Studies on the combinatorial acaricidal efficacy of certain phytoextracts and synthetic pesticides against *Hyalomma anatolicum* (Koch) and *Rhipicephalus microplus* (Canestrini)

Phylum Arthropoda being the largest phylum in animal kingdom, has maximum number of ectoparasites. Among the arthropods, the order Acarina of class Arachnida has most of the ectoparasites represented by ticks and mites infesting our domestic animals. Ticks are the obligatory blood feeders as they are dependent on host’s blood for their survival and reproduction. There are more than 850 species of ticks reported throughout the world. Among them, approximately 180 species are of family Argasidae (soft ticks) and 670 species are of family Ixodidae (hard ticks). Out of them only 104 species are found in Southeast Asia (Ahmed et al. 2007). Among all the vectors, ticks are the most important vectors of diseases affecting both humans and animals worldwide and transmit a greater variety of protozoans, bacterial, rickettsial and viral pathogens than any other arthropod vector (Ahmed et al. 2007).

Problem of ecto-parasites in animals has been quite alarming leading to enormous economic losses through affecting meat and milk production, health status, innumerable skin diseases, transmission of infectious diseases etc. They cause a number of zoonotic diseases that hence are a concern for public health. Heavy infestation of ticks causes a condition known as “tick worry” i.e. manifested by loss of condition and severe degree of anemia. Ticks are also responsible for transmission of various tick borne diseases (Lyme disease, rocky mountain spotted fever, relapsing fever, tularemia, meningoencephalitis, Colorado tick fever, Crimean-congo hemorrhagic fever, babesiosis, cytauxzoonosis, etc.) in animals and man. Diseases transmitted by ticks are a major constraint to livestock industry and continue to pose to be a serious public health problem throughout the world (Willadsen and Kemp 1988; Carrol 2004). In India *Hyalomma anatolicum* and *Rhipicephalus (Boophilus) microplus* are widely prevalent and most economically important ticks infesting dairy animals (Ghosh et al., 2007). The negative impact of these ticks in the cattle industry is a combination of direct and indirect effects.

Synthetic acaricides are the most common tick control method adopted by the cattle owners in India. Various acaricidal compounds have been used for the control of ticks. viz., arsenical compounds, chlorinated hydrocarbon, organophosphorous compounds and pyrethroids, etc. These chemicals are available over-the-counter and are applied on infested animals at
frequent intervals. Indiscriminate use with lower and higher concentrations of synthetic acaricides has probably contributed to the development of resistance in ticks, toxic manifestations in animals, human safety, residual effect on meat and milk and environmental hazards (FAO, 2004). Although, cattle owners have reported treatment inefficiency of these chemicals in field conditions (Kumar et al., 2006, 2010; Vatsya and Yadav, 2011). A systematic broad-spectrum acaricide, Ivermectin, Moxidectin, Doramectin, Abemactin, etc have also been projected as a suitable alternative for topical application, however, not affordable to our farmers. The urgent attention, therefore, been required to find out an effective and safe alternate means of control is felt because of the toxicity related problems with the growing incidences of insect resistance to synthetic insecticides.

The botanical pesticides are more ecofriendly, biodegradable and non-resistible as compared to synthetic pesticides (Prakash and Rao 1997; Prabakar and Jebanesan 2004, Rahuman and Venkatesan 2008). Currently, technocrats have, therefore, diverted their approach towards the development of environmentally safe, biodegradable and target specific botanical acaricides for combating these ectoparasites. In the proposed work, an attempt, therefore, would be undertaken to exploit the certain phytoextracts and synthetic pesticides alone and in their combinations against the common ticks, *Hyalomma anatolicum* and *Rhipicephalus microplus*. Further, the haematological and biochemical changes in the blood of pre & post infested and treated host is also to be studied.
REVIEW OF LITERATURE

Synthetic Acaricides:

Botanical Acaricides:

Shanahan and Hart (1966) studied change in response of *Lucilla cuprina* (Wied) to organophosphorus insecticide in Australia Nature. Regassa (2000) the use of herbal preparation for tick control in Western Ethiopia. Suthrest et al. (1982) studied on tropical legumes of the genus *Stylosanthes* immobilize and kill cattle ticks. Mulla and Su (1999) revealed that botanical insecticides are relatively safe and degradable and are readily available source of bio-pesticides. Abdel-Shafy and Zayad (2002) observed in-vitro acaricidal effect on plant extract of Neem seed oil (*Azadirachta indica*) on egg, immature and adult stages of *Hyalomma anatolicum excavatum* (Ixododea: Ixodidae). Choudhary et al. (2004) evaluated the in vitro effect of *Nicotiana tobaccum* aqueous extract on *Rhipicephalus haemaphysaloides* and concluded the engorged adult *R. haemaphysaloides* were treated with undiluted and 50% diluted aqueous extracted of *Nicotiana tobaccum*. Lori et al. (2005) studied of acaricidal properties of essential oil of *Melalecca alterfolia cheeel* (tea tree oil) against nymphs of *Ixodes ricinus*. Pathak et al. (2006) observed the in vitro effect of indigenous plant extract on ixodid of small ruminant. The efficacy of neem leaves and bark, nochi proved to be the most effective followed by vashambu. Vatsa et al. (2006) observed the in vitro acaricidal effect of some medicinal plants against *Boophilus micro plus* and evaluated that *Ocimum kilimandscharicum* exhibited highest efficacy (98.34%) Followed by *P. glabra* (96.67%), *A. annua* (95%) and *A. vulgaris* (93.34%) significantly lowered. Calmasur et al. (2006) observed insecticidal and acaricidal effect of three Lamiaceae plants essential oils against *Tetranychus urticae* Koch and Besimea tabaci Genn. Ghosh and Azhahiambi (2007) studied laboratory rearing of *Thilerea annulatus* free *Hyalomma anatolicum* ticks. Daurte et al. (2008) observed efficacy and acaricidal activity of *Hyacinhacine analogues* derived from Pyrrolidine alkaloids on eggs hatchability and mortality rates of newly hatched larvae of cattle tick *Rhipicephalus (Boophilus) microplus*. Bagavan et al. (2009) studied adulticidal and larvicidal efficacy of some medicinal plant extracts against cattle ticks *Haemaphysalis bispinosa* Neuman, 1897 (Acarina: Ixodidae) sheep fluke, *Paramphistomum cervi zeder*, 1790, investigated the toxic effect of leaf Hexane, Chloroform, Ethyl acetate and Methanol extract of *Annona squamosa* L. *Centella asiatica*(L.) Urban, *Gloriosa superva* L. *Mukia maderasptensis* (L.) were exposed. Habeeb (2010) studied to the Ethno-Veterinary and medical knowledge of crude plant extracts and its method of application (Traditional and Modern) For tick control of genera *Hyalomma, Rhipicephalus (Boophilus)* Argas and
Orinthodoras are commonly found in Egypt infesting farm animals. The acaricidal activity of crude extracts and fractions from stem and leaves of *Petiveria allicea* (Phytolacaceae) was carried out on larvae and adults of the cattle tick *Rhipicephalus (Boophilus) microplus* using the larval immersion test (LIT) and adult immersion test (AIT) respectively. Pirale et al. (2011) studied the acaricidal effect of *Zataria multiflora* and *Artimisia annua* essential oils on *Rhipicephalus (Boophilus) annulatus*. Ribeiro et al. (2011) tested the acaricidal properties of the essential oil and precocene II obtained from *Calea serrata* less (Asteraceae), an endemic species of South Brazil known as a “quebra-tudo” against the larvae of *Rhipicephalus microplus* (Boophilus). Ravindran et al. (2012) observed acaricidal activity of *cassia alata* against *Rhipicephalus (Boophilus) annulatus*. Politi et al. (2012) evaluated the acaricidal activity of ethanolic extract of *Taget patula* against larvae and engorged adult female of *Rhipicephalus sanguineus* ticks. Gomes et al. (2012) reported the chemical composition and acaricidal activity of essential oil from the leaves of *Lippia sidoides* on *Rhipicephalus microplus* and *Dermacentor nintens*. Felix Nchu et al. (2012) examined the anti-tick properties of essential oil of *Taget minuta* against *Hyalomma rufipes* tick. Juliet et al. (2012) studied the effect of ethanolic extract of leaves of the plant *Jatropha carcus* as a step toward developing a safe and eco-friendly therapeutic agent to combat problems of tick and tickborne diseases.

**Combinatorial studies in ticks control:**

Haematological and Biochemical studies:

Sharma et al. (1997) revealed that twenty four lambs (3-6 months) old naturally infected with *Sarcoptes scabiei* were divided into 3 equal groups. The lambs were treated with either an ointment containing oil of *Cedrus deodara a* suspension containing benzyl benzoate or were untreated controls. Drugs were applied locally on affected areas on alternate days and changes in skin lesions were observed regularly. Blood samples from each group were collected and analyzed for total erythrocytes, leucocytes and haemoglobin concentration every 10 days. The two treated groups responded to the treatment but recovery in the *C. deodara* was faster. Lesions were free mites after 5 applications (10 days) in the *C. deodara* group as compared with 7 applications (14 days) in the benzyl benzoate group. Erythrocyte and leukocyte counts were significantly higher in the *C. deodara* group than in the control group, but oil of *C. deodara* group than in the control group, but haemoglobin concentration was not significantly different between groups. It is concluded that oil of *C. deodara* was more efficacious than benzyl benzoate in controlling sarcoptic mange in sheep. Dalpati et al. (1998) observed that blood and serum from 15 *Demodex* infested goats was examine for glucose, protein, calcium, iron, copper, zinc and inorganic phosphorus, as well as normal haematological parameter. Hair from the same goats was examined for iron copper and zinc. The result showed reduced haemoglobin, total erythrocyte counts and packed cell volume indicating anaemia, increase in eosinophils and lymphocyte and decreased blood glucose, serum, protein, calcium and inorganic phosphorus. Level of iron, zinc in both serum and hair samples also decreased. Naidu and Rao (2000) Observed in 24 semi- intensively managed goats with clinically severs saccoptes scabiei infestation, their blood samples. Showed decreased in haemoglobin, packed cell volume, erythrocyte count, and increase in total leukocyte count, neutrophils, eosinophila and mean corpuscular volume. Two *S/C Ivermectin* injections (0.5 ml/ 25kg body weight) were given at 10 days after the injection, the skin lesions had disappeared and the blood values had returned to normal. Rajendran and Hafeez (2003) observed Haemato -biochemical changes and efficacy of different acaricides in crossbred animals. In Haematological examination he revealed a significant reduction in Hb, PCV, TEC and TLC in tick infested groups as compared to control group while in biochemical analysis he revealed a significant decrease in total serum protein, albumin, but non significant difference in globulin and albumin: Globulin ratio against ivermectin, and pestoban. Kumar et al. (2010) observed effect of ecto-parasite on
haematological and biochemical parameters of goats has been analyzed and compared with normal (control) animals. The experimental tick infected group showed lower values of Hb, PCV, TEC, neutrophil in days 21 when compare to naturally infested group. However first three weeks of study there was no significant difference in eosinophil, and basophil and MCV values between naturally infested village flock and non-infected control group. Biochemical parameters like total protein and globulin were found higher a day 21 in experimental tick infected goats but albumin, AIG ratio, glucose, bilrubin and AST were considerably lower during 7 and day 21 in the experimental group when compare to naturally infested goats. Kumar et al. (2011) evaluated invitro and invivo acaricidal activity of some idigenous plants under organized and farmer flock. The experiment of invivo and invitro effect of adulticidal activity of crude extract Arand, Yellow Kaner and Pudina in natural tick infestation have done. The results indicate that all these extracts had quite similar invivo adulticidal activity on tick population. However invitro results revealed that the mortality percentage was higher as the concentration of the extracts increased and the time interval progress. Statistical analysis of data further revealed that mortality percentage was significantly different (P<0.5) at 4 and 24 hrs interval in case of crude extract of Arand, Yellow Kaner and Pudina.
OBJECTIVES

The main objective of the proposed study is to control ticks infested on goats. This objective would be achieved by screening of certain synthetic pesticides and phytoextracts alone and in their combinations against *Hyalomma anatolicum* and *Rhipicephalus microplus*.

- Evaluation of acaricidal efficacy of certain synthetic pesticides, cypermethrin, deltamethrin and diazinon against *Hyalomma anatolicum* and *Rhipicephalus microplus* and identify the most effective synthetic pesticide.

- Screening of certain Phytoextracts from *Spilanthes acmella*, *Adhatoda vasica* and *Capsicum frutescense* against *Hyalomma anatolicum* and *Rhipicephalus microplus* leads to identify the most potential acaricidal plant extract.

- Chromatographic fractionation of the most potent acaricidal extract to procure the different compounds present in the extract.

- Evaluation of acaricidal efficacy of all the recovered chromatographic fractions against *Hyalomma anatolicum* and *Rhipicephalus microplus* to procure the most potent fraction.

- Combinatorial bioefficacy evaluation of the most potent synthetic pesticides and the most effective chromatographic fraction against the target organisms.

- Hematological and biochemical studies of blood of pre & post infested and treated host.
MATERIALS AND METHODS

A. Materials:

a. Target Organisms:


Phylum: Arthropoda

Class: Arachnida

Order: Ixodida

Family: Ixodidae

Genus: *Hyalomma*

Species: *anatolicum*

Identifying characters:

Female:

Scapular grooves profile is shallow (grooves reach the posterior margin of scutum). Scutum is pale coloured. Scutum posterior margin is smooth. Leg colouration is with pale rings (but the legs are also pale in a patchy or marbled pattern, thus the rings are indistinct). Punctuation size is small. Punctuation distribution is sparse.

Male:

Cervical fields depression is apparent. Conscutum is pale coloured. Lateral grooves are short. Posterior ridges number two (indistinct). Caudal depression is present. Central festoon is dark coloured. Paracentral festoons are separate anteriorly. Posteromedium groove is present (it is long and narrow). Paramedian grooves are small (they may be very indistinct). Leg colouration is with pale rings (but the legs are also pale in a patchy or marbled pattern, thus the rings are indistinct). Punctuation sizes are small. Punctuation distribution is sparse (but with some concentrations of larger punctations at the lateral grooves).

- **Phylum:** Arthropoda
- **Class:** Arachnida
- **Order:** Ixodida
- **Family:** Ixodidae
- **Genus:** *Rhipicephalus*
- **Subgenus:** *Boophilus*
- **Species:** *microplus*

**Identifying characters:**

**Female:**
- Hypostomal teeth are in 4 + 4 columns. In 1st palp internal margin has no protuberance and is short and distinctly concave. In 1st Coxae spurs are distinct. In 2nd & 3rd Coxae spurs are present. Genital aperture posterior lips have a broad U shape.

**Male:**
- Dorsal on left, coxa at top right, and ventral plates at bottom right. (Note: male mouthparts are similar to those shown for the female.) Cornua are distinct. 1st Coxae spurs length is long (also the anterior spurs of 1st coxae is conspicuous dorsally). Ventral plate spurs are indistinct (3 = accessory adanal plate). Ventral plate spurs are indistinct (4 = adanal plate). Caudal appendage is narrow in males. Ventral plate spurs are not visible dorsally.

**b. Parasitic host**

**Goat (*Capra hircus*) Barbari breed:**

It belongs to family Bovidae of order Artiodactyla under class mammalia. Barbari goat is dwarf breed and mainly found in Agra, Mathura, Aligarh, Hathras, Mainpuri, etah and Etawah District. This goat looks like the deer. Whole coat is white and shiny brown spots on the body and has small and beautiful ears. It is of great economic importance as it is good for milk specially for children and meat.
c. Synthetic Pesticides:

1. Deltamethrin:

Deltamethrin is a member of one of the safest classes of pesticides: synthetic pyrethroids. This pesticide is highly toxic to aquatic life, particularly fish, and therefore must be used with extreme caution around water. Although generally considered safe to use around humans, it is still neurotoxic to humans.

Cases of toxicity have been observed in cattle, following use of agricultural deltamethrin preparation in external application in tick control. Symptoms appeared 36 hours after the application, and included muscular tremors that lead to decubitus 12 hours later. After 12 hours, there was spontaneous recovery and the animal could stand up again, although the muscular tremors persisted.

Common Name: Deltamethrin
Chemical Name: (S)-alpha-cyano-3-phenoxybenzyl (IR,3R)-3-(2,2dibromovinyl) 2,2 dimethylcyclopropanecarboxylate,
Chemical Family: Synthetic Pyrethroid.
Chemical Formula: CH\(_{22}\)H\(_{19}\)B\(_2\)NO\(_3\)

![Deltamethrin: Structural formula]

2. Cypermethrin:

Cypermethrin is a synthetic pyrethroid insecticide used to control many pests. It is also used for crack, crevice, and spot treatment to control insect pests in stores. Technical cypermethrin is a mixture of different isomers, each of which may have its own chemical and biological properties. Cypermethrin is light stable. It ia available as an emulsifiable concentrate.
Common Name: Cypermethrin
Chemical Name: (R,S)-alpha-cyano-3-phenoxybenzyl (1 RS) ocic, trans-3-(2,2-dihlorovinyl)-2,2-dimethylcyclopropane-carboxylate.
Chemical Family: Synthetic Pyrethroid.
Chemical formula: C_{22}H_{19}Cl_{2}NO_{3}

Cypermethrin: Structural formula

3. Diazinon:

Diazinon is a nonsystematic organophosphate insecticide use to control cockroaches, silverfish, ants and fleas in residential, non food buildings. Diaznon has veterinary uses against fleas and ticks. It is available in dust, granules, seed dressing, wettable powder and emulsifiable solution formulation.

Common Name: Diazinon
Chemical Name: O,O-diethyl O-2-isopropyl-6-methyl(pyrimidine-4-yl) phosphorothioate
Chemical Family: Synthetic Pyrethroid.
Chemical formula: C_{12} H_{21}N_{2}O_{3}PS

Diazinon: Structural formula
d. Plants Selected:

1. *Spilanthes acmella* (Akarkara): (Family: Asteraceae)

   It is also known as *Spilenthes paniculata, Spilianthes ocymifolia, Bidens acmella, Bidens ocymifolia, Pyrethrum acmella, Verbesina acmella, Blainvillea acmella* (Saraf & Dixit, 2002). It is annual erect or ascending stout herb, 20 to 50 cm high, with opposite leaves, petiolate, broadly ovate, narrowed at base, acute or obtuse at apex. Full bloom with large number of flowers is in March – April. The flower heads are chewed to relieve the toothache and other mouth related troubles, leaves are used externally in treatment of skin diseases. Root decoction is used as purgative. Leaf decoction is used as diuretic and lithotriptic. Whole plant is used in the treatment of dysentery (Agharkar 1991, Verma et al. 1993).

2. *Adhatoda vasica*: Adulsa, Malabar nut (Family: Acanthaceae)

   It is also known as *Justicia adhatoda*. It is well known plant drug in Ayurvedic and Unani medicine. Its leaves used for respiratory disorders, chest affections. Its roots and flowers used as a remedy for cold, cough, bronchitis, asthma, local bleeding due to peptic, piles, menorrhagia, pyorrhea, blood pressure. It is said to be known poisonous to mammals but to kill insects. It is known for possessing, insecticidal, antifeedent and insect repellent in its roots and leaf powder and water extracts against the stored grain pests of rice and paddy (Prakash and Rao1997).

3. *Capsicum frutescense*: (Chili) mirach (Family: Solanaceae)

   It is also known as bird pepper, chilli pepper, cayenne pepper, guinea pepper. It is a short lived evergreen shrub usually 1-1.5 m in height and 1-3 cm in basal stem diameter. Flowers are white with a greenish white or greenish yellow corolla, and either insect or self pollinated. The plant berries typically grow erect ellipsoid-conical to lanceooloid shaped. Leaves are warmed over the fire or boiled and placed on a wound to keep away insects and extract pus. It is used in the treatment of fever including malaria when mixed with *Neurolaena lobata*. The plant possesses insecticidal activity against *Aedes aegypti* larvae (Vinayak et al. 2010).
B. Methodology:

I. Rearing of target organisms:

Ticks will be collected from both farmer’s flocks and some organized farms of different locations of Uttar Pradesh. After proper identification, the fully engorged adults collected from the field will be rinsed in the distilled water and placed on the filter paper, kept inside the tick rearing glass tubes covered with muslin cloth with the help of rubber band. The glass tube will be kept in BOD incubator at 70-80% relative humidity and at 27°C temperature.

II. Extraction of plant extracts:

The selected plants were collected from area adjacent to the campus of Dayalbagh Educational Institute, Agra and the plant, *Spilanthes acmella* will be collected from Botanical garden of the Institute. The selected plant materials will be washed with tap water and allowed to shade dried for about 2-3 weeks. The dried materials will be crushed manually.

The weighed and dried plant materials will be subjected for extraction to soxhlet’s apparatus for extraction in petroleum ether, hexane and methanol subsequently for 72 hours. Extracts will be separated from the solvents by vacuum rotary evaporator to get pure residues and extracts will be finally weighed and kept refrigerator for further use.

III. Preparation of test concentrations:

Cypermethrin, deltamethrin and diazinon will be purchased from the local market from Agra. The stock solutions of desired concentrations will be prepared by diluting the pesticides independently in dechlorinated tap water. Different test concentrations will be prepared by further dilution of the stock solutions for the exposing the ticks.

Pure residues of the plants will be dissolved in ethanol/acetone independently to get stock solutions. Different desired test concentrations will be prepared by diluting these stocks in ethanol/acetone. A range of working test concentrations will be prepared in 500 ml capacity of glass beakers containing 249 ml of tap water and 1 ml of test concentration.
IV. Bioassay:

The engorged female ticks will be collected from animal flocks and washed with water and dries in paper toweling. A group of female will be used in the adult immersion test (AIT) and another group will be incubated at 27±1.5°C and 70-80% relative humidity (Cen et al. 1998) for two weeks until the eggs were laid. These eggs will be provided the larvae and nymph used for the larval immersion test (LIT) and nymph immersion test (NIT).

a). Egg immersion test (EIT):

The eggs of the target organisms will be treated with the synthetic pesticide and phytoextracts independently and will be observed for their developmental profile as per the standard methodology (Elango and Rahuman 2011).

b). Larval immersion test (LIT):

Tick larvae 7-14 days old will be used for experiments. Hatching vials with the highest larval eclosion rate (90-100%) will be selected and placed in the centre of a petri dish that will be subsequently filled with water and soap which prevented their escape. The diluted synthetic pesticides and plant extracts will be transferred to Petri dishes (60 mm x 50 mm) independently. Larvae will be placed between two Whatman No. 1 papers and immersed for 10 minutes. Approximately 100 larvae will be picked with a No. 4 paint brush and gently transferred to clean filter paper packets. The opening of the envelops (treated and control with larval ticks) will be folded with metallic clip, with its identification mark (tested solution and concentration) on the outside. The packets will be placed in the incubator at same temperature and humidity conditions for 48 hours. The envelops will be opened 48 hours post treatment and observed using a stereoscope. The number of live larvae, mortality and any toxicological effects observed will be recorded. The larvae that will be unable to walk forward were considered dead (Elango and Rahuman 2011).

c). Nymph immersion test (NIT):

The nymphs of the target species will be collected from hosts and placed in 9 cm petri dishes. Five groups will be made, four treated and one control in triplicate each. The treated group will be immersed during one minute in prepared test concentration of synthetic pesticides and plants extract independently and the control group will be water with maximum amount of the ethanol.
used in the concentrations. The nymph will be placed individually in to plates with 24 holes and incubated for a period of 15 days at the conditions of temperature 27±1.5 °C and 70-80% relative humidity. Nymphs will be observed with the aid of a stereo-microscope and the mortality rate of each will recorded. The mortality rate will recorded daily by counting dead nymphs. The dead nymphs will be then transferred to Petri dishes and the extract effects on them will be observed. Dead nymph will be identified by the presence of cuticular darkness, lack of malphgian tubes, movement and hemorrhagic skin lesions.

d). Adult immersion test (AIT):

Ten engorged female ticks will be transferred in 9 cm petri dished. Five groups will be made, four treated and one control in triplicates each. The treated group will be immersed during 1 minute in the prepared test concentration of synthetic pesticides and plant extracts independently and the control group will be immersed in water with maximum amount of the ethanol used in the test concentrations. The ticks will be placed individually into a plate with 24 holes and incubated for a period 15 days at the condition of temperature 27±1.5 °C and 70-80 relative humidity. Ticks will be observed with the aid of a stereo-microscope and the mortality rate and weight of produced eggs in each group will be recorded. The mortality rate will be recorded daily by counting dead ticks. The dead ticks will be then transferred to Petri dishes and the extracts effects on them will be observed. Dead ticks will be identified by the presence of cuticular darkness, lack of malphgian tubes, movement and hemorrhagic skin lesions. After 15 days, the number of females laying eggs will be recorded and the eggs of each group will be weighted by using an analytical scale. After that 50 eggs will be placed in 25 mm x 95 mm glass vials at the same conditions. During 21 days the vials will be observed and the hatching rates of the different treatments will be estimated and compared to the controls. The egg laying inhibition and the larval inhibition percentage will be determined for all groups (Elango and Rahuman 2011).

e). Field trial of effective herbal extracts:

Clinical cases after quantification of ticks will be taken for therapeutic studies at different dose levels. The phyto extracts efficacy studies will be repeated for these host groups.
<table>
<thead>
<tr>
<th>Sr. No.</th>
<th>Group</th>
<th>No. of Animals</th>
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<tbody>
<tr>
<td>1.</td>
<td>Pre-infested Group</td>
<td>06 animals</td>
</tr>
<tr>
<td>2.</td>
<td>Post-infested Group</td>
<td>06 animals</td>
</tr>
<tr>
<td>3.</td>
<td>Treated Group</td>
<td>06 animals</td>
</tr>
</tbody>
</table>

**Parameters to be studied:**

1. No. of parasites present on skin
2. Reduction in parasitic load
3. Skin lesions (if any)
4. Recurrence of parasitical load
5. Blood parameters for pre, post infested and treated host

**f). Statistical analysis:**

The average adults, nymph and larval mortality data will be subjected to Probit analysis (Finney 1971) for calculating LC$_{50}$ and LC$_{90}$ and other statistics at 95% confidence limits.

**V. Phytochemical analysis of the most potent crude extract:**

The extracts will be tested for the presence of active principle such as phytosterols, tannins, flavonoids, saponins, alkaloids, glycoside, triterpenoids and proteins. Standard procedures (Column chromatography, TLC, HPLC,) will be used for separation of the different components present in the most potent extract.

**a) Column chromatography of most potent extract:**

The column chromatography will be conducted to isolate each component from the most potent extract. The extract will be treated with silica gel as stationary phase and combinations of different desired solvents as mobile phase for the column chromatography. All fractions will be eluted with silica gel G (80-120 mesh size), as a stationary phase and graded mixture of different desired solvents as a mobile phase. The all recovered fraction will be subjected to egg immersion
test, larval immersion test, nymph immersion test and adult immersion test as per the standard methodology mentioned above.

b) Qualitative analysis:

The most potent fraction will be subjected for their qualitative test and functional group such as phytosterols, tannins, flavonoids, saponins, alkaloids, glycoside, triterpenoids and proteins. Following standard procedures (Debela, 2002).

c) Hematological and Biochemical studies of the host’s blood:

Hematological and biochemical studies of the pre & post infested and treated hosts (goats) will be conducted as per the standard methodology (Jain and Schalm 1975).

**Hematological parameters:**

(i) Erythrocytes count (RBC)
(ii) Total leucocytes count (TLC)
(iii) Differential leucocytes counts (DLC)
(iv) Haemoglobin (Hb)
(v) Erythrocyte sedimentation rate (ESR)
(vi) Platelets counts
(vii) Packed cell volume (PCV)
(viii) Mean corpuscular volume (MCV)

**Biochemical Parameters:**

(i) Glucose (God POD method, Tiez, 1976)
(ii) Total Protein (Modified Biuret and Dumans Method, Duman, 1971)
(iii) Total Lipids
(iv) Albumin (Modified Biuret and Dumans Method, Duman, 1971)
(v) Aspartate transaminase (AST) (24-DNPH Method, Tiez, 1970)
(vi) Alanine transaminase (ALT) (24-DNPH Method, Tiez, 1970)
(vii) Bilirubin (Malloy and Evelyn Method Mally and Evelyn, 1937)