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STUDY ON *IN VITRO* LOUSCIDAL AND ACARICIDAL PROPERTIES OF *CALPURNIA AUREA*, *OTOSTEGIA INTEGRIFOLIA*, *NICOTIANA TABACCUM* AND *JATROPHA CURCAS* AGAINST *BOVICOLA OVIS* AND *AMBLYOMMA VARIEGATUM*

MSc Thesis

By

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June, 2015

Bishoftu, Ethiopia

STUDY ON *IN VITRO* LOUSCIDAL AND ACARICIDAL PROPERTIES OF *CALPURNIA AUREA*, *OTOSTEGIA INTEGRIFOLIA*, *NICOTIANA TABACCUM* AND *JATROPHA CURCAS* AGAINST *BOVICOLA OVIS* AND *AMBLYOMMA VARIEGATUM*

A Thesis submitted to College of Veterinary Medicine and Agriculture of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Tropical Veterinary Parasitology

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June, 2015,

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APPROVAL

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As members of the Examining Board of the final MSc open defense, we certify that we have read and evaluated the Thesis prepared by: Sisay Alemu Mamo entitled STUDY ON *IN VITRO* LOUSCIDAL AND ACARICIDAL PROPERTIES OF *CALIPURNIA AUREA*, *OTOSTEGIA INTEGRIFOLIA*, *NICOTIANA TABACCUM* AND *JATROPHA CURCAS* AGAINST *BOVICOLA OVIS* AND *AMLYOMMA VARIEGATUM* and recommend that it be accepted as fulfilling the thesis requirement for the degree of: Masters of Science in Tropical Veterinary Parasitology.

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DEDICATION

I dedicate this MSc thesis to my baby; I dreamed a little dream, Once upon a time. I dreamed we'd be together one day, Sweet little baby of mine. Sadly that dream was not meant to be, and it's very difficult to know, that now you won't be coming to me. You weren't strong enough to thrive and grow, but I know that you're in heaven now and that's a very good place to be. And I know that when I get there, I'll recognize you, and you'll know me. We'll get to share the love we would have shared here on this earth. And then we'll know without a doubt what all this waiting was worth. Also I dedicate this MSc thesis to my best friend Seyoum Asnake, may God bless him.

STATEMENT OF THE AUTHOR

First, I declare that this thesis is my *bonafide* work and that all sources of material used for this thesis have been duly acknowledged. This thesis has been submitted in partial fulfillment of the requirements for an advanced (MSc) degree at Addis Ababa University, College of Veterinary Medicine and Agriculture and is deposited at the University/College library to be made available to borrowers under rules of the Library. I solemnly declare that this thesis is not submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate.

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Date of Submission:__________________________

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LIST OF ABBREVIATIONS

AAU	Addis Ababa University
CFSPH	Center for Food Security and Public Health
Cpds	Compounds
CSA	Central Statistics Authority
CVM	College of Veterinary Medicine
DDT	Dichloro-Diphenyl-Trichloro-Ethane
DEET	N,N-diethyl-m-toluamide
MoARD	Mnistery of Aagricultural and Rular Developement
NTHNC	National Travel Health and Network Centre
UK	United Kingdom
USD	United States Dollar
WHO	World Health Organization

ABSTRACT

The present study was designed to evaluate the preliminary phytochemicals present in methanolic leaf extracts of *Calpurnia aurea, Otostegia integrifolia, Nicotiana tabaccum* and petroleum ether seed oil extract of *Jatropha curcas* and to assess their *in vitro* louscidal and accaricidal efficacy. Accordingly, each plant was subjected to qualitative phytochemical screening for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tannins, phlobatannins, triterpens and glycosides using standard procedures. The four selected medicinal plants and a commercially used acaricide (0.1% diazinon) were examined for their louscidal and acaricidal activity against *B. ovis* and *A. variegatum* at different time intervals using the *in vitro* adult immersion test at concentrations of 200, 100, 50, 25, 12.5 and 6.25 mg/ml. The findings revealed that extracts of *C. aurea* leaf and *J. curcas* seed oil caused high mortalities of *B. ovis* at all concentrations with no significant difference from the activity of 0.1% diazinon ($P > 0.05$) within 24 hours of exposure. Moreover, at 200 mg/ml concentration, crude extracts of *N. tabaccum* and *O. integrifolia* produced 93% and 63% mortality respectively against *B. ovis* within 24 hours of exposure. The extracts of all four plants were also examined for their acaricidal activity against *A. vareigatum.* Although, the effects of the extracts on this tick species was much lower than what was observed on lice, the extracts of *C. aurea* leaf and *J. curcas* seed oil have shown 56% and 39% mortality at 200 and 100 mg/ml concentrations, respectively within 24 hrs exposure of the parasite and was comparable to the acaricidal activity of 0.1% diazinon ($P > 0.05$). A similar situation was caused by crude extracts of *N. tabaccum* which was 44% and 39% mortality at concentrations of 200 and 100 mg/ml within 24 hrs of exposure, respectively. However, *O. integrifolia* showed significantly lower acaricidal activity ($P < 0.05$) when compared to the reference drug (0.1% diazinon). The current study revealed that crude extracts of *C. aurea* leaf and *J. curcas* seed oil have strong louscidal activity. Moreover, crude extracts *C. aurea* and *J. curcas* showed high mortality at lower concentrations and can be used as a potential alternative to synthetic acaricides to control *B. ovis* infestations. Though, they are not effective at lower concentrations, crude extracts of *C. aurea, N. tabaccum* and *J. curcas* seed oil had better acaricidal effects on cattle ticks at higher concentrations. This is a promising finding to have an alternative means of treatment to substitute the use of synthetic drugs which have a widespread drug resistance especially in developing countries including Ethiopia.

Therefore, further investigation should be made on their safety and *in vivo* efficacy as well as cost effectiveness of the products that exhibited strong louscidal and acaricidal activity with a view of substituting the conventional synthetic acaricides.

Key words: *Bovicola ovis, Amblyomma variegatum, Medicinal plants, Mortality, phytochemical screening,*

1. INTRODUCTION

Ethiopia has a huge livestock resource. The small ruminant population of Ethiopia is about 27.35 million sheep and 28.16 million goats (CSA, 2014). Small ruminants are important contributors to food production in Ethiopia, providing 35% of meat consumption and 14% of milk consumption (Asfaw 1997). Besides, skins from goats and sheep are important economic products contributing for the largest share to the total and agricultural export commodities (Ayele *et al*., 2003). Based on annual off take rates of 7% for cattle, 33% for sheep and 35% for goats, the potential production is estimated at 3.78 million cattle hides, 8.41 million sheep skins and 8.42 million goat skins in 2012/2013 (CSA, 2013). However, their contribution to food production, rural income and export income are still far below the expected potential. This is because small ruminants particularly sheep production in Ethiopia is constrained by the compound effects of diseases, poor feeding and poor management (Hirpa and Abebe, 2008).

In Ethiopia, as many as one-quarter to one-third of all skins processed at tanneries have various defects and are unsuitable for export purposes (Kassa, 1998). Up to 65% of these defects occur in the pre-slaughter stage of production while the animals are alive. Ectoparasites are described as the predominant causes of pre-slaughter skin defects (FAO, 1985). Skin problems caused by ectoparasites such as mange mites, lice, keds and ticks and other skin diseases result in serious economic losses to smallholder farmers, the tanning industry and the country as a whole. According to Kassa (1998), skin problems due to ectoparasites caused 35% of sheep skin and 56% of goat skin rejection. Skin diseases are known to affect the quality of skin. In 1996/97 six tanneries that are found in and around Addis Ababa have rejected 2,037,745 pieces of skins which caused loss of 6.3 million USD (Kassa, 1998) and in 1998/99 three tanneries that are found in Amhara region have reported 443,602 pieces of skin rejection per annum which worth 1.4 million USD (MoARD, 2005).

The infestation of lice on sheep and goats has been reported from different parts of Ethiopia. Lice were considered as causes of cockle following keds and are visible on the skin surface of affected animals leading to skin rejection (Kidanu, 2001). Apart from the occurrence of lice in small ruminanats, higher percentages of tick infestation on cattle have been documented. For instance, in Somali Region of Ethiopia 90% of tick were often obtained from cattle and camels compared

to other pests (Burgdorfer *et al*., 1973). Approximate counts made *in situ* on individual cattle in Borana District, Sidamo Province of Ethiopia, revealed 100 ticks per animal infestation (Walker *et al*., 2000).

Considering the development potential and economic importance of hides and skins, in the last many years, the government of Ethiopia has excuted different development programs aimed at increasing the supply and improving the quality of the raw material. The major intervention tool was the use of acricides such as diazinon, amitraz and other organophosphates and synthetic pyrethroid compounds. Despite these development interventions, hides, skins and the leather industry are still constrained by the poor quality of raw materials (Mahmude, 2000).

Acaricides application is the most widely employed approach and in many African countries it is the only approach towards the control of ectoparasites. It is cheaper and easier to attempt to kill the parasite. However, several reports have shown that the use of some of these acaricides is constrained by growing development of resistance, high toxicity and environmental concerns (De Castro, 1997). In addition, control methods based on the use of synthetic acaricides sometimes fail to keep the number of ectoparasites below economic threshold levels. It is therefore necessary to find alternatives that can minimize negative effects of synthetic acaricides.

The natural world has over the years been a major source of medicinal agents and despite the recent advances in pharmacology and synthetic organic chemistry, plant biomolecules (phytocompounds) continue to provide key lead structures and therapeutic agents for the treatment of many diseases (Phillipson and Wright, 1991; Camacho *et al*., 2000). Plants have provided the basis for traditional treatment for different types of diseases and still offer an enormous potential source of new chemotherapeutic agents. According to an estimate, between 35,000 and 70,000 plant species are used in folk medicine worldwide. Products from hundreds of species are being collected from remote forests and meadows and traded to international markets and consumed (Olsen and Bhattarai, 2005). Similarly, in developed countries 70% to 80% of the populations have used some form of alternative or complementary medicine (WHO, 2008). The identification of novel active plant derived natural compounds could increase the number of available chemotherapeutic agents, thereby reducing the frequency of development of resistance

and providing alternative drugs with greater acceptance, especially in terms of environmental safety (Alawa *et al*., 2003).

In Ethiopia, botanical surveys have already shown that *Calpurnia aurea, Otostegia integrifolia, Nicotiana tabaccum and Jatropha curcas* are traditionally used against ectoparasites of livestock in ethno-veterinary practices (Mirutse and Gobena, 2003; Bekele *et al*., 2012; Teklay *et al.*, 2013). However, limited researches have been done to explore the *in vitro* and *in vivo* effects of such selected medicinal plants against lice and ticks. We hypothesized that since these plants have been used traditionally by the livestock keeping community, their extracts have good acaricidal activity aganst the commonly prevalent ectoparasites, ticks and lice.

Therefore, the objectives of this research were to:

- determine the secondary metabolites present in each study plant extract
- evaluate the *in vitro* louscidal and acaricidal activity of methanolic and petroleum ether extracts of the selected medicinal plants against *B. ovis* and *A. variegatum*.

2. LITREATURE REVIEW

2.1. Ectoprasitism

The term ectoparasites or external parasites refer to "parasites, with few exceptions, that live or burrow into the surface of their host's epidermis" (Wall and Shearer, 1997). Ectoparasites acquire blood meal from their host without penetrating the entire body of their host. It is only the mouthparts that are inserted into the integument of the host. However, there are reports on ticks occurring in the subcuticular regions or deeper regions of the host skin (Sonenshine, 1991). The association between arthropod ectoparasites and vertebrate hosts may take on a variety of forms. In some cases, the parasite may be totally dependent on the host, alternatively, the parasite may feed, or live only occasionally on the host, without being dependent on it (Wall and Shearer, 1997).

The effect of skin parasitism usually depends on the size of invading population, on the manner on which the parasite ekes out its existence and the state of nutrition of the host animal when infected. The damage ectoparasites inflict may be mechanical, but the situation is complicated also by host reactions to the presence of the particular parasite, their secretion and excretion (Peter, 1995). Young animals are generally more susceptible to ectoparasites because of higher region of accessible surface to the body volume and poor grooming behavior (Lehmann, 1993).

2.1.1. Lice Infestation

The lice belong to the order *Phthiraptera* which is divided into four suborders; *Anoplura, Amblycera, Ischnocera and Rhynchophthirina*. *Rhynchophthirina* is a very small suborder that includes just two species, one of which is a parasite of elephants and the other for warthogs. *Amblycera* and *Ischnocera* are known as chewing lice while *Anoplura* are described as sucking lice (Wall and Shearer, 1997).

Both biting and sucking lice affect small ruminants. The important species in sheep and goats are found in the genus *Bovicola* and *Linognathus.* Lice usually are unable to survive for more than 1-2 days off their host and tend to remain with a single host animal throughout their lives. Most species of louse are highly host specific and many species specialize in infesting only one part of

their host body and transfer to new hosts is by body contact, particularly under condition of close confinement (Peter, 1995).

The most common lice affecting sheep are the sheep body louse (*Bovicola ovis*, formerly called *Damalinia ovis*). The adults are about 1.6 mm long and have a pale brown body with dark bands. The young lice (nymphs) are smaller with a cream colored body and a brown head, but no bands on the body (Jenny, 2013). *Bovicola ovis* has typical life cycle. The female deposits about two eggs, attached to the wool or hair next to the skin by a viscid substance, every three days. The egg hatch in 9-10 days, and the nymph matures in about 21 days (Bay and Harris, 1988). Infestations with *B. ovis* occur on over all areas of the body but the upper sides of the animal are favoured. This species move rapidly over the wool fiber but is usually found near the skin (Bay and Harris, 1988).

Obviously, a critical factor in the likelihood of infestations beginning from non-sheep sources is the period for which lice can live away from sheep. Although the standard statement is that lice will not survive for more than 4 to 5 days after removal from sheep, it now seems that the potential period of survival is much longer than this. Laboratory studies were conducted to investigate the relative periods of survival of the different life stage of *B. ovis* and the effects of temperature and the presence of wool on survival period. Large nymphs survived significantly longer than both small nymphs and adults and both age groups of nymphs survived longer than adults. This pattern was consistent across all temperatures. In a comparison of survival, at 4°C, 20°C, 25°C and 36.5°C lice consistently lived longest at 25°C and lice lived significantly longer at both 20°C and 36.5°C than at 4°C. The survival periods measured at 25°C (Crawford *et al.,* 2001). Significant mortalities may also be caused by rapid reversal of temperature gradients in the fleece as sheep walk from shade into sunlight. Eggs fail to hatch at humidities above 90% and if the fleece remains saturated for more than 6 hrs many nymphs and adults can drown (Murray 1963).

Bovicola ovis are susceptible to extremes in temperature and humidity and move up and down the wool fiber to accommodate these changes. They prefer to live at 37°C and 70-90% humidity. Above 39°C the number of eggs laid is reduced, and at 45°C no eggs are laid. On a hot day the fleece temperature on exposed parts of a sheep, with less than 25 mm of wool, may range from

45°C near the skin to 65°C at the wool tip. These temperatures are too hot for eggs and young lice to survive. Also lice and eggs do not survive extended periods of very low temperatures. Lice and their eggs do not survive for very long off the sheep. Survival of lice in wool on fences and in yards is very short. This is due to lack of food, exposure to sunlight and desiccation as well as temperature fluctuations between night and day (Levot *et al*., 2010).

When inspecting sheep for lice, at most times of the year greatest attention should be paid to the sides of the sheep. However, soon after shearing, inspections should also include the neck and lower body regions and areas where longer wool has been left. It should be noted that the chance of detecting lice in the early stages of an infestation is very low. For example, for a sheep with 10 lice, the probability of detecting the infestation by inspecting 10 fleece partings is less than 5%. Even with 40 partings the probability is less than 20% (James *et al*., 2002).

Biting lice feed on the skin and scurf. Being highly active, *B. ovis* is usually considered to be most pathogenic in sheep and it can cause great irritation so that the sheep are restless and have their grazing interrupted. Rubbing and/or bitting leads to wool loss, excoriations and serum exudation. The exuded serum from the wounds cause wool matting and the wound itself may attract blowflies. *B ovis* infestation in sheep is reported to cause an allergic dermatitis referred to as 'scatter cockle' (Pfeffer *et al*., 2010).

Lice infestations in small ruminants in Ethiopia were reported with an overall prevalence of 1.52% in goats and 2% in sheep (Haffeze, 2001) from central Ethiopia; 0.53% in goats and 0% in sheep (Molu, 2002) from southern range land; 14.25% in goats from Komblocha (Numery, 2001); and 2.4% in sheep and 28.8% in goats (Tefera, 2004). The louse species identified were 0.8% *B. ovis* and 1.2% *Linognathus* species in sheep and 1.52 % *Linognathus* species in goats in central Ethiopia (Haffeze, 2001) and 11.54% *Linognathus stenopsis* and 2.71% *B. capre* in goats from Kombolcha (Numery, 2001). However, results obtained from the examination of fresh sheep pelts showed a much higher infestation rate of 89.55% (Ermias, 2000).

The highest prevalences of lice were those recently reported in sheep from Assela by Hailu (2010) who identified *Linognathus* spp. (75.5%) and *B. ovis* (67.1%) as well as Asnake *et al*. (2013) who recorded *Linognathus ovillus* (14.6%) and *B. ovis* (36.1%). Other reports were *B. ovis* infestation in 15.3% sheep and 27.9% goats and *L. ovillus* in 27.9% sheep in Tigray (Mulugeta *et*

al., 2010) and *B. ovis* 26.64% in sheep in Wolayta Sodo (Yacob *et al*., 2008a). In the main, the lice species of sheep and goats identified in several studies conducted so far in Ethiopia were *B. ovis* and *L. stenopsis* (Hailu, 2010).

2.1.2. Ticks Infestation

Ticks are obligate, blood feeding ectoparasites of vertebrates, particularly mammals and birds. They belong to three families *Ixodiadae, Argasidae* and *Nutelielidae*. *Ixodidae*, known as hard ticks, contain almost all the species of ticks of veterinary importance. The second family *Argasidae,* known as soft ticks contains relatively small number of species of veterinary importance (Wall and Shearer, 1997; Okello-onen *et al.,* 1999). According to Wall and Shearer (1997), ticks are primarily parasites of wild animals and only about 10% of the species feed on domestic animals, primarily sheep and cattle.

The body of tick comprises of two main regions: gnathosoma and idiosoma. Gnathosoma includes the basis capituli and mouthparts. The mouthparts consist of a pair of four-segmented palps, a pair of two-segmented chelicerae and a hypostome. Ticks use the chelicerate to penetrate the epidermis of their host and insert the hypostome with retrograde teeth into the wound. The retrograde teeth on the hypostome, together with the cement material secreted by tick's salivary glands, enhances attachment of tick to its host (Sonenshine, 1991).

The life cycle of ticks vary widely. Some species pass their entire life on the host, others pass different stages of the life cycle on successive host, and others are parasitic only at the certain stages. Most ticks spend more time off the host, but are totally dependent on the host for sustenance. They are subjected to microenvironment condition when on the ground and thus tend to be more endemic in specific types of area. Ticks can exist for a long period of time without feeding (Peter, 1995).

Attachment to the host causes irritation of the skin, with subsequent ulceration and sometimes secondary bacterial infections. In addition, tick wounds may become infested by screw-worms or other agents of myiasis, and are also associated with the spread of bovine dermatophilosis (streptotrichosis) caused by bacteria known as *Dermatophilus congolensis.* Heavy infestations of ticks can result in anaemia, particularly in small animals, and the restlessness caused by the

presence of large numbers of ticks can lead to a significant loss of weight and condition (Pegram and Osterwijk, 1990; De Castro, 1997). Ticks are important vectors for diseases like babesiosis, anaplasmosis and erlichiosis in domestic ruminants. They are known to exacerbate non specific disease symptoms like anemia, toxicosis and paralysis (Morel, 1980).

The tropical bont tick, *Amblyomma variegatum* Fabricius, is a three-host tick that originated in Africa (yonow, 1995). It has since spread to several countries, including the Caribbean islands, where it is known as the ‘Senegalese tick’ (CaribVet, 2011b) and the ‘Antigua gold tick’ (Pegram *et al*. 2004). The name 'Senegalese tick' came about because of the suspected introduction of the tick from cattle imports from Senegal to the Caribbean (Barré *et al.*, 1995). They are vividly coloured and decourated ticks, especially the males (CaribVet, 2011b; Merck 2011).

Amblyomma variegatum, the ‘tropical bont tick’, are relatively large and have a bright colouration that makes them easily identifiable (CaribVet, 2001b; Merck, 2011; Pegram *et al*., 2004). They sometimes have bright, yellow-gold colouration that is seen in the males that led to the common name, ‘Antigua gold tick’ (CFSPH, 2006; Pegram *et al*., 2004). Females are usually brown and when fully engorged can be the size of a "nutmeg" (approximately 2 to 3 centimeters long) (CFSPH, 2006). As a member of the family Ixodidae, the tropical bont tick is considered a hard tick and has a scutum (Georgi and Georgi, 1990).

In females the scutum is smaller with a wide posterior angle and straight sides. Due to the scutum being smaller, it only provides partial coverage of the dorsal surface, which, as feeding or engorgement commences, covers a progressively smaller percentage of her body (Georgi and Georgi, 1990). The posterior lips of the female genital aperture forms a wide shaped “U” (Walker *et al*., 2007). In general, ‘tropical bont ticks’ also have long and thick mouthparts that allow them to become firmly embedded in their hosts (Georgi and Georgi, 1990).

The ‘tropical bont tick’ has had a huge effect on the livestock industry, primarily through its transmission of heartwater disease, *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*) (Allan *et al*., 1998; CaribVet, 2011b; OIE, 2009; Parola *et al*., 1999) and their association with dermatophilosis, *D. congolensis* (Allan *et al*., 1998; Barré and Garris, 1990; CaribVet, 2011b; Merck, 2011). The tropical bont tick has also been implicated as a vector or potential vector for several diseases that include Crimean Congo haemorrhagic fever virus, Dugbe virus, yellow fever

virus, *Rickettsia africae* (African tick bite fever) and Jos virus (Merck 2011). The subsequent damage from the mouthparts predisposes the host to infection from various diseases such as dermatophilosis (CaribVet, 2011a; Merck, 2011).

In the Caribbean, only heartwater disease and dermatophilosis have yet been detected in the hosts and have demonstrated clinical symptoms. The testing of ticks and seropositive blood tests of cattle have led to the conclusion that African tick bite fever is widespread in the islands, but there have been few positive human case reports (Kelly *et al*., 2010; NTHNC, 2008). There is a low incidence of documented reports of infection by other diseases in association with the tropical bont tick, and they occur primarily in Central Africa (Merck, 2011).

In Ethiopia, ticks occupy the first place amongst the external parasites by the economic loss they incurred when they infest livestock particularly cattle (Feseha, 1983). Ticks are common in all agro-ecological zones of Ethiopia (Morel, 1980; Pegram *et al*., 1981). Extensive surveys have been carried out, in Ethiopia, on the distribution of tick on livestock in different region of the country (De Castro, 1994; Morel, 1980; Pegram *et al*., 1981) like Gamogofa (Jewaro, 1986), in Gonder (Eshetu, 1988), in Bale (Dejenu, 1988), the highland areas of Harer and Diredawa (Manuri and Tilahun, 1991) and in Jeff, Wellega and Illubabor (De Castro, 1994). *A. variegatum* is the most widely distributed tick species in Ethiopia (Morel, 1980; Pegram *et al*., 1981; De Castro, 1994). Other tick species such as *Rhipicephalus evertsi*, *Hyalomma marginatum rufipes*, *Hyalomma truncatum*, *Amblyomma cohaerens*, *Amblyomma gemma*, *Amblyomma lepidum and Rhipicephalus pulchellus* were also frequently reported in many tick surveys carried out in the country (Solomon *et al*., 2001).

2.1.3. Ectoparasites Control Methods

Farmers mostly rely on the use of chemical acaricides and repellents to control ticks and limit the production losses. In order to reduce contact between ticks and vertebrate hosts, chemical repellents such as N,N-diethyl-m-toluamide (DEET) and permethrin are extensively used (Faulde *et al*., 2003; Klun *et al*., 2003). Acaricides typically are highly lethal to ticks, and field applications generally are quite effective in reducing tick numbers (Stafford and Kitron, 2002; Jernigan *et al*., 2000). Organophosphates (diazinon, fampur, phosmet, dichlorvos), synthetic pyrethroids (resmethrin, deltamethrin, permethrin), carbamates (carbofuran, propoxur), growth

regulator (fenoxycarb, methroprene), amitraz, fipronil and methandiol that are currently being used for tick control. Although clearly effective at reducing transmission of tick-borne pathogens to livestock, repeated heavy applications of pesticides to hosts can cause considerable mortality in non-target arthropods through environmental contamination (Gassner *et al*., 1997). Moreover, evolved resistance to acaricides, which is a well-known problem with mosquitoes, is a persistent issue for tick species such as *Rhipicephallus microplus* that are chronically exposed by virtue of their close association with cattle to which the acaricides are applied (Foil *et al*., 2004, George *et al*., 2004).

2.1.4. Challenges of Ectoparasites Control

Problems posed by synthetic acaricides, resistant tick are on the rise due especially to increased frequency in the application of acaricides (Jonsson *et al*., 2000). For instance, *R. microplus* has developed resistance to synthetic pyrethroids and amitraz (Beugnet and Chardonnet, 1995; Jonsson *et al*., 2000); amitraz, chlorfenvinphos and cypermethrin against *Boophilus decolouratus* (Mekonnen *et al.,* 2002). The resistance mechanism of ticks such as *R. microplus* to acaricides (coumaphos and diazinon) has been linked to an enhanced cytochrome P 450 monooxygenase-mediated detoxification (Li *et al*., 2003).

Environmental pollution is a serious problem posed by the use of synthetic acaricides in tick control. Chemical compounds such as dichloro-diphenyl-trichloro-ethane (DDT), endosulfan and endosulfan sulphate are toxic and bioaccumulate in nature (Bhattacarya *et al*., 2003). Accumulation of these contaminants in water, soil and animals has been reported in Jamaica (Mansingh and Wilson, 1995). In 1961, the breeding number of peregrine falcons fell drastically and this was correlated with abnormally high residual levels of metabolites of DDT and dieldrin found in both the tissue and the carcasses of birds that fed on seeds treated with these compounds (Jarvis, 2000). The accumulation of toxic chemicals obviously has an amplified effect on the food chain leading to magnification of toxic residues in animals occurring at higher levels of the food chain (Boudou and Ribeyre, 1997).

Acaricides were also identified in honey bees using reversed-phase high performance liquid chromatography (Martel and Zeggane, 2002). It is obvious that such a situation is potentially dangerous to humans. Organophosphate accumulation in fatty tissue of mammals can lead to

poisoning in man (Karalliede *et al*., 2003). According to Selim *et al*. (1995), DEET can be absorbed through the skin of humans. Recently, permethrin that was used to repel arthropods has also been implicated in Gulf war related diseases (Riviere *et al*., 2002).

2.1.5. Alterbnative Approaches to Chemical Control

Ethno-veterinary plants use for tick control is very important in Africa and other developing countries since a greater proportion of livestock farmers are small-scale and most of these are in rural areas where cultural practices are still preserved (Madge, 1998). Plant extract preparations are developed by farmers rather than scientists due to lack of finance to purchase synthetic acaricides which force them to depend on traditional methods of tick control (Liang *et al*., 2003) thus making them likely less toxic to the environment and non-targeted species (Castagnoli *et al*., 2002).

Traditional knowledge on the use of ethno-veterinary plants for tick control is fast disappearing due to the lack of documentation since this type of knowledge has been transferred orally. Furthermore, the efficacy of most plants that have been traditionally used hasn't been scientifically tested. Due to the economic and medical importance of ticks, it is necessary to screen some ethno-veterinary plants that have acaricidal properties and could be used widely. Some of the advantages of promoting research on ethno-veterinary include the development of plant-derived semiochemicals which may be easily accessible by the rural communities and their low toxicity and biodegradability; thus, the need for their conservation. Plants are increasingly being recognized as possible sources of anti-tick agents. The use of plants or plant-based products for the control of arthropod ectoparasites on livestock is widespread among small scale livestock keepers in Africa (Lwande *et al*., 1999; Kaaya, 2003, Matlebyane *et al*., 2010). This practice is typically community-based and as a result, the plant species used for such purposes may vary from one community to another. Furthermore, knowledge on such practices is orally transferred from one generation to another and often lacks scientific validation. A number of studies have so far been conducted to validate the use of plants for tick control. For instance, most recently Zorloni *et al*. (2010) demonstrated that extracts of *C. aurea* leaves used by the Borana people of northern Kenya and Southern Ethiopia to treat lice infestations in humans and calves. Besides, Magano *et al*. (2008) and Thembo *et al*. (2010) have described that *C. aurea* had anti-tick properties.

2.2. Hrbal Treatment

2.2.1. Study plants

Plants have provided the basis for the traditional treatment of different types of diseases and still offer an enormous potential source of new chemotherapeutic agents. Different parts of plants have been used to treat ectoparasites both in animals and man. These are roots, barks, leaves and seeds (Teklay *et al*., 2013; Bekele *et al*., 2012; Das *et al.*, 2010). The following are plants selected for this study based on their traditional use by farming communities.

a) *Calpurnia aurea*

Calpurnia aurea belongs to Fabaceae family and is commonly known as *Natal laburnum*. It is a small, multi-stemmed tree (3-4 m) tall. The leaves are about 15-25 cm long, each bearing 5-20 pairs of ovate to oblong leaflets, light green and 2-5 cm long, ending with a terminal one (Figure 1). The flowers are bright yellow, in racemes and the fruits are flat brownish pods (Zorloni, 2007). In southern Ethiopia, it is called 'Chekaby the Borana people'. It is often found in overgrazed areas and is easily cultivated (Germishuizen and Meyer, 2003).

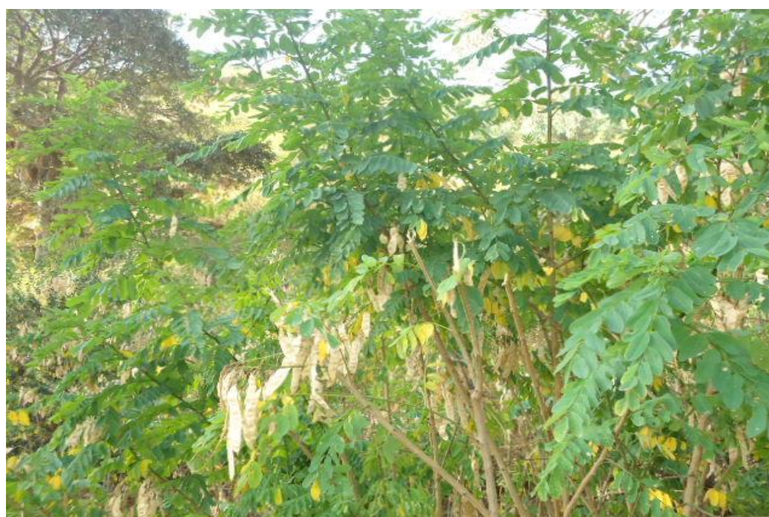

Figure 1: *Calpurnia aurea* (plant)
(Sisay Alemu 02/01/2015)

This plant is widely distributed in Ethiopia. In Southern Ethiopia peoples soak leaves of *C. aurea* in cold water to treat louse infestations (pediculosis) in humans and calves (Heine and

Brenzinger, 1988). In Western Ethiopia, the juice of crushed leaves and bark is used for tick control (Regassa, 2000). In South-Western Ethiopia, the leaves of *C. aurea*, mixed with other plant species, are crushed and squeezed to obtain a juice, which is applied through the auricular route for 2 days to treat earache in humans. In the same area, the plant is traditionally used to treat rheumatism (Yineger *et al*., 2008). Antibacterial and antioxidant activity of *C. aurea* have been reported (Adedapo *et al*., 2008), and the plant has been used to treat bacterial dermatitis (Tadeg *et al*., 2005). It has also been used as a natural pesticide to improve grain storage (Blum and Bekele, 2002).

Calpurnia leaves and powdered roots are used to destroy lice and to relieve itches. Unspecified parts are used to destroy maggots and the leaves are used to treat allergic rashes, particularly those caused by caterpillars. In East Africa, leaf sap is used to destroy maggots in wounds. In Nigeria, the seeds are used to treat abscesses. In Ethiopia, it is used to treat stomach complaints, headache, eye diseases, scabies and skin infection caused by ticks and as an insecticide as well (Asres *et al*., 2001).

Calpurnia aurea extracts are used in southern Ethiopia to protect stock against ticks. Acetone, hexane and water leaf extracts of *C. aurea* collected in southern Ethiopia were tested for repellent/attractant and acaricidal properties on unfed adult *R. pulchellus* ticks. In contrast to many other plant species evaluated, *C. aurea* extracts did not have repellent properties, but rather had a slight attractant capacity. With 20% and 10% acetone extracts, all ticks were either killed or their mobility severely compromised after 1 ml of extract was topically applied on the abdomen. At 5% concentration, 85% of ticks were still affected. However, 10% aqueous solution had a marked effect. The results proved the efficacy of the traditional use of this extract and may lead to a product that can be used commercially to protect animals against tick infestation, under subsistence as well as industrialized conditions (Zorloni, 2007).

Calpurnia aurea is used for the treatment of amoebic dysentery and diarrhoea in animals, killing head lice in humans and ticks in animals, syphilis, diarrhoea, leishmaniasis, tapeworm, trachoma, *Tinea capitis*, wound, scabies, elephantiasis and different swellings (Asres *et al*., 2001). The antioxidant activities of the stem extract of *C. aurea* as determined by the total phenol,

flavonoids, and FRAP methods were higher than that of the leaves. On the other hand, the leaf extract of the plant has higher level of total flavonols and proanthocyanidins.

The leaf extract also has higher radical scavenging activity as shown in 1,1-Diphenyl-2-picrylhydrazyl (DPPH), and 2,2¿-azinobis-3- ethylbenzothiazoline-6-sulfonic acid (ABTS) assay. The leaf extract showed activity against seven of the bacterial organisms (Adedapo *et al*., 2008). *C. aurea* are important to most terrestrial arthropods, and this may apply even to those whose major habitat is not vegetation. Ticks demonstrate those newly hatched larvae move up vegetation to assist contact with a passing mammalian host. This tick behaviour can be exploited with success for off-host semiochemical-assisted tick control strategy (Sonenshine, 2006).

b) *Otostegia integrifolia*

There are more than 20 species of *Otostegia*. Many of these species have circumboreal distribution in Mediterranean region and Asia. Only a small number of the most common species with the most obvious fruiting bodies have been evaluated for biological activity. There are more than 65 new and novel compounds have been isolated. Naturally, the species of this genus traditionally been used as an ophthalmia, mosquito repellency, antimicrobial activity, antihyperglyceamic activity and antioxidant activity which prevent different kind of ailment (Zorloni, 2007).

Otostegia integrifolia is widely distributed in Ethiopia and a very common shrub in overgrazed hillsides or old and abandoned farms. The plant is easily recognized and stands out due to the grayish color of the leaves (Figure 2). The yellow flowers are also showy when in full bloom. Medicinally, the genus *Otostegia* is very important. Also this plant have genuine antiplasmodial activity along with its safety profile observed in the study could make the leaf extract of *O. integrifolia* a potential addition to the ant malarial armamentarium, and also provide scientific support for the ethno-medicinal use of the plant (Abyot *et al*., 2013).

Figure 2: *Otostegia integrifolia*

(Sisay Alemu 08/06/2015)

Five species of this genus, *Otostegia*, have been reported to occur in the flora of Ethiopia (Sebald, 2006). *O. integrifolia* Benth, commonly known by its vernacular name 'Tinjut', is claimed to have insecticidal properties and often used as fumigant for pots and houses (Karunamoorthi *et al*., 2009). Other reports also indicated the application of the leaves of the plant for the treatment of tonsillitis, uvulitis, lung diseases, stomachache, malaria and hypertension (Giday *et al*., 2007; Andemariam, 2010). Moreover, species of genus *Otostegi*a have shown to possess antiulcer, antispasmodic, antidepressant, anxiolytic and sedative activities (Giday *et al*., 2007).

In Northern Ethiopia, *Otostegia* is commonly used to smoke utensils for sterilization. It is also a ritual custom for a mother to cleanse herself with the smoke on the tenth day after giving birth to a child before leaving her confinement to resume normal daily activities (Getahun, 1976). Various ethno-botanical surveys have shown the traditional use of the plant as antimalarial (Giday *et al*., 2007), anthelmintic (Parvez and Yadav, 2010), anticough, antirheumatic, antiabortifacient and its application against acute fibrile illnesses and infertility (Abebe and Ayehu, 1993). It is also used as an insect repellant, a claim that has been experimentally proved (Waka *et al*., 2004; Karunamoorthi *et al*., 2009). Even, some compounds have been identified and isolated from the essential oil and the chloroform extract of air-dried leaves of *O. integrifolia* (Tesso and Konig, 2004).

c) *Jatropha curcas*

Jetropha curcas Linn belongs to the family Euphorbiaceae and are used in traditional folklore medicine to cure various aliments in Africa, Asia and Latin America (Burkill *et al*., 1994). The plant is native to North America but now thrives well in Africa and Asia. It is easy to establish as it grows relatively quickly with high yields (Willis, 1967). It is one of the promising biodiesel plants (Figure 3).

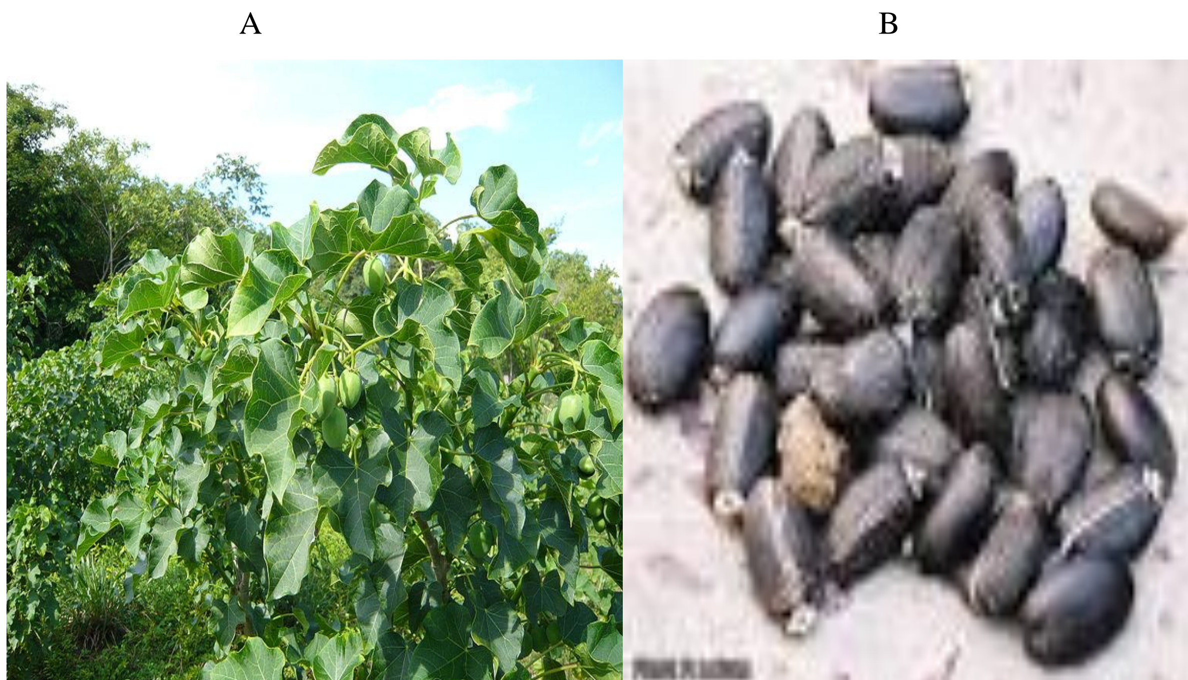

Figure 3: *Jatropha curcas*
A: Plant; B: Seed.

Traditionally, it is used to cure diseases like cancer, piles, snake bites, paralysis and dropsy (Okujagu *et al*., 2006). The antimicrobial and larvicidal activities of the leaves of the plant (Kalimuthu *et al*., 2011), stem and bark (Igbinosa *et al*., 2009) and the insecticidal property (Adebowale and Adedire, 2006) of *J. curcas* seed oil were already reported. There were many previous reports on the acaricidal activity of essential oils from these plants against *R. annulatus* (Martinez *et al*., 2010) and *R. microplus* (Ribeiro *et al*., 2011).

Acaricidal activity of crude extracts from stem and leaves of different plants against the cattle ticks were also reported (Kaaya *et al*., 2009). However, on acaricidal properties of *J. curcas* against *R. annulatus*, the commonest tick species is reported (Rajamohanan, 1982). The

antiovipositional and ovicidal effects of *J. curcas* against *Callosobruchus maculatus* Fab were also reported. It is speculated that suffocation and/or lethal chemical poisoning due to *Jatropha* oil application prevented the adult emergence from the bruchid, *C. maculatus* (Jadhau and Jadhua, 1984). The larvicidal effect of methanolic leaf extract of *J. curcas* against the first and fourth instar larvae of *Culex quinquefasciatus* (Kalimuthu *et al*., 2011) was reported. In another study, the acetone extract of *J. curcas* leaves extended the duration of the various larval instars and of pupation of *Aedes aegypti* even at very low concentration and showed toxicity at higher concentrations (Joish and Pathipati, 2009).

Jatropha curcas has long been implicated in traditional medicine and also used as an insect repellant, a mollusicide and a rodenticide (Duke, 1985). The insecticidal and acaricidal activities of *J. curcas* seed oil were previously reported (Dimri and Sharma, 2004). The oil of *J. curcas* seed was identified as efficacious in controlling sarcoptic mange in sheep when combined with ascorbic acid (Dimri and Sharma, 2004). *J. curcas* seeds showed high content of unsaponifiable matter and the insecticidal activity of seed oil was attributed to the presence of sterols and triterpene alcohols (Openshaw, 1986). The by-product after extraction of oil from the seeds of the plant also contained insecticides (Openshaw, 1986).

The extract of *J. curcas* was highly effective in controlling hatching of eggs laid by the treated ticks. The eggs laid by the treated ticks were apparently glossy in their appearance. Thus, it is evident from the results of study that the oviposition was not at all inhibited but the eclosion was prevented. The ethanolic extracts of *J. curcas* induced a significant concentration dependent decrease in egg mass production and complete blocking of the hatching of the laid ova (Ravindran, 2011). The hexane extract of aerial parts of the plant *Calea serrata* inhibited hatching of *B. microplus*e ggs, when they were immersed in the dilution of the extract and the activity was attributed to chromenes present in the plant especially the precocene II (Ribeiro *et al*., 2008). Similarily, *Lysilomala tisilquum* extract also showed an inhibitory effect on egg hatching at concentration of 19, 200 µg/ml (Fernandez *et al*., 2011).

The moulting hormones, ecdysteroids play an important role in the regulation of salivary gland function, production of pheromones, oogenesis and oviposition (Velayutham *et al*., 2012) in ticks. Ecdysone is metabolized by specific cytochrome P450 isozymes to the active form, 20

hydroxy ecdysone (20 HE). This is then transported through haemolymph to the target cells, where it binds to the ecdysone receptor to cause gene transcription (Osadebe *et al*., 2012) several phytochemicals, in particular, the flavones have the potential to interact with the vertebrate estrogen receptor as agonists or antagonists. Some of the flavones like luteolin, quercetin, apigenin and chrysin were reported to inhibit ecdysone mediated gene expression in an ecdysone responsive cell line, CL8 (Oberdorster*et al*., 2001). Apigenin inhibit both mammalian and insect cytochrome P450 isozyme expression and activity. The flavones, apigenins (apigenin 7-O-β-D-neohesperidoside, apigenin 7-O-β- D-galactoside), orientin, vitexin, vicenin II and the biflavone di-C-β-Dglucopyranoside-methylene-(8, 8')-biapigenin were isolated from the leaves of *J. curcas* (Abd-Alla *et al*., 2009). The ethanolic extract of the leaves of *J. curcas* at low concentrations can significantly inhibit the hatching of laid eggs and can be considered as a possible alternative for the control of ticks (Sanis *et al*., 2012).

d) *Nicotiana tabaccum*

Tobacco belongs to the large family Solanaceae, genus *Nicotina*. They are mostly stout herbs of ten feet or more, with a number of them being annual or perennial in growth (Figure 4). One outstanding feature of the tobacco plant is the extensive leaf area it produces. A number of tobacco genus have found their use in cigar manufacture and as nicotine for use as an insecticide. Local farmers in South-Western Nigeria have found an extract of tobacco plant to be useful in the treatment and prevention of lice (Wightman, 1978).

Figure 4: *Nicotiana tabaccum* (plant)

In traditional medicine, *N. tabaccum* is used for the treatment of diseases like toxic-shock syndrome, pneumonia, boils, carbuncles, etc. However, its extracts might also be used in healing of wounds, be effective against methicillin-resistant *Staphylococcus aureus* (MRSA) and controlling certain plant diseases (Patil *et al*., 2014). The ethyl acetate extract of *N. tabaccum* effective against *Ervinia carotovora* (Bakht and Shafi, 2012). It is also used as an insecticide in agriculture for the last several years (Rosell *et al*., 2008). The plant originated from America, however, now, it has been frequently cultivated in the Indian sub-continent where it is called as 'tambaku'. The plant is known throughout the world due its narcotic chattels. Whole herb, dried leaves and stalks of *N. tabaccum* are traditionally used in the subcontinent for its emetic, purgative, analgesic, antispasmodic, sedative and insecticidal properties (Murray *et al*., 1992). *N. tabaccum* is also used in the ethno-veterinary practice as an anthelmintic (Iqbal *et al*., 2006). Dipeolu and Ndungu (1991) have demonstrated acaricidal efficacy of a natural product based on ground mixture of *N. tabaccum* leaves and a mineral salt mined from around Lake Magadi of Kenya. Larvae and nymphs of *R. appendiculatus* were killed on the ears of calves within 24 hrs, and large numbers of adult ticks were found dead *in vitro* within 2–3 days of application of the product. Tobacco is well known for its anti-parasitic effects (Potenza *et al*., 1999) reported the acaricidal activity of tobacco against two spotted spider mite *Tetranychus urticae*. Anthelmintic activity of *N. tabaccum* extract has been reported by Mansingh and Williams (1998) and Iqbal *et al*. (2006).

Hot water, acetone, chloroform, and methanol extracts of the leaf of *N. tabaccum* were tested against the larvae of *C. quinquefasciatus* (Rahuman *et al*., 2009a). The crude aqueous and methanol extracts of *N. tabaccum* were investigated *in vitro* and *in vivo* for anthelmintic activity against *Haemonchus contortus* (Iqbal *et al*., 2006). *N. tabaccum* extracts were tested for pesticidal activity against *Tribolium castaneum*, and shown to be very active against *B. microplus* (Williams and Mansingh, 1993; Mansinghand and Williams, 1998).

2.2.2. Plant extraction and phytochemical screening tests

a) Plant extraction

Plant-derived substances have recently become of great interest owing to their versatile applications. Medicinal plants are the richest bio-resource of drugs of traditional systems of

medicine, modern medicines, nutraceuticals, food supplements, folk medicines, pharmaceutical intermediates and chemical entities for synthetic drugs (Ncube *et al*., 2008). Extraction (as the term is used pharmaceutically) is the separation of medicinally active portions of plant and animal tissues using selective solvents through standard procedures. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form, and are intended for oral or external use. These include classes of preparations known as decoctions, infusions, fluid extracts, tinctures, pilular (semisolid) extracts or powdered extracts. Such preparations have been popularly called galenicals, named after Galen, the second century Greek physician (Remington and Remington, 2008).

The purpose of standardized extraction procedures for crude drugs (medicinal plant parts) is to attain the therapeutically desired portions and to eliminate unwanted material by treatment with a selective solvent known as menstrum. The extract thus obtained, after standardization, may be used as medicinal agent as such in the form of tinctures or fluid extracts or further processed to be incorporated in any dosage form such as tablets and capsules. These products contain complex mixture of many medicinal plant metabolites such as alkaloids, glycosides, terpenoids, flavonoids and lignans (Handa *et al*., 2008). The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave-assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents). For aromatic plants, hydrodistillation techniques (water distillation, steam distillation, water and steam distillation), hydrolytic maceration followed by distillation, expression and enfleurage (cold fat extraction) may be employed. Some of the latest extraction methods for aromatic plants include headspace trapping, solid phase micro-extraction, protoplast extraction, microdistillation, thermomicrodistillation and molecular distillation (Handa *et al*., 2008).

The basic parameters influencing the quality of an extract are plant part used as starting material, solvent used for extraction and extraction procedure. While effect of extracted plant phytochemicals depends on the nature of the plant material, its origin, degree of processing, moisture content and particle size whreas the variations in different extraction methods that will

affect quantity and secondary metabolite composition of an extract depends upon type of extraction, time of extraction temperature, nature of solvent, solvent concentration and polarity (Ncube *et al*., 2008).

Fresh or dried plant materials can be used as a source for the extraction of secondary plant components. Many authors had reported about plant extract preparation from the fresh plant tissues. The logic behind this came from the ethno medicinal use of fresh plant materials among the traditional and tribal people. But as many plants are used in the dry form (or as an aqueous extract) by traditional healers and due to differences in water content within different plant tissues, plants are usually air dried to a constant weight before extraction. Other researchers dry the plants in the oven at about 40°C for 72 hrs. In most of the reported works, underground parts (roots, tuber, rhizome, bulb etc.) of a plant were used extensively compared with other above ground parts in search for bioactive compounds possessing antimicrobial properties (Das *et al*., 2010).

Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure. Properties of a good solvent in plant extractions includes, low toxicity, ease of evaporation at low heat, promotion of rapid physiologic absorption of the extract, preservative action, inability to cause the extract to complex or dissociate. The factors affecting the choice of solvent are quantity of phytochemicals to be extracted, rate of extraction, diversity of different compounds extracted, diversity of inhibitory compounds extracted, ease of subsequent handling of the extracts, toxicity of the solvent in the bioassay process, potential health hazard of the extractants (Eloff, 1998). The choice of solvent is influenced by what is intended with the extract. Since the end product will contain traces of residual solvent, the solvent should be non-toxic and should not interfere with the bioassay. The choice will also depend on the targeted compounds to be extracted (Das *et al*., 2010).

b) Phytochemical screening

Phytochemicals are bioactive chemicals of plant origin. They are regarded as secondary metabolites because theplants that manufacture them may have little need for them. They are naturally synthesized in all parts of the plant body; bark, leaves, stem, root, flower, fruits, seeds,

etc. i.e. any part of the plant body may contain activecomponents (Tiwari *et al*., 2011). The quantity and quality of phytochemicals present in plant parts may differ from one part to another. In fact, there is lack of information on the distribution of the biological activity in different plant parts essentially related to the difference in distribution of active compounds (or active principles) which are more frequent in some plant parts than in others (Lahlou, 2004).

Phytochemicals have been recognized as the basis for traditional herbal medicine practiced in the past and currently in vogue in parts of the world (Lalitha and Jayanthi, 2012). In the search for phytochemicals that may be of benefit to the pharmaceutical industry, researchers sometimes follow leads provided by local healers in a region (Das *et al*., 2010). Following such leads, plantparts are usually screened for phytochemicals that may be present. The presence of a phytochemical of interest may lead to its further isolation, purification and characterization. Then it can be used as the basis for a new pharmaceutical product. Successful determination of biologically active compounds from plant material is largely dependent on the type of solvent used in the extraction procedure (Tiwari *et al*., 2011). This, therefore, underscores the need to try as much solvents as possiblein screening plant parts for phytochemicals.

3. MATERIALS AND METHODS

3.1. Study Areas

The experiment was conducted in two sites where evaluation of the *in vitro* louscidal efficacy test was carried out at Addis Ababa University, College of Veterinary Medicine and Agriculture (CVMA), Parasitology Laboratory at Bishoftu while evaluation of the *in vitro* acaricdal efficacy and phytochemical screening tests were conducted at Haramaya University, College of Veterinary Medicine (CVM), Parasitology and Biochermistry Laboratory respectively at Haremaya.

3.2. Study Design

In vitro experimental studies were carried out from November, 2014 to April, 2015 to evaluate the acaricidal efficacy of methanolic leaf extracts of *C. aurea, O. integrifolia* and *N. tabaccum* as well as petrolium ether seed oil extracts of *J. curcas* against *B. ovis* and *A.variegatum*.

3.3. Study Parasites

Adult sheep lice, *B. ovis*, were collected from sheep and adult ticks, *A. variegatum*, were collected from cattle for the *in vitro* and acaricidal efficacy test using the four selected medicinal plants. Coat brushing technique was used for collection of lice from sheep. The parasites were maintained in plastic cups into which water soaked cottons were placed to increase the humidity of the air found in the cups. The cups were covered by gauze to allow the free circulation of air into the cups and then the parasites were transported. Identification of the parasites was conducted under a stereoscopic microscope according to the descriptions of Wall and Shearer 1(997). Only, adult lice were used in these experiments. Ticks were collected from animals using forceps at main body sites namely: head, dewlap, brisket, belly and back, udder or scrotum, anogenital, leg and tail during. Adult ticks collected from each of the main body sites were maintained in universal bottles separately and then transported to the Parasitology Laboratory of College of Veterinary Medicine, Haramaya University for identification and *in vitro* test efficacy test. Identification and recording of tick samples took place within a few hours of collection. Ticks were identified using stereomicroscope following the standard identification procedures described by Walker *et al*. (2003).

3.4. Plant Material Collection and Extraction

The plant species used in this study, *N. tabaccum* (leaf), *C. aurea* (leaf) *O. integrifolia* (leaf) and *J. curcas* seeds were selected based on the information obtained from previous botanical surveys (Teklay *et al*., 2013; Bekele *et al*., 2012; Mrutse and Gobena, 2003). People traditionally used those plants for the control of ectoparasites. The selected plants of *N. tabaccum* (leaf) and *C. aurea* (leaf) were collected from Eastern Harerege Zone Haramaya and Garamuleta woredas respectively while *O. integrifolia* (leaf) were collected from North Shewa Fiche. The plants were identified and verified at Aklilu Lemma Institute of Pathobiology (ALIPB) voucher No SALD/94/05, SALD/96/05 and SALD/97/05 respectively. To reduce possible contamination, especially by fungi, latex gloves were worn when leaves were collected. The plant material was spread out on paper sheets in the shade at room temperature separately to dry for two weeks. When desiccated, all selected plant leave were stored in sealed containers separately and transported to ALIPB, Addis Ababa for extraction. The dried plant materials were crushed in an electric grinder to coarse powder. One hundred grams (100 g) of powdered material were soaked in four hundred ml (400 ml) of methanol separately for 48 hrs on an orbital shaker. Extracts were filtered using a Buckner funnel and Whatman (No 1 filter paper). Each filtrate was concentrated to dryness under reduced pressure at 40°C using a rotary evaporator (Eloff, 1999).

In addition, mature *J. curcas* seeds were collected from Babile woreda of Eastern Harerege Zone of Oromia Region. The plants identified and verified at ALIPB voucher No SALD/95/05. The mature seed was powdered using a mortar and pestle. The powdered seeds (200 g) were extracted by (Soxhlet) with petroleum ether (boiling point 60–80°C) for 6 hrs. The solvent after extraction was removed by distillation on a water bath (Manash *et al*., 2013).

The extraction rates (%) were calculated in accordance with Eloff (1999) as follows:-

$$\text{Extraction rate (\%)} = \frac{\text{Weight of extracts (g)}}{\text{Weight of the plant material (g) before extaction}} \times 100$$

3.5. Preliminary Phytochemical Screening of Solvent Extracts

The crude methanol leaf extracts and petrolium ether extract seed oil were screened for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tannins, phlobatannins, triterpens and glycosides using standard procedure (Hymete, 1986; Mbata *et al*., 2006).

Crude oil extracts were considered positive for the formation of frothing indicated the presence of saponins. While green precipitate indicates the presence of condensed tannins. Whereas, the upper layer turns red and should yellow with green fluorescence this indicates the presence of steroids. A red or orange color indicates the presence of flavonoids. Green to black precipitate indicated the presence of phenolic compound. Occurrence of orange-red precipitate indicated the presence of alkaloids while the appearance of red color indicates the presence of triterpenes. Moreover, the deposition of a red precipitate was taken as an evidence for the presence of phlobatannins (Hymete, 1986; Mbata *et al*., 2006).

3.6. *In Vitro* Louscidal and Accaricidal Efficacy Test

For the louscidal and accaricidal efficacy test, adult immersion test was used as described by Negero *et al*. (2014). The dried extracts of *C. aurea*, and *O. integrifolia* were diluted in distilled water and 3% ethanol, respectively while that of *N. tabaccum* and *J. curcas* oil were diluted in 2% Tween80 at the concentrations required for the bioassays (20%, 10%, 5%, 2.5%, 1.25% and 0.65%). The *in vitro* tests were started within one hour after lice collection (Heukelbach *et al*., 2006). Ten active lice in three replications were put into petridishes and 1 ml of each concentration were directly added to the three replicate petridishes and incubated at 27-28°C and 75-80% relative humidity for 24 hrs. These three replicates were treated with distilled water, 3% ethanol, and 2% Tween80 as negative and using 0.1% diazinon 60 EC as positive controls (Jadhav *et al*., 2007). The test solutions, positive (0.1% diazinon 60 EC) and negative (distilled water 3% ethanol, and 2% Tween80) controls were removed just after one minute contact time, using whatman No. 1 filter paper. Each louse in each petridish was closely observed for death under stereomicroscope (Appendix 2) at 30 min, 1 hr, 2 hrs, 3 hrs, 6 hrs and 24 hrs time intervals (Nanaa *et al*., 2010).

The criteria used for death of lice were extremely strict. If any signs of life such as movement of antennae, gut cells or minimal legs movements were observed with stimulation by niddle, the lice were categorized as alive. The lice were judged as dead, if there were no signs of movment at all (Jadhav *et al*., 2007). The number of deaths was recorded in pre-prepared format. The percent mortality rates of lice were calculated as per Abotts (1925) as cited by (Pamo *et al*., 2005).

$$\text{Corrected mortality} = \frac{\text{\% Treated mortality - \% Negetive control mortality}}{\text{100 - \% Negetive control mortality}} \times 100$$

Mortality in the petridishes treated with crude extracts of plants were corrected to take account of control mortality using Abbott's correction. Louscidal effects were classified as follows: strong, mortality >80%; moderate, mortality 80–60%; weak, mortality 60–40%; little or no activity, mortality <40%.

The dried extracts of *C. aurea* and *O. integrifolia*, were diluted in distilled water and 3% ethanol, respectively and that of *N. tabaccum* and *J. curcas* seed oil were diluted in 4% Tween80 at the concentrations required for the bioassays (20%, 10%, 5%, 2.5%, 1.25% and 0.65% mg/ml). A total three replicates of ten ticks were used for each dilution of the extract (Sanis *et al*., 2012). of 960 ticks were used in this experiment. A group of ten ticks were immersed for 2 min into the respective dilution (10 ml) in a 50 ml beaker with gentle agitation (Sanis *et al*., 2012), and three replicates were treated either with distilled water, 3% ethanol and 4% Tween80 as negative and 0.1% diazinon 60 EC as positive controls. Ticks were recovered from the solution, dried using tissue paper towels and placed in separate plastic specimen tube (25 × 50 mm). The tubes were incubated at 28°C and 80% relative humidity (Sanis *et al*., 2012) then transfered into petridishes and each tick in each petridish was closely observed for death under stereomicroscope (Appendix 3) at 30 min, 1 hrs, 2 hrs, 3 hrs, 6 hrs and 24 hrs time intervals. The criterias used for death of ticks were extremely strict. If any minor signs of life such as minimal legs movement and phalengial reflexes were observed with stimulation by forceps, the ticks were categorized as alive. The ticks were judged as dead when there were no vital signs of life at all (Jadhav *et al*., 2007).

The numbers of deaths were recorded in a pre-prepared format (Annex II). The percent mortality rate of ticks was calculated as per Abbots formula cited by Krishnaveni and Venkatalakshmi (2014).

$$\text{Mortality \%} = \frac{\text{No. of mortality}}{\text{Total number of ticks}} \times 100$$

3.7. Data Analysis

The collected raw data were stored in Microsoft Excel Spreadsheet following edition. Statistical software package called SPSS windows version 17.0 was used for data analysis. Analyses of variance (one-way ANOV, Tukey test) were used to compare the means of different treatments (concentrations) of the extracts and controls in different time used for *in vitro* efficacy studies of medicinal plants. $P < 0.05$ at 95% level of confidence was used as the level of significance.

4. RESULTS

4.1. Physicochemical Characteristics and Yield of Plant Extracts

Physical characteristic features of extracts and percentage yield have been depicted in Table 1. Methanolic crude extract of *C. aurea* (leaves) was green powder which is soluble in organic solvents and fairly soluble in distilled water. Crude extracts of *O. integrifolia* (leaves) and *N. tabaccum* (leaves) are semisolid whereas petroleum ether extracted oil of *J. curcas* (seed) consisted of golden yellow colour oil.

Table 1: Physical characteristics and percentage yield of crude/oil extracts of the study medicinal plants

Plants	Local name	Parts of the plant extracted	Solvents for extraction	Constituent of extract	Colour of extract	Weight of dry powder (g)	Weight of dry extract (g)	%	Means of dilution
C. aurea	Degeta	Leaf	Methanol	Powder	Green	1200	204	17	Distilled water
O. integrifolia	Tunget	leaf	Methanol	Semi solid	Greenish brown	1200	232	19.3	3% ethanol
J. curcas	Gulo ferengy	Seed	Petroleum ether	Oil	Golden yellow	1200	90	7.5	2% Tween80
N. tobaccum	Tembo	Leaf	Methanol	Semi solid	Brown	1200	122	10.18	2% Tween80

Chemical analysis of different extracts contains alkaloids, saponins, phlobotanin, steroids flavonoids, glycosides, tannins and triterpens (Table 2). Pictures of different secondary metabolites from oil/crude extracts are presented in Figure 5.

Table 2: Qualitative determinations of active ingredients in crude/oil extract of the study plants

Secondary metabolities	*C. aurea*	*O. integrifolia*	*N. tobaccum*	*J. curcas*
Saponin	+	+	+	-
Tanin	+	+	+	+
Phenolic cpds	+	+	+	+
Steroids	+	-	+	+
Flavonoids	+	-	-	+
Phlobotanin	+	+	-	ND
Glycosides	+	+	+	-
Triterpens	-	-	-	+
Alkaloids	+	-	+	-

Source: +: Positive; -: Negative; ND: Not done

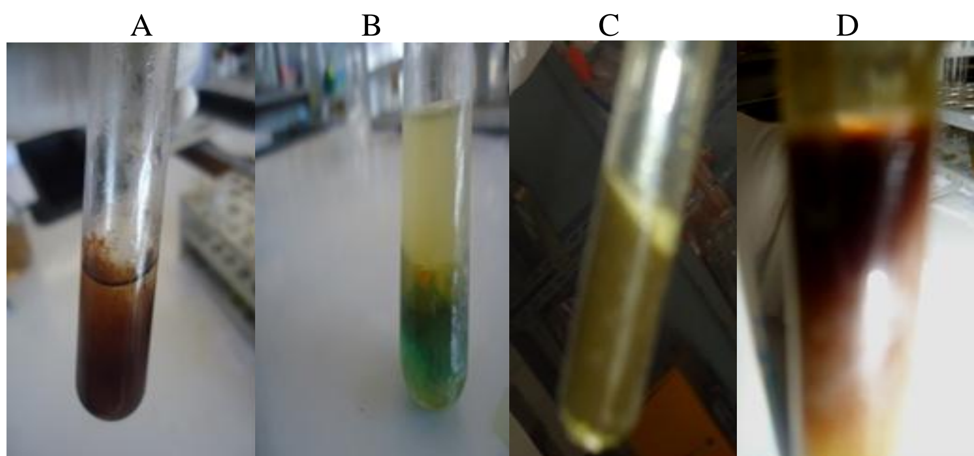

Figure 5: Pictures of different secondary metabolites from crude/oil extracts
(A) Deposition of a red precipitate indicating the presence of phlobatannin; (B).Green precipitate indicating the presence of condensed tannins; (C).The formation of froth indicates the presence of saponins; (D) Red upper layer and yellow lower layers indicates the presence of steroids.

4.2. Evaluation of *In Vitro* Louscidal Activity

A total of four extracts from four different medicinal plants were tested for their louscidal activity against *B. ovis.* Mortalities for the lice treated with the different concentrations of *C. aurea* crude extract are shown in Figure 6. *C. aurea* crude extract at 100 mg/ml concentration showed higher louscidal activity of 70% efficacy after 30 minutes of exposure. After 6 hrs of exposure 200 mg/ml concentration of *C. aurea* extract showed 100% mortality which was significantly ($P < 0.05$) higher mortality as compared to other concentrations except 100 mg/ml which showed 90%. After 24 hrs of exposure with the exception of the lowest concentration of 6.25 mg/ml, all test concentrations caused 100% mortality of lice. Moreover, there was no statistically significant difference ($P > 0.05$) in the louscidal activity among different concentrations after 24 hrs of exposure when compared to the reference drug (0.1% diazinon).

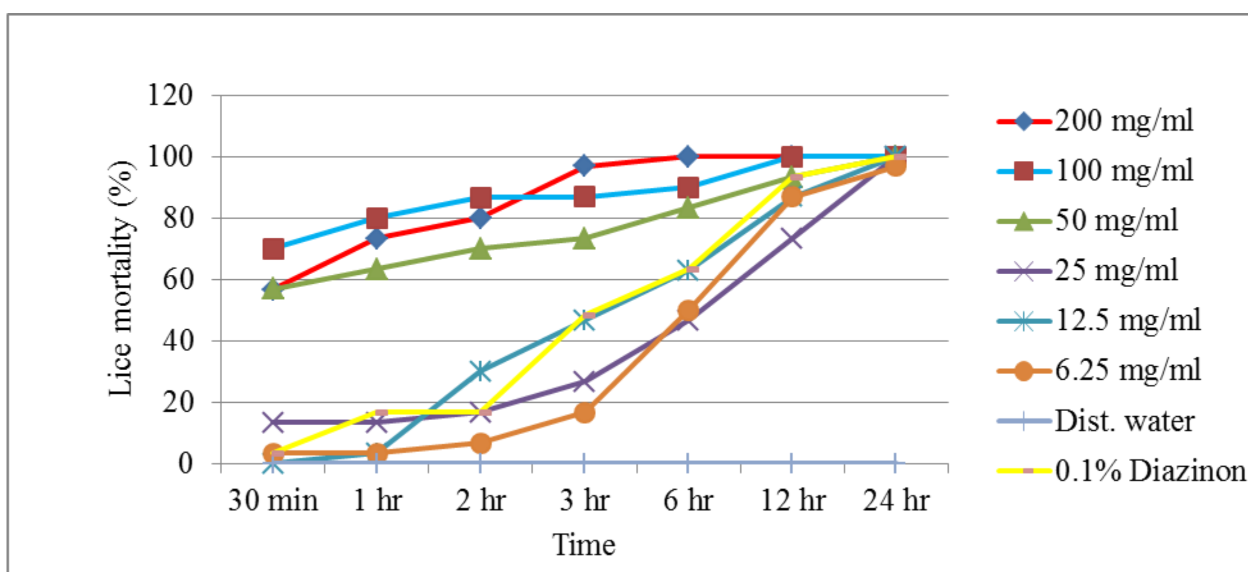

Figure 6: Mortalities of *B. ovis* treated with crude extracts of *C. aurea* (leaf)

Mortalities for the lice treated with the different concentration of *O. integrifolia* crude extract are shown in Figure 7. All concentrations of *O. integrifolia* leaf extracts showed no louscidal activity at 30 minutes. At 12 hrs, higher mortality percentage up to 50% was recorded on lice exposed to 200 mg/ml concentration. All concentrations of *O. integrifolia* extract showed no statically significant ($P > 0.05$) difference in their activity. Moreover, *O. integrifolia* had significantly ($P < 0.05$) lower louscidal activity as compared to positive control (0.1% diazinon). While after 24 hrs exposure at aconcentrations of 200 mg/ml and 100 mg/ml showed mortality of 63% and 53%

respectively which was statistically significant difference ($P < 0.05$) when compared to the reference drugs.

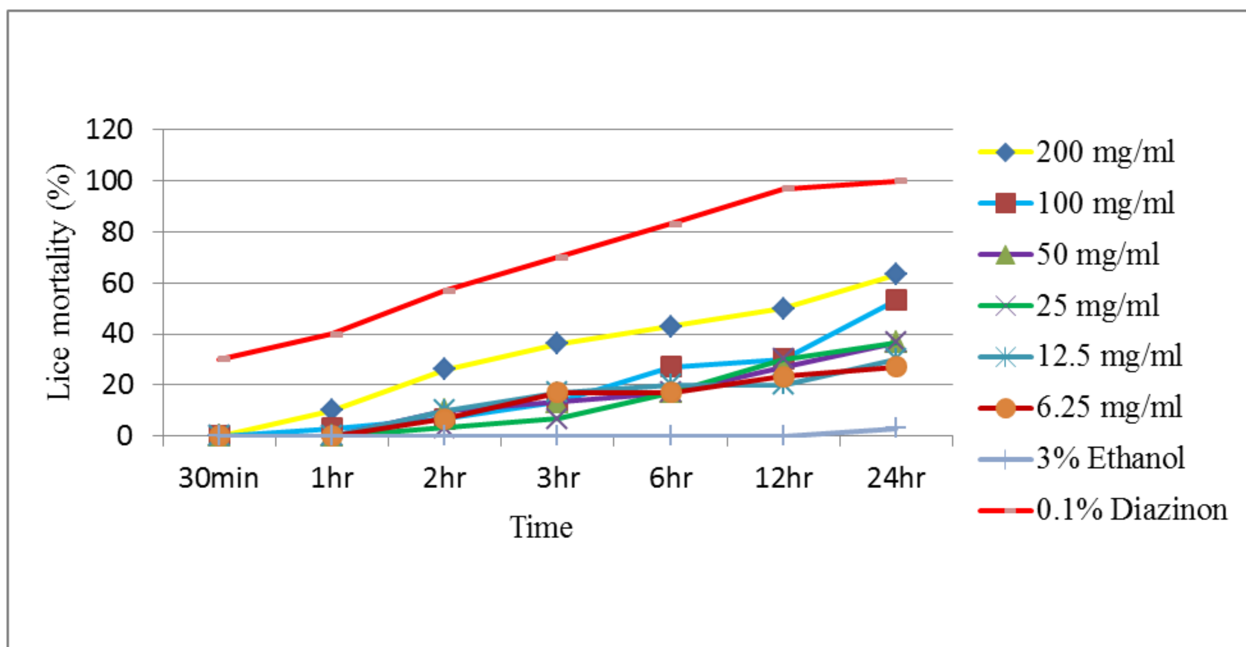

Figure 7: Mortalities of *B. ovis* treated with crude extracts of *O. integrifolia* (leaf)

Mortalities for the lice treated with the different concentration of *N. tobaccum,* extract are shown in Figure 8. 200 mg/ml concentration of *N. tabaccum* crude extract showed higher louscidal activity of 33% after 30 minutes of exposurewhich was significantly ($P < 0.05$) higher mortality as compared to all <100 mg/ml concentrations. After 12 hrs of exposure, 200 mg/ml concentration of *N. tabaccum* extract showed 83% mortality which was statistically not significant ($P > 0.05$) as compared with other concentrations except at 12.5 mg/ml and 6.25 mg/ml. While after 24 hrs exposure, of 200 and 100 mg/ml concentrations of *N. tabaccum* extracts caused mortality of 93% and 87% respectively which was statistically insignificant difference ($P > 0.05$) when compared to the reference drugs (0.1% diazinon).

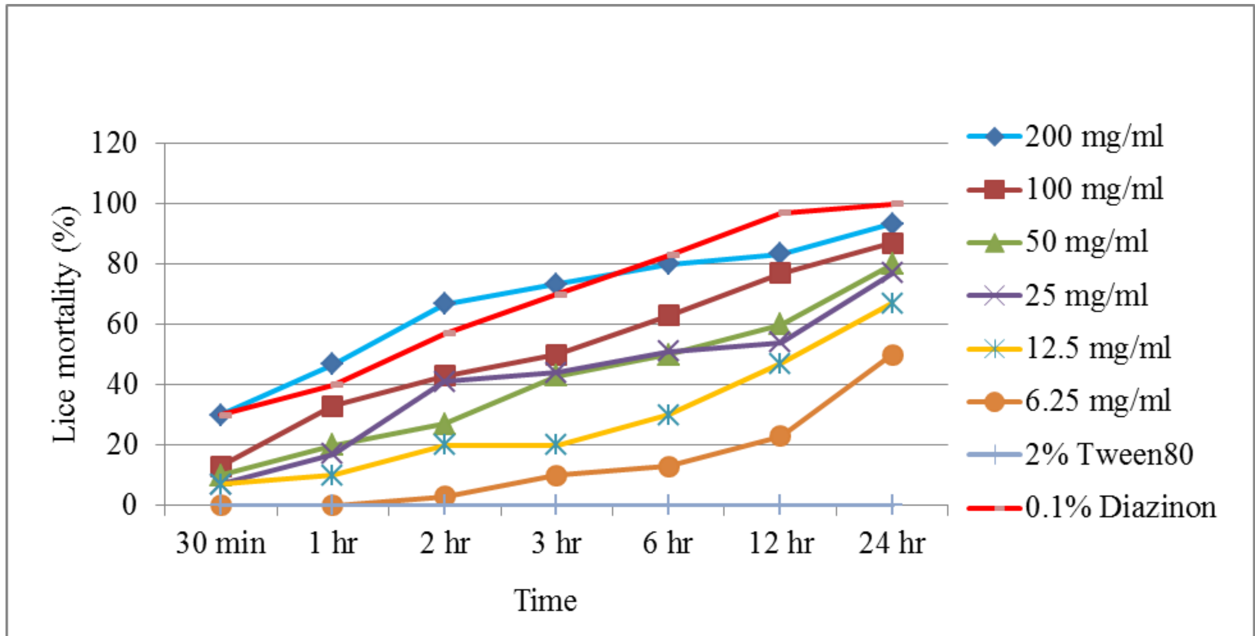

Figure 8: Mortalities of *B. ovis* treated with the crude extracts of *N. tabaccum* (leaf)

Mortalities for the lice treated with the different concentration of *J. curcas* oil extract are shown in Figure 9. *J. curcas* crude extract at 200 mg/ml concentration showed higher activity 50% after 30 minutes of exposure which was significantly ($P < 0.05$) higher mortality as compared to 25, 12.5 and 6.25 mg/ml concentrations. After 24 hrs of exposure 200 mg/ml concentration of *J. curcas* crude extract showed 100% mortality which was significantly ($P < 0.05$) higher mortality as compared to the lower concentration of 6.25 mg/ml. Moreover, there was no statistically significant difference ($P > 0.05$) in the louscidal activity among different concentrations after 24 hours exposure except lower concentration of 6.25 mg/ml when compared to the reference drugs (0.1% diazinon).

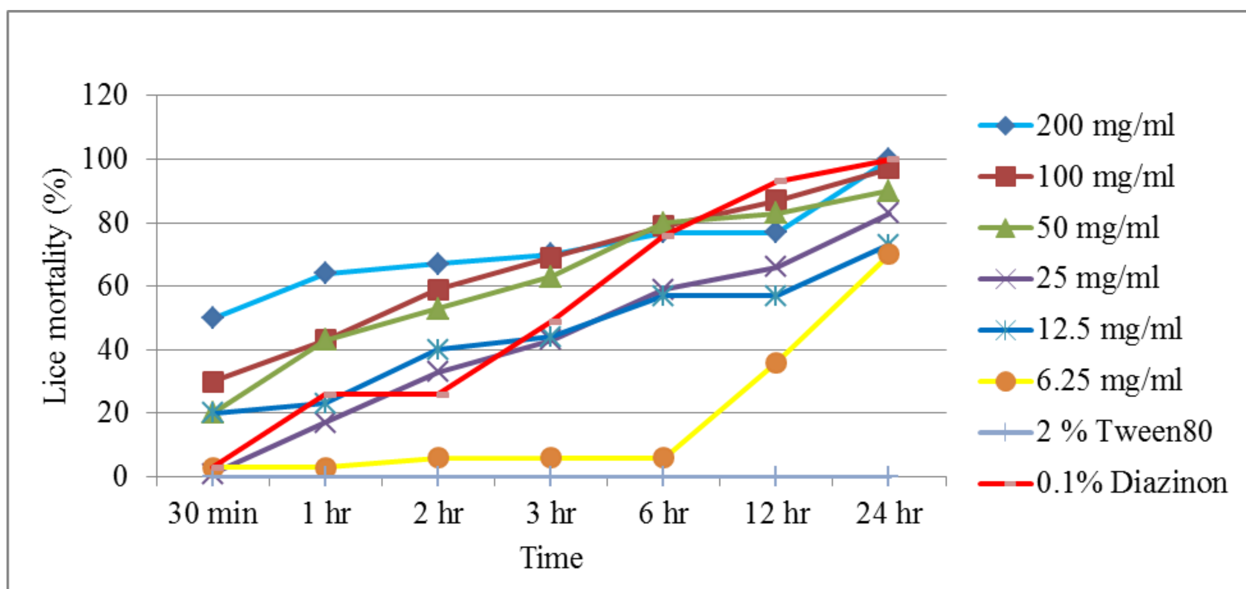

Figure 9: Mortalities of *B. ovis* treated with the *J. curcas* oil extract.

Comparative *in vitro* activity of crude extracts of *C. aurea, N. tabaccum* and *O. integrifolia* at 6 hrs, 12 hrs and 24 hrs has been given in Table 3. The results revealed that 100 and 50 mg/ml crude extracts of *C. aurea* showed statistically significant ($P < 0.05$) high louscidal activity after 6 hrs of exposure than *N. tabaccum* and *O. integrifolia*. In addition, 100 and 50 mg/ml crudes extract of *C. aurea* exhibited statistically significant ($P < 0.05$) high louscidal activity after 12 hrs f exposure than *N. tabaccum* and *O. integrifolia* while after 24 hrs exposure exept 200 mg/ml and 6.25 mg/ml concentrations of *N. tabaccum* and all concentrations of *C. aurea* crude extracts had statistically significant ($P < 0.05$) higher louscidal activity than *N. tabaccum* and *O. integrifolia* crude extracts. In addition, *N. tabaccum* after 24 hrs of exposure showed statistically significant ($P < 0.05$) higher louscidal activity than *O. integrifolia* at 200 100, 50 and 25 mg/ml concentrations. However, because of the different extraction methods used, *J. curcas* effect is not included in comparison.

Table 3: Comparisons on louscidal activity of different extracts on *B. ovis* at different times of exposure

Dose (mg/ml)	Mean mortality rate (%)±SE								
	6 hrs			12 hrs			24 hrs		
	CA	NT	OI	CA	NT	OI	CA	NT	OI
200	100±5.9[a]	80±11[a]	43.3±3.3[b]	100±0.0[a]	83.3±8.8[a]	50±0.0[b]	100±0.0[a]	93.3±3.3[a]	63.3±3.3[b]
100	90±6.7[a]	63,3±8.8[b]	26.7±.33[c]	100±0.0[a]	76.7±8.8[b]	30±0.0[c]	100±0.0[a]	86.3±3.3[b]	53.3±3.3[c]
50	83±13[a]	50±5.8[b]	16.7±8.8[c]	93±6.7[a]	60±0.0[b]	26.7±3.3[c]	100±0.0[a]	80±5.8[b]	36.7±3.3[c]
25	46.7±8.8[a]	50±0.0[a]	16.7±6.7[a]	73±6.7[a]	53.3±3.3[b]	30±0.0[c]	100±0.0[a]	76.7±3.3[b]	36.6±3.3[c]
12.5	63.3±5.7[a]	26.7±12[a]	20±10[a]	86.7±6.7[a]	36.7±8.8[b]	20±10[b]	100±0.0[a]	66.7±3.3[ab]	66.7±3.3[b]
6.25	50±18[a]	13.3±8.8[a]	16.7±16.7[a]	86.7±6.7[a]	23.3±12[b]	23.3±12[b]	96.7±3.3[a]	50±5.8[b]	26.7±12[b]
NC	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±.0.0[a]	0.0±0.0[a]	0.0±0.0[a]	00±0.0[a]	3.3±3.3[a]
PC	63.8±6.8[a]	83.3±8.8[a]	93±6.7[a]	93±3.3[a]	96.7±3.3[a]	96.7±3.3[a]	100±0.0[a]	100±0.0[a]	100±0.0[a]

Mean values with different letters in the same column are significantly different ($P < 0.05$).

CA = *Calpurnia aurea*; NT = *Nicotiana tabaccum*; OI = *Otostegia integrifolia*

4.3. Evaluation of *In Vitro* Acaricidal Activity

The results of *in vitro* effect of acaricidal activity revealed that there was no mortality of ticks in all plant extracts until 6 hrs. Mortalities for the tick treated with the different concentration of *C. aurea,* crude extract are shown in Figure 10. The crude extract *C. aurea* at 200 mg/ml concentration showed higher acaricidal activity (56% mortality) after 24 hrs of exposure which was significantly ($P < 0.05$) higher mortality as compared with other concentrations except with 100 mg/ml. After 24 hrs of exposure 200 and 100 mg/ml concentrations of *C. aurea* extract showed comparable acaricidal efficacy ($P > 0.05$) with the reference drug (0.1% diazinon).

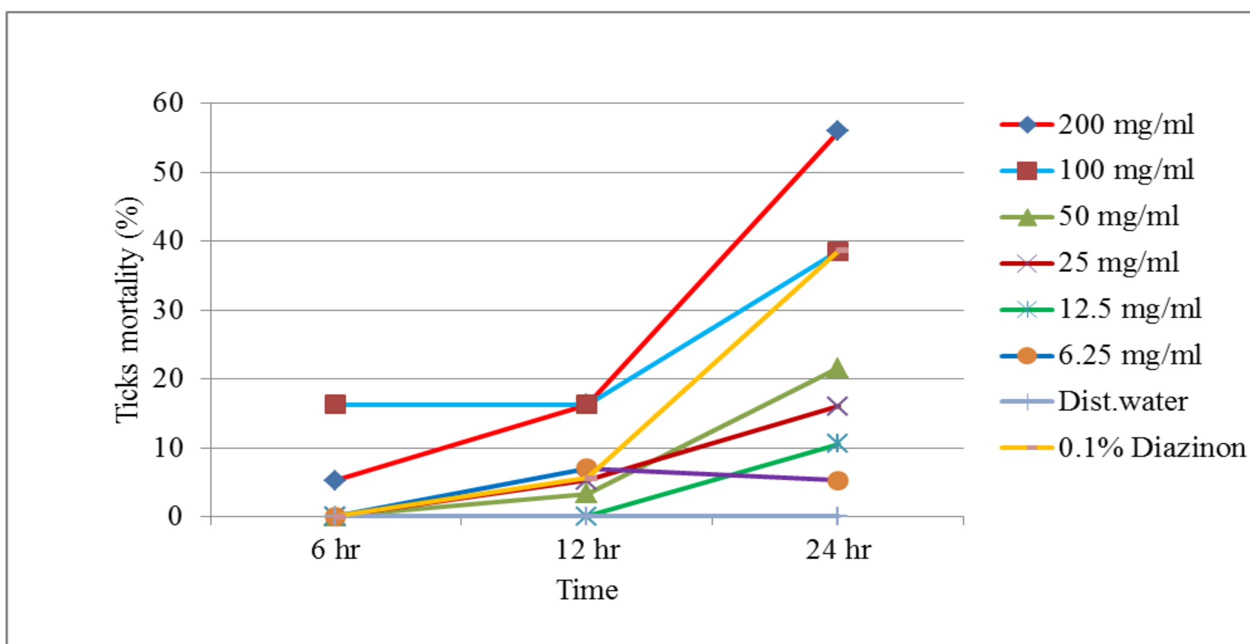

Figure 10: Mortalities of *A. varegatum* treated with the crude extracts of *C. aurea*

Generally, the efficacy of *O. integrifolia* at all concentrations was low against *B. ovis*. Surprisingly, a similar trend was also noted for diazinon. Mortalities for the tick treated with the different concentration of *O. integrifolia* crude extract are shown in Figure 11. Crude extracts *O. integrifolia* at 200 and 100 mg/ml concentrations exhibited higher acaricidal activity (27%) after 24 hrs of exposure which was statically significant ($P < 0.05$) higher mortality as compared with low concentrations. After 24 hrs of exposure the above concentration of *O. integrifolia* crude extract showed comparable acaricidal efficacy ($P > 0.05$) with 0.1% diazinon (the reference drug).

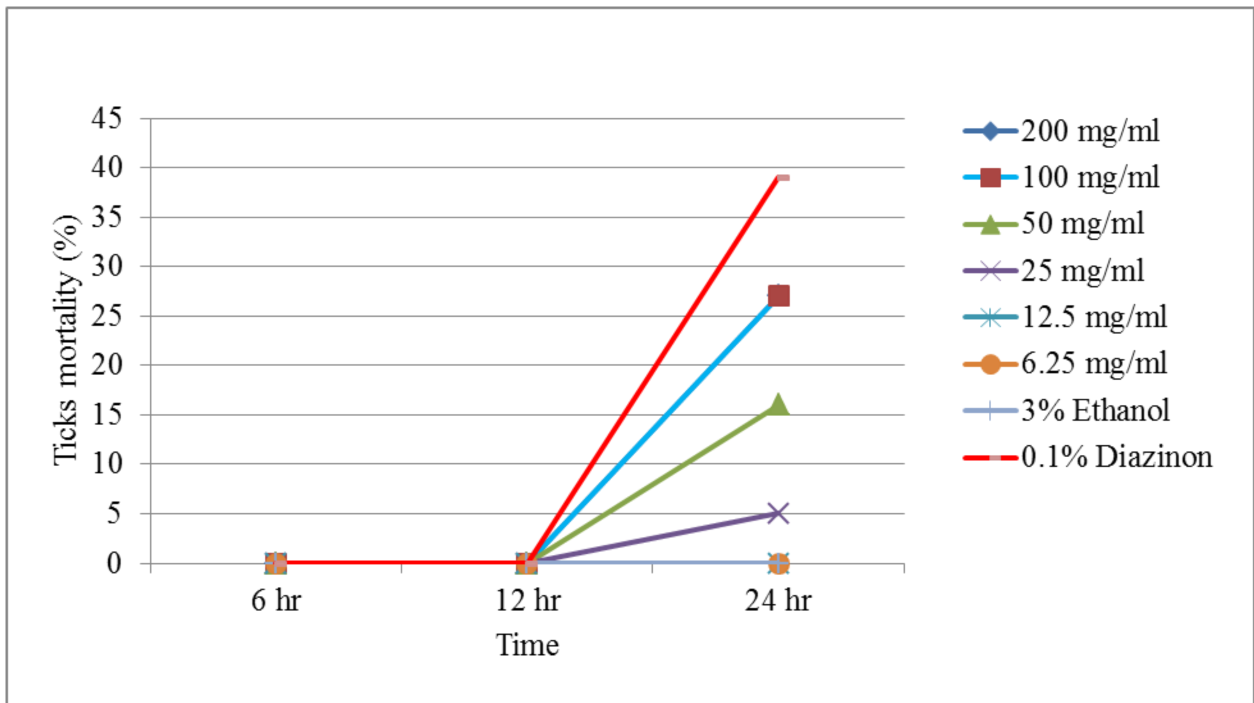

Figure 11: Mortalities of *A. varegatum* treated with the crude extracts of *O. integrifolia*
Crude extracts of *O. integrifolia* at 200 mg/ml and 100 mg/ml as well as at 12.5 mg/ml, 6.25 mg/ml, and 3% ethanol concentrations exhibited identical mortality percentage (lines overlap).

After 24 hrs of contact, *N. tabaccum* crude extract showed similar acaricidal efficacy ($P > 0.05$) to the reference drug (0.1% diazinon) in causing tick mortality at concentrations higher than 100 mg/ml (Figure 12). But compared to the negative control (4% Tween80) the two higher concentrations showed statistically significance difference ($P < 0.05$) to cause mortality of tick (Figure 12).

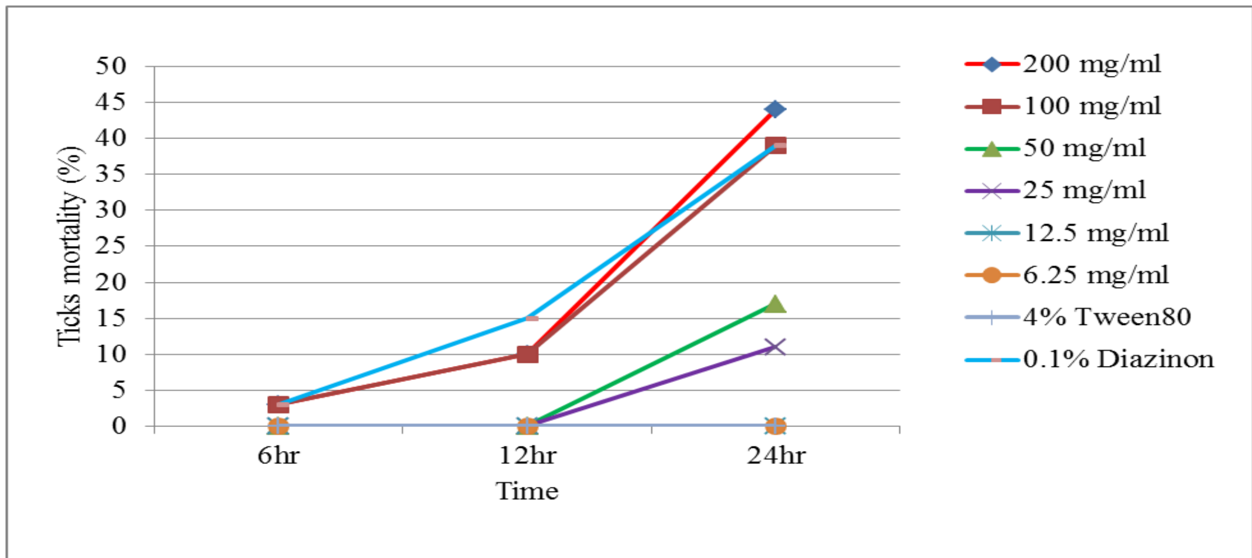

Figure 12: Mortalities of *A. variegatum* treated with the crude extracts of *N. tobaccum*
N. tabaccum crude extract at 12.5 mg/ml, 6.25 mg/ml, and 4% Tween80 concentrations had identical mortality (lines overlap).

Mortalities for the tick treated with the different concentration of *J. curcas* oil extract are shown in Figure 13. The effect of 200 mg/ml concentration of *J. curcas* crude extract (56%) was higher than for all other concentrations excepting for 100 mg/ml and 0.1% diazinon at 24 hrs post exposure.

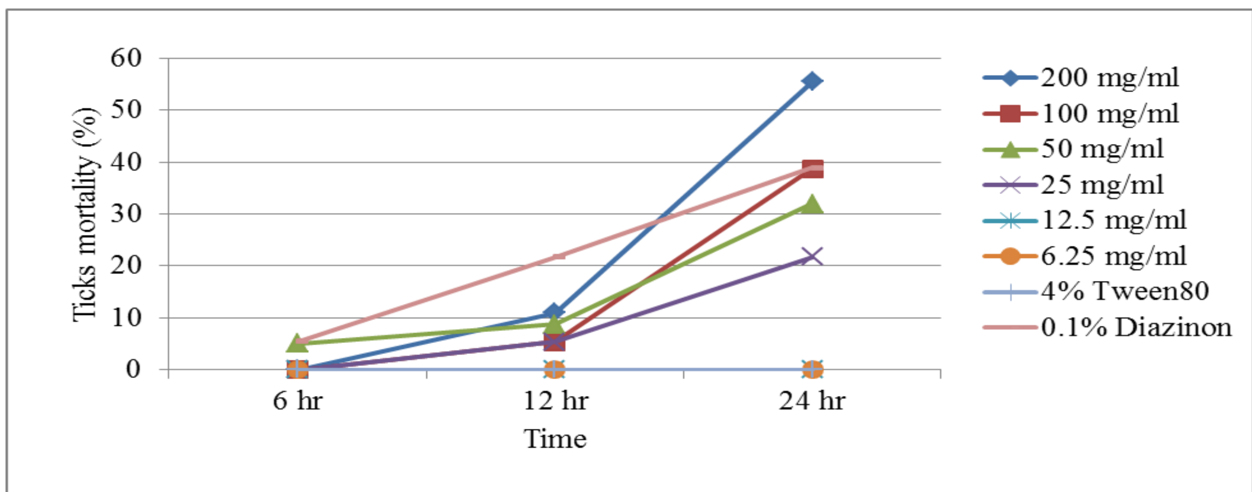

Figure 13: Mortalities of *A. varegatum* treated with the *J. curcas* oil extract
The oil extract at 12.5 mg/ml, 6.25 mg/ml, and 4% Tween 80 showed similar mortality (lines overlap)

Comparative *in vitro* acaricidal activity of different crude extract of *C. aurea*, *N. tabaccum* and *O. integrifolia* has been given in Table 4. The results revealed plant *C. aurea* and *J. curcas* showed 56% mortality wich was significantly ($P < 0.05$) higher mortality than by *N. tabaccum (*44%) and *O. integrifolia* (27%) after 24 hrs of exposure at 200 mg/ml concentrations. However, because of the different extraction method used, *J. curcas* effect is not included in the comparative table below. When the impacts of the different plant extracts were compared between the two parasite species, they are more effective against lice than the tick species studied ($P < 0.05$) exept at lower concentration of *O. integrifolia* (Table 5).

Table 4: Comparisons on acaricidal activity of different concentrations of crude extracts on *A. variegatum* after 6 and 12 hrs exposure

Dose (mg/ml)	Corrected mortality rate (%)±SE								
	6 hr			12 hr			24 hr		
	CA	NT	OI	CA	NT	OI	CA	NT	OI
200	5.3±5.3[a]	5.3±5.3[a]	0.0±0.0[a]	16.3±9.5[a]	16.3±9.5[a]	0.0±0.0[a]	55.5±5.5[a]	44.3±5.7[b]	27±5.7[b]
100	16.3±.9.5[a]	5.3±.5.3[a]	0.0±0.0[a]	163±9.5[a]	16±0.0[a]	0.0±0.0[a]	38.7±5.7[a]	38.7±5.7[a]	27±5.7[a]
50	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	3.3±3.3[a]	0.0±0.0[a]	0.0±0.0[a]	21.7±5.7[a]	16±0.0[a]	16.6±0.0[a]
25	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	5.3±5.3[a]	0.0±0.0[a]	0.0±0.0[a]	16±0.0[a]	11 ±5.3[a]	5.3±5.3[a]
12.5	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	16±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
6.25	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	11±5.3[a]	0.0±0.0[a]	0.0±0.0[a]	11±5.3[a]	0.0±0.0[a]	0.0±0.0[a]
-ve control	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±.0.0[a]	0.0±0.0[a]	0.0±0.0[a]	0.0±0.0[a]
+ve control	5.3±5.3[a]	0.0±0.0[a]	6.7±3.3[a]	11±5.3[a]	0.0±0.0[a]	13.3±6.7[a]	38.7±5.7[a]	38.7±5.7[a]	38.7±5.7[a]

Values are expressed as mean ± SD. Mean values with different letters in the same column are significantly different ($P < 0.05$).

CA = *Calpurnia aurea*; NT = *Nicotiana tabaccum*; OI = *Otostegia integrifolia*

Table 5: Comparisons on louscidal and acaricidal activity of different concentrations of crude extracts after 24 hrs exposure

Dose (mg/ml)	Plant	Corrected mortality rate (%)		
		Ticks	Lice	*P*–Value
200	*C. aurea*	55±5.3	100±0.0	0.001
	N. tobaccum	44.3±5.7	93.±3.3	0.000
	O. integrifolia	27.3±5.7	63±3.3	0.001
	J. curcas	55.5 ±5.3	100±0.0	0.001
100	*C. aurea*	39±5.8	100±0.0	0.000
	N. tobaccum	39±5.7	86.7±3.3	0.000
	O. integrifolia	27.3±5.7	53±3.3	0.001
	J. curcas	39±5.8	96.7±3.3	0.001
50	*C. aurea*	22±5.8	100±0.0	0.000
	N. tobaccum	16±0.0	80±5.8	0.000
	O. integrifolia	16±0.0	40±5.8	0.007
	J. curcas	32±10	90±5.7	0.001
25	*C. aurea*	16±0.0	100±0.0	0.000
	N. tobaccum	11±5.3	76.7±5.8	0.000
	O. integrifolia	5.3±0.0	36.7±3.3	0.002
	J. curcas	21.6±5.7	83±8.8	0.004
12.5	*C. aurea*	16±0.0	100±0.0	0.000
	N. tobaccum	0.0±0.0	66.7±5.8	0.000
	O. integrifolia	0.0±0.0	30±15	0.121*
	J. curcas	0.0±0.0	73±3.3	0.000
6.25	*C. aurea*	11±.5.3	10.7±5.3	0.000
	N. tobaccum	0.0±0.0	50±10	0.000
	O. integrifolia	0.0±0.0	2.67±1.2	0.091
	J. curcas	0.0±0.0	70±5.7	0.000

*Not significant

5. DISCUSSION

5.1. Phytochemical Constituents

A systematic work on louscidal and acaricidal activity of herbal product is a major alternative approach for the control of ectoparasite infestation in animals. In the present study, methanolic crude extract of *C. aurea* (leaves), *O. integrifolia* (leaves), *N. tabaccum* (leaves) were extracted in a rotary evaporator and petroleum ether extract *J. curcas* (seed) oil in soxhlet apparatus. Among all four medicinal plants *O. integrifolia* yielded high percentage of extract (19.3%) (Table 1). Similar to this finding, Zewdneh *et al*. (2015) obtained a high percentage yield of extract (19%) through methanol extraction *O. integrifolia* leaf.

The percentage yield of extracts of *C. aurea* and *N. tabaccum* were 17% and 10.18%, respectively (Table 1). However, in Ethiopia, there were no reports on extraction of both these plants. The seed of *J. curcas* produced 7.5% yield. This finding is supported by Srivastava *et al*. (2010) who reported 7.45% yield. In the present study, differences on the percentage yield of extracts among the four selected medicinal plants might be due to variation on the nature of plant species, solvents used and method of extraction employed. In support of this, Perucci *et al.* (1995) described that solubility of various plant ingredients depends on the extraction methods and the solvent used.

On qualitative phytochemical screening test of plant extracts, *C. aurea* (leaves) was found positive for alkaloids, saponins, phlobotanin, steroids, flavonoids, glycosides, tannins and phenolic compound, while negative for triterpens. This finding is in agreement with Umer *et al*. (2013) who reported the presence of alkaloids, tannins, flavonoids and saponinson methanol extract of *C. aurea* leaf. Similarly, other study showed that water extract of *C. aurea* leaves contained alkaloids (Zorloni *et al*., 2010).

The present finding revealed that crude extract of *O. integrifolia* (leaves) was positive for phlobotanin, saponins, glycosides, tannins and phenolic compound while negative for alkaloids, steroids, flavonoids and triterpens. In line with this finsing, Zewdneh *et al*. (2015) have reported that methanolic extract of *O. integrifolia* (leaves) was positive for saponins and phenolic compound while negative for alkaloids, steroids and flavonoids.

The chemical analysis in this study revealed that the leaves of *N. tabaccum* were positive for alkaloids, glycosides, saponins, tannins, phenolic and steroids compound but negative for flavonoids, phlobotanin and triterpens. This finding is in concordance with Bowman and Rand (1980) who disclosed the presence of alkaloid in *N. tabaccum* leaves and Rahul *et al*. (2015) who reported that ethyl acetate extracted *N. tabaccum* (leaves) was positive for steroids. However, this is different from previous study conducted by Chitra and Sivaranjani (2012) who described that water extracted *N. tabaccum* (leaves) were positive for steroids, flavonoids, while negative for glycosides and Sunil *et al.* (2010) who noted that alcohol extracted *N. tabaccum* (leaves) were positive for flavonoids. The inconsistency among those and the present findings might be due to differences in the solvents used for extraction. Parts of the plant extracted and the solvents used for extraction determine the medicinally active portions of plant (Solomon *et al*., 2013).

The chemical analysis of petroleum ether extracted oil of *J. curcas* (seed) was found positive for tannins, triterpens, phenolic compound, flavonoids, and steroids, while negative for saponins, glycosides, and alkaloids. This finding is similar with the study which states that ethanolic extract of *J. curcas* seeds had high content of unsaponifiable matter, sterols and triterpene (Jazeen, 1977). In contrast to the present findings, Srivastava *et al.* (2010) noted that acetone extract of *J. curcas* (seed) contained saponins but not triterpens and steroids. In addition, the presence of alkaloids and saponins in *J. curcas* leaves have been reported (Uche and Aprioku, 2008).

5.2. Louscidal Efficacy

The present study aimed to assess the relative efficacy of extract of the four selected plants: *C. aurea*, *J. curcas*, *N. tabaccum* and *O. integrifolia*. Accordingly, *C. aurea* crude extract and *J. curcas* seed oil showed pronounced louscidal activity with total mortality (100%). *N. tobaccum*, on the other hand, showed a moderate louscidal activity. Relatively lower louscidal activity was exhibited by *O. integrifolia*. However, the available scientific research on the cidal effect of these plants is limited to sheep keds. In Ethiopia, Negero *et al*. (2014) reported mortality percentage of sheep keds to be 80%, 47% and 57% when exposed to aqueous leaf extract of *C. aurea*, *N. tabaccum* and *J. curcas* plants respectively. This finding is in agreement with the present study indicating that *C. aurea* has high insecticidal activity equivalent to diazinon as compared to the other plants. The potency of the extracts was concentration and exposure time dependent. This difference in mortality perecentage might be due to variability in the amount or composition of

secondary metabolites present in the plants at the time of extraction as, the effectiveness of the plants dependes on complex mixture of secondary metabolites, such as alkaloids, glycosides, terpenoids, and flavonoids (Handa *et al*., 2008).

Comparative *in vitro* louscidal activity of different crude extracts of the four plants revealed that *C. aurea, J. curcas* and *N. tabaccum* at 200 and 100 mg/ml concentrations and 0.1% diazinon had demonstrated the highest *B. ovis* mortality of about 87-100% at 24 hours post exposure whereas *O. integrifolia* at all concentrations showed the least activity. Moreover, *C. aurea* has shown extraordinary quality in that it has achieved 100% mortality of lice even at concentrations as low as 12.5 mg/ml. The difference in in efficacy might be due to variability in the biochemical composition of the plant materials. In this regards, assessment of secondary metabolites in our plat extracts has revealed that the best active plant, *C. aurea* has steroids, flavonoids, alkaloids, phlebotanin and glycosides whereas the least active plant didn't constitute the first three secondary metabolites. Lower louscidal activity of *O. integrifolia* is might hence be attributed to lower quantity of secondary metabolites and absence of alkaloids as higher mortality of the parasite resulted due to complex mixture of secondary metabolites as described by Koul (2008). The highest mortality of *B. ovis* was observed by *C. aurea* which is considered as the best louscidal agent among the investigated and compared plants. The relative efficacy of *J. curcas* is not compared to the other medicinal plants in the study because of the diferenece in the extraction solvent used.

5.3. Acaricidal Efficacy

Another important finding of the curent study was evaluating the efficacy of the four selected plants against *A. variegatum* tick.The results revealed that contrary to our expectation, diazinon at 0.1% concentration has caused only 39% *A. varigatum* mortality in all tests. Moreover, different from their effect on lice, early mortality was not documented before at lest three hours post exposure. On the other hand, although not statistically different from the reference drug, our plant extracts have shown variable efficacy ranging between 27% and 56%. The findings of this study varied from previous studies in the percentage of mortality. Zorloni *et al*. (2010) reported that acetone extracts of *C. aurea* leaf at 5% concentration killed 85% of ticks. On the contrary, Regassa (2000) reported 10% mortality with aqueous extracts of *C. aurea* leaf and bark at 5 hrs exposure time of engorged female *B. decolouratus*. These differences may arise from differences

in the method of extraction, exposure time and parasite species tested. Studies have shown that organic solvent extracts show greater biological activity than the aqueous extract (Parekh and Chanda, 2007).

There is no available published scientific document on the effect of *O. integrifolia* against ticks. However, the importance of O. *integrifolia* has been documented previsouly by Abyot *et al*. (2013), who reported hydro alcoholic leaf extract of *O. integrifolia* possesses potent activity against *Plasmodium berghei* malaria parasite invivo with a maximum percent chemosuppression of 80.5 at a dose of 600 mg/kg/day. Zorloni (2007) also reported that *O. integrifolia* leaf had mosquito repellency, antimicrobial, antihyperglyceamic and antioxidant activity which prevent different kind of ailment.

At higher concentrations of 200 mg/ml and 100 mg/ml *N. tabaccum* demonstrated comparable efficacy to the reference drug (0.1% diazinon). Ratan (2004) has already reported that engorged adult *Rhipicephalus haemaphysaloides* treated with undiluted and 50% diluted aqueous extract of *N. tabaccum* have immediate effect on mortality and long term effect on fecundity parameters. Mansingh and Williams (1993) also reported *N. tabaccum* is effective against *Boophilus microplus.*

At higher concentrations of 200 mg/ml and 100 mg/ml *J. curcas* crude extract showed comparable efficacy with the positive control (reference drug) against *A. varigatum*. The present finding is not in line with Sanis *et al.* (2012), who reported ethanolic leaf extract of *J. curcas* didn't reveal any cidal effect on adult *Rhipicephalus annulatus* engorged female ticks at a concentration of 100 mg/ml. This difference might be due to the very low content of secondary metabolites in *Jatropha* leaves compared to seed oil (Joish and Pathipati, 2009). Similarly, the difference may arise from the difference in the tick species, physiological status of tick and extraction solvent used among the studies.

Comparative *in vitro* acaricidal activity of different crude extracts of the four plants revealed that *C. aurea, J. curcas* and *N. tabaccum* at 200 and 100 mg/ml concentrations and 0.1% diazinon had demonstrated the highest *A. varigatum* mortality of about 39-56% at 24 hrs post exposure where as *O. integrifolia* at all concentrations showed least activity. Moreover, *C. aurea*, and *J. curcas*, has shown extraordinary quality in that it has achieved 56% mortality of tick. The difference in in

efficacy might be due to variability in the biochemical composition of the plant materials. In this regards, assessment of secondary metabolites in our plant extracts have revealed that the best active plant, *C. aurea* has steroids, flavonoids, alkaloids, phlebotanin and glycosides whereas the least active plant is not constituted of the first three secondary metabolites. Lower acaricidal activity of *O. integrifolia* is might hence be attributed to lower quantity of secondary metabolites and absence of alkaloids, which act synergistically with glycosides when present and gave anti-tick activity (Ghosh *et al*., 2015). Studies indicate the presence of alkaloid glycosides and phenol are important chemicals to initiate the mechanism of action *in vitro* and *in vivo* activity causing mortality against ticks (Kumar *et al*., 2011). Ticks (*A. varigatum*) generally appear less susceptible to the effects of the plant extracts compared to lice (*B. ovis).* This might be attributed to thickness of the integument in ticks as compared to lice which interfers with absorption of the active ingredients present in the extracts of the study medicinal plants

6. CONCLUSION AND RECOMMENDATIONS

This study aimed to demonstrate the relative efficacy of extracts of four medicinal plant species against *B. ovis* and *A. varigatum*. The findings unequivocally demonstrated that three of the four plants, *C. aurea, N. tabaccum* and *J. curcas* have potent louseicidal activity comparable to the commercial acaricide, diazinon and the effect of *C. aurea* was appreciable enven at lower concentrations and at a very early stage of contact with the parasite. *O. integrifolia* had very minimal effect on both lice *B. ovis* and *A. varigatum* suggesting that it lacks essential elements to kill the parasites. On the other hand, the efficacy of the above three plants was moderate at higher concentrations up to 200 mg/ml and the effect was started very late after exposure. An additional finding of this study which is worth noting is the low efficacy of diazinon on *A. varigatum* which deserves further study on the possible development of resistance. In general, *C. aurea, N. tabaccum* and *J. curcas* have much better efficacy on lice than on ticks and hence can be further exploited to explore lead compounds.

Based on the above concluding remarks, the following recommendations are forwarded:-

- Further studies on isolation and characterization of the responsible active components of the selected plant materials should be conducted.

- Further investigation need on their safety and efficacy *in vivo* as well as cost effectiveness of the products that exhibited strong louscidal and acaricidal activity with a view of substituting the conventional synthetic acaricide drugs.

- The low efficacy of diazinon should be further investigated to elucidate the activity range of the compound and suspected prevalence of resistance by not only *A. varigatum* but also other species of ticks.

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8. APPENDIXES

Appendix 1: Preliminary phytochemical screening of solvent extracts

A) Preliminary phytochemical screening of solvent extracts (Option 1)

The crude methanol extract was screened for the presence or absence of secondary metabolites such as alkaloids, steroidal compounds, phenolic compounds, flavonoids, saponins, tanninsPhlobatannins and glycosides using standard procedures (Hymete, 1986; Mbata *et al*., 2006).

1) Saponins (Frothing test)

Extract (300 mg) was boiled with 5 ml water for two minutes. The mixture was cooled and mixed vigorously and left for three minutes. The formation of frothing indicated the presence of saponins.

2) Tannins (Ferric chloride test)

About 0.5 g of the extract was boiled in 10 ml of water in a test tube and then filtered. A few drops of 0.1% ferric chloride (or dilute ferric chloride solution) was added and observed for brownish green or a blue-black coloration indicating the presence of tannins. Formation of a blue-black precipitate indicates hydrolyzable tannins and green precipitate indicates the presence of condensed tannins.

3) Flavonoids (Shinoda test)

To one ml of the extract, few drops of NaOH were added. An intense yellow color was produced in the extract which becomes colorless on addition of a few drops of dilute acids indicates the presence of flavonoids. Or, about 0.5 g of the extract was dissolved in 1.5 ml of 50% methanol and warmed on steam bath. Metallic magnesium and 5 drops of concentrated hydrochloric acid were added. A red or orange color indicates the presence of flavonoids aglycone. Or, the alcoholic extract (5 ml, corresponding to 1 g of plant material) was treated with a fewdrops of

concentrated HCl and magnesium turnings (0.5 g).The presence of flavonoids wasindicative if pink or magenta-red color developed within 3 min.

4) Steroids (Lieberman-Burchardís test)

Approximately,1 mg of the extracts was dissolved in 10 ml of HCl and equal volume of conc. sulphuric acid was added by the sides of the test tube. The upper layer turns red and sulphuric acid layer should yellow with green fluorescence. This indicates the presence of steroids.

5) Test for Triterpens (Lieberman-Burchardís test)

Extract (300 mg) was mixed with 5 ml HCl and warmed for 30 minutes. Few drops of concentrated sulphuric acid was added and mixed well. The appearance of red color indicates the presence of triterpenes.

6) Test for phenolic compounds

To 2 ml of filtered solution of the aqueous macerate of the plant material, 3 drops of a freshly prepared mixture of 1 ml of 1% ferric chloride and 1 ml of potassium ferrocyanide was added to detect phenolic compounds. Formation of bluish-green color was taken as positive.

7) Test for glycoside ($FeCl_3$ test)

To about 0.5 g of the extract/fraction, 5 mL of conc. H_2SO_4 was added and boiled for 15 min. This was then cooled and neutralized with 20% KOH. The solution was divided into two portions. Three drops of ferric chloride solution was added to one of the portions, and a green to black precipitate indicated phenolic aglycone as a result of hydrolysis of glycoside.

8) Test for alkaloids (Dragendoffís test)

To about 0.5 g of each extract, 1% diluted HCl (20 ml) was added in a conical flask, heated on asteam bath and then filtered. The filtrate was made alkaline with 28% NH_3 solution and then extracted with chloroform (3×5 cm^3). The combined $CHCl_3$ extracts were concentrated and treated with equal volume of 1% HCl. Dragendorffis reagents (2 ml) were added and occurence of orange-red precipitate indicated the presence of alkaloids. Or, the alcoholic extract (corresponding to 2.5 g of plant material) was evaporated to drynessand the residue was heated

on a boiling water bath with 2N HCl (5 ml).After cooling, theinto two equal portions. One portion was treated with a few drops of Mayer's reagent andthe other with equal amounts of Wagner's reagent (3). The samples were then observed forthe presence of turbidity or precipitation. A (+) score was recorded if the reagent produced onlya slight opaqueness; a (++) score was recorded if a definite turbidity, but no flocculation wasobserved and a (+++) score was recorded if a definite heavy precipitate or flocculation was produced.

Test for free anthraquinones (Bontragerís test)

Small portion of the extract was shaken with 10 ml of benzene and filtered. Five milliliters of 10% NH_3 solution was added to the filtrate and stirred. The production of a pink-red or violet color indicates the presence of free anthraquinones.

Option 2: Phytochemical Screening Procedure (option 2)

1) Cardiac glycosides (Lieberman's test)

Dissolved a 0.5 g of the plant extract in 2 ml of acetic anhydride and cool well in an ice-bath. Add carefully concentrated sulfuric acid. A colour change from purple to blue to green indicated the presence of aglycone portion of the cardiac glycosides.

2) Saponins (Frothing test)

The presence of saponins is determined by Frothing test. Extract (300 mg) with 5 ml water for two minutes. Cool the mixture and mixed vigorously and left for three minutes. The formation of frothing indicated the presence of saponins.

3) Tannins (Ferric chloride test)

Boil about 0.5 g of the extract in 10 ml of water in a test tube and then filter. Add A few drops of 0.1% ferric chloride (or dilute ferric chloride solution) and observe for brownish green or a blue-black coloration indicating the presence of tannins. Formation of a blue-black precipitate indicates hydrolyzable tannins and green precipitate indicates the presence of condensed tannins.

4) Flavonoids (Shinoda test)

A) Reaction with sodium hydroxide:

To 1 ml of the extract, few drops of NaOH were added. An intense yellow color was produced in the extract which becomes colorless on addition of a few drops of dilute acids indicates the presence of flavonoids.

B) Test for free flavonoids

A 5 ml of ethyl acetate was added to a solution of 0.5 g of the extract in water. The mixture was shaken, allowed to settle and inspected for the production of yellow colour in the organic layer which is taken as positive for free flavonoids.

5) Test for alkaloids

A) Preliminary test

A 100 mg of plant extract was dissolved in dilute hydrochloric acid. Solution was clarified by filtration. Filtrate was tested with Dragendroff's and Mayer's reagents. The treated solutions were observed for any precipitation.

B) Confirmatory test

A 5 g of the plant extract was treated with 40% calcium hydroxide solution until the extract was distinctly alkaline to litmus paper, and then extracted twice with 10 ml portions of chloroform. Chloroform extracts were combined and concentrated in vacuum to about 5 ml. Chloroform extract was then spotted on thin layer plates. Solvent system (n-hexane-ethyl acetate, 4:1) was used to develop chromatograms and detected by spraying the chromatograms with freshly prepared Dragendroff's spray reagent. An orange or dark colored spots against a pale yellow background was confirmatory evidence for presence of alkaloids.

6) Steroids (Lieberman-Burchardís test)

A 1 mg of the extracts was dissolved in 10 ml of HCl and equal volume of conc. sulphuric acidwas added by the sides of the test tube. The upper layer turns red and sulphuric acid layer should yellow with green fluorescence. This indicates the presence of steroids.

7) Test for triterpenes (Lieberman-Burchardís test)

Extract (300 mg) was mixed with 5 ml HCl and warmed for 30 minutes. Few drops of concentrated sulphuric acid was added and mixed well. The appearance of red color indicates the presence of triterpenes.

8) Test for phenolic compounds

To 2 ml of filtered solution of the methanol and acetone macerate of the plant material, 3 drops of a freshly prepared mixture of 1 mL of 1% ferric chloride and 1 ml of potassium ferrocyanide was added to detect phenolic compounds. Formation of bluish-green color was taken as positive.

9) Test for anthraquinones (Borntrager's test)

Small portion of the extract was shaken with 10 ml of benzene and filtered. A 5 ml of 10% NH_3 solution was added to the filtrate and stirred. The production of a pink-red or violet color indicates the presence of anthraquinones.

10) Test for proteins (Millon's test)

Crude extract when mixed with 2ml of Millon's reagent, white precipitate appeared which turned red upon gentle heating that confirmed the presence of protein.

11) Test for Carbohydrates (Fehling's test)

Equal volume of Fehling A and Fehling B reagents were mixed together and 2ml of it was added to crude extract and gently boiled. A brick red precipitate appeared at the bottom of the test tube indicated the presence of reducing sugars.

12) Test for phytosterol

The extract was refluxed with solution of alcoholic potassium hydroxide till complete saponification takes place. The mixture was diluted and extracted with ether. The ether layer was evaporated and the residue was tested for the presence of phytosterol. The residue was dissolved in few drops of diluted acetic acid; 3 ml of acetic anhydride was added followed by few drops of Concentrated H_2SO_4. Appearance of bluish green colour showed the presence of phytosterol.

13) Test for phlobatannins

About 2 ml of aqueous extract was added to 2 ml of 1% HCl and the mixture was boiled. Deposition of a red precipitate was taken as an evidence for the presence of phlobatannins.

14) Iodine Test

Crude extract was mixed with 2 ml of iodine solution. A dark blue or purple coloration indicated the presence of the carbohydrate.

Appendix 2: Parasites used for the *in vitro* adulticidal activity

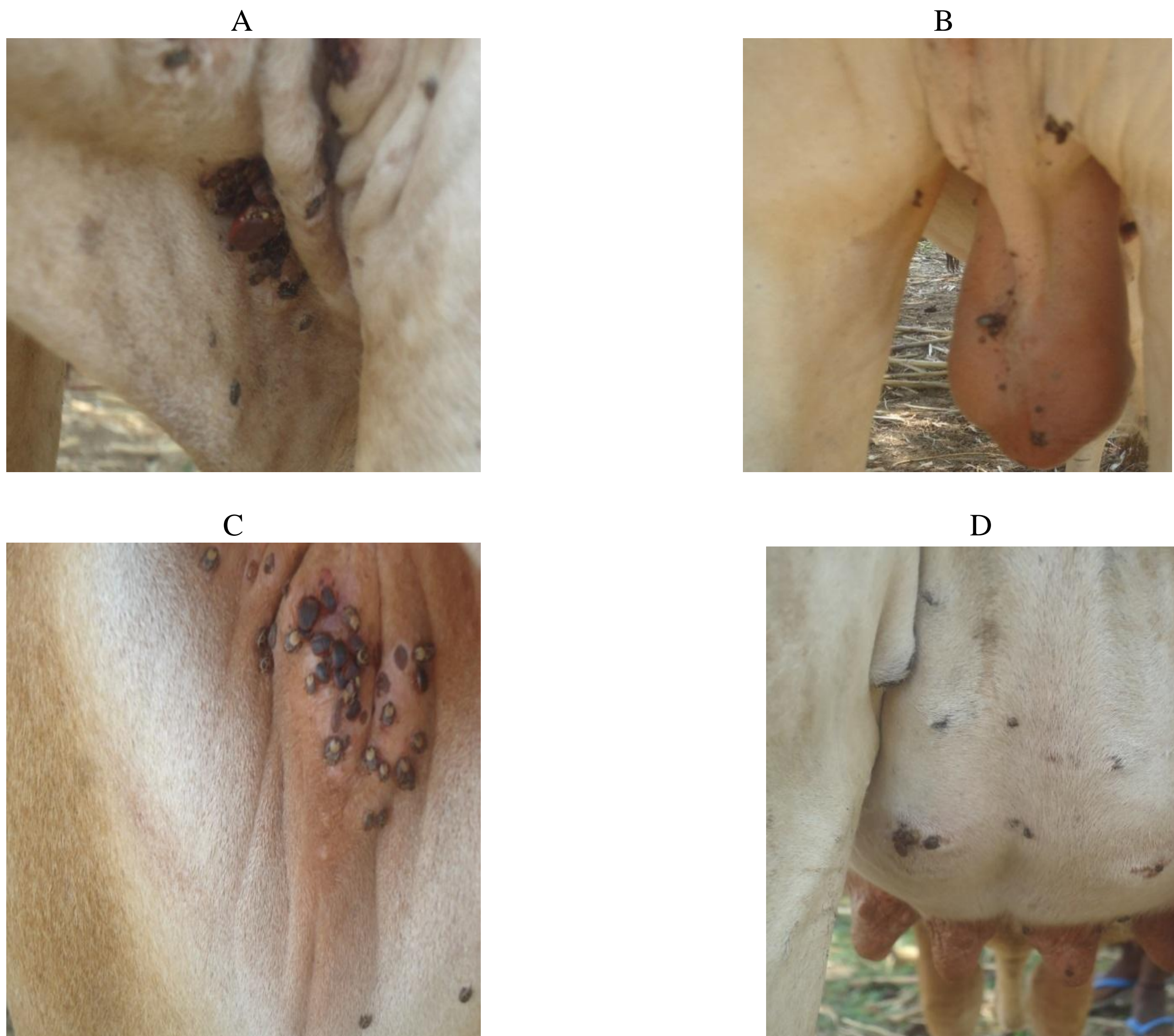

Figure 14: *Amblyomma variegatum* Fabricius attached on different body parts of of a bull and cow

A) Dewlap (bull); B) Scrotum (bull): C) Vulva (cow); and D) Udder.

(Sisay Alemu, 12-01-2015)

Appendix 3: Cheking the mortality of the lice and ticks under microscope

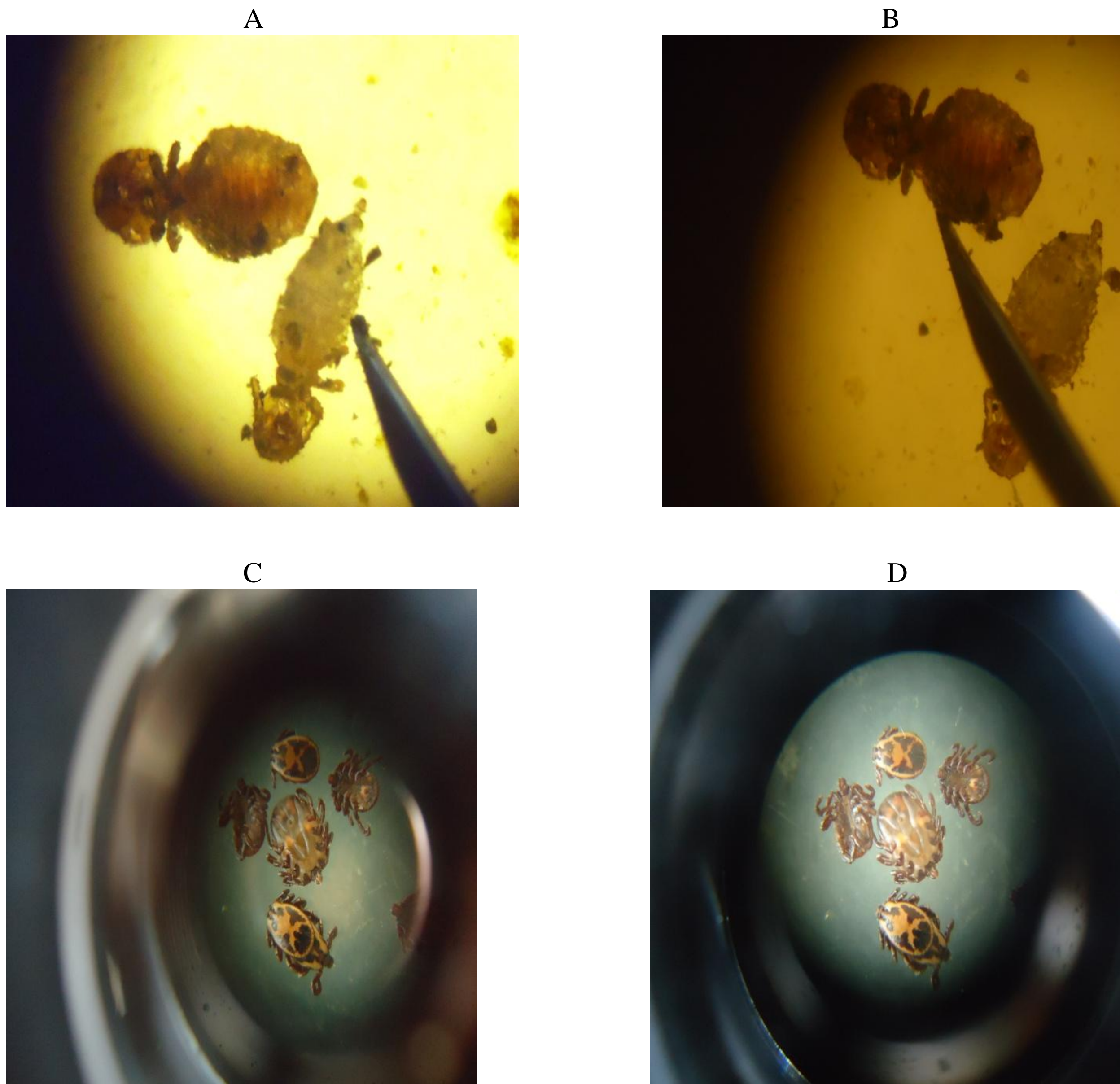

Figure 15: Appearane of dead lice and ticks under a stereiomicroscope

A and B: dead lice; C and D dead ticks

(Sisay Alemu, 12-01-2015)

Appendix 4: *In vitro* adultcidal recording format sheet

Concentration	Replication	No of parasites								Total died in 24 hrs	%
		Immersed	Died after								
			30 min	1 hr	2 hrs	3 hrs	6 hrs	12 hrs	24 hrs		
20%	1										
	2										
	3										
10%	1										
	2										
	3										
5%	1										
	2										
	3										
2.5%	1`										
	2										
	3										
1.25%	1										
	2										
	3										
6.25%	1										
	2										
	3										
-ve control	1										
	2										
	3										
+ve control	1										
	2										
	3										