An *In Vitro* Acaricidal Efficacy Assesment Of Crude Methanolic And Ethanolic Extracts And Latex Secretion Of *Cryptostegia Grandiflora* Against *Amblyomma* And *Ornithodorus* Tick Species

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Abstract: Experimental study was carried out from February 2016 to April 2016 with the aim of *in vitro* acaricidal efficacy evaluation of *C. grandiflora* against *Amblyomma* and *Ornithodorus species*. The acaricidal activities of crude aqueous methanolic and ethanolic extracts of the leaves and stems and latex secretions of *C. grandiflora* were assessed against adults of *Amblyomma* and *Ornithodorus species* by using adult immersion test (AIT). Three concentrations of the crude extract (200 mg/ml (20%), 100 mg/ml (10%) and 50 mg/ml (5%) with three replicates for each were used. The result obtained from this study indicated that the crude stem ethanol and leaf methanolic extracts of *C. grandiflora* at higher concentration (200mg/ml) and latex secretion in its pure form used, were lethal to *Amblyomma* and *Ornithodorus species* of ticks and comparable to the positive control. The mean mortality rate was varied from 26.67% to 86.67% for crude extracts and 93.33% for pure latex secretion formulation at 24 hours after treatment. The mortality was increased with increasing concentrations (50 mg/ml (5%), 100 mg/ml (10%), (200 mg/ml (20%).The efficacy of extracts and latex against tested ticks was statistically significant ($p<0.05$). There was a significance difference ($p<0.05$) in acaricidal efficacy between extracts and different concentrations tested. These results stipulated that leaf methanolic and stem ethanolic extracts, and latex secretion of *C. grandiflora* may serve as tick control even in their crude and pure secretion form, respectively. To apply on ticks, it should be essential to fractionate the extracts and test each component separately to determine the active compounds responsible for the killing effect of the potent extracts. The overall findings of the current study indicated that most of *C. grandiflora* extracts and its latex had potential acaricidal effect warranting further *in vitro* and *in vivo* evaluation. Additional comprehensive research is highly recommended.

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1. Introduction

Tick-borne infectious diseases are a growing and very serious world health problem and a major obstacle for animal health and production especially in developing countries (Rajput *et al*., 2006).In fact, ticks are second to mosquitoes as vectors of human pathogens and the most important vectors of pathogens affecting cattle in tropical and subtropical countries (Peter *et al*., 2005). Environmental and climatic global change is currently exerting a strong impact on the transmission and distribution of tick-borne pathogens (El Kammah *et al.*, 2007).

Ticks are vectors and reservoirs of zoonotic bacteria such as *Borrelia spp.*, spotted fever and typhus group *Rickettsia spp*., *Ehrlichia spp*., *Coxiella burnetii*, *Tularaemia spp*. and *Bartonella spp.* which cause emerging zoonoses in humans. Ticks are considered to have more veterinary significance than medical importance in the country. Recent studies have indicated an increase in the spectrum of tick borne pathogens affecting humans and animals (Bersisa *et al*., 2014).

All these direct forms of damage together with tick-transmitted diseases (including Babesiosis, Theileriosis, Anaplasmosis and Cowdriosis) cause important economic losses to the livestock industry, mainly affecting tropical and subtropical countries, where ticks constitute one of the main difficulties for the development of the livestock breeding industry (Jongejan and Uilenberg, 2004; Rajput *et al.*, 2006).

The economically most important ixodid ticks of livestock in tropical regions belong to the genera of *Hyalomma*, *Boophilus*, *Amblyomma* and *Rhipicephalus* (Frans, 2000).

The tropical bont tick, *Amblyomma variegatum,* is considered one of the most detrimental of the tick species present in Africa and now the Caribbean (Carib Vet, 2011b; Stachurksi and Lancelot, 2006). It can result in severe economic losses due to hide damage, milk production reduction, and death of livestock (Norval *et al*., 1992, Walker, 1996). The two

primary diseases of concern that are associated with the tropical bont tick are dermatophilosis and heartwater (Carib Vet, 2011b; Merck, 2011).

Ornithodoros species transmits African swine fever virus which affects only porcine species and causes African swine fever (ASF), highly lethal to pigs, which is one of the most important viral diseases of swine included in the A list of the OIE (Kleiboeker and Scoles, 2001; Labuda and Nuttall, 2008). They act not only as vectors but also as reservoirs of relapsing fever spirochetes, which seem to be quite vector-specific without crossed infections (Shanbaky and Helmy, 2000).

Environmental pollution is a serious problem posed by the use of synthetic acaricides in tick control. Chemical compound such as endosulfan and endosulfan sulphate are toxic and bioaccumulate in nature (Bhattacarya *et al*., 2003). Organophosphate accumulation in fatty tissue of mammals can lead to poisoning in man (Karalliede *et al*., 2003).

Herbal medicine used to be the best alternative, as most antimicrobial agents were highly toxic and resistance development is in an increasing sequence (Mohammed *et al*., 2013).

The application of botanicals to livestock to control ectoparasites of veterinary importance is widespread in the developing countries (Robert *et al*., 2010). In contrast, to chemical acaricides, botanical acaricides have many advantageous features of being degraded in the environment, do not remain in livestock, are not as prone to resistance, and are relatively safe for humans, animals, the environment (Alawa *et al.,* 2003).

Ethno veterinary medicine is frequently used for treating of livestock diseases by many different ethnic groups in Ethiopia. Nearly 90% of livestock population in the country use plant based traditional medicines as their major health care system (Endashaw, 2007). Ethno veterinary medicine plays an important role in animal production and livelihood development. It provides valuable alternatives to and complements western-style veterinary medicine (Shen *et al*., 2010) and is accessible and easy to prepare and administer, at little or no cost to the farmer (Jabbarm *et al*., 2005). However, limited research work is conducted in Ethiopia to exploit this potential.

Some of the plants documented in Ethiopia to treat same/similar livestock diseases (Seifu *et al*., 2006, Giday *et al.,* 2003, Sori *et al*., 2004). These include *Acacia nilotica* (used to treat diarrhoea); *Acalypha indica* (used against anthrax), *Aloe trichosantha* (used against anthrax, contagious caprine pleuropneumonia and contagious bovine pleuropneumonia), *Balanites aegyptiaca* (used against anthrax), *Calotropis procera* (used against blackleg) and *Dobera glabra* (used against tick infestation) (Mirutse and Tilahun, 2013).

C. grandiflora (Roxb) R. Br. (Family: Asclepiadaceae) (Halemero, Am.) is widely distributed throughout tropical Africa, Madagascar and some parts of India (Kirtikar and Basu, 1975). Asclepiadaceae and Capparidaceae took the better share of the reported plants followed by Euphorbiaceae and Solanaceae in Amibara, Afar (Mirutse and Tilahun, 2013). Rubber vine, Halemero, is poisonous and if cattle accidentally eat the leaves, they die (Matthew *et al*., 2016).

C. grandiflora, also known as the Indian rubber vine is said to contain cardiac glycosides responsible for producing a digitalis like toxicity upon consumption of its leaves. Digitalis toxicity produces a toxidrome characterized by gastrointestinal, neurologic, electrolyte, hematologic and cardiac manifestations (Salmaan *et al*., 2012).

Different extracts of *C. grandiflora* (Roxb) Rbr. leaves were investigated for their antibacterial potential against *Pseudomonus, cepacia*, *Bacillus, megatorium*, *Staphylococcus aureus*, *Escherichia coli*, *Bacillus subtilis* and *Bacillus coagulans* (Pulok *et al*., 1999).

This plant species is also reported to possess various biological activities like antioxidant (De Freitas *et al*., 2010), antitumor (Doskotch *et al*., 1972), antiviral (Vijayan *et al*., 2004) and control the schistosomiasis (Adewunmi, 1984). The aqueous solution of ethanol extract of aerial parts (Sharma *et al*., 1967, Sharma and Shukla, 1977) and the latex derived from this plant have proteolytic, bacteriolytic activity and possess relevant enzymatic activities against pathogenic related proteins (Pant and Srivastava, 1966; Cleverson *et al.,* 2010).

However, acaricidal activity of *C. grandiflora*extract and latex scientifically was not evaluated. This indicates the need for the scientific evaluation of acaricidal efficacy of *C. grandiflora* plant against different species of ticks and thus, recent information is important to understand the acaricidal efficacy of this medicinal plant.

Therefore, the general objective of this study was to evaluate the acaricidal efficacy of crude methanolic, ethanolic extracts and latex secretion of *C. grandiflora* with different concentrations against *Amblyomma and Ornithodorus* tick species

2. Materials And Methods

2.1. Site and Collection of the Study Plant

Selection of plant is based on literature survey on traditional complaint of poisonous in Ethiopia and other parts of the World. The study plant parts that are at seed stage were collected in February 2016 from Afar, Amibara district, Ethiopia (Figure 1). It is

geographically located between 39°34' and 42°28' East Longitude and 8°49'and14°30'North Latitude (CSA, 2008). Due to wide ecological adaptation, the plant occurs on large varieties of soils and over wide range of altitudes. The climate of its natural range is characterized by hot temperature averaging 20°c, but the whole temperature ranges from -1.5 to 500 °c (Pasiecznik *et al*., 2001). The stem and leaf of Halemero were collected from the area as mentioned above by latex glove worn hand and transferred to pre-prepared clean basket. Specimens of the plant were identified by local afar pastoralists as "Halemero" and further identified at spp. level by using its flower at Aklilu Lemma Institute of Pathobiology (Annex 1). A voucher specimen was deposited at the Herbarium, Department of Botany, and Addis Ababa University.

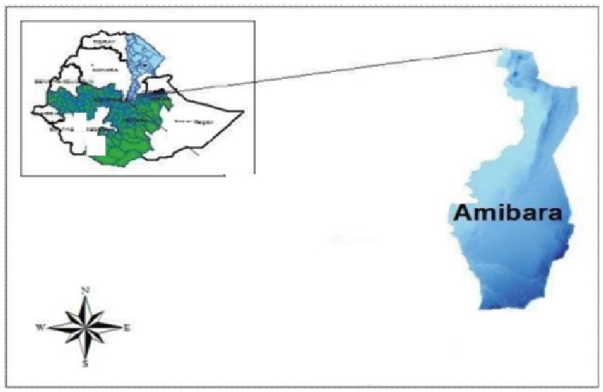

Figure 1: Map of the study plant collection area
Source: (Birtukan, 2015)

2.2. Description of the Study Plant

Cryptostegia grandiflora (Halemero, Am.), is a self-supporting, scrambling, many-stemmed vine that grows to two meters tall with long trailing whips. A milky soap oozes from stems, leaves and seedpods when cut or broken. Leaves are dark green and glossy, 6-10cm long, 3-5cm wide and in opposite pairs. Roots have been found at a depth of 13 meters in mine shafts. Roots of seedlings are twice as long as shoots. The growth form of the vine differs depending on the surrounding conditions. They can form dense canopies of overlapping plants with long whips, form towers upto 30meters high, the height of native trees grows as freestanding shrubs in the absence of other vegetation. Flowers are large and showy, with five white to light purple petals in a funnel shape. The seedpods are rigid, 10-12cm long, 3-4cm wide and grow in pairs at the end of a short stalk.

2.3. Study Design

The experimental study was conducted from January to April 2016 to evaluate efficacy of *Cryptostegia grandiflora* aqueous methanolic and ethanolic extracts and latex secretion against *Amblyomma* and *Ornithodorus species* by using adult immersion test *in vitro* using the protocol described by Askale (2015). Extractions of the medicinal plant using aqueous ethanol and methanol methods was carried out following a standard protocol prepared by Gizachew *et al*. (2013) and Gemechu *et al*. (2013).

2.4. Study Methodology

2.4.1. Preparation and extraction of the plant

The preparation of the plants and extractions were carried as described by Gemechu *et al*. (2013) (Annex 3). Briefly, after collection leaves and stems of *C. grandiflora* (Halemero, Am.) were washed with distilled water to remove dirt and soil particles. The plant was cut into small pieces, spread out on paper sheets, dried in shaded area at room temperature by turning parts up and down to prevent fungal growth for two weeks and finely powdered after desiccation by using pestle and mortar after thorough agitation with distilled water. 111g and 237g leaves were soaked in 80% methanol and 70% ethanol, respectively and 155g and 145g stem of *C. grandiflora* (Halemero, Am.) were soaked as above, respectively to 1:4 in separate flask and shaken and macerated for 72hrs by automatic orbital shaker (Magano *et al*., 2011). The mixture was later strained using a muslin cloth and filtered using a Whatman filter paper No. 1: 125mm (Camlab, UK) and the filtrate was concentrated in 3000 rev/min in a vacuum rotary evaporator at 60°C and was evaporated to dryness in dry air oven at 40°C (Annex 4). The filtrates were stored in capped labeled bottles and kept in the refrigerator at 4°C until use (Bagavan *et al*., 2009).

2.4.2. Extraction Solvents used for the study

The higher concentrations of more bioactive flavonoid compounds were detected with ethanol 70% due to its higher polarity than pure ethanol. Since nearly all of the identified components from plants active against microorganisms are aromatic or saturated organic compounds, they are most often obtained through initial ethanol or methanol extraction (Prashant *et al*., 2011). Methanol was chosen for extraction in this study because it has wide solubility properties for low molecular and moderately polar substances, including the antioxidant-active phenolic compounds (Ming *et al*., 2011).

2.5. Extract and Control Dilution to Prepare Working Concentrations

The concentrations such as 20%, 10% and 5% of the aqueous methanolic and ethanolic extracts and 100% latex secretions were used for accaricidal efficacy test. 2% tween80 was used as negative control while 0.1% diazinon used as positive control (Annex 6). The choice of positive control was based on their commercial availability and patronage by livestock keepers and veterinary clinics in the area. Diazinon 60% EC with an active ingredient of 600g/L was

obtained from Adami Tulu Pesticides processing share company, Addis Ababa, Ethiopia. It was manufactured in October 31, 2015 and has one years of shelf life from date of manufacturing. A positive control of 0.1% v/v diazinion 60 EC was diluted in distilled water according to the manufacturers recommendation (1:1000) before being used for further experiment (Heukelbach *et al.*, 2006). Tween80 at concentration of 2% v/v was used because at this concentration it was able to dissolve the extracts but with no harm on the parasites.

2.6. Study Parasites Collection, Transportation and Identification

Hard ticks from naturally infested cattle pastured on local grazing land and soft ticks from deep soil that arise when a person or animal stands nearby were collected by applying gentle rotational movement using hand worn latex glove and apply antiseptics on animals which the tick was taken in Afar, Amibara district, Ethiopia. Collected ticks were put in sterile vials separately for soft and hard ticks and the vials with ticks were wrapped in cotton net gauze for oxygen supply and taken immediately to the laboratory. All collected ticks were examined under stereomicroscope and identified to the genus level using the taxonomic key described by Kaiser (1987) in Horror Agricultural Research Center, Parasitology laboratory, Afar. For our experimental study ticks were selected depending on morphological keys described by Gerald and Robert (1965). Adult *Amblyomma* and *Ornithodorus species* were used for the *in vitro* bioassays.

2.7. *In vitro* Acaricidal Efficacy Test

2.7.1. Adult immersion test

Three replicates for each concentration of five adult ticks were immersed in the respective dilutions of extracts and control solutions for two minute of exposure. After immersion, the ticks were recovered from tube and filtered with filter paper and placed in separate Petri dishes (Zaman *et al*., 2012). Equal milliliters of 2% tween80 and 0.1% diazinion 60 EC were used as negative control and positive control, respectively. The petridishes were incubated at 30^{o}C, which is the room temperature of the area where the study conducted, with 80% relative humidity and death of ticks were recorded after 30 min, 1hr, 2hr, 3hr, 6hr, 12hr and 24hr intervals (Annex 7). The viability of ticks was checked regularly by stimulation with a needle and observing under stereomicroscope and ticks were recorded as dead depending on the reaction shown (Askale, 2015). Efficacy of extract to kill the adult ticks was determined against negative control. The total percentage of mortality within 24 hrs was calculated by the formula previously used by Krishnaveni and Venkatalakshmi (2014) as follows.

$$\text{Mortality } \% = \frac{\text{no.of dead ticks}}{\text{total no.of ticks}} \times 100$$

2.7.2. Definition of test scores for crude extracts

Definition of test scores was adopted from those reported by Rosado-Aguilar *et al*. (2010) as follows. Activity of crude plant extracts were classified in mean % of mortality of adult ticks as; high mortality (80-100%); relatively high mortality (64-79%); moderate mortality (50-65%); low mortality 31-49%; and non-significant activity of mortality (0-30%).

2.8. Dose Response Bioassay

From the stock solution, different concentrations ranging from 20% to 5% for ticks were prepared (Annex6). Based on the preliminary screening results, different crude solvent extracts prepared from the leaf and stem of *C. grandiflora* were subjected to dose response bioassay against *Amblyomma* and *Ornithodorus species* for each. The numbers of dead ticks were counted after 24 h of exposure, and the percentage mortality was reported from the average of three replicates. However, at the end of 24h, the selected test samples turned out to be equal in their lethality potential.

2.9. Data Analysis

Data from experimental results on acaricidal effect of the extract with the lethal dose concentrations were entered into MS excel 2016 spread sheets database system used for data management. SPSS software version 20 was used for data analysis. Results of the study were expressed using descriptive statistics as a mean of mortality percentage ± standard error (Mean ± SE). Statistical significance was determined by one-way analysis of variance (ANOVA) with multiple comparison tests (Post Hoc/Tukey's test/HSD) to compare parameters within and between groups tested. All significant levels set at $P<0.05$ of 95% confidence interval.

3. Results

3.1. Physical Characteristic Features and Percentage Yield of Extracts

Physical characteristic features and percentage yield of the extracts are shown in table 1. Crude methanol and ethanol extracts of *C. grandiflora* leaves and stems have darkish green and yellowish green color, respectively; semi solid and sticky nature. The latex secretion of *C. grandiflora* has whitish color and oil in consistency.

Table 1: Physical characteristics features and percentage yield of the extracts

Botanical Name	Local Name	Plant parts used	Color of extracted product	Characteristics of extracted material	Aqueous solvent used	% yield
C. grandiflora	Halemero (Am.)	Stem (dry)	yellowish green	semi solid and sticky	Methanol	1.29
					Ethanol	1.37
		Leaf (dry)	Darkish green	semi solid and sticky	Methanol	1.8
					Ethanol	1.7
		Latex (milky)	whitish	oily	None	25

Am= Amharic

3.2. Acaricidal Activity of Crude Extracts against *Amblyomma* tickspecies

Mortalities of *Amblyomma species* treated with different concentrations of *C. grandiflora* crude 80% methanolic and 70% ethanolic stem and leaf extracts, respectively and its latex secretion were screened for their acaricidal activity by using adult immersion test and both extracts and the latex had showed acaricidal activity against *Amblyomma species*. Statistical increase in tick mortality was started 12hr post exposure with positive control and 24hr post exposure with 200mg/ml, 100mg/ml and 50mg/ml concentrations of *C. grandiflora* extract. At 24hr post exposure period, latex had caused significantly higher tick mortality than the 100mg/ml or less concentration of the extract ($P<0.05$). There was mean percentage difference in mortality of ticks between positive control and 200mg/ml concentration of extracts. Moreover, there is significant difference ($P<0.05$) between the three higher concentrations (≥50mg/ml). Both the positive control (0.1% diazinon) and stem methanolic extract (10%) had equal acaricidal activity against *Amblyomma species*. Similarly, the 20% ethanolic extracts of stem and leaf of *C. grandiflora* had equal acaricidal activity. The observation of the lethal effect of C. *grandiflora* latex secretion was greater (80%±20) as compared to the reference drug (Table 2).

Table 2: Mortality percentage of *Amblyomma species* post 24 hours' exposure

Conc. %100	Mortality %± SE					
	Stem ethanol	Stem methanol	Leaf ethanol	Leaf methanol	Latex80±20^{e}	Diazinon
20	60±20.0^{a}	53.33±11.54^{ab}	60±20^{ac}	66.67±11.54^{ad}		
10	53.33±11.54^{b}	73.33±11.54^{ba}	40±20^{bb}	53.33±30.55^{bc}		
5	53.33±11.54^{c}	33.33±11.54^{ca}	26.67±11.54^{cb}	26.67±11.54^{cc}		
Positive control	0	0	0	0	0	73.33±11.54^{d}
Negative control						

Key: The missing value (100%) indicated that no experiment was conducted. The value (73.33±11.54) for positive control indicated that it was conducted for all extracts tested. Values are expressed as mean of mortality % ± SE. Mortality percentage of values with different letters in the same and different row and column for each extract and concentration indicated that they are significantly different ($P < 0.05$).

3.3. Acaricidal activity of crude extracts against *Ornithodorus species*

Latex secretion of *C. grandiflora* had observed higher acaricidal activity as early as 2hrs post exposure with pure formulation while this effect was delayed by 12 hours for the positive control. Within 24 hours post exposure, plant extract with 200mg/ml, 100mg/ml and latex secretion had caused higher mortality. Moreover, there was significant difference between the reference drug and all concentrations of the extract ($p<0.05$). In conclusion, all 200mg/ml extract concentrations and latex secretion of *C. grandiflora* tested had strong (>80%) Ornithodorocidal activity at 24hr post exposure as compared to the reference drug (Table 3).

Table 3: Mortality percentage of *Ornithodorus tick species* 24 hours' post exposure

Conc.%100	Mortality% ± SE					
	Stem ethanol	Stem methanol	Leaf ethanol	Leaf methanol	Latex93.33±11.5^{e}	Diazinon
20	86.67±11.5^{a}	66.67±23.1^{ab}	86.67±23.1^{ac}	86.67±11.5^{ac}		
10	60±0.0^{b}	40±20.0^{ba}	46.67±11.6^{bb}	60±20.0^{bc}		
Positive control	0	0	0	0	0	66.67±11.5^{d}
Negative control						

Key: The missing value (100%) indicated that no experiment was conducted. The value (66.67%± 11.5) for positive control indicated that it was conducted for all extracts tested. Values are expressed as mean of mortality % ± SE. Mortality percentage of values with different letters and star in the same and different row and column for each concentration and extract indicated that they are significantly different ($P < 0.05$).

3.4. Comparative Efficacy of Extracts

Stem ethanol and leaf methanol extracts and latex secretion had revealed greater acaricidal effect at higher concentration (200mg/ml) whereas methanolic extracts had moderate to strong acaricidal effect at lower (100mg/ml) and higher (200mg/ml) concentrations, respectively. Moreover, there had significant difference ($P<0.05$) between extracts and even the same extract with different concentrations.

4. Discussion

In contrast, to chemical acaricides, botanical acaricides have many advantageous features of being degraded in the environment, do not remain in livestock, are not as prone to resistance, and are relatively safe for humans, animals and the environment.

The current study was aimed to assess the acaricidal efficacy of extracts of *Cryptostegia grandiflora* and to evaluate its efficacy against selected tick species. Diazinon was used as a positive control to evaluate the efficacy.

The current study revealed that there was a difference percentage in the yield of extracts among the different stem and leaf parts. The leaf of *C. Grandiflora* gave the highest yield (1.8%) among extracts while the lowest (1.27%) was observed for the stem of *C. grandiflora*. Habtemariam *et al*. (1994) reported that, apart from the difference in species, plant parts used, age, harvest season and habitat could also contribute to the variation in biochemical profiles and yields.

The results obtained in the current study indicated that *C. grandiflora* had significant ($p<0.05$) acaricidal efficacy on *Amblyomma* and *Ornithodorus* tick species. Moreover, stem ethanolic and leaf methanolic extracts and latex secretion of *C. grandiflora* had strong accaricidal activity against *Amblyomma* and *Ornithodorus*tick *species* at 200mg/ml and pure formulation, respectively compared to control. This had indicated that the acaricidal efficacy of it was subjected to dose response bioassay. There had moderate acaricidal activity at 50mg/ml and 100mg/ml. This also stipulated some toxic level even at lower concentrations. There had no significance difference between the tick species tested against different formulations of extracts. This indicated that this botanical acaricide can act as a broad spectrum acaricide. However, the significance difference ($P<0.05$) between the efficacy of extracts and even the same extract by different concentrations may be attributed to the difference in phytochemical constituents and dose response relationship. It could also be related to the nature of the plant at the time of collection. Surprisingly, the latex had much better acaricidal effect (80% to 93.33%) compared to the reference acaricide (66.67% to73.33%) the putting the latter under the suspect of acaricidal resistance.

The experimental result showed that, different concentrations of *C. grandiflora* crude80% methanolic and 70% ethanolic stem and leaf extracts had mortality effects on *Amblyomma species* and its latex secretion upon screening for their acaricidal activity. An increase in tick mortality was started 12hr post exposure with positive control and 24hr post exposure with 50mg/ml, 100mg/ml and 200mg/ml by increasing concentrations of *C. grandiflora* extract but the overall mortalities were higher for the extracts within 24 hrs post exposure. This stipulated that the extracts had a slow but longer half-life that remained its toxicity dose responsible for the killing effect. At 24hr post exposure period, latex had caused significantly higher tick mortality than the 100mg/ml or less concentration of the extract ($P<0.05$). This had supported by literatures and traditional cattle owners that the latex had highly poisonous and lethal.

Furthermore, latex secretion of *C. grandiflora* had revealed statistically higher acaricidal activity as early as 2hrs post exposure with pure formulation while this effect was delayed by 12 hours for the positive control. Within 24 hours post exposure, plant extract with 200mg/ml and latex secretion had caused significantly ($p<0.05$) higher mortality against *Ornithodorous* ticks. This could be due to the surface area of soft ticks allowed easy diffusion of higher amounts of solutes fastly responsible for the lethal effect because of absence of Scutum. However, there was no significant difference ($P<0.05$) between the two ticks tested. This result suggested that there was no difference in the sensitivity of these two ticks to the extract.

However, it was not possible to compare the findings of the current study with others, since no similar research works were conducted on extracts of *C. grandiflora* against ticks as far as our search is concerned. But to highlight how extracts of *C. grandiflora* had a toxicological and lethal potential there were a few reports. Salmaan *et al*. (2012) reported that the crude methanol extract of *C. grandiflora* revealed the presence of alkaloids, glycosides, flavonoids, steroids, saponins, tannin and phenolic compounds. Thakur *et al*. (2014) have reported that the actions of secondary metabolites such

as flavonoids, glycosides and alkaloids played a major role in toxicity activity. Moreover, cardiac glycosides are responsible for producing a digitalis like toxicity upon consumption of its leaves. Digitalis toxicity produces a toxidrome characterized by gastrointestinal, neurologic, electrolyte, hematologic and cardiac manifestations.

Shashikala *et al*. (2015) have reported that leaves of *C. grandiflora* medicinal potential is as purgative, analgesic, wound healing remedies, antioxidant, antiviral, treatment of schistosomiasis and as molluscicide. MISC (2002) have also reported that when ingested the latex secretion of *C. grandiflora* is caused heart malfunction as well as both stomach and intestinal disorders in both humans and animals due to the presence of toxic glycosides.

Conclution And Recommendations

Tick resistance to acaricides is on the rise due especially to increased frequency and uncontrolled applications of commercial acaricides. In contrast, to chemical acaricides, botanical acaricides have many advantageous features of being degraded in the environment, do not remain in livestock, are not as prone to resistance, and are relatively safe for humans, animals and the environment. One way to control acaricidal resistance problem is through the development of alternative uses of acaricides by screening and testing medicinal plants for their possible acaricidal effects. Based on the above fact, the present preliminary study was conducted on extracts of *C. grandiflora* by 80% methanol and 70% ethanol and its pure latex secretion form and tested *in-vitro* against *Amblyomma* and *Ornithodorus* tick species for their killing efficacy at different concentrations. It was observed that both extracts tested and latex secretion had moderate to strong acaricidal activity. Moreover, stem ethanol and leaf methanol extracts at higher concentration(200mg/ml) and with its pure latex formulation were greater acricidal effect against tested ticks compared to the effect of positive control (0.1% diazinon). Efficacy of the extracts increases with increasing concentration and exposure time. Our results stipulated the acaricidal activity of *C. grandiflora* leaves, stem and latex for the first time and supported its traditional complaint of poisonous. Therefore, the present study concluded that extracts and latex formulation of *C. grandiflora* against ticks could be used as potential alternative to substitute commercially available drugs.

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

❖ Further study is recommended to elucidate the acaricidal effect of the selected plant by using different extraction methods and other parts of the plant.

❖ It should be essential to fractionate the extracts and test each component separately to determine the active compounds responsible for the killing effect of the potent extracts.

❖ *In vivo* toxicity and efficacy test must be done to determine their safety and determination of dosage level and its pharmacodynamics in animal models.

❖ Comprehensive research should be done to validate the importance of the materials for future development of lead compounds towards producing alternative acaricides for those extracts that have shown promising results.

❖ The low efficacy of diazinon on ticks as compared to the extracts and latex secretion needs attention. Hence, acaricidal resistance study against both the tested tick species and other species of ticks is recommended and the extracts of *C. grandiflora* should be tested and evaluated against oviposition and larval inhibition efficacy against ticks.

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Annexes

Annex 1**: Study plant Identification Keys using Flower and Collection Format**

Figure 2: Image of *C. grandiflora* flower used for identification

Study plant identification keys

- Flowers with 5 united yellow, white, green, or pink;
- Five sepals; petals and sepals are folded down;
- Five erect, colored corona hoods on top;
- Leaves opposite and simple

Study plant collection format

Local name of plant	Parts of plant collected	place of collection

Figure 3: Plant parts used for the study with its latex

Annex 3: Preparation of plant material for extraction

Materials and reagent

-Leaves and stems of *Cryptostegia grandiflora (*Halemero*,* Am.*)*

-Water for washing the plant

-Table for dispersing and drying

-Wooden pistol and Mortar

- Latex gloves

- Universal bottle

Procedure

- The latex secretion, leaves and stems of *Cryptostegia grandiflora (*Halemero,Am.*)* were brought from Afar, Amibara District
- The latex was oozed out by applying pressure using latex glove because the local afar pastoralists said that it is very poisonous; and added to universal bottle for later use
- The leaves and stems then washed with clean water to remove any dirty from it
- The plant then put on a clean table and turned up and down to avoid fungal growth with in a room to air dry for at least two weeks.
- The dried plants were then crushed with wooden pistol and mortar and filter with sieve.

Annex 4: Extraction of the plant material

Material and reagents

- Powders of the plant
- 80% Methanol - Rotary evaporator - Clay plates

- 70% Ethanol -Shaker - Latex gloves
- Flasks -Filter paper - Bakery
- Balance -Dry oven

Procedure:

- The dried powder of 111g and 237g leaves and 155g and 145g stem of *Cryptostegia grandiflora* (Halemero, Am.) weighed with digital balance and were soaked in 1:4 methanol and ethanol in separate flask.
- The plant material with the solvent was put on automatic shaker for 72 hours to allow mixing of the plant with the solvent for crude yield.
- After 72 hours the plant material was macerated using gauze.
- The macerated part was filtered with Whatman no.1 filter paper to bakery.
- This filtered material was then extracted with rotary evaporator separating the solvent from crude extract of the plant and sit it on dry oven for up to 2 weeks until it dried.

Annex 5: Extract physical characteristics, yield and dilution format recording format

Name of plant	Color of extracted product	Characteristics of extracted material	Solvent used	Percentage yield %

Annex 6: Preparation of working solution

Material and reagent

- Crude extract - 2% Tween 80
- 10 ml syringe - Test tubes
- Latex glove

Procedure:

❖ Preparation of working solution

- To prepared 20%(20 gm/ 100 ml) take 1gm of crude extract
- Mixed with 5ml of 2% Tween 80 (Tween80 was prepared by v/v ratio using distilled water)
- To made serial dilution for 10% and 5% working solutions of the above mixed solution

✓ Divided equally the above 20% into two test tubes to get 10% working solution

✓ Then added 2.5ml of 2%Tween 80

✓ Then also divided the 10% working solution into two test tubes to get 5% working solution

✓ 2.5 ml of 2% Tween 80 was added

✓ Positive control 0.1% Diazinon was prepared by v/v ratio using distilled water

✓ Stored in refrigerator at + 4 ^{o}C until used

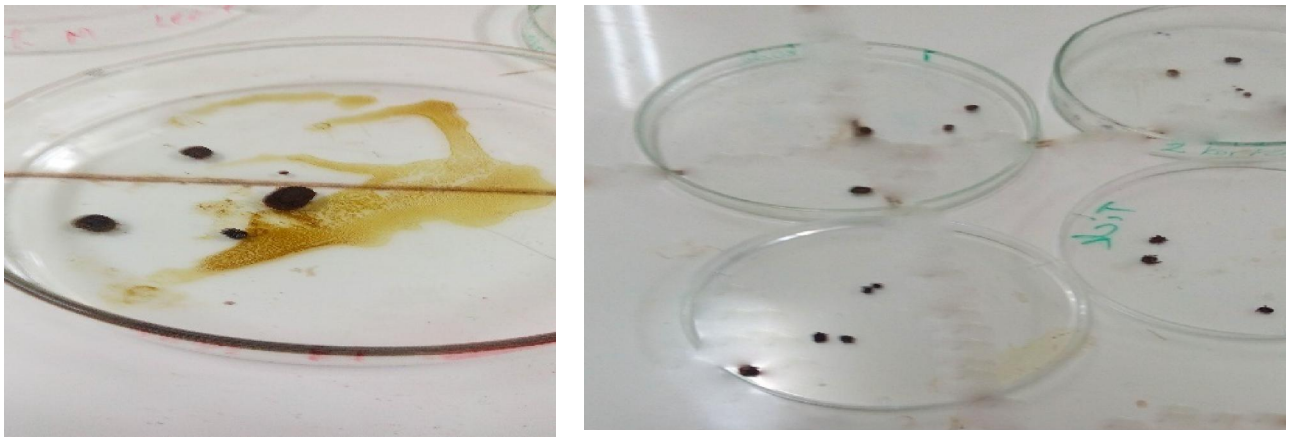
❖ The latex secretion was directly applied after collection

Annex 7: *In vitro* acaricidal efficacy test and recording format

Adult immersion test (AIT)

1. Three replicates for each concentration of 5 adult ticks was immersed in the respective dilutions of extracts and control solutions for 2 min of exposure.
2. After immersion, the ticks were recovered from tube and filtered with filter paper and placed in separate Petri dishes (Zaman *et al*., 2012).
3. Five millilitres of 2% tween80 and distilled water and 0.1% diazinion 60 EC were used as negative control and positive control respectively.
4. The petridishes were incubated at room temperature of 30^{o}C with 80% relative humidity and death and immobilized ticks were recorded after 30 min, 1hr, 2hr, 3hr, 6hr, 12hr and 24hr interval
5. The viability and immobility of ticks were checked regularly by stimulation with a needle and observing under stereomicroscope to check their respiratory movement and ticks were recorded as dead if no reaction and respiratory movement were shown.
6. The percentage mortality of total ticks within 24 hrs was calculated by the formula previously used by Krishnaveni and Venkatalakshmi (2014) as follows:

$$\text{Mortality } \% = \frac{\text{no.of dead ticks}}{\text{totall no.of ticks}} \times 100$$



a **b**

Figure 3: Adult *Ornithodorus* and *Amblyomma spp.* Of ticks incubated at room temperature after extract immersion, respectively.

In vitro acaricidal efficacy test recording format

	Concentration	**Replication**	**No. of immersed ticks**	**No. of dead tick/s after/post incubation**						
				30 min	1hr	2hrs	3hrs	6hrs	12hrs	24hrs
Plant Extract	20%	1	5							
		2	5							
		3	5							
	10%	1	5							
		2	5							
		3	5							
	5%	1	5							
		2	5							
		3	5							
Positive control	0.1% Diazinon	1	5							
		2	5							
		3	5							
Negative control	2% Tween 80	1	5							
		2	5							
		3	5							

3/19/2017