CHAPTER 4 - MATERIALS AND METHODS
Materials and methods

4 (a) Materials-

1. Experimental plant- *Carissa carandas*

   Taxonomic classification-
   - **Kingdom**: Plantae
   - **Order**: Gentianales
   - **Family**: Apocynaceae
   - **Sub-family**: Rauvolfioideae
   - **Genus**: *Carissa*
   - **Species**: *carandas*

Description- *C. carandas* is found throughout India mainly in the semi-arid regions. *C. carandas* plant is widely cultivated in the home gardens, farmer’s fields and orchards as hedge plant. *C. carandas* is commonly used as a condiment or additive to Indian pickles and spices. It is a very hardy, drought-tolerant plant that thrives well in a wide range of soils. *C. carandas* is an evergreen deciduous small to big shrub usually 2-4 m tall. The stem is rich in white latex and the branches contain sharp spines. Flowers are small, measuring 3-5 cm in diameter, with white color. The fruit is a berry, which is formed in clusters of 3-10 fruits. The fruit is broad ovoid in shape and contains many seeds. Young fruits are pinkish white and become red to dark purple when ripe. Ripe fruit color varies from white, green and pinkish red depending on the genotype. Flowering starts in the month of January-February and fruits mature in May-June. Fruits are generally harvested at immature stage for vegetable purpose, fully ripen fruits are consumed fresh or processed.
Importance and uses- Leaves of *C. carandas* is used against fever, diarrhoea, and earache. The roots serve as a stomachic, remedy The *C. carandas* fruit is an astringent, antiscorbutic and as a remedy for biliousness and useful for cure of anemia. In traditional medicine the fruit is used to remove worms from the intestinal tract. The fruit has anti-microbial and antifungal properties and its juice used to clean old wounds which have become infected. The fruit have an analgesic action as well as an anti-inflammatory one. The juice can be applied to the skin to relieve any skin problems. Traditionally *C. carandas* has been used to treat anorexia and in sanity for itches and insect repellent.

Traditional medicinal uses of *C. carandas*- Traditional healers having expertise in treatment of different types of cancer from *C. carandas*. They use its different plant parts to dress the cancerous wounds and to kill the maggots. To prepare the *C. carandas* decoction, its roots, flowers, spines, leaves and fruits are mixed in equal proportion and crushed to make an aqueous paste. This paste is applied at very initial stages. This paste is boiled in water and when half quantity of water remains, the boiling is stopped and lukewarm decoction is used to wash the cancerous wounds. The healers claim that this decoction is having immense potential to heal the wound and make it infection free. In many ways, it acts in more promising ways than *Azadirachtaindica* plant parts. Many healers boil the aqueous paste in Mustard seed oil and when all watery contents evaporate, the boiling is stopped and special oil is used for wound dressing.
2. Experimental animal- *Rhipicephalus microplus* (identified using standard keys)

Kingdom: Animalia
Phylum: Arthropoda
Sub-phylum: Chelicerata
Class: Arachnida
Order: Acari
Suborder: Metastigmata
Family: Ixodidae
Genus: *Rhipicephalus*
Species: *microplus*

**Identification-** *Rhipicephalus microplus* is a member of the family Ixodidae (hard tick). This tick was formerly known as *Boophilus microplus*, however, *Boophilus* has recently become a subgenus of a genus *Rhipicephalus*. Hard ticks have a dorsal shield (scutum) and their mouth parts (capitulum) protrude forward when they are seen from above (An illustrated laboratory manual- R.M.Cable, 1985). *Boophilus* ticks have a hexagonal basis capitulum. The spiracular plate is rounded or oval and palps are very short, compressed and ridged dorsally and laterally. Males have aadanal shields and accessory shield. Anal groove is absent or indistinct in females, and faint in males. There are no festoons or ornamentation (Essentials of Parasitology- Gerald *et al.*, 1988).

**Geographic distribution-** The cattle tick is widely distributed in Central and South America, parts of the southern USA, Africa, Asia (India), and northern Australia. The distribution of the cattle tick is largely determined by climatic factors. *B.*
*microplus* requires high humidity and ambient temperatures of at least 15-20 °C for egg laying and hatching.

Disease spread- Cattle tick infestation causes:

- Damage to hides
- Loss of production
- Anemia and death
- Weakness leading to greater mortalities during droughts.

**Biology** - Ticks are not insects; they belong to the class Arachnida, which also includes mites, spiders and scorpions. Ticks differ from insects in that they have one body region, eight legs and no antennae. Ticks are the largest members of the order Acarina and are virtually the only members of that order you can see without magnification. The order Acarina (ticks and mites) to which they belong differ from other arachnids in that their bodies are not conspicuously segmented, but the abdomen and cephalothorax are fused into one body region. They cannot fly, run, hop or jump; they can only slowly climb up and perch on an object until some host passes by; then they either climb on or fall on to the unfortunate creature. They feed entirely on blood of vertebrates with barbed, piercing organs; they take a firm grip on the skin and suck blood from 15 minutes to several days. Ticks are further divided into two families: hard ticks in the family Ixodidae, and soft ticks in the family Argasidae. Hard ticks have a hard, smooth shield on their backs and are tapered at the front with an apparent head; they are the ticks most readily recognized by most people. Female hard ticks feed once and lay as many as 10,000 eggs or more. Soft ticks lack the shield-like plate on their upper surface, have a tough, leathery, pitted skin and no distinguishable head and look like animated pieces of bark or debris. Some soft tick females can
feed several times and lay 20 - 50 eggs after each meal. Both groups can swell to considerable size after a blood meal. Ticks have 4 stages in their life cycle: egg, larva, nymph and adult.

4 (b) Methodology-

1. **Prevalence Study** - Fortnightly survey was performed in order to study the abundance of ticks. The study was done on selected cows and calves (naturally infested) and their different body parts were screened for ticks. Month wise prevalence (Graph1), Age wise prevalence (Graph 2 and 3) and overall prevalence (Table1) was calculated as follows:

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   \text{Prevalence\%} = \frac{\text{infested animals}}{\text{total animal observed}} \times 100
   \]

2. **Collection of cattle ticks** - Ticks were collected from local areas of Dayalbagh, Maithan, Heerabagh, Indrapuri, Agra. Mainly engorged female ticks from the proximal parts of both infected calves and cows were taken (Plate10). Ticks were transported to the laboratory in a wooden box having holes.

3. **Collection of Carissa carandas plant leaves** - Leaves were collected from Dayalbagh surroundings (Plate1). The leaves were weighed, washed with distilled and milli Q water, finely chopped and heated in microwave for 10 minutes. The extract was filtered with whatman no.1 filter followed by Millipore 0.45 micron filter (Plate4).

4. **Preparation of Silver nanoparticles synthesized from Carissa carandas aqueous leaf extract** - 1 mM AgNO₃ was prepared freshly and mixed with aqueous leaf extract of *C. carandas* (Plate 2). Then
verification of formation of AgNPs was done using UV-vis spectrophotometry (Figure1) and TEM (Plate3).

5. **In-vitro efficacy of AgNPs and Carissa carandas aqueous extract by adult immersion test (AIT)**- Engorged females were weighed individually to form homogeneous groups of 10 animals. Then these animals were immersed for 5 minutes in varying concentrations of synthesized AgNPs. After this period, females were removed from the solution, dried on whatman paper and placed in properly identified petridishes bearing double sided tape. Observation was done after 5hrs, 24 hrs, 48hrs and 72 hrs (Plate 9). Three replicates were performed by each concentration. Positive and negative control (Plate 5) was run in parallel to each experimental group. Same procedure was followed with aqueous extract (Plate 8).

Effect on Reproductive Index- R.I.= egg mass/female mass.

6. **Statistical analysis**- Results were represented as mean+S.E and students ‘t’ test was done to compare means.

7. **Microphotography**- Ticks were photographed with Olympus stereomicroscope from the vector control laboratory of the Department.