ANTI-ACETYLCHOLINESTERASE ACTIVITIES OF LEAF EXTRACTS OF *Carphalea glaucescens* AND *Gnidia glauca* FROM MBEERE NORTH SUBCOUNTY, KENYA ON *Chilo partellus* LARVAE

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DECLARATION

I, Anne Wangithi Njoroge, duly declare that the work presented in this thesis is my original work and has not been presented for a degree or any other award in any other university or any other institution

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DEDICATION

This thesis is dedicated to my husband Dr. James Kariithi because of his emotional and financial support. To my precious daughter Sheila Njeri for encouraging me to press on

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ABBREVIATIONS AND ACRONYMS

ACh	Acetylcholine
AChE	Acetlycholinesterase
ACTI	Acetylthiocholide Iodide
ANOVA	Analysis of Variance
BSA	Bovine serum albumin
Bt	*Bacillus thuringiensis*
ChE	Cholinesterase
CMs	Carbamates
DCM	Dicholomethane
DDT	Dichlorodiphenyltrichloroethane
DMSO	Dimethylsulphoxide
DTNB	5, 5' dithiobis-2-nitrobenzoic acid
EDTA	Ethylenediaminetetraacetic acid
EPN	Enthomopathogenic Nematode
EU	European Union
FAO	Food and Agriculture Organization
FAOSTAT	Food and Agriculture Organization Statistics
IC_{50}	Inhibition concentration (50%)
ICIPE	International Centre of Insect Physiology and Ecology
MDGs	Millennium Development Goals
Ms excel	Microsoft excel
OPs	Organophosphates
Ph	Hydrogen potential
SEM	Standard error of mean
SSB	spotted stem borer
WHO	World Health Organization

ABSTRACT

The stem borer *(Chilo partellus)* is one of the major constraints in maize and sorghum production worldwide. Control of *Chilo partellus* is mainly done through synthetic insecticides which are very expensive and have negative effects to environment and non-target organisms. Farmers in Mbeere use *Carphalea glaucescens* and *Gnidia glauca* in control of *Chilo partellus* because of the high cost of conventional insecticides. However, there is lack of scientific information on the their mode of action is not known. This study was designed to gather preliminary data that can be used to develop a bio-insecticide to control the *Chilo partellus*. Aqueous and dichloromethane leaf extracts from *Carphalea glaucescens* and *Gnidia glauca* were assayed *in vitro* for their activities against *Chilo partellus* anti-acetylcholinesterase activity. Acetylcholinesterase is one of the most efficient enzymes of nervous system which is concentrated at the cholinergic synapses and at neuromuscular synapses where it rapidly hydrolyses the neurotransmitter acetylcholine. Acetylcholinesterase plays a critical role in terminating synaptic transmission so that the next nerve impulse can be transmitted across the synapse. Plants were collected from Siakago, Mbeere North sub-county in Embu County, Kenya. *Chilo partellus* were obtained from KALRO (Katumani) and the crude enzyme acetlycholinesterase extracted through homogenization. Activity of the isolated crude enzyme was determined as described by Ellman *et al*. (1961). Acetylthiocholine iodide was used as a substrate in the assay. The optical density (OD) was measured at 412 nm by spectrophotometer. The experiments were done in triplicates. This study bioassay six extracts concentrations for both aqueous and DCM extracts of *C. glaucescens* and *G. glauca.* Cyclone was used as the standard drug and normal control lacked the inhibitor. This design was followed for aqueous and dichloromethane of the two plants. The aqueous leaf extracts of *C. glaucescens* percent enzyme inhibition was between 86.67% – 47.57% while DCM extracts of *C. glaucescens* percent inhibition was between 73.64% - 34.54%. Aqueous extracts of *G. glauca* percent inhibition was between 90.00% - 33.63% and DCM extracts of *G. glauca* percent inhibition was between 96.97% - 35.09%. Cyclone percent inhibition was 96.97%. Results also showed that the extracts had tannins, phenols, flavonoids, terpenoids, saponins, alkaloids, cardiac glycosides and steroids which have been associated with AChE inhibition activity. Therefore, the study has revealed that aqueous and DCM leaf extracts from *C. glaucescens* and *G. glauca* have the potential of anti-acetlycholinesterase activity. Hence the studied extracts can further be purified and developed into plant- derived biopesticides.

CHAPTER ONE

INTRODUCTION

1 Background Information

Maize (*Zea mays*), a monoecious plant is a staple food grown throughout the globe and belongs to grass family (*Poaceae*). Maize plant grows annually. It accounts for about 40% daily calories (FAO, 2015) and has per capita consumption of 98 kilograms; this translates to between 30 and 34 million bags (2.7 to 3.1 million metric tonnes) of annual maize consumption in Kenya. Maize is major source of carbohydrates, mineral salts and proteins. According to Chaudhary (1983), maize grains have great nutritional value as they contain 72% starch, 10% protein, 4.8% oil, 8.5% fibre, 3.0% sugar and 1.7% ash. Maize is used in many different ways such as human food, as fodder for animals, as raw materials for manufacture of products like corn oil, corn starch, products of fermentation, in production of biofuel among many other uses. In Kenya, maize is ground to powder and consumed as ugali or porridge. It can also be consumed when boiled or roasted when fresh on the cob. *Zea mays* is the most important cereal fodder and grain crop under both irrigated and rain fed agricultural system in the semi-arid and arid tropics (Hussan *et al*., 2003).

It is estimated that in 2012, the total world production of maize was 875,226,630 tons, (FAO, 2012) with United States, China and Brazil harvesting 31%, 24% and 8% of total production of maize respectively. Developed countries produce more maize or corn than the developing

countries. In Kenya the counties that produce large maize surplus are in the Rift Valley region mainly Trans Nzoia and Uasin Gishu Counties primarily on medium and large farm. According to FAOSTAT (2010), the demand for maize is projected to increase by at least 40% over the next ten years in East and Southern Africa. One of the Millennium Developments Goals (MDGs) is to fight hunger worldwide therefore more need to be done at the global level to reduce loss of maize production and increase its production for food security to be achieved. Eradicating hunger is the key to development for any nation.

In order to meet this demand, Kenya needs to increase maize production. However, there are many constraints affecting maize production. The abiotic and biotic factors affects maize production which include diseases, temperature, light, type of seed planted, moisture, pests among others. Among the insect pests attacking cereal crops in Africa, the lepidopteran stem borers are by far the most injurious (Youdeowi, 1989). Maize stem borer is a global plant pest that causes great losses in crop production. The yield losses reported due to stem borers vary greatly. Melaku *et al.* (2006) reported 49% grain yield losses due to stem borers in northern Ethiopia but on average yield losses can be estimated between 20% and 50%.

Two of the economically important cereal stem borers in Africa are introduced species: *Chilo partellus* and *C. sacchariphagus* (Bleszynsky, 1970). *C. partellus* is one of the stem borers that reduce the production of maize. *C. partellus,* also known as spotted stem borer (SSB) entered Africa

from India (Overholt *et al*., 1996). *Chilo partellus* is very invasive and once it invades an area it displaces native species and is widely distributed. In coastal Kenya, there is evidence that *C. partellus* has partially displaced the indigenous stem borer, *Chilo orichalcociliellus* (Ofomata *et al*., 1999a; 1999b; Ofomata *et al*., 2000). The distribution of C. partellus now includes Ethiopia, Sudan, Somalia Kenya, Tanzania, Uganda, Mozambique, South Africa, Swaziland, Lesotho, Zimbabwe, Zambia,, Malawi, and Bostwana (Igram, 1983; Harris, 1989). In Mozambique, larvae of third-generation *C. partellus,* the most important stem borer, were reported to infest 87% of cobs of late-planted maize and severely damage 70% of grain (Berger, 1981). In Kenya, 18% yield losses were attributed to *C. partellus,* and *C. orichalcociliellus* in maize (Warui, and Kuria, 1983) and 88% in sorghum owing to *C. partellus* (Seshu, 1988)

Farmers worldwide are trying to increase maize production in order to achieve one of the MDGs by reducing loss of production caused by stem borers. There are various conventional methods used to control stem borers globally. One of these control methods is use of synthetic pesticides to kill the stem borers and reduce their infestation. In order to achieve the MDG on food security, large quantities and varieties of pesticides are currently in use to increase crop production than in the past. According to Aktar *et al.* (2009), about 25-35% loss in agricultural produce is caused by pests and diseases which can be controlled by use of pesticides. Overuse of pesticides can cause environmental problems. A study by Koul *et al*. (2008) observed that about

2.5 million tons of pesticides are used on crops annually and the worldwide damage caused by pesticides reaches $100 billion each year. Currently, consumers are more aware of environmental and potential health hazards associated with heavy use of synthetic pesticides hence the need to develop a pesticide that is environmentally friendly. Consumers are demanding food of high quality, which is safe and with long shelf life.

The management of *C. partellus* has been typically carried out by chemical insecticides, which are associated with environmental contamination, high levels of resistance and damage to non-target organisms (Roditakis *et al*., 2009). The synthetic insecticides are the largest class of pesticides in use today. This is because farmers can access them easily over the counter. However, extreme use of these synthetic pesticides has created a number of problems in the world like bioaccumulation, toxicity against beneficial organisms and also increased insect resistance. According to Jacobson (1989), due to increasing problems by the use of synthetic pesticides, researchers are taking more interest to use microbial or plant based bio-pesticides because these are narrow spectrum, bioactive, biodegradable and environmentally safe in nature. Insecticides can be synthetic or naturally occurring chemicals. Cyclone is a synthetic broad spectrum insecticide. Acetylcholinesterase, involved in the synaptic transmission of impulse, is the target for insecticides belonging to organophosphorus and carbamate group (Yu, 2008). Organophosphates act upon the nervous system of the pests for example, Cholinesterase (ChE) inhibition while others act as growth regulators or endotoxins.

The second method is cultural control which is commonly used by farmers who do not have money to buy synthetic chemicals. Cultural control is cheap to apply because one does not require buying equipment to use and effects on the environment are also minimal. Cultural controls are management tools and activities that make the crop habitat less favorable for pests to survive and cause damage (Horne and Page, 2008). Techniques of cultural control include crop rotation, intercropping, varying planting dates, and using different methods of cultivation. Intercropping crops of preference crop and susceptible crop increases the yield because stem bores will be attracted to the preference and leave the susceptible. A recent study from Kenya has reported the effectiveness of intercropping maize with the non host molasses grass, *Melinis minutiflora* (Khan, 1997a; 1997b).

Trap plants like napier grass are also used to trap *C. partellus* to prevent them from attacking maize. A study by Zayaur *et al*. (2006) demonstrated that *Pennisetum purpureum* had the potential to be used as trap crop in *C. partellus* management because they were more preferred by the borer moths for oviposition. However, the use of trap plants has disadvantage of competing for food with the susceptible crop resulting in reduced yield. Manipulating planting dates is affected by constraint like rainfall which determines when planting should be done. And some farmers are illiterates and only know the planting seasons therefore by changing the dates of planting might not contributes to very high crop yield. This is because majority of small and medium scale farmers rely on rainfall and there are seasons for them to planting and harvesting maize hence failure to plant when rains start can reduce production. Though the cultural method is cheap to use however it has a demerit in that it is labor

intensive, some farmers do not even have calendar to manipulate the dates of planting.

Biological control is the third method that farmers use to control *C. partellus.* Attempts have been made to import exotic parasitoids into Kenya for biological control of pests but cost is high and this limits wide use of this method of importing parasitoids. The high cost and inefficacy of insecticidal control of *C. partellus* brought about the initiation of a biological control programe using exotic parasitoids as a possible control method against the borer (Kfir, 1991; 1992).

Mechanical or physical control is where farmers inspect crops with pests and uproot them or pick the pest and kill them. Diseased plants are also uprooted and burnt. Biotic factors like nitrogen deficiency in the soil affects the destruction of maize by stem borers. A Study indicates that nitrogen fertilizer can be applied as an integrated pest management tactic in control of *C. partellus* population development and infestation on maize crop (Arshad *et al.,* 2013).

Microbial insecticides are insecticides which contains microorganisms like bacteria, viruses among others or toxins they produce. A good example microbial insecticide is the *Bacillus thuringiensis* (*Bt*) which is composed of the bacterium *B. thuringiensis.* The *Bt* has been used successfully in South Africa and work best in area with high moisture and undisturbed environments. However the effectiveness of *Bt* is reduced by conditions such as heat and dryness. Evidence of *Bt* resistance in many pest insects has compelled the search for new insect control agents (Tabashnik, 1994). More

studies need to be done on plants so that the ones with pesticidal component can be known and exploited to make pesticides. In his study, Rajwinder (2010) observed that it is likely that novel and potent molecules that can be used for pest management still remain to be discovered from many plant species.

Natural control is the fourth method used in control of stem borers and other pests. Currently more and more researches are been done to meet the consumers demands. The shift is towards biopesticides, which are known to be eco-friendly and less harmful to non target organisms. The shift, from conventional synthetic insecticides to biological control agents, has been due to environmental concerns and difficulties with insecticides resistance, thus bio-insecticides are virtually considered to be safe and environment friendly insect control agents (Miranpuri and Kachatourian, 1993). Many studies are currently being done on plant extracts to develop biopesticides and other herbal drugs because plants are able to synthesize many different chemicals compounds to prevent their colonization by pests and animals. According to Saxena (1998), more than 6000 species of plants have been screened and about 2500 plant species belonging to 235 families possessed biological activity against various categories of pests. Botanical insecticides have long been touted as attractive alternatives to synthetic chemical insecticides for pest management (Cox, 2002).

The alternative sources of insect-control are plants because they contain a range of bioactive compounds and many of which are selective. Harmful effect from plants on non-target insects is very little if any. In Africa and

elsewhere, plants extracts are still widely used in the treatment of many ailments and up to 80% of the African population use traditional medicines for health care (WHO, 2002). Plants provide medicines which are not expensive that needed to prevent and even cure diseases. Plants are also environment friendly pest control which reduces crop losses caused by pests. Many insecticides have been made from plants such as nicotine**.** Pyrethrin and neem alkaloids are also used against insects. Currently, natural plants products have been considered as one of the most promising sources of biorational products with new modes of actions to manage phytophagus insects (Dayan *et al.,* 2009).

Plants are sedentary in nature and they are exposed to many factors in the environment where their habitat. In their natural habitat there are bacteria, viruses, pests, animals among others which contribute greatly to reduction in productivity. The above challenges make plants to synthesis secondary metabolites in order to protect themselves against herbivorous animals and even pathogens. Secondary metabolites are by products of pathways in plants and they vary widely in their distribution. Secondary metabolites play a major role in the adaptation of plants to the environment and in overcoming stress conditions in their habitat. Plant secondary metabolites are unique sources for pharmaceuticals, food additives, flavors, and industrially important biochemicals (Ramakrisha *et al*., 2011).

It was against this background that this study was designed to bioscreen aqueous and dichloromethane leaf extracts of *Carphalea glaucescens* and

Gnidia glauca for *in vitro* anti acetylcholinesterase (AChE) activity in *C. partellus*, as a preliminary step towards development of a plant derived insecticide that is safe in the environment, biodegradeable, can reduce the *C. partellus* population, increase food production and the mode of action is known.

1.2 Problem statement

Food security is a key objective for any government. However, maize productivity is greatly reduced by *C. partellus*. According to Prem *et al.* (2010), Chilo *partellus* is one of the major biotic stresses reducing maize productivity. Kenya loses an estimated 13.5% of its maize production, the major staple food, to pests annually (De Groote, 2002). *Chilo partellus* are mainly controlled use of synthetic insecticides which are expensive and have negative effects especially in the environment making production of maize to be expensive in the long run. Nearly all insecticides have the ability to change ecosystems; many are toxic to humans; some concentrate along the food chain and others kill non target organisms. In recent past, many studies have pointed out some of the negative effects of synthetic pesticides both to the environment and non target organisms. FAO (2008) has been concerned about ill health associated with synthetic residue pesticides in foods.

Although many plants have been used for control of crop pests including *C. partellus*, their bioactivity has not been scientifically investigated and the modes of actions are not known

1.3 Hypothesis

Aqueous and dichloromethane leaf extracts of *C. glaucescens* and *G. glauca* have no *in vitro* anti acetylcholinesterase activity in *C. partellus*.

1.4 Objectives

1.4.1 General objective

To bioscreen aqueous and dichloromethane leaf extracts of *C. glaucescens* and *G. glauca* for *in vitro* anti acetylcholinesterase activity in *C. partellus* larvae.

1.4.2 Specific objectives

i. To determine *in vitro* antiacetylcholinesterase activity of aqueous leaf extracts of *C. glaucescens* and *G. glauca* in *C. partellus* larvae.
ii. To determine *in vitro* antiacetylcholinesterase activity of dichloromethane leaf extracts of *C. glaucescens* and *G. glauca* in *C. partellus* larvae.
iii. To determine the qualitative phytochemical composition of aqueous and dichloromethane leaf extracts of *C. glaucescens* and *G. glauca.*

1.5 Justification and significance of the study

Due to increasing public concerns of environmental pollution by the conventional synthetic pesticides and the development of insecticide resistance in some insect groups (Azmathullah, 2011), studies have shown that plants have compounds that are bioactive against pests however the mode of action is not known. It is necessary to bioscreen plants which have traditionally been found by farmers to control *C. partellus* and whose mode of

action is unknown. It is also important to confirm traditional use of these plants against *C. partellus*. Understanding how these plants kill *C. partellus* is important because it will help in determining whether they are toxic to non target organism or not.

This study gathered preliminary data that would result in the development of a cheap, environmentally safe but effective intervention against stem borer whose mechanism of action is known. This study also generated information regarding bioactivity of the aqueous and dichloromethane leaf extracts *C. glaucescens* and *G. glauca* that complemented the ongoing effort on stem borer infestation in crops. Data collected on phytochemical composition provided information about insecticidal phytochemicals which can further be purified and developed into plant- derived biopesticides. In addition, this study revealed research gaps that can be subjected to further research. These have been outlined in chapter five of this thesis.

CHAPTER TWO

LITERATURE REVIEW

2.1 *Chilo partellus*

2.1.1 Biology and description

Chilo partellus, the spotted stem borer, is a moth belonging to the order *Lepidoptera* in the family *Crambidae*. It was described by Swinhoe in 1885. *C. partellus* is a pest that was introduced to Africa most likely from India in the early 20th century and became established in Eastern Africa in the early 1930s (Mohamed, 2004). A survey by Le Ru *et al.* (2006) reported existence of a total of 136 stem borer species from 75 wild host species in East and Southern Africa. Though they are many species, *C. partellus* is the most invasive, a notorious pest of maize and plays havoc role all over the world (Atwal, 1976). Two to three weeks old plants are at risk of attack by the stem borer larvae.

Photo 1: *Chilo partellus.* Source: www.nbair.res.in

Moths are most active at night and lay eggs on undersides of maize leaves, mostly near mid-ribs. They hatch after four to ten days. *Chilo* larvae are about 25 mm long when mature, with a red brown head. The body is cream white to yellow brown with dark brown spots along the back which give them a spotted appearance (www.infonet-biovision.org.). Initially they feed in the leaf whorl, and then they crawl up the plant into the funnel where they feed on leaves for 2 to 3 days and then either move to other plants or enter inside the maize stem. After the larvae bore into the maize stems, they feed and grow within the stems for 15 to 20 days (www.infonet-biovision.org). When fully grown, they cut a hole in the side of the stem before pupating within the tunnel inside the maize stem.

The total larval period is usually 23 days when conditions are favorable during the growing season. However, during dry or cold weather, *Chilo* larvae enter into a resting period (diapause) of 6 months or more in stems, stubble and other plant residues. With the beginning of the rains, the larvae pupate within the stems. Pupation normally takes 5-14 days after which adult moths emerge (Harris, 1989). The adults have a wingspan of about 20 to 25 mm. *Chilo partellus* normally develops continuously all year-round indicating that their population increase towards the end of the year.

The *Chilo* larvae feed on various grass species especially sorghum, millet and maize. The larvae feed on terminal leaves of maize, thus producing holes on the tissue that has been eaten away. They even feed on the growing points of plant causing ‘dead heart’. Older larvae tunnel deeply into the stem

hence weakening the stem and even interfering with the proper flow of nutrients (Le Ru *et al.,* 2011). This result in weak plants and poor harvest hence reduced maize productivity.

2.1.2 Economic importance

Chilo Partellus is one of the economically most damaging pests in Asia and Africa attacking all parts of the plant except the roots (Kamala *et al*., 2012). The economic importance of an insect is determined by the damage it causes. *Chilo Partellus* is the most serious pest damaging crops from 26.5% to 80.4% in different agro-climatic regions of India (Chatterji, *et al.,* 1969) Every year, they destroy a great deal of the maize crop. Research conducted in most West African countries has shown that lepidopteran stem and ear borers are among the most economically important pests of maize (Polaszek, 1998). Two to three weeks old plants are at risk of attack by the stem borer larvae. According to Kfir *et al*. (2002), *Chilo partellus* feeding habits result in yield losses of up to 88%. Studies have shown that stem borers are the most damaging group of insect pests in maize cultivation and account for an estimated average annual loss of 18% (Bekele *et al.*, 2011).

2.2 Conventional control of crop pests

2.2.1 Chemical control

This is the most commonly used method against crop pests. Their world wide use has increased in recent years with 2.5 million tonnes of pesticides being used annually (Koul *et al*. 2008). Some of the challenges facing use of synthetic pesticides include the fact that they are not selective and kill even

the non targeted organisms. Over 98% of sprayed insecticides and 95% of herbicides exert their effect on non targeted species because they spread across the whole agricultural field (George, 2004). Even though this method is effective, repeated use of these chemicals may cause environmental hazards and various biochemical changes in non-target animals (Markowitz, 1992).

The majority of insecticides have mode of action that can be classified into three categories namely: neurotoxins, respiratory inhibitors and growth regulators (Song and Scharf, 2008). Organophosphate insecticides can result in acetylcholinesterase inhibition in human through overexposure. These pesticides combine with acetylcholinesterase at nerve endings in the brain and nervous system, and with other types of cholinesterase found in the blood. This allows acetylcholine to build up, while protective levels of the cholinesterase enzyme decrease which result to symptoms of poisoning from pesticides cholinesterase inhibition. A 25 to 35 percent drop signals moderate poisoning, and a 35 to 50 percent decline in the cholinesterase readings indicates severe poisoning (Paul, 1987).

Insecticides are classified into two major groups according to the method of application and the way they enter the insect's body. Contact insecticides are sprayed or dusted on the insect's body. The poison is absorbed through the body wall. Most soft-bodied insects are vulnerable to contact insecticides. Contact insecticides have no residue activity unlike systemic insecticides. Systemic insecticides are absorbed by plant tissues, and become distributed systematically throughout the whole plant so that when insects feed on the sap they are poisoned. These insecticides have residual or long-term activity. A

study by Chagnon (2015) revealed evidence of the negative impacts of systemic insecticides on decomposition, nutrient cycling, soil respiration, and invertebrate populations valued by humans.

In recent past, pharmaceutical companies have increased development of pesticides that target a wide range of pests. Cothran *et al.* (2013) in their study observed that increased quantity and frequency of pesticide application have posed a major challenge to the targeted pests causing them to either disperse to new environment or adapt to novel conditions. Though synthetic pesticides have advantage of killing pests very fast and in large numbers, their successful use is greatly affected by resistance of the mutant pests. The conventional insecticides contain chemicals which causes mutation of insects and pests. Biopesticides would not result to resistant of pests because they are readily biodegradable and are not stored in plant and animal tissues therefore there are no concerns in regards to residual levels.

Insecticides are effective in destroying disease-carrying insects and insects that damage crops and property; however, they also invoke resistance over time. Repeated application of pesticides like DDT leads to increase in pest resistance and resurgence of other non- targeted species. Resistance has been found in different insecticide groups for example, 291 species have developed cyclodiene resistance, followed by DDT (263 species), organophosphates (260 species), carbamates (85 species), pyrethroids (48 species), fumigants (12 species), and other (40 species) (Dhaliwal *et al.*, 2006). Besides, synthetic pesticides have negative effects on environment and excessive use of insecticides destroys many beneficial insects, birds and small mammals. This

is occasioned by the high mobility of most chemicals through runoff and wind which carry and spread them to other locations (Damalas and Eleftherohorinos, 2011).

2.2.1 Mode of action of pesticides

The mode of action is the one that describe how the pesticide kills the pests or make them inactive. Mode of action according to Bloomquist (2008), refers specifically to which biological process the pesticide interrupts. Different pesticides have different modes of action. Understanding the mode of action is important because one can know whether an insecticide will be toxic to non target organisms such as fish, birds and mammals or not. In addition to those they intended to kill some insecticide kill other non target organism even from other species. For example birds maybe harmed or even killed when they feed on insecticide granules mistaking them for food. Important insects like bees that pollinate plants can be killed by insecticide and this would result in reduction in crop yields.

Acetylcholine is one of the major molecules by which nerve impulses are transmitted from nerve cell or involuntary muscles (Lopez and Pascual-Villalobos, 2010). A pesticide that can bind, or inhibit, acetylcholinesterase, making it unable to breakdown acetylcholine, is called "acetylcholinesterase inhibitor," or "anti acetylcholinesterase agent." Organophosphates (OPs) and the carbamates (CMs) are the two main classes of cholinesterase inhibiting pesticides. While the effects of cholinesterase inhibiting products are intended for insect pests, this is not always the case because these chemicals can also be harmful to humans in some situations and the environment.

Acetylcholinesterase inhibitors are chemicals that bind to the enzyme, cholinesterase, and prevent it from breaking down the neurotransmitter, acetylcholine. This results in the accumulation of the neurotransmitter, acetylcholine at the synapses which causes rapid twitching of voluntary muscles and even paralysis. Organophosphate insecticides include some of the most toxic pesticides.

2.2.2 Cultural control

Cultural control methods are practices that reduce pest establishment, reproduction, dispersal, and survival. Intercropping maize with non host crop can reduce the *C. partellus* population. For example, intercropping with aromatic herbs *Allium cepa* (onion) and *Allium sativa* (garlic) is one of the cultural methods of pest control, which is used to repel insects. Push-pull strategy is another method of controlling insect pests and increasing crop production (Hassanali *et al.*, 2008**).** This method uses trap plants which attract the stem borers so that they do not destroy the host crop. Adjusting row spacing is also an effective cultural control measure for reducing corn earworm infestations. By using narrow row spacing, the canopy closes over the soil faster, reducing the attractiveness of the crop to host-seeking corn earworm moths (Tounou, 2013). Farmers can also control pests by killing a pest directly or making the environment unsuitable for it. Traps for rodents are examples of mechanical control and barriers such as screens to keep birds or insects away.

2.2.3 Biological and natural control

According to Dreistadt (2007), biological control is the beneficial action of predators, parasites, pathogens, and competitors in controlling pests and their damage. Over recent past interest in biological control has increased. Consumers increasingly demand products that are grown in a sustainable manner and are free of insecticide residue (O'Daherty *et al*., 2011). Unlike synthetic chemical method, which kills non target species, cause detrimental health effects to human beings and pollute the environment, biological control is safe, species specific and has long-term action on the target pests (Nafiu and Mustapha, 2014).

Biological control was started by International Centre of Insect Physiology and Ecology (ICIPE) in an attempt to reduce the cost of maize production. A research at ICIPE, Kenya by Niassy *et al.* (2012) successfully developed a fungus-based biopesticide for management of thrips *Frankliniella occidentalis* (Pergande) from *Metahrizium anisopliae*. In India, *Metahrizium anisopliae* and Entomopathogenic Nematode (EPN) are used as biological control agents against termites and are reported to be effective (Girma, 2011). Regulatory barriers is one many challenges that appear to limit the success of botanicals as alternative pest control. The larval parasitoid *Cotesia flavines* Cameron was introduced from Asia into Kenya and was released at the coast in 1993 to control the invasive exotic maize stem borer *C. partellus* (Swinhoe) however, research by Kipruto *et al*. (2009) revealed that the economic value of releasing parasitoid for control of *Chilo partellus* was too high.

Botanical pesticides are the important alternatives to minimize or replace the use of synthetic pesticides as they possess an array of properties including toxicity to the pest, repellency, antifeedant, insect growth regulatory activities against pests of agricultural importance (Prakash and Rao, 1989; Prakash *et al.*, 1990). In fact botanical pesticides are in use in Indian agriculture for over a century to minimize losses caused by pests and diseases (Prakash *et al*., 1990; Prakash *et al*., 2008)

2.3 Role of medicinal plants in control of crop pest

Medicinal plants are considered as a resource of new drugs (Gayathri and Gayathri, 2014). Ninety-one plants, with claimed medicinal properties against a total of 34 human and livestock ailments, have been reported and botanically identified as belonging to 57 genera and 33 plant families (Ketema, 2013). Farmers are a lot of pressure from EU to reduce or stop using synthetic insecticides due to the negative effects of these insecticides. European Union market is demanding organically grown produce from farmers. Therefore, more research is required in the development of more eco-friendly insecticides particularly from plant extracts.

More need to be done globally to increase production and use of biopesticides. To protect crops in modern agriculture and in an increasingly regulated world, natural plant-based insecticides can be a feasible plant pest management method and an attractive alternative to synthetic chemical insecticides because botanicals reputedly pose little threat to the environment, non-target organisms or to human health (Isman, 2006). Botanical control of termites has been

reported by Getahun and Bekele (2006), in whose study they concluded that extracts of seed powder of *M. ferruginea* and *A. indica*, fresh stem bark of *C. Macrostachyus* showed higher toxic effects on different termite casts.

Plant extracts contain naturally occurring phytochemicals with biopesticidal activities. They are readily biodegradable and are not stored in plant and animal tissues and because of this there are no concerns in regards to residual levels. Botanical extracts such as neem have been effectively used to control stem borers including *C. partellus*. Small amounts of neem leaves powder mixed with sawdust at the ratio of 1:1 and placed in the funnel of the plant during sensitive growth phase with treatment repeated eight to ten days were found to be efficient. In his study, Ahmand (2007) reported that phytochemicals protect plants from insect attacks. However, production of phytochemicals varies from plant to plant. Natural plant products have been considered as the most promising sources of biorational products with new modes of action to manage phytophagous insects (Rattan, 2010). However, the mechanism of pesticide action against stalk borers is unknown.

2.4 Pesticidal phytochemicals

Secondary metabolites do not play a direct role in growth and development of plants but they facilitate the primary metabolism in plants. Plants are totipotent unlike animals that can escape from predators by moving away plants are stationary. Due to this plants have devised ways of interacting with the environment even under harsh conditions. To enable plants to survive they

produce secondary metabolites which protect them from stresses in their habitat.

Some secondary metabolites are antfeedents and prevent predators from feeding on them. A number of plants produce polyphenols called tannins which confer the bitter taste on such plants and consequently herbivores stay away from eating such plants (Okwute and Nduji, 1992). Terpenoids have important functions in plant defence against herbivores (Keeling and Bohlmann, 2006; Mumm and Hilker, 2006; Cheng *et al.,* 2007).

Alkaloids have historically been used as sources of drugs, beverages, poultices and poisons (Roberts and Wink, 1998). Caffeine and quinine are examples of alkaloids. Most alkaloids are believed to function as defensive elements against predators, especially mammals because of their general toxicity and deterrence capability (Hartmann *et al*., 1991; Harborne, 1998). Plants containing alkaloids are known to cause death of livestock. Modes of action of alkaloids vary. Some interfere with components of the nervous system, especially the chemical transmitters; others affect membrane transport, protein synthesis and miscellaneous enzyme activities (Creelman and Mullet, 1997)

Saponins possess important biological functions such as insecticidal activities (Jonathan *et al*., 2004). Saponins are known to function in plant defenses, protecting the plants from phytopathogenic migroorganisms, phytophagus mammals and insects (Pelah, 2002; Chaieb, 2010). Saponins and terpenoids are the most numerous and structurally diverse plant natural products and have

been exploited by humans in the pharmaceutics (Zwenger and Basu, 2008). According to Sparg *et al*. (2004), saponin has been reported to have anti parasitic and antibacterial capabilities.

Flavonoids are the largest metabolites synthesized by plants. They are soluble in water and belong to the polyphenol family. Their role in plant is cell signaling pathways and antioxidant effects formed in vegetables like grapes and blackberries. Tannins and flavonoids in this case are mainly associated with antioxidant properties of plant extracts (Huda *et al*., 2009).

2.5 *Gnidia glauca* and *Carphalea glaucescens*

2.5.1 Description and geographical distribution

Gnidia glauca also known as fish poison belongs to the family *Thymelaeaceae***.** It is a tree of five to ten meters of height. The bark is grey or brown and it is smooth. Inner bark is very fibrous and pale yellow in colour with fragrance. Leaves arrangement is alternate, forms clusters at the upper part of branches. The type of leaf is simple with entire margin. The size of leaf is 7-8 cm long to 1-2 cm wide. Flowers are bisexual and regular. Flowers are usually yellow fading to brown, numerous and in heads (Kereru *et al*., 2008). They have superior ovary and fruit is enclosed by the persistent base of the calyx tube. Seeds of this plant are black in color.

Photo 2: *Gnidia glauca* (Local name in Mbeere *Muthira*). Source www.biotik.org

In Kenya *G. glauca* flowers in February to March and May to July. The plant is widely distributed in tropical Africa, from Nigeria eastward to Sudan and Ethiopia and southward to Malawi and Zambia (Kereru *et al*., 2008). It also grows in southern India and in Sri Lanka.

Carphalea glaucescens belongs to the family *Rubiceae*. It is a shrub of about 2m tall. Flowers which are pale pink are in dense inflorescences of about 5 cm across and long corolla tube of 1.5-3 mm long and are sweetly- scented. The fruit has green- purple color with an enlarged calyx (Neuwinger, 2000). Seeds are brown and 2.3-2.7 mm long and 1.1-1.2 mm wide. Aerial part can be used as food by wild animals (Neuwinger, 2000).

Photo 3: *Carphalea glaucescens* (local name in Mbeere *Murema Muthua*). Source: wildernessdiary.squarespace.com

2.5.2 Medicinal uses

Gnidia glauca maybe used in many ways: A decoction of the boiled root is drunk in East Africa for treatment of indigestion. In Ethiopia, root powder mixed with skimmed milk and taken orally for seven days for treatment of rabies (Peterson, 2006). The bark is made into arrow poison in Kenya; the bark is boiled in water for several hours and the residue is smeared on arrow tips. *G. glauca* (Family-Thymelaeceae) A study by Amarajeewa *et al*. (2007) observed that *G. glauca* is used as traditional phytomedicine for treating sore throat, abdominal pain, wounds, burns, and snake bites. Leaves have been applied to treat the swellings, back ache, and joint aches (Kareru *et al.*, 2007). It also has agrochemical applications as a molluscicide, insecticides, piscicide and even larvicide (Borris and Cordell, 1984; Franke, *et al*., 2002;

Javaregowda and Naik, 2007). It has been shown that several *Gnidia* species possess remarkable antineoplastic activity (Kupchan *et al., 1976*). It is used as antiviral agent against rabies in Ethiopia (Teklehaymanot and Giday, 2007).

The *G. glauca* is used to treat many ailments. A decoction of the boiled root is drunk in East Africa for treatment of indigestion. In Ethiopia, root powder mixed with skimmed milk is taken orally for seven days for treatment of rabies (Peterson, 2006). The bark is made into arrow poison in Kenya; it is boiled in water for several hours and the residue is smeared on arrow tips. In Mbeere, the locals use *G. glauca* to kill fish in ponds. In India, the barks and leafs are used for treatment of blisters, swellings and contusions. Leaf extracts are used as insecticide while the stem, bark and leaves are used as fish poison (Teklehaymanot and Giday, 2007). The bark is said to be poisonous. Alcoholic extract of the root showed strong *in vivo* inhibitory activity against P-388 leukemia in mice according to Peterson (2006).

In Mbeere peasant farmers use *G. glauca* and *C. glaucescens* to manage stem borers but the mode of action is not known. In Mbeere sub counties, leaves of *C. glaucescens* are used by the locals as anti-termite (Kereru *et al*., 2008). The aim of the study is to bioscreen aqueous and dichloromethane leaf extracts of *C. glaucescens* and *G. glauca* for possible *in vitro* anti cholinesterase in *C. partellus* in order to determine the mode of action.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection and preparation of the plant materials

Fresh leaves of *C. glaucescens* and *G. glauca* were harvested from Siakago division, Mbeere North Sub County, in Embu County guided by ethnobotanical information from local farmers and herbalists. An acknowledged taxonomist authenticated the identity of the two plants and voucher specimens for each plant collected were deposited at Kenyatta University herbarium for future reference. The leaves were transported to Kenyatta University, where the study was undertaken. They were dried at room temperature until dry following which they were ground to powder using an electric mill. The ground samples were weighed, packed separately in plastic bags, labeled and stored at room temperature (25^{o}C) ready for extraction. A kilogram of each plant sample was stored.

3.2 Chemicals

Dichloromethane, acetylthiocholine iodide (ACTI) , coomassie Brilliant Blue G-250, bovine serum albumen (BSA), 5-5 dithiobis-2-nitrobenzoic acid (DTNB), cyclone, ethanol, phospholic acid sodium dihydrogen phosphate and sodium hydrogen phosphate were procured from Sigma chemical Co. All chemicals used were of analytical grade.

3.3 Extraction procedure

For aqueous extraction, a sample of 500 g of each powdered plant leaves were added to 5 litres of distilled water and heated for 6 hour in a water bath at 60^{o}C. It was then cooled, decanted, and filtered under pressure using a

vacuum pump. The filtrate was freeze dried using AdVantage Pro Freeze Dryer-SP to obtain the powder material. The material was stored at 4^{o}C until use in bioassay.

For organic extraction, a sample of 500 g of each powdered plant leaves were soaked in 5 litres of dichloromethane for 24 hours. The mixture was then filtered under pressure using a vacuum pump. The filtrate was concentrated using a rotary evaporator at 40^{O}C to obtain dry extract which was stored at 4^{O}C until use in bioassay.

3.4 Preparation of extract concentrations

This study used eight independent treatments of the aqueous and organic leaf extracts of *C. glaucescens* and *G. glauca.* For the bioassays, extracts were diluted to six concentrations; 2.5mg/ml, 5.0 mg/ml, 7.5mg/ml, 25 mg/ml, 50 mg/ml and 75 mg/ml.

3.5 Crude acetlycholinesterase extraction procedure

Crude acetlycholinesterase (AChE) enzyme was extracted from *C. partellus* obtained from KALRO (Katumani). Third and fourth instar larvae were used for this study. After washing the larvae at least three times with distilled water, they were homogenized for 3 minutes (0.5 g) in 6 ml sodium phosphate buffer (pH 8, 0.1 M, containing 1.0 M Na_2HPO_4 and 1.2 M NaH_2PO_4). After filtration through whatman number 1 filter paper, the homogenate was centrifuged at 2,000 g for 20 min at 4^{o}C. The supernatant was used as a crude enzyme extract. After crude AChE was extracted from the larvae of *C.*

partellus, the concentration was determined by the method described by Bradford (1976) and bovine serum albumen (BSA) was used as the standard for protein. The dye was made by dissolving 100mg Coomassie Brilliant Blue G-250 in 50ml 95% ethanol followed by 100ml 85% (w/v) phosphoric acid. Absorbance was measured at 595nm and the absorbance values were used to plot the standard curve. The standard curve was used to determine the concentration of the AChE.

3.6 Experimental design

The study bioassayed six extracts concentrations for both aqueous and dichloromethane leaf extract of *C. glaucescens* and *G. glauca.* To accomplish this, treatments were set up into eight different groups as follows:

i. Group I (blank): Comprised all reagents (Table 3.1) except the plant extract.
ii. Group II (positive): Comprised of all the reagents (Table 3.1) and cyclone - the reference drug (concentration 2 µl /ml).
iii. Group III: Comprised of all the reagents (Table 3.1) and extract concentration of 2.5 mg/ml.
iv. Group IV: Comprised of all the reagents (Table 3.1) and extract concentration of 5.0 mg/ml.
v. Group V: Comprised of all the reagents (Table 3.1) and extract concentration of 7.5 mg/ml.
vi. Group VI: Comprised of all the reagents (Table 3.1) and extract concentration of 25 mg/ml.

vii. Group VII: Comprised of all the reagents (Table 3.1) and extract concentration of 50 mg/ml.

vii. Group VIII: Comprised of all the reagents (Table 3.1) and extract concentration of 75 mg/ml.

This design was followed for both aqueous and organic leaf extracts of the two plants. The experiments were conducted in triplicates. This design is as shown in the table below (Table 3.1).

Table 3.1: Treatment protocol for the determination of *in vitro* anti-acetyl cholinesterase activities of *C. glaucescens* and *G. glauca* on *C. partellus*

Treatment Group	Treatment
Normal control(blank)	None + buffer + crude enzyme extract(AChE)+ATCI+ DTNB
Positive control	Cyclone +crude enzyme (AChE) + buffer+ ATCI + DTNB
Experimental group A	2.5 mg/ml of plant extract +crude enzyme extract(AChE) + buffer + ATCI + DTNB
Experimental group B	5 mg/ml of plant extract +crude enzyme extract(AChE) + buffer + ATCI + DTNB
Experimental group C	7.5 mg/ml of plant extract +crude enzyme extract(AChE) + buffer + ATCI + DTNB
Experimental group D	25 mg/ml of plant extract +crude enzyme extract(AChE) + buffer + ATCI + DTNB
Experimental group E	50 mg/ml of plant extract +crude enzyme extract(AChE) + buffer + ATCI + DTNB
Experimental group F	75 mg/ml of plant extract +crude enzyme extract(AChE) + buffer + ATCI + DTNB

Key: ACTI Acetylthiocholine iodide
DTNB 5-5 dithiobis-2-nitrobenzoic acid)

3.7 Determination of anti- acetylcholinesterase activity

Acetylcholinesterase activity was determined as described by Ellman *et al*. (1961), with some modification that allowed the use of 1 ml cuvette glass. The principle of this method is the measurement of the rate of production of thiocholine from the hydrolysis of acetylthiocholine iodide. Thiocholine, then reacts with 5,5' dithiobis (2-nitrobenzoic acid) (DTNB) to form a yellow anion 5–thio-2-nitrobenzonic acid, which can be detected at 412nm. The absorbance is read at 412 nm with spectrophotometer. A 150 µl volume of 0.1 M sodium phosphate buffer (pH 8) was transferred to an eppendorf tube, and 10 µl of the plant extract was added, followed by 20 µl larvae homogenate (crude enzyme). A 10 µl volume of 14 mM of acetythiocholine iodide was added and the mixture was incubated for 30 minutes at 25ºC. Thereafter 10 µl of 10 mM of DTNB was added and the reaction mixture was then incubated for 5 minutes at room temperature (25ºC).

The blank (normal control) constituted the same reagents except the plant extracts. The optical density (OD) was read after one and four minutes. Then the change optical density with time (OD/min) was recorded after 3 minutes at room temperature (25º) to estimate substrate hydrolysis overtime. One unit of AChE activity is defined as 1 µl of substrate hydrolyzed per minute. The activity of the AChE was calculated as follows;

$$\text{Enzyme activity} = \frac{(\Delta As - \Delta Ac)\text{X vol. of cuvette X } 10^6}{\varepsilon \text{ X time X vol. of the sample X protein conc.}}$$

Where;

ΔAs Represents the change in the absorbance of test sample from the beginning to the end of the measurement period

ΔAc	Represents the change in the absorbance of normal control from the beginning to the end of the measurement period
Cuvette vol.	absorbance is proportional to concentration and must be multiplied by the volume to calculate the absolute number of moles product formed,
10^6	This converts the moles of ε to moles.
ε	The molar extinction coefficient converts absorbance values to concentrations.
Time	Enzymatic activity is expressed per unit time and this represents the time interval the absorbance was measured.
Sample vol.	The sum was divided by the sample volume so that the activity is expressed per unit volume of the sample,
Protein	Dividing by the protein concentration will provide information about the amount of enzyme activity per unit protein.

3.8 Acetylcholinesterase sensitivity to different concentrations of the extract

For sensitivity tests, different concentrations of the aqueous and organic leaf extracts of *C. glaucescens* and *G. glauca* were tested to determine the extract concentration that inhibited 50% of the enzyme activity. The value of median inhibition concentration (IC_{50}) for the inhibitors was calculated based on log (inhibitor concentration) versus probit (percentage of inhibition) linear regression. Concentration response curve of change in absorbance against concentration was plotted to show the sensitivity of the enzyme to the extracts. The percent inhibition was calculated by;

$$\text{Percent inhibition} = \frac{(\text{Rate without inhibitor} - \text{Rate with inhibitor})}{\text{Rate without inhibitor}} \text{X } 100$$

3.9 Qualitative phytochemical screening

Qualitative phytochemical screening was done to determine the presence of selected secondary metabolites of aqueous and dichloromethane leaf extracts of *C. glaucescens* and *G. glauca*. Phenols, flavonoids, Cardiac glycosides, tannins, alkaloids, terpenoids, saponins and steroids were

screened according to the standard methods as described by Harborne (1998) and Kotake (2000).

3.9.1 Phenols

About 1ml of ferric chloride solution was added to 2 ml of each plant extract. The formation of blue to green colour signifies the presence of phenolics.

3.9.2 Flavonoids (Sodium hydroxide test)

About 0.5 g of plant extract was dissolved in 2 ml of the solvent in a test tube and 2 ml of dilute sodium hydroxide was added to the test tube with the extract solution. A golden yellow precipitate indicated presence of flavonoids.

3.9.3 Alkaloids

A 5 ml volume of each plant extract was first acidified with 1M hydrochloric acid. This acidic medium was heated and then treated with Dragendroff's reagents. A reddish brown precipitate show positive results for the presence of alkaloids.

3.9.4 Terpenoids (Salkowski test)

About 0.5 g of each of the plant extract was added to 1 ml of ethyl acetate and then mixed with 2 ml chloroform. 3 ml concentrated sulphuric acid was added carefully by the sides of the test tube forming a layer. Formation of a reddish brown colour at the interface was regarded as positive test for the presence of terpenoids.

3.9.5 Saponins (Froth test)

About 0.5g of each plant extract was put in a test tube. A few drops of sodium bicarbonate solution was added to the test tube and shaken vigorously. The mixture is then allowed to stand for about 20 minutes and froth indicates presence of saponins and no froth indicates absence of saponins

3.9.6 Cardiac glycosides (Keller-Killian test)

About 0.5 g of the plant extract was dissolved in 2 ml glacial acetic acid containing 2 drops of 10% ferric chloride solution. Then it is under-layered with 1 ml of concentrated sulphuric acid. A brown or violet ring at the interface indicates presence of de-oxy sugar characteristic of cardenolides.

3.9.7 Steroids

Plant extract was screened for steroids, by putting 0.5 g of each extract in a test tube and dissolving it in 2 ml of chloroform. Then 3 ml of concentrated sulphuric acid was added carefully by the sides of the test tube forming a layer. Formation of a reddish brown colour at the interface indicated steroidal ring.

3.9.8 Tannins

About 0.5ml of extract solution put in a test tube, to which 1ml of water and 2 drops of ferric chloride were added. Blue colour was observed positive test for presence of tannins.

3.10 Data collection, management and statistical analysis

The effect of the aqueous and dichloromethane leaf extracts of *C. glaucescens* and *G.glauca* on acetylcholinesterase was recorded. Data collected included the efficacy of the extract against the AChE, change in absorbance, and qualitative phytochemical screening was also done. Data on change in optical OD was loaded on excel spreadsheet for analysis. Data on different extract concentrations, and controls were analyzed using one way ANOVA. This was followed by Tukey's post hoc for means separation and pair-wise comparison and to establish the levels of significance among the different treatment groups a $P \leq 0.05$ was considered statistically significant.

Data on phytochemical was analyzed on a table showing presence or absence of the phytochemicals in both aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca.* Minitab version 17 software was used for statistical analysis.

CHAPTER FOUR

RESULTS

4.1 *In vitro* anti acetylcholinesterase activity of aqueous leaf extracts of C. *glaucescens* and *G. glauca* in *C. partellus*

Results in table 4.1 indicated that normal control had the highest mean change in absorbance (0.032) while positive control had the lowest mean change in absorbance (0.001). The mean change in absorbance increased from 0.001 for 2.5mg/ml extract concentration to 0.015 for 75mg/ml extract concentration of aqueous leaf extract of *C. glaucescens.* Calculation of percent inhibition enzyme activity versus log concentrations of the extract, it was found that the IC_{50} of the extract was 79.43mg/ml (Figure 4.1). As table 4.1 shows, increase in extract concentration was accompanied by a decrease in acetylcholinesterase activity.

Generally, the aqueous leaf extract of *C. glaucescens* showed efficacy against AChE activity in *C. partellus* larvae (Table 4.1). This was indicated by percent inhibition of the enzyme activity by various extract concentrations (Table 4.1). The percent inhibition of acetyl cholinesterase activity by the aqueous leaf extracts of *C. glaucescens* at concentrations of 2.5mg/ml, 5mg/ml, 7.5mg/ml and 25mg/ml was almost as effective as the reference pesticide (cyclone) ($P>0.05$; Table 4.1). The extract concentration with the highest inhibition was 2.5 mg/ml with a value of 86.67±6.48 % (Table 4.1).

However, the percent inhibition of the enzyme by extract concentrations of 50mg/ml and 75mg/ml was significantly less than that of the reference

pesticide (P<0.05; Table 4.1). The extract concentration of 75 mg/ml had the lowest enzyme inhibition of 47.57±4.85 (Table 4.1). The normal control group had no effect against the enzyme activity. As figure 4.1 shows, the higher extract concentrations had lower anti acetylcholinesterase activity.

Table 4.1: The anti-acetylcholinesterase activity of various concentrations of aqueous leaf extract of *C. glaucescens* on *C. partellus*

Treatment (mg/ml)	Mean change in absorbance	Enzyme activity (µmoles/min/mg protein)	Mean % inhibition
Normal control	0.032±0.058	0.000	0.00±0.00
Positive control	0.001±0.055	3.744	96.97±1.84[a]
2.5	0.002±0.008	5.797	86.67±6.48[ab]
5	0.004±0.003	2.705	84.97±8.55[ab]
7.5	0.005±0.033	1.739	78.80±13.50[ab]
25	0.011±0.008	0.406	62.73±7.27[b]
50	0.012±0.006	0.193	61.50±16.80[b]
75	0.015±0.002	0.110	45.57±4.85[b]

Values are expressed as mean ± SEM for triplicate readings. Values with different superscripts are significantly different calculated by one way ANOVA followed by Tukey's post hoc test (p>0.05).

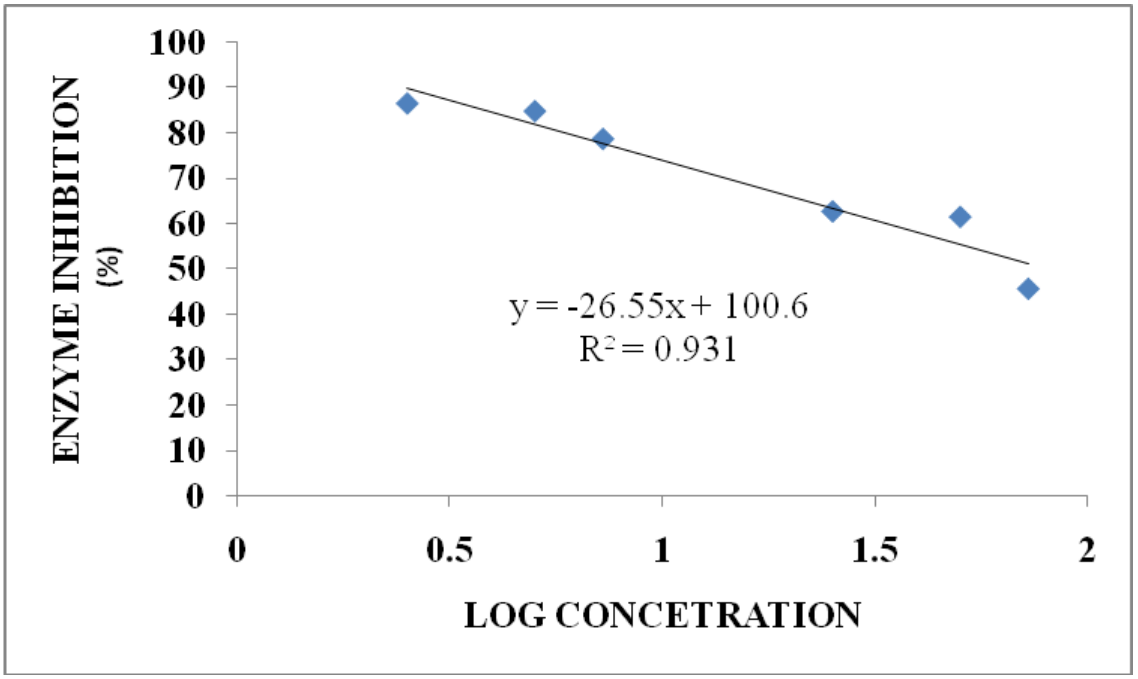

Figure 4.1: The percent inhibition by aqueous leaf extracts of *C. glaucescens* on acetylcholinesterase of *C. partellus*

Results in table 4.2 indicated that normal control had the highest mean change in absorbance (0.032) while positive control had the lowest mean change in absorbance (0.001). The mean change in absorbance increased from 0.003 for 2.5mg/ml extract concentration to 0.016 for 50mg/ml extract concentration of aqueous leaf extract of *G. glauca*. However the extract concentration of 75mg/ml had lower mean absorbance of 0.013.

Similarly, the aqueous leaf extract of *G. glauca* exhibited *in vitro* anti acetyl cholinesterase activity in *C. partellus* (Table 4.2). The percent inhibition of acetyl cholinesterase activity by the aqueous leaf extracts of *G. glauca* at concentration of 75mg/ml was significantly different from that of the reference pesticide (cyclone) ($P>0.05$; Table 4.2). However, the percent inhibition by extract concentrations of 2.5mg/ml, 5mg/ml, 7.5mg/ml, 25mg/ml and 50mg/ml was not significantly different from the reference pesticide (cyclone) ($P<0.05$;

Table 4.2). The normal control group had no effect against the enzyme activity.

The percent inhibition of aqueous leaf extract of *G. glauca* that had the highest inhibition was extract concentration of 2.5 mg/ml with a value of 90.00±0.91% (Table 4.2). The extract concentration with the lowest inhibition was of 75mg/ml with a value of 33.63±9.09 % (Table 4.2). The IC_{50}, calculated from percent inhibition of enzyme activity versus log concentrations of the extract, was found to be 63.10 mg/ml (Figure 4.2). In this study, a change in absorbance was associated with inhibition of enzyme activity. The enzyme activity was showed to decreased as the extract concentration increased (Table 4.2).

Table 4.2: The anti-acetylcholinesterase activity of various concentrations of aqueous leaf extract of G. *glauca* on *C. partellus*

Treatment (mg/ml)	Mean change in absorbance	Enzyme activity (µmoles/min/mg protein)	Mean % Inhibition
Normal control	0.032±0.058	0.000	0.00±0.00
Positive control	0.001±0.055	3.744	96.97±1.84[a]
2.5	0.003±0.000	5.990	90.00±0.91[a]
5	0.005±0.002	2.609	85.70±5.79[a]
7.5	0.006±0.003	1.675	83.03±7.90[a]
25	0.008±0.004	0.245	75.80±10.90[ab]
50	0.016±0.011	0.155	59.10±21.90[ab]
75	0.013±0.019	0.122	33.63±9.09[b]

Values are expressed as mean ± SEM for triplicate readings. Values with different superscripts are significantly different calculated by one way ANOVA followed by Tukey's post hoc test ($p>0.05$).

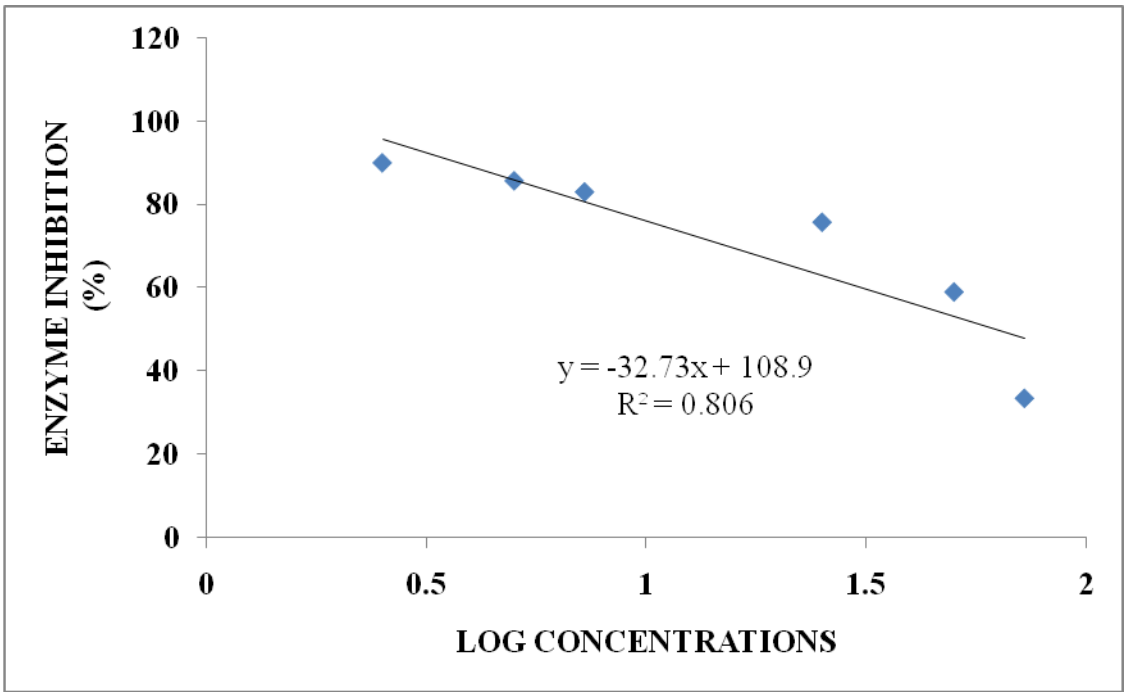

Figure 4.2: The percent inhibition by aqueous leaf extracts of *G. glauca* on acetylcholinesterase of *C. partellus*

4.2 *In vitro* anti acetylcholinesterase activity of DCM leaf extracts of *C. glaucescens* and *G. glauca* on *C. partellus*

Results in Table 4.3 indicated that normal control had the highest mean change in absorbance (0.032) while positive control had the lowest mean change in absorbance (0.001). The mean change in absorbance increased from 0.009 for 2.5mg/ml extract concentration to 0.037 for 75mg/ml extract concentration of DCM leaf extract of *C. glaucescens.*

Generally, the DCM leaf extract of *C. glaucescens* showed efficacy against AChE activity on *C. partellus* (Table 4.3). The efficacy was also indicated by percent inhibition of the enzyme activity by various extract concentrations (Table 4.3). The percent inhibition of acetylcholinesterase activity by the DCM leaf extracts of *C. glaucescens* at the concentrations of 2.5mg/ml, 5mg/ml and 7.5mg/ml showed no significant difference from the reference

pesticide (cyclone) ($P>0.05$; Table 4.3). Among the six different extract concentrations, the 2.5 mg/ml had the highest percent inhibitory effects on activity of acetylcholinesterase with a value of 73.64±8.67% (Table 4.3).

However, the percent inhibition by extract concentrations of 25mg/ml, 50mg/ml and 75 mg/ml was significantly less than the reference pesticide ($P<0.05$; Table 4.3). The lowest percentage enzyme activity inhibition of 34.54±6.05 % was caused by extract concentration of 75mg/ml (Table 4.3). The normal control group had no effect against the enzyme activity. As Table 4.3 shows, lower extract concentration had higher enzyme activity. From this study, any change in absorbance was also associated with inhibition of enzyme activity. The IC_{50} for DCM leaf extract of *C. glaucescens* was computed and found to be 12.02 mg/ml, which was the lowest among the four studied extracts (Figure 4.3). At lower extract concentrations of 2.5mg/ml, 5mg/ml, and 7.5 mg/ml the extract was more sensitive to enzyme activity than at higher concentration of 25, 50 and 75 mg/ml (Table 4.3).

Table 4.3: The anti-acetylcholinesterase activity of various concentrations of DCM leaf extract of *C. glaucescens* on *C. partellus*

Normal control	0.032±0.058	0.000	0.00±0.00
Positive control	0.001±0.055	3.744	96.97±1.84[a]
2.5	0.009±0.003	4.444	73.64±8.67[ab]
5	0.013±0.028	1.836	64.80±28.00[ab]
7.5	0.018±0.002	0.902	45.45±6.19[ab]
25	0.021±0,004	0.021	35.50±13.4[b]
50	0.028±0.036	0.039	35.09±4.66[b]
75	0.037±0.033	0.032	34.54±6.05[b]

Values are expressed as mean ± SEM for triplicate readings. Values with different superscripts are significantly different calculated by one way ANOVA followed by Tukey's post hoc test (p>0.05).

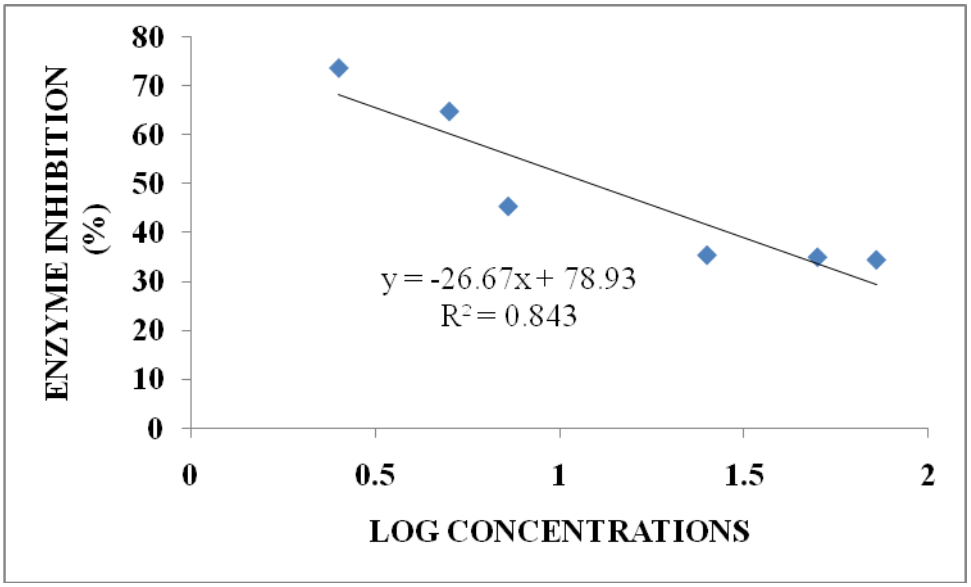

Figure 4.3: The percent inhibition by DCM leaf extracts of *C. glaucescens* on acetylcholinesterase of *C. partellus*

Table 4.4 exhibited that normal control had the highest mean change in absorbance (0.032) while positive control had the lowest mean change in absorbance (0.001). The mean change in absorbance increased from 0.001 for 2.5mg/ml extract concentration to 0.025 for 50mg/ml extract concentration of

DCM leaf extract of *G. glauca.* However, for this plant extract 75mg/ml extract concentration it had a lower a mean change in absorbance of 0.010.

Similarly, the DCM leaf extract of *G. glauca* exhibited *in vitro* anti acetylcholinesterase activity in *C. partellus* compared to the blank (Table 4.4). This was also indicated by percent inhibition of the enzyme activity by various extract concentrations (Table 4.4). The percent inhibition of acetylcholinesterase activity by the DCM leaf extracts of *C. glauca* at the concentrations of 2.5mg/ml, 5mg/ml and 7.5mg/ml was not significantly different from the reference pesticide (cyclone) ($P>0.05$; Table 4.4). The concentration of *C. glauca* of 2.5mg/ml exhibited the highest percent inhibition against acetylcholinesterase with a value of 96.97±1.84% (Table 4.4).

However, the percent inhibition by extract concentrations of 25mg/ml, 50mg/ml and 75mg/ml was significantly different from that of the 2.5mg/ml, 5mg/ml, 7.5mg/ml and reference pesticide (cyclone) ($P<0.05$; Table 4.4). The percent inhibition by extract concentrations of 25mg/ml, 50mg/ml was less significantly effective than that of 75mg/ml ($P<0.05$; Table 4.4). As expected, the normal control group had no effect against the enzyme activity. The extract concentration which exhibited the lowest percent inhibition was 50 mg/ml with a value of 35.09±8.02% (Table 4.4). The IC_{50} of DCM leaf extract of *G. glauca* was calculated as 39.81 mg/ml (Figure 4.4). As table 4.4 shows, the lower the extract concentrations, the higher the enzyme activity.

Table 4.4: The anti-acetylcholinesterase activity of various concentrations of DCM leaf extract of *G. glauca* on *C. partellus*

Treatment(mg/ml)	Mean change in absorbance	Enzyme activity (µmoles/min/mg protein)	Mean % inhibition
Normal control	0.032±0.058	0.000	0.00±0.00
Positive control	0.001±0.055	3.744	96.97±1.84[a]
2.5	0.001±0.001	9.807	96.97±1.84[a]
5	0.003±0.001	2.802	90.91±3.67[a]
7.5	0.004±0.004	1.804	84.90±10.70[a]
25	0.018±0.011	0.271	55.50±13.50[b]
50	0.025±0.018	0.068	35.09±8.02[c]
75	0.010±0.048	0.142	42.40±10.10[c]

Values are expressed as mean ± SEM for triplicate readings. Values with different superscripts are significantly different calculated by one way ANOVA followed by Tukey's post hoc test (p>0.05).

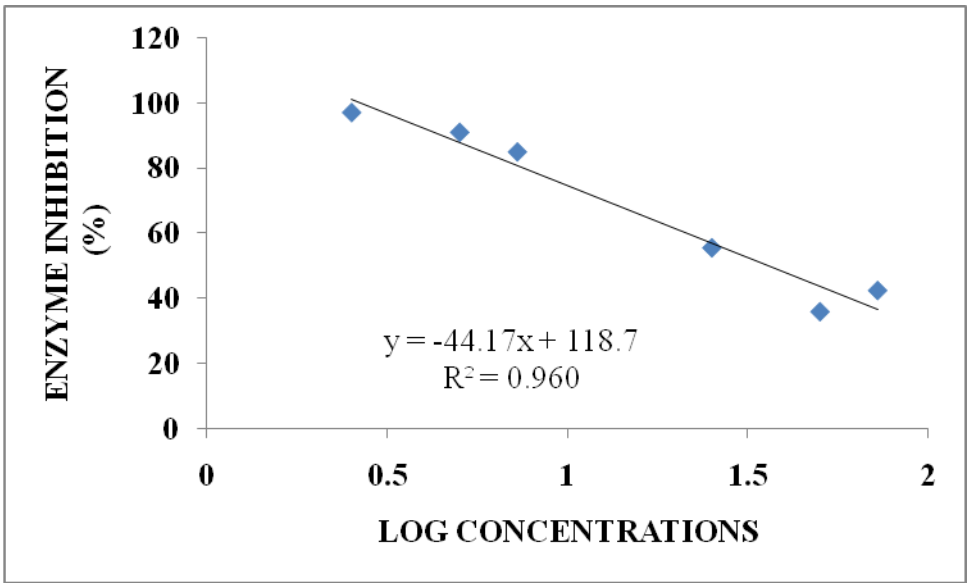

Figure 4.4: The percent inhibition by DCM leaf extracts of *G. glauca* on acetylcholinesterase of *C. partellus*

The aqueous leaf extracts of *C. glaucescens* exhibited higher percent inhibition on anti-acetylcholinesterase of *C. partellus* at all extract concentrations than the DCM leaf extract (Table 4.1; Table 4.3; Figure 4.5). Therefore, aqueous

leaf extracts was a better inhibitor than organic leaf extracts. At 2.5 mg/ml, the aqueous leaf extracts had a percent inhibition of 86.67% compared to 73.64% of the DCM leaf extract. However, at 75 mg/ml extract concentration, the aqueous leaf extract had a percent inhibition of 47.57% compared to 34.54% of the DCM leaf extracts.

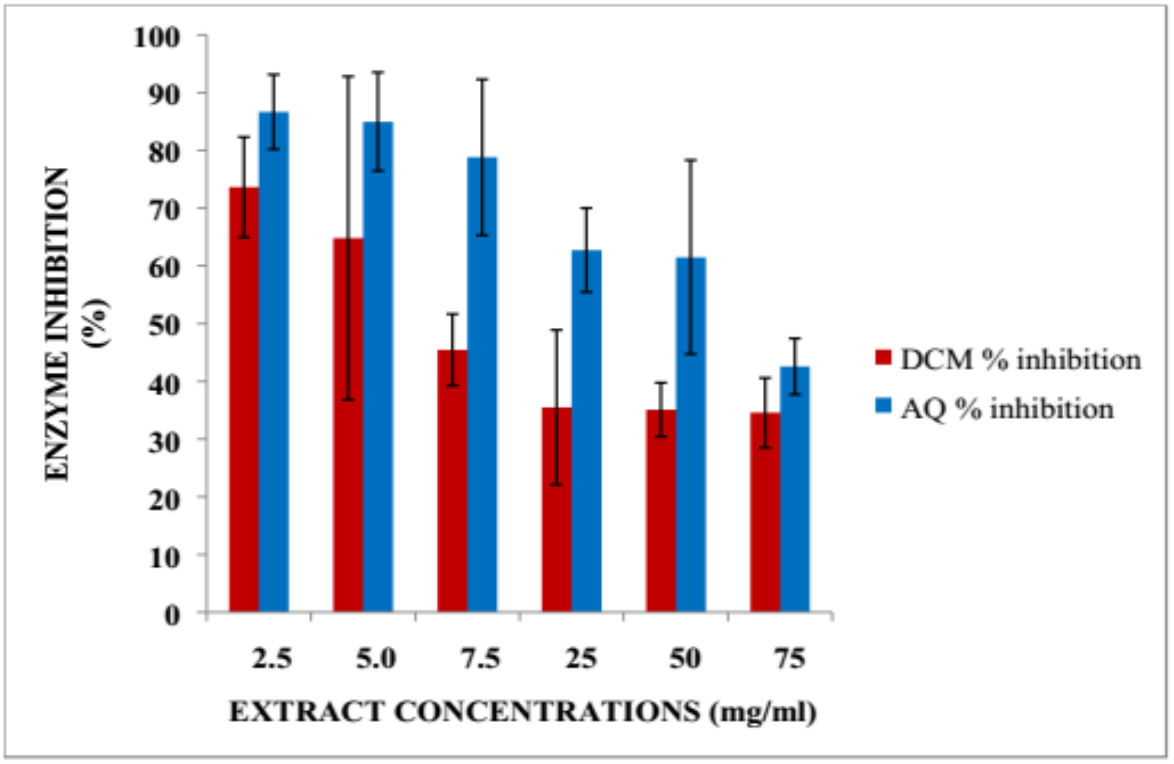

Figure 4.5: Comparison of percent enzyme inhibition at various concentrations of aqueous and DCM leaf extracts of *C. glaucescens* on acetylcholinesterase of *C. partellus*

The comparison of percent enzyme inhibition at various concentrations of DCM and aqueous leaf extracts of *G. glauca* showed that at all extract concentrations except 25mg/ml and 50 mg/ml, the DCM leaf extract exhibited higher percent enzyme inhibition than the aqueous leaf extract (Table 4.2; Table 4.4; Figure 4.6). At a concentration of 2.5 mg/ml, the DCM leaf extracts

had a higher percentage inhibition of acetylcholinesterase activity (96.97%) than the aqueous (90.00%). However, the DCM leaf extract had the lowest percent inhibition value of 35.09% at 50mg/ml while the concentration of the aqueous extract caused percent enzyme inhibition of 33.63% at a concentration of 75mg/ml.

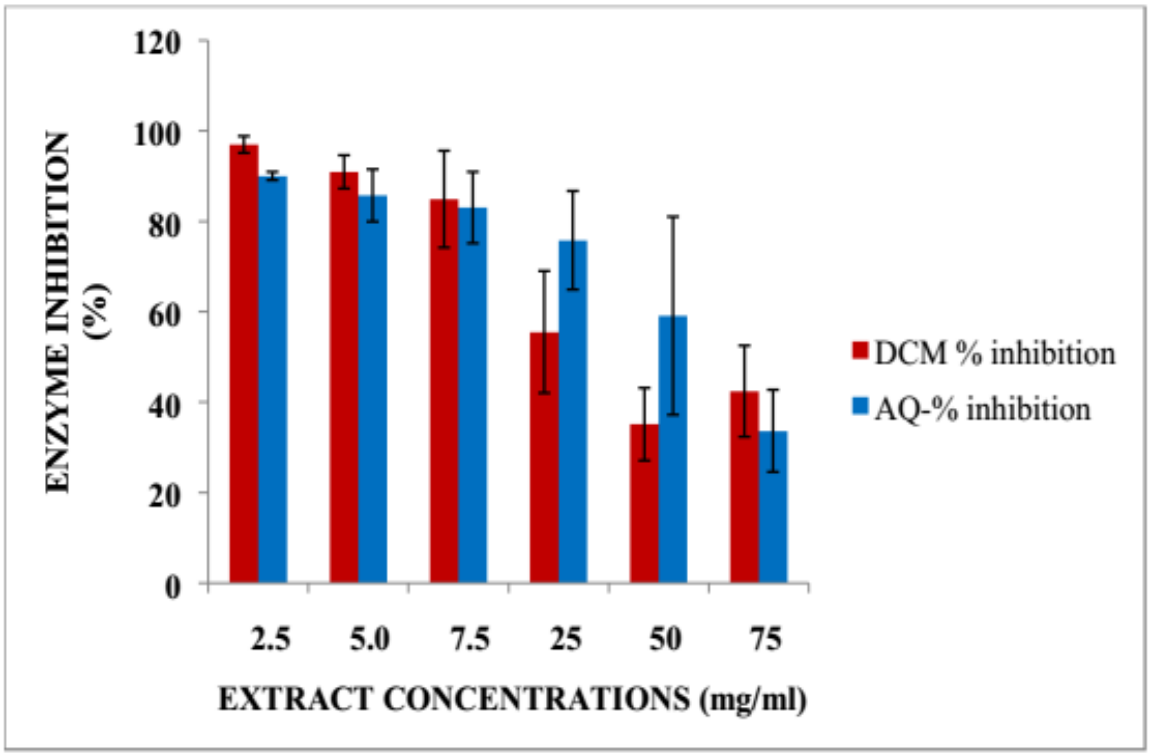

Figure 4.6: Comparison of percent enzyme inhibition of various concentrations of aqueous and DCM leaf extracts of *G. glauca* on acetylcholinesterase of *C. partellus*

In terms of enzyme inhibitions it was apparent that at concentrations of 2.5, 5 and 7.5 mg/ml, the DCM leaf extracts of *G. glauca* exhibited a higher enzyme inhibition than all the other extracts (Figure 4.7). However, at the highest concentration of 75 mg/ml there was increased percent enzyme inhibition. The extracts that exhibited the lowest enzyme inhibition at all concentrations was the DCM leaf extract of *C. glaucescens* (Figure 4.7). Among the two aqueous

leaf extracts, *C. glaucescens* exhibited higher inhibitory activity against the acetylcholinesterase than *G. glauca* (Figure 4.7).

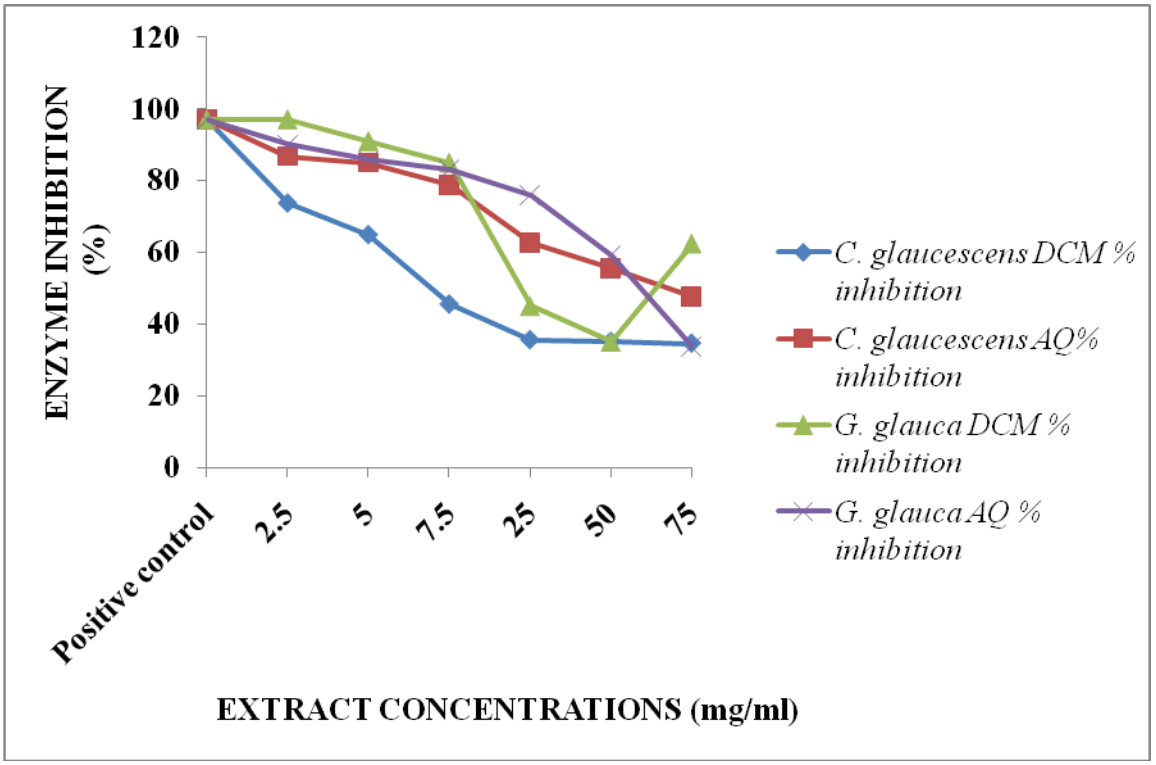

Figure 4.7: Comparison of percent enzyme inhibition of various concentrations of aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca* on acetylcholinesterase of *C. partellus* larvae

The activity of the acetylcholinesterase enzyme after exposure to various concentrations of aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca* was compared and this revealed that the DCM leaf extracts of *G. glauca* had the highest enzymatic activity at all concentrations than all the other extracts (Figure 4.8). On the other hand, the DCM leaf extracts of *C. glaucescens* exhibited the lowest enzyme activity at all concentrations. The extract concentration with the highest enzymatic activity was 2.5 mg/ml of the DCM leaf extract of *G. glauca* with a value of 9.8 µmoles/min/mg protein. The lowest enzymatic activity was observed at a concentration of 25 mg/ml of

the DCM leaf extracts of *C. glaucescens* with a value of 0.021 µmoles/min/mg protein.

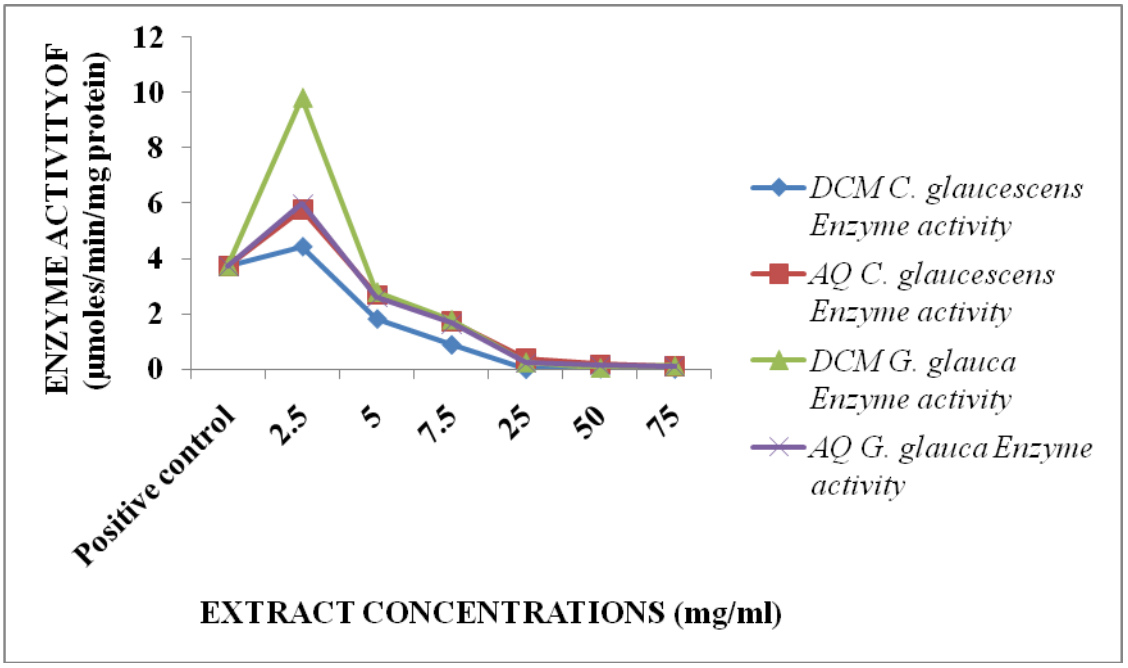

Figure 4.8: Comparison of enzymatic activity of aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca* on acetylcholinesterase of *C. partellus*

4.3 Qualitative phytochemical screening

Both aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca* were qualitatively screened for phytochemicals. The aqueous extract of *G. glauca* had tannins, phenols, flavonoids, terpenoids, saponins, alkaloids and steroids however, cardiac glycosides were absent. The phytochemicals present in DCM extract of *G. glauca* were tannins, phenols, flavonoids, terpenoids, saponins, alkaloids and steroids but cardiac glycosides and steroids were absent. In *C. glaucescens* tannins, phenols, flavonoids, terpenoids, cardiac glycosides and alkaloids were present in aqueous extract however, saponins and steroids were absent. In the DCM extract of *C. glaucescens* tannins,

phenols, steroids, flavonoids, terpenoids and alkaloids were present but saponins, and cardiac glycosides were absent (Table 4.5).

Table 4.5: Qualitative phytochemical screening of *G. glauca* and *C. glaucescens*

Phytochemical	***G. glauca***		***C. glaucescens***	
	Aqueous extract	**DCM extract**	**Aqueous extract**	**DCM extract**
Phenols	+	+	+	+
Flavonoids	+	+	+	+
Terpenoids	+	+	+	+
Saponins	+	+	-	-
Alkaloids	+	+	+	+
Cardiac glycosides	-	-	+	-
Steroids	+	-	-	-
Tannins	+	+	+	+

KEY: + = Present - = Absent

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

This study was designed to determine the effects of aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca* on anti-acetylcholinesterase of *C. partellus* larvae. It was apparent, in this study that small changes in absorbance were associated with higher inhibition enzyme activity. Results revealed that as mean change in absorbance increases as concentration increased however, the enzyme activity of acetylcholinesterase decreased. The principle of Ellman's method is the measurement of the rate of production of thiocholine from the hydrolysis of acetylthiocholine iodide. Thiocholine, then reacts with 5,5' dithiobis (2-nitrobenzoic acid) (DTNB) to form a yellow anion 5–thio-2-nitrobenzonic acid, this would be due less product formation as concentration increased.

In this study, it was observed that as the aqueous extract concentration of *C. glaucescens* increased from 2.5mg/ml to 75mg/ml, the percent enzyme inhibition decreased from 86.67% to 45.57% (Table 4.1) and for *G. glauca* from 90% to 33.63% this phenomenon could be due to high concentration of substrate which made diffusion of molecules in solution difficult. In high concentrations (75mg/ml) enzymes molecules were not free to bind with the substrate lowering the enzymatic activity (Table 4.1) however, at lower extract concentration (2.5mg/ml) enzyme activity was high.

Similarly, the percent enzyme inhibition of aqueous leaf extract of *G. glauca* decreased from 90% to 33.63% (Table 4.2) as concentration increased from 2.5mg/ml to 75mg/ml. If concentration of substrate is high the excess of substrate combine generating an invalid complex of substrate-substrate complex. These findings are in line with studies carried out by Junquig *et al*. (2011) and Qian *et al.* (2008) which showed that high substrate concentration generated inhibition to the enzyme. These results were similar to a study conducted by Orhan *et al*. (2004), which demonstrated that *Fumaris* species have potent inhibitory activity against AChE. Foliar application of semi-solid crude extract of *T. orientalis* on maize was also found to be effective against *C. partellus* (Anju and Sharma, 1999). All the concentrations tested for aqueous leaf extracts of *C. glaucescens and G. glauca* had percent enzyme inhibition an indication that the two plants have *in vitro* anti-acetylcholinesterase activity on *C. partellus*.

The DCM leaf extract of *C. glaucescens* and *G. glauca* exhibited dose dependent response (Table 4.3; Table 4.4). This could be due to insufficient bioactive compounds at higher concentration as compared to lower concentrations. The DCM leaf extract of *G. glauca* had the highest percent inhibition with a value of 96.97% (Table 4.4; Figure 4.6). These findings of anti-acetylcholinesterase activity were comparable to a study done by Kiendrebeogo *et al.* (2011), which revealed that the *Eucalyptus camaldulensis* and *Ocimum canum* had percent acetylcholinesterase inhibition of 83% and 72% respectively. In addition, crude ethanol extract of *P. emblica* was found to inhibit 62.18% of AChE and 59.17% of BUCHE at a concentration of 100

µg/ml whcich indicated it was a potential source of cholinergic inhibitor (Kushal *et al.,* 2015).

The study revealed that DCM leaf extracts of *G. glauca* had a higher percentage enzyme inhibition than aqueous leaf extracts in all concentrations except 25 and 50mg/ml. Perhaps the difference is due to phytochemicals of the two aqueous leaf extracts concentrations acting in synergy that resulted in higher percent enzyme inhibition. Another reason for the difference could be the constituents phytochemicals were in higher concentrations for larvicidal. Cyclone an organophosphate was used as the reference control for this study. A number of experimental studies have indicated that organophosphate pesticides inhibit AChE activity in organisms and they show different sensitivities to these pesticides (Manju *et al*., 2014).

A low IC_{50} value indicates that an extract is a good inhibitor of the enzyme because only a small amount will be required for maximum inhibition. In this study, the DCM leaf extracts of *C. glaucescens* had the lowest IC_{50} value (12.02 mg/ml) indicating that DCM is a better solvent for extraction of bioactive compounds with possible anti-AChE activities compared to aqueous leaf extracts (79.43 mg/ml) of the same plant. The higher activity of the organic extracts might suggest that organic solvents are able to extract more active compounds with possible AChE inhibitory activity than water (Deepak *et al*., 2009). On the other hand, the DCM leaf extract of *G. glauca* also had a lower IC_{50} value (39.81mg/ml) than aqueous (63.10mg/ml) of the same plant. This shows that *C. glaucescens* and *G. glauca* leaves contain non-polar compounds which dissolve in DCM solvent easily. All the tested plant

extracts of aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca* as well as the positive control contained a degree of inhibitory activity against acetylcholinesterase.

In this study results indicated that extracts of aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca* had tannins, phenols, flavonoids, terpenoids, alkaloids and steroids present which are comparable to another study which observed that the carbohydrates, saponins, phytosterols, phenols, flavonoids and tannins had mosquito larvicidal activity (Khanna and Kannabiran, 2007). Similarly, prenylated xanthones, tetracyclic phenols and saponins are reported to be effective in controlling mosquito *Aedes aegypti*, the vector of yellow fever (Marston *et al*., 1993).

The observed bioactivity of the extracts in this study can be attributed to the constituent phytochemicals in the extracts (Table 4.5). Most likely, the presence of phenols and flavonoids in the extracts could have contributed to the inhibition of AChE activity. These results are comparable with those reported by Wang *et al*. (2011), who reported a positive correlation of phenolics and flavonoids with acetylcholinesterase inhibitory activities. Similarly, a study conducted by Orhan *et al.* (2004) found out that since most of the acetylcholinesterase inhibitors are known to contain nitrogen, the higher activity of these extracts may be due to their rich alkaloidal content.

In this study, all the leaf extracts of the two plants were found to contain phenols and flavonoids. Research by Kadri *et al.* (2010) evaluated *C.*

schoenanthus and found they were rich in phenols and flavonoids and they had good AChE inhibitory activity comparable to butylated hydroxytoluene (BHT), a known standard. Therefore the fact that the two phytochemicals were present in the plants was an indication that they can be good AChE inhibitors. In addition, phenols and flavonoids play important role in plant defence against pathogens and herbivorous predators (Puupponen-Pimia *et al.,* 2008).

In another study, four diterpenes; dihydrotanshinone, cryptotanshinone, tanshinone I and tashinone IIA were isolated from the acetone extract of the dried root of *Salvia militiorrhiza* and it was concluded that these compounds contributed to the anti- acetylcholinesterase activity of the plant (Ren *et al.,* 2004; Orhan and Aslan, 2009). Therefore, the fact that terpenoids were found in aqueous leaf extracts and DCM leaf extracts of *C. glaucescens* and *G. glauca* is an indication that they may be responsible for the anti-acetylcholinesterase activity against *C. partellus* in this study.

The aqueous leaf extracts of *C. glaucescens* exhibited better enzyme activity inhibition than the DCM leaf extract. Hence, for extraction of the active compounds for AChE inhibition from *C. glaucescens,* water would be the best extraction solvent. To extract active compounds with acetylcholinesterase inhibitory activity, from *G. glauca,* water would be a good extraction solvent because it has a high percent enzyme inhibition of 90%. The fact that the aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca* showed potent larvicidal effect against *C. partellus* larvae is a confirmation of reported success in use of the two plants by peasant farmers in traditional control of *C. partellus* in Mbeere Sub-county.

5.2 Conclusion

In conclusion, this study has revealed that the aqueous leaf extracts of *C. glaucescens* and *G. glauca* have the potential of *in vitro* anti acetylcholinesterase activity in *C. partellus*. Results showed that percent enzyme inhibition for the reference pesticides was 96.97%, at 2.5mg/ml aqueous extract concentration, of *C. glaucescens* and *G. glauca* had 86.67% and 90%, respectively an indication that there was no significant difference in percent enzyme inhibition. Similarly, the DCM leaf extracts of *C. glaucescens* and *G. glauca* also have the potential of *in vitro* anti acetylcholinesterase activity in *C. partellus*. Results revealed that at all concentrations (2,5mg/ml, 5mg/ml, 7.5mg, 25mg/ml, 50mg/ml and 75mg/ml) of DCM of *C. glaucescens* and *G. glauca* had degree of enzyme inhibition

The two plants extracts had phytochemicals associated with acetylcholinesterase activity like tannins, phenols, flavonoids, terpenoids and alkaloids. Therefore the two plants can be used as biopesticide in the control of *C. partellus.* This study confirms and supports traditional use of the two plants in control of *C. partellus* by farmers. The null hypothesis of this study is thus rejected.

5.3 Recommendations

From this study, the following recommendation can be made:

i. Use of these leaf extracts as biocontrol agents against *C. partellus* because results showed that both plants have *in vitro* anti-acetylcholinesterase activity on *C. partellus.*

ii. Use of DCM as extraction solvent for *G. glauca* because the study results revealed that this extract had the lowest IC_{50}.

iii. Use of water as extraction solvent for *C. glaucescens* because aqueous extract had the highest percent enzyme exhibition.

5.4 Suggestions for further studies

i. Further study to use lower extract concentrations (lower than 2.5mg/ml) of aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca* than those used in this study because results revealed lower concentrations have higher enzyme inhibition. This will test whether lower concentration result to higher enzymatic activity.

ii. To carry out an *In vivo* study using the aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca* to confirm the observations obtained from this *in vitro* study.

iii. Determinations of other parameters like mortality and growth of *Chilo partellus* larvae upon exposure to aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca* which can go a long way in the management of *Chilo partellus*.

iv. Bioassay-guided isolation and purification of active phytochemicals of the aqueous and DCM leaf extracts of *C. glaucescens* and *G. glauca* with the aim of identifying which specific phytochemicals have acetylcholinestrase activity on *Chilo partellus*.

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APPENDICES

APPENDICES I: Comparison of readings from spectrophotometer of aqueous leaf extract of *C. glaucescens* and *G. glauca* on anti-acetylcholinesterase of *C. partellus*

Treatment (mg/ml)	Time in minutes	Mean Readings from spectrophotometer	
		C. glaucescens	*G. glauca*
I Normal control	1st min	0.846±0.029	0.846±0.029
	4th min	0.878±0.050	0.878±0.050
II positive control	1st min	3.565±0.070	3.565±0.070
	4th min	3.564±0.079	3.564±0.079
III 2.5 mg/ml plant extract	1st min	1.673±0.007	1.755±0.010
	4th min	1.676±0.006	1.751±0.010
IV 5.0 mg/ml plant extract	1st min	1.777±0.022	1.747±0.005
	4th min	1.775±0.019	1.742±0.004
V 7.5mg/ml plant extract	1st min	1.731±0.023	1.760±0.005
	4th min	1.726±0.050	1.754±0.003
VI 25 mg/ml plant extract	1st min	1.942±0.004	1.901±0.057
	4th min	1.931±0.002	1.893±0.053
VII 50 mg/ml plant extract	1st min	2.102±0.011	2.489±0.083
	4th min	2.090±0.006	2.476±0.070
VIII 75 mg/ml plant extract	1st min	2.210±0.082	2.574±0.056
	4th min	2.196±0.082	2.559±0.053

Values are expressed as mean ± SEM for triplicate readings

APPENDICES II: Comparison of readings from spectrophotometer of DCM leaf extract of *C. glaucescens* and *G. glauca* on anti-acetylcholinesterase of *C. partellus*

Treatment (mg/ml)	Time in minutes	Mean readings from spectrophotometer	
		C. glaucescens	*G. glauca*
I Normal control	1st min	0.846±0.029	0.846±0.029
	4th min	0.878±0.050	0.878±0.050
II positive control	1st min	3.565±0.070	3.565±0.070
	4th min	3.564±0.079	3.564±0.079
III 2.5 mg/ml plant extract	1st min	1.704±0.002	1.821±0.046
	4th min	1.695±0.001	1.820±0.047
IV 5.0 mg/ml plant extract	1st min	2.522±0.029	1.822±0.002
	4th min	2.507±0.042	1.819±0.003
V 7.5mg/ml plant extract	1st min	2.197±0.069	1.824±0.021
	4th min	2.179±0.068	1.821±0.025
VI 25 mg/ml plant extract	1st min	3.337±0.050	2.780±0.063
	4th min	3.329±0.050	2.756±0.055
VII 50 mg/ml plant extract	1st min	4.015±0.021	3.253±0.027
	4th min	3.987±0.057	3.228±0.027
VIII 75 mg/ml plant extract	1st min	3.783±0.002	2.152±0.006
	4th min	3.746±0.034	2.121±0.049

Values are expressed as mean ± SEM for triplicate readings

Appendix III: Analysis of readings from spectrophotometer of aqueous leaf extract of *C. glaucescens* on anti-acetylcholinesterase of *C. partellus*

Descriptive Statistics: Min 4 Reading

DCM extract *C. glaucescens*

Min 4 Reading

Variable	Mean	SE Mean	StDev
I Normal	0.878	0.050	0.087
II Positive control	3.564	0.079	0.136
III 2.5 mg/ml plant extract	1.695	0.001	0.002
IV 5 mg/ml plant extract	2.507	0.042	0.073
V 7.5 mg/ml plant extract	2.179	0.068	0.117
VI 25 mg/ml plant extract	3.329	0.050	0.087
VII 50 mg/ml plant extract	3.987	0.057	0.098
VIII 75 mg/ml plant extract	3.746	0.034	0.060

One-way ANOVA: Min 4 Reading versus DCM extractS *C. glaucescens*

Source	DF	SS	MS	F	P
DCM extract *C. glaucescens*	7	25.563	3.652	444.26	0.000
Error	16	0.132	0.008		
Total	23	25.695			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0907	99.49%	99.26%	98.85%

DCM extract *C. glaucescens*	N	Mean	StDev	95% CI
I Normal	3	0.878	0.087	(0.767, 0.989)
II Positive control	3	3.564	0.136	(3.453, 3.675)
III 2.5 mg/ml plant extract	3	1.695	0.002	(1.584, 1.806)
IV 5 mg/ml plant extract	3	2.507	0.073	(2.396, 2.618)
V 7.5 mg/ml plant extract	3	2.179	0.117	(2.068, 2.290)
VI 25 mg/ml plant extract	3	3.329	0.087	(3.218, 3.440)
VII 50 mg/ml plant extract	3	3.987	0.098	(3.876, 4.098)
VIII 75 mg/ml plant extract	3	3.746	0.060	(3.635, 3.857)

Tukey Pairwise Comparisons

DCM EXTRACT C. glaucescens	N	Mean	Grouping

VII 50 mg/ml plant extract	3	3.987	A
VIII 75 mg/ml plant extract	3	3.746	A B
II Positive control	3	3.564	B C
VI 25 mg/ml plant extract	3	3.329	C
IV 5 mg/ml plant extract	3	2.507	D
V 7.5 mg/ml plant extract	3	2.179	E
III 2.5 mg/ml plant extract	3	1.695	F
I Normal	3	0.878	G

Pooled StDev = 0.0907
Means that do not share a letter are significantly different.
Tukey Method and 95% Confidence

Appendix IV: Analysis of spectrophotometer reading of aqueous leaf extract of *G. glauca* on anti-acetylcholinesterase of *C. partellus*

Descriptive Statistics: Min 4 Reading_3

Variable	Aqueous extract *G. glauca*	Mean	SE Mean	StDev
Min 4 Reading_3	I Normal	0.878	0.050	0.087
	II Positive control	3.564	0.079	0.136
	III 2.5 mg/ml plant extr	1.751	0.010	0.018
	IV 5 mg/ml plant extract	1.742	0.004	0.006
	V 7.5 mg/ml plant extrac	1.754	0.003	0.005
	VI 25 mg/ml plant extrac	1.893	0.053	0.093
	VII 50 mg/ml plant extra	2.476	0.070	0.121
	VIII 75 mg/ml plant extr	2.559	0.053	0.091

One-way ANOVA: Min 4 Reading_3 versus AQ EXTRACT G. glauca

Source	DF	SS	MS	F	P
Aqueous extract G. glauca	7	13.190	1.884	260.33	0.000
Error	16	0.116	0.007		
Total	23	13.306			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0851	99.13%	98.75%	98.04%

Aqueous extract *G. glauca*	N	Mean	StDev	95% CI
I Normal	3	0.878	0.087	(0.774, 0.982)
II Positive control	3	3.564	0.136	(3.460, 3.668)
III 2.5 mg/ml plant extract	3	1.751	0.018	(1.647, 1.856)
IV 5 mg/ml plant extract	3	1.742	0.006	(1.638, 1.846)
V 7.5 mg/ml plant extract	3	1.754	0.005	(1.650, 1.858)
VI 25 mg/ml plant extract	3	1.893	0.093	(1.789, 1.997)
VII 50 mg/ml plant extract	3	2.476	0.121	(2.372, 2.580)
VIII 75 mg/ml plant extract	3	2.559	0.091	(2.455, 2.663)

Tukey Pairwise Comparisons

Aqueous extract *G. glauca*	N	Mean	Grouping
II Positive control	3	3.564	A
VIII 75 mg/ml plant extract	3	2.559	B

VII 50 mg/ml plant extract	3	2.476	B
VI 25 mg/ml plant extract	3	1.893	C
V 7.5 mg/ml plant extract	3	1.754	C
III 2.5 mg/ml plant extract	3	1.751	C
IV 5 mg/ml plant extract	3	1.742	C
I Normal	3	0.878	D

Pooled StDev = 0.0851
Means that do not share a letter are significantly different
Tukey Method and 95% Confidence

Appendix V: Analysis of spectrophotometer reading of DCM leaf extract of *C. glaucescens* on anti-acetylcholinesterase of *C. partellus*

One-way ANOVA: Min 1 Reading versus DCM extracts *C. glaucescens*

Tukey Pairwise Comparisons

DCM extract *C. glaucescens*	N	Mean	Grouping
VII 50 mg/ml plant extract	3	4.015	A
VIII 75 mg/ml plant extract	3	3.783	B
II Positive control	3	3.565	C
VI 25 mg/ml plant extract	3	3.337	D
IV 5 mg/ml plant extract	3	2.522	E
V 7.5 mg/ml plant extract	3	2.197	F
III 2.5 mg/ml plant extract	3	1.704	G
I Normal	3	0.846	H

Means that do not share a letter are significantly different.

Tukey Method and 95% Confidence

One-way ANOVA: Min 4 Reading versus DCM extracts *C. glaucescens*

Tukey Pairwise Comparisons

DCM EXTRACT C. glaucescens	N	Mean	Grouping
VII 50 mg/ml plant extract	3	3.987	A
VIII 75 mg/ml plant extract	3	3.746	A B
II Positive control	3	3.564	B C
VI 25 mg/ml plant extract	3	3.329	C
IV 5 mg/ml plant extract	3	2.507	D
V 7.5 mg/ml plant extract	3	2.179	E
III 2.5 mg/ml plant extract	3	1.695	F
I Normal	3	0.878	G

Means that do not share a letter are significantly different.

Tukey Method and 95% Confidence

Appendix VI: Analysis of spectrophotometer reading of DCM leaf extract of *G. glauca* on anti-acetylcholinesterase of *C. partellus*

Descriptive Statistics: Min 1 Reading_1

Variable	DCM extract *G. glauca*	Mean	SE Mean	StDev
Min 1 Reading_1	I Normal	0.878	0.050	0.087
	II Positive control	3.565	0.070	0.121
	III 2.5 mg/ml plant extr	1.821	0.046	0.080
	IV 5 mg/ml plant extract	1.822	0.002	0.003
	V 7.5 mg/ml plant extrac	1.824	0.021	0.037
	VI 25 mg/ml plant extrac	2.780	0.063	0.110
	VII 50 mg/ml plant extra	3.253	0.027	0.047
	VIII 75 mg/ml plant extr	2.152	0.006	0.010

One-way ANOVA: Min 1 Reading_1 versus DCM extracts *G. glauca*

Source	DF	SS	MS	F	P
DCM EXTRACT G. glauca	7	16.365	2.338	422.72	0.000
Error	16	0.089	0.006		
Total	23	16.454			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0744	99.46%	99.23%	98.79%

DCM extract *G. glauca*	N	Mean	StDev	95% CI
I Normal	3	0.878	0.086	(0.787, 0.969)
II Positive control	3	3.565	0.121	(3.474, 3.656)
III 2.5 mg/ml plant extract	3	1.821	0.080	(1.730, 1.912)
IV 5 mg/ml plant extract	3	1.822	0.003	(1.731, 1.913)
V 7.5 mg/ml plant extract	3	1.824	0.037	(1.733, 1.915)
VI 25 mg/ml plant extract	3	2.780	0.110	(2.689, 2.871)
VII 50 mg/ml plant extract	3	3.253	0.047	(3.162, 3.344)
VIII 75 mg/ml plant extract	3	2.152	0.010	(2.061, 2.243)

Tukey Pairwise Comparisons

DCM EXTRACT G. glauca	N	Mean	Grouping
II Positive control	3	3.565	A
VII 50 mg/ml plant extract	3	3.253	B

VI 25 mg/ml plant extract	3	2.780	C
VIII 75 mg/ml plant extract	3	2.152	D
V 7.5 mg/ml plant extract	3	1.824	E
IV 5 mg/ml plant extract	3	1.822	E
III 2.5 mg/ml plant extract	3	1.821	E
I Normal	3	0.878	F

Pooled StDev = 0.0744
Means that do not share a letter are significantly different.
Tukey Method and 95% Confidence

Descriptive Statistics: Min 4 Reading_1

Variable	DCM extract *G. glauca*	Mean	SE Mean	StDev
Min 4 Reading_1	I Normal	0.878	0.050	0.087
	II Positive control	3.564	0.079	0.136
	III 2.5 mg/ml plant extr	1.820	0.047	0.081
	IV 5 mg/ml plant extract	1.819	0.003	0.004
	V 7.5 mg/ml plant extrac	1.821	0.025	0.043
	VI 25 mg/ml plant extrac	2.756	0.055	0.095
	VII 50 mg/ml plant extra	3.228	0.027	0.047
	VIII 75 mg/ml plant extr	2.121	0.049	0.084

One-way ANOVA: Min 4 Reading_1 versus DCM extracts *G. glauca*

Source	DF	SS	MS	F	P
DCM extract *G. glauca*	7	16.180	2.311	351.42	0.000
Error	16	0.105	0.007		
Total	23	16.285			

Model Summary

S	R-sq	R-sq(adj)	R-sq(pred)
0.0811	99.35%	99.07%	98.55%

DCM extract *G. glauca*	N	Mean	StDev	95% CI
I Normal	3	0.878	0.087	(0.779, 0.977)
II Positive control	3	3.564	0.136	(3.465, 3.663)
III 2.5 mg/ml plant extract	3	1.820	0.081	(1.721, 1.919)
IV 5 mg/ml plant extract	3	1.819	0.004	(1.720, 1.918)
V 7.5 mg/ml plant extract	3	1.821	0.043	(1.721, 1.920)

VI 25 mg/ml plant extract 3 2.756 0.095 (2.657, 2.855)
VII 50 mg/ml plant extract 3 3.228 0.047 (3.129, 3.327)
VIII 75 mg/ml plant extract 3 2.121 0.084 (2.022, 2.220)

Tukey Pairwise Comparisons

DCM EXTRACT G. glauca	N	Mean	Grouping
II Positive control	3	3.564	A
VII 50 mg/ml plant extract	3	3.228	B
VI 25 mg/ml plant extract	3	2.756	C
VIII 75 mg/ml plant extract	3	2.121	D
V 7.5 mg/ml plant extract	3	1.821	E
III 2.5 mg/ml plant extract	3	1.820	E
IV 5 mg/ml plant extract	3	1.819	E
I Normal	3	0.878	F

Pooled StDev = 0.0811
Means that do not share a letter are significantly different.
Tukey Method and 95% Confidence

Appendix VII: Analysis of the change in absorbance of aqueous leaf extracts *C. glaucescens* on anti-acetylcholinesterase of *C. partellus*

Variable	Aqueous extract *C. glaucescens*	Mean	SE Mean	StDev
Reading	I Normal	0.032	0.058	0.101
	II Positive control	-0.001	0.055	0.100
	III 2.5 mg/ml plant extr	-0.002	0.008	0.014
	IV 5 mg/ml plant extract	-0.004	0.003	0.005
	V 7.5 mg/ml plant extrac	-0.005	0.033	0.057
	VI 25 mg/ml plant extrac	-0.011	0.008	0.014
	VII 50 mg/ml plant extra	-0.012	0.006	0.010
	VIII 75 mg/ml plant extr	-0.015	0.002	0.004

One-way ANOVA: Reading versus aqueous leaf extract *C. glaucescens*

Source	DF	SS	MS	F-Value	P-Value
Aqueous extract *C. glaucescens*	7	0.004	0.001	0.21	0.977
Error	16	0.046	0.003		
Total	23	0.051			

Model Summary: S=0.0538 R-sq=8.57% R-sq=0.00% R-sq=0.00%

Significance level $\alpha = 0.05$

Aqueous extract *C. glaucescens*	N	Mean	StDev	95% CI
I Normal	3	0.032	0.101	(-0.033, 0.098)
II Positive control	3	-0.001	0.096	(-0.067, 0.065)
III 2.5 mg/ml plant extract	3	-0.002	0.014	(-0.067, 0.064)
IV 5 mg/ml plant extract	3	-0.004	0.005	(-0.070, 0.061)
V 7.5 mg/ml plant extract	3	-0.005	0.057	(-0.071, 0.061)
VI 25 mg/ml plant extract	3	-0.011	0.014	(-0.070, 0.061)
VII 50 mg/ml plant extract	3	-0.012	0.010	(-0.078, 0.054)
VIII 75 mg/ml plant extract	3	-0.015	0.004	(-0.081, 0.051)

Tukey Pairwise Comparisons

AQ EXTRACT C. glaucescens	N	Mean	Grouping
I Normal	3	0.032	A
II Positive control	3	-0.001	A
III 2.5 mg/ml plant extract	3	-0.002	A
IV 5 mg/ml plant extract	3	-0.004	A
VI 25 mg/ml plant extract	3	-0.005	A

V 7.5 mg/ml plant extract	3	-0.011	A
VII 50 mg/ml plant extract	3	-0.012	A
VIII 75 mg/ml plant extract	3	-0.015	A

Pooled StDev = 0.0538

Means that do not share a letter are significantly different.

Tukey Method and 95% Confidence

Appendix VIII: Analysis of the change in absorbance of aqueous leaf extracts extracts *G. gnidia* on anti-acetylcholinesterase of *C. partellus*

Variable	Aqueous extract *G. glauca*	Mean	SE Mean	StDev
reading 1	I Normal	0.032	0.058	0.101
	II Positive control	-0.001	0.055	0.096
	III 2.5 mg/ml plant extr	-0.003	0.000	0.001
	IV 5 mg/ml plant extract	-0.005	0.002	0.003
	V 7.5 mg/ml plant extrac	-0.006	0.003	0.004
	VI 25 mg/ml plant extrac	-0.008	0.004	0.006
	VII 50 mg/ml plant extra	-0.016	0.011	0.019
	VIII 75 mg/ml plant extr	-0.015	0.019	0.033

One-way ANOVA: reading 1 versus aqueous extract *G. glauca*

Source	DF	SS	MS	F	P
Aqueous extract *G. glauca*	7	0.005	0.001	0.26	0.962
Error	16	0.042	0.003		
Total	23	0.047			

Model Summary: S = 0.0512 R-sq = 10.15% R-sq(adj)= 0.00% R-sq(pred)= 0.00%

Significance level $\alpha = 0.05$

Aqueous extract *G. glauca*	N	Mean	StDev	95% CI
I Normal	3	0.032	0.101	(-0.032, 0.095)
II Positive control	3	-0.001	0.096	(-0.064, 0.062)
III 2.5 mg/ml plant extract	3	-0.003	0.001	(-0.066, 0.060)
IV 5 mg/ml plant extract	3	-0.005	0.004	(-0.068, 0.058)
V 7.5 mg/ml plant extract	3	-0.006	0.005	(-0.068, 0.057)
VI 25 mg/ml plant extract	3	-0.008	0.006	(-0.071, 0.055)
VII 50 mg/ml plant extract	3	-0.016	0.019	(-0.079, 0.047)
VIII 75 mg/ml plant extract	3	-0.015	0.033	(-0.078, 0.048)

Tukey Pairwise Comparisons

Aqueous extract G. glauca	N	Mean	Grouping
I Normal	3	0.032	A
II Positive control	3	-0.001	A
III 2.5 mg/ml plant extract	3	-0.003	A
IV 5 mg/ml plant extract	3	-0.005	A
V 7.5 mg/ml plant extract	3	-0.006	A
VI 25 mg/ml plant extract	3	-0.008	A

VIII 75 mg/ml plant extract	3	-0.015	A
VII 50 mg/ml plant extract	3	-0.016	A

Pooled StDev = 0.051

Means that do not share a letter are significantly different.

Tukey Method and 95% Confidence

Appendix IX: Analysis of the change in absorbance of DCM leaf extracts *C. glaucescens* on anti-acetylcholinesterase of *C. partellus*

Variable	DCM extracts *C. glaucescens*	Mean	SE Mean	StDev
Reading	I Normal	0.032	0.058	0.101
	II Positive control	-0.001	0.055	0.096
	III 2.5 mg/ml plant extr	-0.009	0.003	0.005
	IV 5 mg/ml plant extract	-0.013	0.028	0.048
	V 7.5 mg/ml plant extrac	-0.018	0.002	0.004
	VI 25 mg/ml plant extrac	-0.021	0.004	0.008
	VII 50 mg/ml plant extra	-0.028	0.036	0.063
	VIII 75 mg/ml plant extr	-0.037	0.033	0.057

One-way ANOVA: Reading versus DCM extracts *C. glaucescens*

Source	DF	SS	MS	F	P
DCM extract *C. glaucescens*	7	0.009	0.001	0.36	0.911
Error	16	0.058	0.004		
Total	23	0.067			

Model Summary: S = 0.0602 R-sq = 13.71% R-sq(adj)= 0.00% R-sq(pred)= 0.00%

Significance level $\alpha = 0.05$

DCM extracts *C. glaucescens*	N	Mean	StDev	95% CI
I Normal	3	0.032	0.101	(-0.042, 0.106)
II Positive control	3	-0.001	0.096	(-0.075, 0.073)
III 2.5 mg/ml plant extract	3	-0.009	0.005	(-0.082, 0.065)
IV 5 mg/ml plant extract	3	-0.013	0.048	(-0.087, 0.061)
V 7.5 mg/ml plant extract	3	-0.018	0.004	(-0.092, 0.056)
VI 25 mg/ml plant extract	3	-0.021	0.008	(-0.095, 0.052)
VII 50 mg/ml plant extract	3	-0.028	0.063	(-0.101, 0.046)
VIII 75 mg/ml plant extract	3	-0.037	0.057	(-0.110, 0.037)

Tukey Pairwise Comparisons

DCM extracts *C. glaucescens*	N	Mean	Grouping
I Normal	3	0.032	A
II Positive control	3	-0.001	A
III 2.5 mg/ml plant extract	3	-0.009	A
IV 5 mg/ml plant extract	3	-0.013	A
V 7.5 mg/ml plant extract	3	-0.018	A
VI 25 mg/ml plant extract	3	-0.021	A

VII 50 mg/ml plant extract	3	-0.028	A
VIII 75 mg/ml plant extract	3	-0.037	A

Pooled StDev = 0.0602273

Means that do not share a letter are significantly different

Tukey Simultaneous 95% CIs

Appendix X: Analysis of the change in absorbance of DCM leaf extracts *G. gnidia* on anti-acetylcholinesterase of *C. partellus*

Variable	DCM extracts *G. glauca*	Mean	SE Mean	StDev
Reading 1	I Normal	0.032	0.058	0.101
	II Positive control	-0.001	0.055	0.096
	III 2.5 mg/ml plant extr	-0.001	0.001	0.001
	IV 5 mg/ml plant extract	-0.003	0.001	0.002
	V 7.5 mg/ml plant extrac	-0.004	0.004	0.007
	VI 25 mg/ml plant extrac	-0.018	0.011	0.019
	VII 50 mg/ml plant extra	-0.025	0.018	0.031
	VIII 75 mg/ml plant extr	0.010	0.048	0.083

One-way ANOVA: Reading 1 versus DCM extracts *G. glauca*

Source	DF	Adj SS	Adj MS	F-Value	P-Value
DCM extract *G. glauca*	7	0.006	0.001	0.25	0.963
Error	16	0.055	0.003		
Total	23	0.061			

Model Summary: S = 0.0588 R-sq = 10.00% R-sq(adj)= 0.00% R-sq(pred)= 0.00%

Significance level $\alpha = 0.05$

DCM extract *G. glauca*	N	Mean	StDev	95% CI
I Normal	3	0.032	0.101	(-0.041, 0.103)
II Positive control	3	-0.001	0.096	(-0.073, 0.071)
III 2.5 mg/ml plant extract	3	-0.001	0.001	(-0.073, 0.071)
IV 5 mg/ml plant extract	3	-0.003	0.002	(-0.075, 0.070)
V 7.5 mg/ml plant extract	3	-0.004	0.007	(-0.076, 0.068)
VI 25 mg/ml plant extract	3	-0.018	0.019	(-0.090, 0.054)
VII 50 mg/ml plant extract	3	-0.025	0.031	(-0.097, 0.047)
VIII 75 mg/ml plant extract	3	0.010	0.083	(-0.062, 0.082)

Tukey Pairwise Comparisons

DCM extract *G. glauca*	N	Mean	Grouping
I Normal	3	0.031	A
VIII 75 mg/ml plant extract	3	0.010	A
III 2.5 mg/ml plant extract	3	-0.001	A
II Positive control	3	-0.001	A
IV 5 mg/ml plant extract	3	-0.003	A
V 7.5 mg/ml plant extract	3	-0.004	A

VI 25 mg/ml plant extract	3	-0.018	A
VII 50 mg/ml plant extract	3	-0.025	A

Pooled StDev = 0.0588

Tukey Method and 95% Confidence

Means that do not share a letter are significantly different.