu *et al.* 2013). Plant composition does not only rely on the solvent used during the plant compounds extractions but many factors such as genetic, environmental and the geographic origin (climate, elevation and topographic), harvest time, drying and extraction methods of a plant should be taken into consideration. Plants growing at different environment and suppressed under different stress tend grow ordinarily at different rates, size and development stages (Pirbalouti & Mahommadi 2013).

Components observed from the water extracts in this study were the saponins, phenolic components and tannins and no alkaloids for all plants. The outcomes of this study suspecting the anthelmintic activity by medicinal plants containing saponins, phenolic compounds and tannins is supported from literature (Wang *et al.* 2010; Ali *et al.* 2011; Hussain *et al.* 2011). Saponins, phenolic compounds and tannins have already been documented with antiflammatory, anthelmintic, analgesic, anticancer and antidiabetic activities. Saponins are a large family of structurally related heterosides compounds of steroid linked to one or more oligosaccharide moieties by glycosidic linkages. According to Ali *et al.* (2011) the parasiticidal activity observed on their study was comparable to the anthelmintic commercial drug albendazole at concentrations of 10 and 20 mg/ml, however at 40 mg/ml saponins showed anthelmintic activity better than that of the commercial drug by 1.32 times and it was therefore stated that saponins possess excellent anthelmintic activity. From observed results in Chapter 2, *N. glauca* unlike *C. orbiculata* and *H. depressa* water extract needed a prolonged time to lead to total death of the experimental

larvae. It has been observed from the current study that *N. glauca* water extracts possesses no saponins while *C. orbiculata* and *H. depressa* water extracts possesses no alkaloids. Wang *et al.* (2010) stated that saponins activity results from their membrane permeabilizing property and pore formation on the parasites which has also been observed from anthelmintic commercial drugs such as praziquantel and toltrazuril.

Acetone extracts from the current study showed the presence of saponins and tannins. The *C. orbiculata* and *H. depressa* acetone extracts showed no presence of phenols and alkaloids, whilst *N. glauca* had none of the tested constituents which might be the reason for a prolonged time it took to lead to a total death of all the larvae in larval mortality assay. The general alkaloid test showed the absence of alkaloid, however, *N. glauca* has been documented with toxic nicotine and anabasine which are currently still suspected for the anthelmintic activity observed from this plant (Mhinana *et al.* 2010). Nicotine is the main alkaloid of tobacco plants from genus *Nicotiana* and it is used in improvement of respiration at lower concentrations but can lead to respiratory problems in higher concentrations. This constituent is suspected to have provided the anthelmintic activity documented from *N. glauca* for it has earlier been documented with ectoparasiticidal and anthelmintic properties (Iqbal *et al.* 2006; Hadaruga *et al.* 2010). Anabasine is stated as a pyridine alkaloid which might not have been detected since the phytochemical test conducted from the current was not specifically on pyridine alkaloid. Anabasine possess insecticidal and antimicrobial properties as stated by Baikenova *et al.* (2004). All in all water extracts possessed many constituents than did the acetone extracts which might explain the better activity of water extracts as compared to acetone extracts. Flavonoids and terpenoids were not tested for in this study, however, literature shows that they are possible constituents containing anthelmintic and anticancer activities (Patel *et al.* 2010).

In chapter 6 the aim of the study was to determine anthelmintic activity and cytotoxicity effects of potassium permanganate in comparison with Tramisol®, Noromectin® and Valbazen® representing anthelmintic groups namely levamisole, ivermectin and albendazole on the parasitic gastrointestinal nematodes, brine shrimps and the bovine kidney cells. Parasitic gastrointestinal nematode egg is the stage disseminated into the environment and is therefore the most protected stage in

the whole life cycle which can survive for decades regardless of variation in environmental conditions (Hounzangbe - Adote 2005; Molefe *et al.* 2012). In the current study the 3 tested anthelmintic drugs Tramisol®, Noromectin® and Valbazen® inhibited the egg hatchability at the range of 83 to 95%, 66 to 79% and 89 to 94%, respectively, whereas less than significant percentages were observed with potassium permanganate where the egg inhibition percentages ranged from 1 to 7% which are lower percentages as compared to the 3 drugs tested. No ovicidal activity was therefore observed in potassium permanganate as compared the negative control. The results obtained from the egg hatch assay showed the high effectivity of the 3 anthelmintic groups. Failer to inhibit egg hatchability by potassium permanganate might be due to the hydrophobic nature of the egg shell as well as the ability to withstand various climatic conditions (Gill *et al.* 1995; Salih 2003; Hounzangbe- Adote 2005).

In larval development assay the inhibition percentages ranged from 93 to 96%, 84 to 92% and 89 to 92% with Tramisol®, Noromectin® and Valbazen®, respectively. These are significant inhibition percentages for tested commercial anthelmintic drugs. However, potassium permanganate inhibited 85 to 98% of larvae from developing at all concentrations as compared to positive and negative controls with only 15% of larvae that did not develop to the infective stage in the negative control. The larvicidal activity of potassium permanganate was comparable to that of the tested standard drugs used in the study, levamisole, ivermectin and albendazole. These standard drugs are effective on broad helminth infections which include the roundworms, hookworms, whipworms and pinworms (Gaikwad *et al.* 2011). The possible reason for the low egg inhibition might be the fact that eggs are protected with thick cuticle which makes them resistant to various environmental conditions. High effectivity of potassium permanganate on the larval development and larval mortality assays could be due to easy transcuticular absorption of it into the larval body (Getachew *et al.* 2012)

Conclusions

The objective of the study was to determine the anthelmintic and anticancer activity of *C. orbiculata, H. depressa, N. glauca* and potassium permanganate; however, toxicity features were also evaluated for live cell safety. Results observed in this study have shown that extracts of *C. orbiculata, H. depressa* and *N. glauca* have the potential to be considered for use as remedies against parasitic gastrointestinal parasite in infections of small stock. Furthermore, these plant extracts have also showed to possess anticancer activity. This means that they can be considered as potential remedies for cancer. Water extracts appeared to be more effective in most experiments. This could be advantageous for resource poor farmers as these plant extracts are cost effective. Plants are environmentally friendly since they need no chemicals to be manufactured, however, other plants cannot be consumed by animals due to their bitterness and sourness. In this case the extracts would need to be mixed with flavourants for oral administration or injection routes should be considered. On the other hand pharmacologist will be aware that certain plants contain anthelmintic and anticancer activity and therefore cheaper and accessible drugs may be manufactured from natural substances. The possible use of the experimental plant extracts as remedy for parasitic infections and cancer is further supported by the fact that these extracts have proved to express very low toxicity or no toxicity at all to mammalian cells.

Potassium permanganate was also tested on the egg hatch, larval development and larval mortality of the parasitic gastrointestinal nematodes in comparison to standard registered commercial anthelmintic drugs, Tramisol®, Noromectin® and Valbazen®. No egg hatch inhibitions were observed at all concentration, however, high inhibition percentages were observed from larval development and larval mortality at the lowest concentration tested as compared to commercial drugs used. Potassium permanganate was highly toxic on the brine shrimps whereas it was concentration dependent on the normal mammalian cells. This study supports the usage of potassium permanganate as prophylaxis at low concentration in treatment of nematode larval infection of small stock.

Recommendations

- Potassium permanganate could still be used as prophylaxis of pathogens such as viruses, bacteria, fungi and parasites causing diseases such as Avian Influenza, Cholera and intestinal worm infection in animals; however, correct dosages should be prepared because it is toxic in higher concentrations and can leave hot spots if it is not totally dissolved in appropriate water volume. There is therefore a need for further studies that will result in formation of correct dosages per animal species e.g.: sheep/goats/cattle.

- None of tested substances were extremely toxic on the MDBK cell line but for better safety measures, other normal mammalian cell lines should be tested. Other cytotoxicity tests such as the Aspartate Transaminase (AST) should also be conducted to determine if plant extracts would pose any toxic effect on the animal host.

- The brine shrimp lethality tests in the current study were established with minor problems, the crude extracts prepared for the study were dissolved to correct concentrations in distilled water and acetone according to the solvents used when preparing the extracts and all the *Artemia salina* nauplii total death was observed in 24 hours. It is therefore recommended that brine shrimp lethality tests water and acetone may be used to extract crude extracts but saline water should be used in concentration preparations.

- Chemical constituents from the plants should be extracted separately and tested individually for treatment of nematode infections and cancer cells in order to determine which constituents are responsible for anthelmintic or anticancer activity.

- Chemical constituents of experimental plants from various regions should be tested because similar plants may have different constituents due to exposure to different conditions such as climate.

- Essential oils have been documented to possess anthelmintic and anticancer activity. Essential oils of *C. orbiculata, H. depressa* and *N. glauca* should be extracted and tested individually for anthelmintic and anticancer activities as well as cytotoxicity.
- Farmer education workshops on usage of medicinal plants in treatment of infections should be conducted. These will even teach farmers on how to prepare correct dosages per animal weight.

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