Plants as Sources of Natural and Effective Acaricides

Against Ixodes ricinus  (Acari: Ixodidae)

FAWZEIA ELMHALLI
Ticks and tick-borne diseases are major health hazards worldwide, with increasing numbers of cases of Lyme disease and tick-borne encephalitis reported yearly. Meanwhile, concerns about the environmental impact and safety of chemical acaricides are driving research into alternative control methods, such as plant-derived acaricides. I evaluated eight plant species for their toxicity and repellency against nymphs of *Ixodes ricinus* (Acari: Ixodidae), the most important life cycle stage of tick-borne infection of humans.

Paper I examines the toxicity of the principal active component of the essential oil (EO) of lemon eucalyptus (*Corymbia citriodora*), *p*-menthane-3,8-diol (PMD). At 4 h of exposure time (ET), lethal PMD concentrations for 50% mortality (LC$_{50}$) were 0.035–0.037 mg/cm$^2$ and for 95% mortality (LC$_{95}$) were 0.095-0.097 mg/cm$^2$. For 0.1 mg/cm$^2$, lethal times for 50% mortality (LT$_{50}$) were 2.1-2.8 h and for 95% mortality (LT$_{95}$) were 3.9-4.2 h. An open filter assay gave the most consistent results of five methods tried. Paper II investigated the toxicity of ylang-ylang oil (YYO) and star anise oil (SAO), two naturally occurring, commercially available and inexpensive EOs. Oils were tested at 0.05, 0.1, 0.2, and 0.4 μl/cm$^2$, and dead nymphs counted at 30-min intervals up to 5h and then at 24, 48 and 72h. For YYO, an exposure of 4.4h resulted in LC$_{95}$ for 0.4 μl/cm$^2$ and LC$_{50}$ for 0.2μl/cm$^2$. The LT$_{95}$ was 3h for 0.4 μl YYO/cm$^2$ and 4.3 h for 0.2 μl/cm$^2$. For SAO, the highest concentration (0.4 μl/cm$^2$) only reached LC$_{50}$ at 14 h and LT$_{95}$ was 24h. Thus, YYO is a much stronger acaricide but SAO still showed significant toxicity.

Paper III investigated two plants of traditional medicinal or economic importance in Libya - *Salvadora persica* (Miswa or Miswak) and *Rosmarinus officinalis* (Libyan Rosemary). EOs were extracted from wild-collected leaves by steam distillation. Oils were tested on *I. ricinus* nymphs and their chemical composition analysed by GC-MS. *R. officinalis* EO at 0.5 and 1μl/cm$^2$ exhibited 20% and 100% mortality, respectively, after about 5h of ET. The LC$_{50}$ and LC$_{95}$ for 1μl/cm$^2$ *R. officinalis* oil were 0.7 and 0.95 μl/cm$^2$, respectively. *S. persica* oil at 1μl/cm$^2$ gave 95% repellency up to 1.5h, reducing to 50% at around 5.45 h, but no significant mortality even after 24h ET. GC-MS analysis showed both oils to be rich in the monoterpens 1,8 cineol, α-pinene and β-pinene with values of 20.8%, 5.9% and 16.8 %, respectively, for *S. persica* and 24.07%, 13.03% and 2.45%, respectively, for *R. officinalis*.

Paper IV investigated EOs extracted from leaves of three additional native Libyan plants - *Artemisia herba alba* (white wormwood), *Origanum majorana* (oregano) and *Juniperus phoenicea* (Ar-aar). At 1μl/cm$^2$, the LT$_{95}$ for both *A. herba* and *J. phoenicea* EO was 2h versus 72 h for *O. majorana* oil. GC-MS analyses gave plant specific combinations of the monoterpenoids α-pinene, 1,8-cineol, camphor, linalool, terpinene-4-ol, α-terpinol, β-caryophyllene and β-thujanone. EO of *A. herba alba* contained most of the oxygenated monoterpens, which all are all known to have insecticidal activity.

Taken together, all the EOs used in this study show a broad spectrum of effects against *I. ricinus* nymphs, making them good candidates for controlling ticks and, thereby, the diseases they carry.

**Keywords:** Acaricidal, *Ixodes ricinus*, Libyan plants, essential oils, Plant-derived acaricides.

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In the name of God the most merciful.

For those who in my heart and mind always...
for the beautiful influence in everything..

To my children...
Abubaker,
Joud,
Mouhammed,
Jouri.
List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.


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# Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>11</td>
</tr>
<tr>
<td>Ticks</td>
<td>11</td>
</tr>
<tr>
<td>Hard Ticks (<em>Ixodidae</em>)</td>
<td>13</td>
</tr>
<tr>
<td>Hard Tick Biology</td>
<td>13</td>
</tr>
<tr>
<td>Life cycle</td>
<td>13</td>
</tr>
<tr>
<td>Tick Feeding Process</td>
<td>14</td>
</tr>
<tr>
<td>Why study <em>Ixodes ricinus</em>?</td>
<td>15</td>
</tr>
<tr>
<td>Why focus on the nymphal stage?</td>
<td>16</td>
</tr>
<tr>
<td>Medical importance of <em>I. ricinus</em></td>
<td>17</td>
</tr>
<tr>
<td>Climate change effects on disease activity</td>
<td>21</td>
</tr>
<tr>
<td>Controlling ticks</td>
<td>22</td>
</tr>
<tr>
<td>Biological control</td>
<td>22</td>
</tr>
<tr>
<td>Chemical control</td>
<td>24</td>
</tr>
<tr>
<td>Natural acaricides</td>
<td>25</td>
</tr>
<tr>
<td>Plant-derived acaricides</td>
<td>25</td>
</tr>
<tr>
<td>Thesis aims</td>
<td>30</td>
</tr>
<tr>
<td>Materials used in the study</td>
<td>31</td>
</tr>
<tr>
<td>Tick collection and preservation.</td>
<td>31</td>
</tr>
<tr>
<td>Plant Material</td>
<td>31</td>
</tr>
<tr>
<td>Commercial essential oil (EOs)</td>
<td>33</td>
</tr>
<tr>
<td>Method development</td>
<td>35</td>
</tr>
<tr>
<td>Summary of papers</td>
<td>37</td>
</tr>
<tr>
<td>Summary of Paper I</td>
<td>37</td>
</tr>
<tr>
<td>Summary of paper II</td>
<td>38</td>
</tr>
<tr>
<td>Summary of Paper III</td>
<td>40</td>
</tr>
<tr>
<td>Summary of Paper IV</td>
<td>42</td>
</tr>
<tr>
<td>Concluding remarks</td>
<td>44</td>
</tr>
<tr>
<td>Svensk sammanfattning</td>
<td>45</td>
</tr>
<tr>
<td>Acknowledgements</td>
<td>48</td>
</tr>
<tr>
<td>References</td>
<td>51</td>
</tr>
</tbody>
</table>
## Abbreviations and definitions

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>Adenosine di-phosphate</td>
</tr>
<tr>
<td>BP</td>
<td>Tick-born pathogens</td>
</tr>
<tr>
<td>CDC</td>
<td>Centre for Disease Control (USA)</td>
</tr>
<tr>
<td>ECDC</td>
<td>European Centre for Disease Prevention and Control</td>
</tr>
<tr>
<td>EFSA</td>
<td>European Food Safety Agency</td>
</tr>
<tr>
<td>EM</td>
<td>Erythema Migrans</td>
</tr>
<tr>
<td>EO</td>
<td>Essential oil</td>
</tr>
<tr>
<td>ET</td>
<td>Exposure time</td>
</tr>
<tr>
<td>GC-MS</td>
<td>Gas Chromatograph Mass Spectrometer</td>
</tr>
<tr>
<td>LB</td>
<td>Lyme Borreliosis</td>
</tr>
<tr>
<td>LC</td>
<td>Lethal concentration</td>
</tr>
<tr>
<td>LT</td>
<td>Lethal Time</td>
</tr>
<tr>
<td>NIST</td>
<td>National Institute of Standards and Technology</td>
</tr>
<tr>
<td>PMD</td>
<td>Para-menthane 3,8-diol</td>
</tr>
<tr>
<td>PGI₂</td>
<td>Prostaglandin I. 2</td>
</tr>
<tr>
<td>RC</td>
<td>Repellency concentration</td>
</tr>
<tr>
<td>RH</td>
<td>Relative humidity</td>
</tr>
<tr>
<td>SAO</td>
<td>Star Anise oil</td>
</tr>
<tr>
<td>TBD</td>
<td>Tick-born disease</td>
</tr>
<tr>
<td>TBE</td>
<td>Tick-born encephalitis</td>
</tr>
<tr>
<td>TBEIR</td>
<td>TBE incidence rate</td>
</tr>
<tr>
<td>TBEV-Eu</td>
<td>TBE virus: the European subtype</td>
</tr>
<tr>
<td>TBEV-FE</td>
<td>TBE virus: the Far-eastern subtype</td>
</tr>
<tr>
<td>TBEV-Sib</td>
<td>TBE virus: the Siberian subtype</td>
</tr>
<tr>
<td>TBP</td>
<td>Tick born (non-bacterial) parasites</td>
</tr>
<tr>
<td>TFPI</td>
<td>Tissue Factor Pathway inhibitor Ixolaris</td>
</tr>
<tr>
<td>TNFα</td>
<td>Anti-Tumour Necrosis Factor alpha</td>
</tr>
<tr>
<td>YYO</td>
<td>Ylang-ylang oil</td>
</tr>
</tbody>
</table>
Introduction

Ticks and tick-borne diseases have gained increasingly serious attention in recent years because of their impact on public health. Ticks affect human and animal health worldwide and are the cause of significant economic losses. Ticks are responsible for damage caused directly due to their feeding behaviour, and the impact of this on the global economy is considered high. (Estrada-Peña and Jongejan 1999; Jongejan and Uilenberg 2004; Murray et al. 2015; Smith and Guégan 2010). Diseases such as Lyme disease and European tickborne encephalitis (TBE) have received increasing attention as the number of cases increases every year (Centers for disease control and prevention (cdc) 2017). At the same time, public concerns about the environmental impact and safety of chemical acaricides are driving research into alternative, sustainable methods for their control. Thus, the discovery of effective and environmentally safe acaricides, such as plant-based acaricides is urgently needed.

Ticks

“Tick” is the common name for a group of small arachnids in the arthropod order Ixodida (Figure 1). Ticks, exclusively blood-feeding ectoparasites, along with mites constitute the group Acarina, they have a worldwide distribution and mainly feed on the blood of mammals, birds and occasionally on reptiles and amphibians. In addition to the direct damage caused to their hosts, ticks are the most important arthropod vectors of disease agents to humans and animals in the northern hemisphere (Sonenshine 1993). Ticks may cause problems ranging from skin irritation, minor allergic reactions, to severe, sometimes fatal anaphylactic reactions, as well as, indirect infections due to transmission of viruses, bacteria, protozoa and nematodes (Estrada-Peña and Jongejan 1999; Jongejan and Uilenberg 2004). There are about 907 species in the order Ixodida (Bowman and Nuttall 2008), and these are divided taxonomically into three families: Argasidae, Ixodidae and Nuttalliellidae.
The soft ticks (Argasidae)
Soft ticks have 5 genera and about 186 species, and together these have an almost worldwide distribution (Bowman and Nuttall 2008). They have an oval or pear-shaped outline, with the anterior body region broadly rounded. The mouthparts are difficult to see from a dorsal view (Anderson and Magnarelli 2008; Sonenshine et al. 2014). Most of the medically important soft ticks belong to the genus *Ornithodoros* and *Argas*, compared to more than six genera of medically important hard ticks (Service 2008). All life stages can feed on the same host so the opportunity for transmitting disease is less than for Ixodidae.

The hard ticks (Ixodidae)
Ixodidae have a worldwide distribution, but are more common in temperate regions than soft ticks (Service 2008). This is the largest family of acarida, and has 13 genera and 720 species (Figure 1). Medically the most important genera are *Ixodes, Dermacentor, Amblyomma, Haemaphysalis, Rhipicephalus* and *Hyalomma* (Gaugler 2016). External morphology of hard ticks is flattened dorsoventrally in the unfed state and the mouthparts are clearly visible. A sclerotized dorsal plate (scutum) is evident in all stages, which is absent in soft ticks (Anderson and Magnarelli 2008).

The Nuttalliellidae (no common name)
This genus contains only a single described species, *Nuttalliella namaqua* that is of no medical importance (Service 2008). For long times only females found in the Afrotropical region were known (Sonenshine 1991). It has not been collected for many years (Bowman and Nuttall 2008). Recently, males and immatures have been described (Latif et al. 2012).
Hard Ticks (*Ixodidae*)

Hard ticks possess many features that contribute to their remarkable success as disease vectors. Two of the most outstanding are their longevity and their reproductive potential, which together make them substantial pathogen reservoirs in the field (Ghosh et al. 2007). Another important feature for several species is the fact that they are able to feed on a very broad host spectrum. Lastly, they consume a very large quantity of blood over a relatively long period, which increases the chance of both ingesting and transmitting a pathogen.

Life cycle

Ticks can be one-, two-, or three-host feeders. The majority of ixodids are classed as three-host ticks, and the hosts may or may not be of the same species. The life cycle of ixodids can take from 1 to 6 years and involves 4 developmental stages: egg, larva, nymph, and adult (Figure 2). After hatching, the larva seeks a host, feeds and then drops off to moult to the nymphal stage. The nymph then seeks another host, feeds, drops off and mouls to the adult stage.
Females drop off from their host to start oviposition in a sheltered microenvironment, laying up to several thousand eggs (Sonenshine 1991).

Figure 2. The life cycle of hard ticks. This usually takes two years.

Tick Feeding Process

The Ixodidae or hard ticks possess the most complex feeding biology of all hematophagous arthropods (Liu et al. 2014). Compared to other blood feeding arthropods, feeding in ixodids ticks is a slow and complex process. It takes several days to reach repletion and then detachment, which necessitates extended control over the host’s immune response. During this feeding process, ticks inject saliva and ingest blood in an alternating pattern. Many proteins present in tick saliva dampen host defences to insure adequate feeding time (Kotal et al. 2015). This in turn creates a favourable environment for survival and propagation of tick-borne pathogens, which are transmitted via saliva (Kazimirová and Stibraniiová 2013; Wikel 1999). Hard ticks also secrete cement into and above the gradually expanding lesion that acts as a kind of glue. This further strengthens the bond with the skin anchoring and sealing the parasite’s hypostome to the host’s skin. Both saliva and cement are secreted by the salivary glands.

The first phase of the feeding cycle involves attachment and penetration of the host, and it may take between 1 and 2 days before the tick begins to feed. The tick will then feed for several additional days, depending upon the life stage and the tick species. During the feeding process, ticks alternate blood
sucking and secretion of saliva into the wound through the same channel. Injected saliva contains various compounds. These molecules are used to counteract the host haemostatic, inflammatory and immune systems creating a repertoire of potent bioactive salivary molecules. These molecules include vasodilators, e.g. prostacyclin PGI$_2$ in *Ixodes* spp. increases blood flow (Ribeiro 1989), anti-coagulants, e.g. *Ixolaris*, a novel recombinant tissue factor pathway inhibitor (TFPI) from the salivary gland of *Ixodes scapularis* (Francischetti et al. 2002), and anti-platelets, e.g. apyrase enzyme which destroys platelets (Ribeiro et al. 1985). Ticks saliva has also been found to have anti-inflammatory factors such as anti-tumour necrosis factor alpha (TNFα) from *I. ricinus* saliva (Konik et al. 2006).

Ticks are “pool feeders”, meaning they take in fluids that are exuded into the wound generated by the bite. Due to the synthesis of fresh expandable cuticle, hard ticks are able to imbibe enough blood to increase their size as much as 100 times (Anderson and Magnarelli 2008; Sonenshine et al. 2014). The red blood cells are lysed in the lumen of the tick midgut. However, in contrast to other hematophagous arthropods, the digestion of the proteins and other molecules in the blood is an intracellular process that takes place within the epithelial cells of the midgut. A large capacity for food storage in the midgut explains the tick’s ability to wait for a vertebrate host up to several years without feeding (Lara et al. 2005).

The duration of tick feeding and its attachment site on the host will influence the risk of pathogen transmission and development of infection (Wilhelmsson et al. 2013). The duration of tick feeding including salivation and blood ingestion is important for trasmission of pathogens. For example, ticks carrying the TBEV will begin to transmit virus particles one hour after attachment (Alekseev et al. 1996). In contrast the risk of transmission of Lyme borreliosis (LB) develops more slowly and increases with the duration of feeding time (Crippa et al. 2002). *Borrelia*, *Anaplasma* and *Rickettsia* are the main pathogenic bacteria transmitted by ticks. These bacteria need time for maturation in the tick midgut and subsequent migration to the salivary glands (Bernard et al. 2018). The duration of feeding time can be estimated from the tick’s scutal and coxal indices (Gray et al. 2005).

**Why study *Ixodes ricinus***?

*Ixodes ricinus* belongs to family Ixodidae, subfamily Ixodinae (Figure 1). It is commonly known as the sheep tick or the castor bean tick, and is the most important and most common tick in northern Europe. *I. ricinus* is responsible for many diseases affecting humans, pets and economically important animals. It occurs over a wide geographical range, from Ireland in the west to the
Ural Mountains (Russia) in the east, and from Scandinavia in the north to Morocco and Egypt in the south (Estrada-Peña et al. 2018). *I. ricinus* usually feeds once per life cycle stage (Figure 2), using different hosts from a wide range of species. The tick is therefore well adapted to the seasonal patterns in the northern hemisphere. In most of its geographical range, *I. ricinus* feeds from March to October. Two sub-populations occur in most areas; the larger one is active in spring and early summer and a smaller one in late summer and autumn. The life cycle usually takes 2-3 years, but in some conditions it can take from 1-6 years. Blood feeding occurs once in each stage and for a period of a few days. Digestion of the blood meal and development to the next stage occurs during the midsummer period while the tick is hidden deep in the vegetation to avoid dry conditions in the hot season. The eggs are laid in batches of about 2000, and the larve are ready to feed within a few days of hatching. All stages of *I. ricinus* quest for hosts using an ambush technique whereby they climb up vegetation and wait for a host to pass by (Gern et al. 2008; Sonenshine 1993).

*I. ricinus* are able to detect changes within the environment like light levels, temperature, carbon dioxide, humidity and vibration. For this, they possesses light-sensitive cells on their dorsum (Perret et al. 2003). They also have sensory organs such as Haller’s organs, subtle receptors that are located on the forelegs Haller’s organs make ticks able to smell in stereo (A. Lakos, personal communication) indicating the best time to quest for potential hosts (Sonenshine and Roe 2013). During questing, ticks lose water but they actively reabsorb water vapour from the atmosphere in litter zones (Rudolph and Knülle 1974).

Why focus on the nymphal stage?

The nymphal stage of *I. ricinus* is the most medically important (Figure 3). This is because nymphs can transmit diseases as easily as adults can, but they
are both more abundant and less noticeable. Nymphs are small (less than 2 mm in length), which makes them difficult to spot. This makes them more likely to stay on the host longer without being detected giving them more opportunities for attachment as well as the longer time of attachment before detection. Together these features result in increased opportunity to transmit pathogens while feeding and therefore, nymphs pose more risk (Randolph 1998). In contrast, the larger size of adult ticks makes them much easier to detect and more likely to be removed before transmission occurs.

Nymphs are very active from spring through early summer, which coincides with the peak of outdoor activity for most people (Jaenson et al. 2012b). They feed for a few days during spring-summer before dropping to the ground and moulting to adult ticks. The hosts of preference of nymphal ticks are medium-larg sized ungulates. However, their multi-host behaviour gives them considerable opportunities to spread pathogens among numerous animal species, including humans, and to transmit infections that can be extremely serious (Iori et al. 2005). The human risk of getting tick-borne diseases transmitted by *I. ricinus* has been broadly linked to tick nymph density (Lindgren et al. 2000). For these reasons, I focused on the nymphal stage in my studies as it is considered the most medically important stage, particularly as a vector of virus and microorganisms causing disease in humans.

**Medical importance of *I. ricinus***

*Ixodes ricinus* is responsible for many diseases affecting humans, pets and production animals. In addition to TBE, TBD caused by viruses include Louping-ill for which the affected hosts are sheep, cattle, goats, grouse, and, possibly, humans. The clinical manifestation of Louping-ill is shared with TBE, which is also caused by a virus and well known to affect humans (Pritt et al. 2016). In addition to viruses, ticks can carry bacteria, particularly *Borrelia burgdorferi* s. l., *Anaplasma phagocytophilum* and *Rickettsia helvetica*. *Borrelia* is a spirochete bacterium that causes LB in humans, but can infect other species like dogs and horses. *I. ricinus* transmitted LB affects 85,000 people every year in Europe (ECDC. 2018; Lindgren and Jaenson 2006; Pållson et al. 2008). Anaplasmosis mainly affects ruminants, dogs, horses and humans. *Staphylococcus aureus* is responsible for tick pyaemia disease in lambs. Finally, *Rickettsia helvetica* causes rickettsiosis in rodents and humans. Ticks can also be vectors of disease-causing protozoa. These include babesiosis, caused by *Babesia divergens* and *Babesia microti*, which occurs in cattle, humans and rodents (Aberer 2009). *I. ricinus* has also been implicated in the transmission of tularaemia and Q-fever.
Lyme disease
Lyme disease, named for the town of Lyme (Connecticut, USA) where it was first recognized, is the most common tick-borne illness. It has been reported from at least 26 European countries and is the most important vector-borne disease of the northern hemisphere as well as Asia (Service 2008). It is caused by the spirochete *Borrelia burgdorferi*, a bacterium belonging to the order of Spirochaetales and family of Spirochaetaceae. (Steere et al. 1985). Most cases of LB are reported from temperate regions and coincide with the distribution of the principal vector, ticks. Only hard ticks, particularly *Ixodes spp.* transmit *Borrelia burgdorferi*, which of course includes *I. ricinus* (Gray 1998).

European Lyme borreliosis
Lyme borreliosis was first reported in the United state in the late of 1970s (Steere et al. 1978), and had been reported throughout Europe by 1982. LB is the most common tick-borne infection in Europe, as well as in the USA where 300,000 cases per annum are reported (Mead 2015). Despite increasing awareness of the disease and increasing attention in the media, there are few published data suggesting an increase in LB prevalence in Europe. Nonetheless, the number of reported cases in Europe has been rising from the early 1990s (Tälleklint and Jaenson 1998) as well as expanding in geographic distribution, and there are now an estimated 85,000 cases of LB in Europe each year (Lindgren et al. 2000; Slack et al. 2011). Several studies have reported that the incidence of LB in Sweden and The Netherlands has increased distinctly over the last 10 years (Rizzoli et al. 2011; van Kampen et al. 2013). The highest number reported in southern Sweden is 464 cases per 100,000 person-years, and the mean number reported in 2016 provided a rate of 22.05/100,000 person-years (Makiello and Sykes 2016). Other studies have shown that the prevalence of LB varies considerably among different European countries, with an overall increasing prevalence from west to east (Wilking and Stark 2014).

The first local clinical manifestation of LB is the so-called erythema migrans EM, which corresponds to a skin inflammation at the site of tick’s bite, often in the form of a halo surrounding the bite site. In the absence of an efficient host immune response, the bacteria can then spread via the blood and the skin to distant organs such as heart, joints, central nervous system, and distant skin (Stanek et al., 2012; Steere et al., 2016). EM (single or multiple) occurs in approximately 90% of patients that subsequently develop Lyme disease. Systemic symptoms, such as fever, myalgia, arthralgia, headache, fatigue, and/or carditis are common in the later stages of Lyme disease. Other common symptoms include cranial nerve palsies, especially facial nerve palsy, and meningitis, sometimes accompanied by papilledema and increased intracranial pressure. In rare cases, LB can also cause a complete heart block, which may cause syncope (Lantos 2011).
Tick-Borne Encephalitis (TBE)

Tick-borne encephalitis is caused by tick-borne encephalitis virus (TBEV), an RNA virus in the Flavivirus genus. Today, it is universally acknowledged that the TBEV is polytypic and is genetically and phenotypically diverse both within populations and between geographic location (Korenberg and Kovallevsky 2000). Six virus subtypes have been identified, three of which are most prevalent (Ecker et al. 1999; Zlobin et al. 2001; Demina et al. 2010). These three subtypes of the TBEV are the European subtype (TBEV-Eu), which is mainly transmitted by *I. ricinus*, the Far-eastern (TBEV-FE) and Siberian (TBEV-Sib) subtypes, both of which are mainly transmitted by *I. persulcatus* (Süss 2011). Recent findings from Finland suggest that *I. ricinus* can also transmit TBEV-Sib (Jääskeläinen et al. 2016). In Europe, most cases are infected by TBEV-Eu but cases infected with TBEV-FE have been reported in Estonia and Latvia, and TBEV-Sib in Estonia and Finland (Beauté et al. 2018). Presently in Europe, TBE is most prevalent in southern Germany, Switzerland, Austria, the Czech Republic, Slovakia, Hungary, Slovenia, the Baltic countries, Poland, parts of Scandinavia, and Russia (Heinz et al. 2013; Kunze 2013) (Figure 4).

TBE is a viral disease that attacks the host nervous system, sometimes resulting in long-term neurological symptoms and even death (Lewis and Glaser 2005; Reisdorff 1999). The disease can cause both mild and severe illnesses, with permanent consequences such as concentration problems, paralysis and depression. The incubation period lasts between 7 and 14 days on average. The disease exhibits the traditional process of an infectious illness, beginning acutely with a rapid rise in body temperature, chills, headache, muscle pain and nausea. Disorders of consciousness are also often reported. Typical symptoms include slight paralysis (decreased muscle tone) and shoulder and neck palsy (Cherkassky 1994). Approximately 1% of cases result in patient death (Pettersson et al. 2014). Longitudinal surveillance in Austria shows TBE emergence in previously unaffected regions. Similar findings have also been reported from Norway, Sweden, and Denmark (Fomsgaard et al. 2009; Johan et al. 2006; Skarpaas et al. 2004).

In fact, the prevalence of TBE has increased in Europe by approximately 318% in just the last 30 years, with more than 10,000 cases reported each year (Zavadskas et al. 2013). The TBE incidence rate (TBEIR) in a recent Russian study indicated that during 1980–1989 the average number of TBE cases per year was 1.6 (TBEIR = 0.1 per 100,000), while in 2008–2017 it was 64.4 (TBEIR = 5.4 per 100,000). Thus the TBEIR was 54 times higher in 2008-2017 versus 1980-1989 (Tokarevich et al. 2019). The overall EU/EEA TBE notification rate also increased in 2016 compared with 2015 (0.4 cases per 100,000 population). Increases of over 40% in the notification rate were reported for the Czech Republic (62%), France (49%), Germany (57%), Italy (862%), Lithuania (91%), Poland (83%) and Slovakia (111%). Decreases in
notification rates of 30% or more were mostly observed among countries that also reported a lower number of cases (Croatia, Estonia, Hungary and Latvia) (ECDC. 2018). A strong upsurge of TBE in Europe in recent years has been associated with climatic, ecologic, and human behavioral changes that could increase the risk for virus exposure (de Graaf et al. 2016; Jaenson et al. 2018; Jahfari et al. 2017) (Figure 4).

Figure 4. Distribution of TBE cases per 100,000 population, according to EEU/EEA at 2014 and 2016. European Centre for Disease Prevention and Control. Tick-borne encephalitis. Acknowledgment to: ECDC. Annual epidemiological report for 2016. Stockholm: ECDC; 2018.

Figure 5. The map shows presence/absence of *Ixodes ricinus* in Europe, RED The species is known to have been present at least in one municipality within the administrative unit. YELLOW The species has been introduced in the administrative unit without confirmed establishment. LIGHT GREY No information is available on the existence of field studies on ticks. Acknowledgment to: European Centre for Disease Prevention and Control (ECDC), http://ecdc.europa.eu/en/pages/legalnotice.aspx, last update 22 Feb 2017.
Climate change effects on disease activity

*I. ricinus* appears to have increased its geographical distribution and density-activity in many areas of Europe during the last decades (Jaenson et al. 2012a; Lindgren et al. 2000; Medlock et al. 2013). This is reflected in an increasing number of reported cases of tick-borne diseases (Michelet et al. 2014). In Scandinavia a correlation between changes in tick distribution or abundance (Mannelli et al. 2012) and the incidence of LB and TBE has been demonstrated (Lindgren and Gustafson 2001). Studies from the Czech Republic also indicate that in recent decades the occurrence of *I. ricinus* and TBE cases has shifted to include higher altitudes, probably due to a prolonged vegetation period, in particular milder autumns (Mannelli et al. 2012). In 2014, a collaborative working group was organized under the project VectorNet with help from the European Food Safety Agency (EFSA) and the European Centre for Disease Prevention and Control (ECDC). This group started compiling maps of tick distribution based on existing tick collecting data. The project also supports the collection of data on vectors related to both animal and human health. This work has recognised the wide distribution of *I. ricinus* spp. in Europe (Figure 5).

LB is transmitted by hard ticks belonging to the genus *Ixodes*, including mostly *Ixodes scapularis* / *pacificus* in North America, *Ixodes ricinus* in Europe, and *Ixodes persulcatus* in Asia, all of which are affected by local environment. The transmission cycle of LB depends on the variety and distribution of mammal and bird hosts, as well as tick species. LB has a global distribution in temperate regions of North America, Europe, and Asia (Jongejan and Uilenberg 2004) combination of milder winters and extended spring and autumn seasons would be expected to result in increased time for tick activity (Jaenson et al. 2012b; Kilpatrick and Randolph 2012; Léger et al. 2013; Lindgren et al. 2000; Madder and Pascucci 2013; Rizzoli et al. 2011). Likewise, warming is expected to extend the transmission season for TBE in Europe. In Sweden, warmer winters have been accompanied by an increase in the tick population and the annual number of cases of TBE reported (Jaenson et al. 2012b). Although most TBE transmission to humans is by the nymphal ticks, all tick stages have well-defined seasons of feeding activity, which vary geographically and may be prolonged with milder winters (Lindgren and Gustafson 2001). However, there are many factors involved in the changing of *I. ricinus* distribution within its prior endemic zones. Climatic change is one of them but there is also the changing distribution of tick hosts or other ecological changes and anthropogenically induced changes. These factors are strongly interlinked and important to assess the risk of the spread of infections transmitted by this vector.
Controlling ticks

Various strategies for control of ticks have been developed and adjusted to the particular geographical area and circumstances. These can be classified in two main ways - biological control and chemical control. Chemical control can use synthetic materials, natural chemicals extracted from plants or pheromones extracted from the target organism. Biological control, which uses natural pathogens or predators, tends to be more environmentally safe but expensive and harder to achieve. In addition, there has been some success in controlling tick borne diseases through vaccination.

Biological control

Pathogens and Predators

Biological control of ticks has been difficult, as ticks do not have many natural enemies. In nature, some bacterial and fungal pathogens as well as predators such as spiders, ants, beetles, rodents, and birds contribute significantly towards limiting tick populations, as does the grooming activities of tick hosts. Several attempts have been made to test various predators and entomopathogenic fungi for use against ticks (Perez-Perez et al. 2010; Samish et al. 2008). Fungi of the genera Beauveria and Metarhizium, and nematodes in the families Steinernematidae and Heterorhabditidae have so far shown the most promising potential for tick biocontrol (Samish et al. 1999). However, of the nematodes studied to date the only ones found to be effective so far are Steinernema carpocapsae (Weiser) and Steinernema glaseri (Steiner). These are effective against engorged female black-legged ticks (Ixodes scapularis), but these have already fed on and thus possibly infected a host (Zhioua et al. 1995). Moreover, the nematodes tested were unable to survive at colder temperatures making them of limited value against ticks, especially in more northern or higher altitude habitats (Xuejuan and Hominick 1991).
Vaccinations
Vaccines can be aimed either at killing or repelling the ticks themselves or at combatting the pathogens that the ticks may carry or the diseases these pathogens may cause. Direct control of tick-borne diseases through vaccination has many advantages as it reduces environmental pollution and avoids resistance of ticks to acaricides resulting from repeated application and exposure. The development of vaccines against ticks also has the potential to allow the inclusion of multiple types of antigens that can be aimed at a wide range of ticks (De la Fuente and Kocan 2006).

The vaccine prevention of tick-borne disease in humans through vaccination is at present far from being satisfactory. Current vaccination against TBE, a potentially severely debilitating or even fatal neurological disease, provides a high level of protection (90%–99% efficacy) and even cross-protection between the three TBE subtypes (Loew-Baselli et al. 2011). However, no vaccine exists for the most prevalent TBD, LB, which, while not usually fatal, can still be a very debilitating disease. The protozoan parasites Babesia spp., which cause various kinds of babesiosis, are currently controlled in animals using antiprotozoal drugs and acaricides. However, efforts to control Babesia bovis and Babesia bigemina by vaccination with attenuated parasites are generally unsatisfactory as this carries the risks of contamination and adverse reactions (Bock et al. 2004). The development of vaccines against tick-born (non-bacterial) parasites (TBP) is hampered by limited understanding of host immunity to this group, TBP strain diversity and the possible transmission of multiple parasites by the same tick species. Therefore, vaccine strategies against TBPs that target tick antigens seem to hold the most promise of affording broad protection against TBD, and these strategies are increasingly being required (Nuttall et al. 2006; Willadsen 2004). A protein first labeled 4D8 and now called subolesin was identified through an Ixodes scapularis cDNA expression library as a potential tick-protective antigen, and the protein was later found to be conserved among several ixodid tick species (Almazán et al. 2005; Almazán et al. 2003). However, the identification of protective antigens is still the limiting step in the development of an effective anti-tick vaccine.

Although there has been some recent progress in identifying tick-protective antigens, only a few antigens have been tested. The outcome of trials involving hybridization of protein in vaccines have so far failed to show evidence that they can prove effective against a wide range of different ticks species. Even with the discovery of protective antigens, it is still necessary to develop vaccine formulations, conduct field studies and commercialize the resulting product. These are all challenges that make it unlikely to realise this option soon as well as more expensive than natural acaricides from plants. Nonetheless, vaccination is still generally considered as a good option and also might be safer for the environment.
Chemical control

Chemicals used as acaricides are mainly poisonous compounds and can be roughly classified as synthetic or natural; natural also includes pheromones which also have potential as chemical control agents against ticks. To date, synthetic acaricides have had limited effectiveness in reducing tick invasion and are also often accompanied by several negative aspects. These include a selection of acaricide-resistant ticks and environmental contamination. In addition, the development of new acaricides is a long and expensive process. Together these factors emphasize the need for alternative approaches to tick control, such as natural acaricides.

Synthetic acaricides

Synthetic acaricides are one of the main and most commonly used tools for controlling tick populations and for reducing the transmission of tick-borne human pathogens. Control programmes are largely based on the use of commercially available chemicals such as the arsenicals, chlorinated hydrocarbons, organophosphates, carbamates, formamidines, pyrethroids, macrocyclic lactones (Andreotti et al. 2011). However, the use of chlordimeform as an acaricide was discontinued in 1976 because of proven carcinogenic effects (Ware Jr 2000).

Tick Resistance to Synthetic acaricides

Resistance has been reported to all the synthetic acaricides listed above, and for most tick species and different localities around the world. Resistance to arsenicals was the first reported in Australia for the Asian blue tick, *Boophilus microplus* (Compagno 1984). However, resistance to arsenical acaricides in *Boophilus decoloratus* was actually detected much earlier in South Africa in 1941 (Baker et al. 1978). Toxaphene resistance has been reported from Kenya, Malawi, South Africa, Uganda, Zambia and Zimbabwe with *Boophilus decoloratus* (FAO 1984) and from Ethiopia (Regassa and de Castro 1993). In a study done in commercial farms on some tick species, *B. decoloratus*, *Rhipicephalus evertsi evertsi*, *Amblyomma hebraeum* and *Rhipicephalus appendiculatus* showed high levels of resistance to amitraz, chlorfenvinphos and cypermethrin (Mekonnen et al. 2002). Populations of *B. microplus* from Mexico were found to have developed resistance to many classes of acaricide including chlorinated hydrocarbons (DDT), pyrethroids, organophosphates, and formamidines (amitraz) (Foil et al. 2004). Currently, there is no precise information about resistance to these acaricides in *I. ricinus*, but considering that this is a close relative of species that developed resistance, it is likely that *I. ricinus* can also develop resistance to these synthetic acaricides.

The tick control programs that depend on the use of synthetic acaricides, appear to have failed to prevent the development of resistance in ticks. In fact,
tick populations that develop acaricide resistance become more difficult to control (Corson et al. 2001).

Natural acaricides

Pheromones

Pheromones are compounds that are produced and released by one individual to influence the behavior of another individual of the same species. These compounds are volatile molecules (Norval et al. 1991). Pheromones have been used as biological control agents, e.g. to disrupt the reproduction of the target (Sonenshine and Hamilton 1989). However, pheromone alone cannot control ticks; they need to be used in combination with a true acaricide. For example, a pheromone+acaricide impregnated device was developed to attract and kill male ticks before they could mate (Sonenshine 2006). So far, only synthetic acaricides have been employed in combination with pheromones, so there is the potential that pheromones may be effectively used together with extracts from natural oils. Both compounds would then have to be incorporated into a slow delivery device due to their volatility (evaporation properties). Otherwise, any improvement in short-term protection could not be considered effective. Various techniques are available to produce the delay in release required for pheromones, for example, incorporation into plastics, adhesives, paraffin, gelatin or microcapsules. Development of a tick control device or system using pheromones is also dependent on how it will be applied, for example, to vegetation in the host environment or to the host itself. Other things that need to be considered are the solubility of the pheromone in the coupling device (carrier), the required range of attraction, and the duration of the activity. All of these contribute to the effectiveness of this method of control (Sonenshine 2006).

Plant-derived acaricides

The relationship between plants and arthropods is chemically mediated by secondary metabolites. Such bioactive plant products have been used in various ways since ancient times, especially in cultures with a strong herbal tradition (Secoy and Smith 1983). Essential oils (EOs) are a concentrated hydrophobic liquid containing volatile (easily evaporated at normal temperatures) chemical compounds from plants which have low molecular weight. EOs are also known as volatile oils, ethereal oils, aetherolea, or simply as the oil of the source plant. Oils are usually extracted by distillation, often using steam. Other extraction processes include expression, solvent extraction, resin tapping, wax embedding, and cold pressing. EOs usually include major terpene
or terpenoid components, which can constitute up to 30% of the oil (Bakkali et al. 2008).

Many plant EOs show a broad spectrum of effects against arthropods pests, including anti-feedant (adversely affecting insects or other animals that eat them), repellent, growth regulatory and anti-vector activity. The insecticidal or acaricidal efficacy is often attributed to the oil’s major component(s); however, there is also evidence that the various oil components may work in synergy (Yang et al. 2004). This can occur because some oil components aid cellular accumulation and absorption of other toxic components (Cal 2006). EOs are often rich in monoterpenoid compounds (a class of terpenes that consist of two isoprene units and have the molecular formula C_{10}H_{16}) (Breitmaier 2006; Gershenzon and Croteau 1993). Monoterpenoid compounds cause death in arthropods by inhibiting acetyl-cholinesterase activity in the nervous system (Houghton et al. 2006), some of the most efficacious ones were α-pinene, β-pinene, 1,8-cineol camphor, linalool, terpinene-4-ol, α-terpinol, β-thujone and 15 compounds more, which were found to have an acetylcholinesterase activity in insects (Jankowska et al. 2017). There are many studies that have proofed the effect of these compounds on octopamine receptors, which are specific for invertebrates – including Acari, hence interfering with their octopaminergic nervous system (Bischof and Enan 2004; Jankowska et al. 2017; Kostyukovsky et al. 2002). Thus, there is a neurotoxic rather than simply a mechanical way in their mode of action. This also has the advantage of being a target site not shared with mammals. In fact, most EOs are relatively nontoxic to mammals and fish in toxicological tests, and thus meet the criteria for “reduced risk” pesticides (Stroh et al. 1998).

Due to their high chemical diversity and consequent potential for bioactivity, EOs have received significant research interest as potential sources of novel and natural alternatives to synthetic pesticides for many applications including agriculture and apiculture, as well as veterinary and human medicine (Isman 2006). An extensive account of research into EOs for pest control is given by Bakkali et al. (Bakkali et al. 2008), including the potential of these products to target viruses, fungi, bacteria and protozoa.

Plants contain unique repertoires of diverse chemical structures, many of which have been successfully exploited by man. Various plant products, crude extracts and EOs have also been used against ticks with encouraging results. This includes all the life cycle stages - adult, nymph and larva - of several economically important tick species, including *Amblyomma cajennense*, *Dermacentor nitens*, *Hyalomma anatolicum*, *Ixodes ricinus*, *Ixodes scapularis*, *Rhipicephalus* (*Boophilus*) *microplus*, *Rhipicephalus sanguineus* and *Rhipicephalus turanicus* (Clemente et al. 2010; Coskun et al. 2008; Daemon et al. 2009; de Monteiro et al. 2012; Dolan et al. 2014; Elmhalli et al. 2018; Elmhalli et al. 2009; Ghosh et al. 2011; Godara et al. 2015; Godara et al. 2014; Kamaraj

The potential of both EOs and extracts against a range of ectoparasites of veterinary significance has been recently reviewed (Ellse and Wall 2014; George et al. 2008). This includes examples of their use against insect or acarid pests of livestock, poultry and domestic animals. For example, application of a floral extract of camomile produced 100% mortality against the mite *Pseudoperopotes cuniculi*, a pest of domestic rabbits (Macchioni et al. 2004), and against the red spider tick, *Dermanyssus gallinae*, a pest of domestic fowl. Further studies demonstrated the presence of numerous EOs in these extracts (Kim et al. 2004) (George et al. 2010). Extracts from *Achyranthes aspera*, *Anisomeles malabarica*, *Gloriosa superba*, *Psidium guajava*, *Ricinus communis*, and *Solanum trilobatum Lants* (Zahir et al. 2009) similarly showed effectiveness in targeting multiple ectoparasites of veterinary and medical significance, in this case the larve of *Rhipicephalus (Boophilus) microplus* ticks and the larvae of two different genera of mosquitoes, *Anopheles subpictus* and *Culex tritaeniorhynchus*. Several plant derived pesticides have been commercially developed and shown to be effective against fleas, ticks and mange mites in cats and dogs, as well as against ectoparasitic mites infesting honeybees (George et al. 2008). A recent review of tick prevention (Kiss et al. 2012) devoted multiple sections to tick prevention using botanicals, noting research undertaken with both EOs and extracts targeting larvae and adults alike. Importantly, this study highlighted the potential influence of factors such as solvent selection and the life-stage of the target organism on efficacy, with younger stages typically more susceptible than adults (Kiss et al. 2012).

However, research into the use of EOs as control agents is still at a preliminary stage. Wide-ranging field trials, standardization of components, standardization of extraction techniques, standardization of experimental design and mammalian toxicology reporting, as well as further investigation into the residual activities and length of shelf-life of these oils are all required before their potential can be fully explored. Nonetheless, the use of EOs in the control of medical ectoparasites is a promising field that holds huge potential for the future of green pesticides. Thus the potential for an alternative solution for controlling acarid ticks using plant-derived acaricides is real and recognized.

**Preparation of essential oils (EOs)**

An EO is a concentrated hydrophobic liquid containing volatile chemical compounds from plants, known also as volatile oils, ethereal oils and aetherolea. In contrast to fatty oils, EOs typically evaporate completely. Moreover, there is a significant difference between plant EOs and plant extracts. The majority of EOs are produced from plant material in which they occur by different kinds of distillation or by cold pressing in the case of the peel oils from citrus fruits.
In contrast, extracts that are obtained by solvent extraction with different organic solvents, such as liquid carbon dioxide via supercritical fluid extraction, may not be considered as true EOs (Baser and Buchbauer 2015).

**Essential oils extraction processes:**

The hot steam helps to release the aromatic molecules from the plant material since the steam forces open the pockets in which the oils are kept in the plant material. The molecules of these volatile oils then escape from the plant material and evaporate into the steam. The temperature of the steam needs to be carefully controlled - just enough to force the plant material to let go of the EO, yet not too hot as to burn the plant material or the EO.

The steam containing the EO is then passed through a cooling system to condense the steam, which forms a liquid from which the EO and water are then separated. The steam is produced at greater pressure than the atmosphere and therefore boils at above 100°C Celsius this facilitates the removal of the EO from the plant material at a faster rate and in doing so prevents damage to the oil. This technique uses temperature to separate the aromatic oil from an organic source. Some oils - as lavender oil - are heat sensitive (thermolabile) and with this extraction method, the oil is not damaged. In addition to distillation at atmospheric pressure, high-pressure steam distillation is most often applied in European and American field stills, and the increased temperature applied significantly reduces the time of distillation. Generally, the process of steam distillation is the most widely accepted method for the production of EOs on a large scale. In this study we used a Clevenger-type apparatus for steam distillation which returns the hydrosol to the still and maintains the essential oil phase but only for essential oils that are less dense than water and therefore float.

Expression or cold pressing is a process in which the oil glands within the peels of citrus fruits are mechanically crushed to release their content these processes lead to products that are not entirely volatile, because they may contain coumarins, plant pigments, and so on. However, they are nevertheless acknowledged as EOs by the International Organization for Standardization and, the different pharmacopoeias (Baser and Buchbauer 2015; Hüsnü and Buchbauer 2015).

There are more types of steam distillation in EO manufacture, such as the hydro diffusion, where the steam is fed in from the top onto the botanical material instead of from the bottom. Cohobation extraction, is another method, that, for example is used with rose oil; after extraction, the essential oil was mixed with water (Braga et al. 2005). Rectification in extraction of EOs is usually used when an EO contains any impurities (Castillo-herrera et al. 2007), This method uses re-distillation for purification, in this case the extract is marketed as "double-distilled" (Zhang et al. 2011). In addition, fractional
distillation, is a normal distillation process, but instead of the EO being collected continuously, it is collected in batches (the fractions that are referred to). Ylang-ylang EO is for example usually extracted in this way (Timcik and Fergeus 2006).

**Gas chromatography–mass spectrometry (GC-MS)**

The device consists of two instruments: a gas chromatograph connected to a mass spectrometer. A capillary column with defined dimensions (length, diameter, thickness of the film) is placed in the gas chromatograph and connected to the injector and to the outlet of the mass spectrometer. The molecules that are passing through the column with the help of a carrier gas will move at different rates depending on the GC-temperature and their affinity for the film properties, and thus will elute from the column at different times, called retention time. Molecules with the same retention time are passed on to the downstream mass spectrometer where the molecules are ionized and fragmented in the ion source, separated based on the mass to charge ratio and detected in an ion collector (Sparkman et al. 2011). The obtained mass spectra are then identified by comparison with mass spectra in the Nist database, a set of available standards and their retention times (Rt) to identify the components. While the quantitative analysis runs by calculating the area of the peak using the mathematical function of integration, the area under the peak is proportional to the amount of analyte present in the chromatogram. Concentration can be calculated using a calibration curve created by finding the response for a series of concentrations of analyte, or by determining the relative response factor of a compound. Recently, computer software is used to draw and integrate peaks, and match MS spectra to library spectra.
Thesis aims

The overall aims of my PhD studies were to evaluate compounds from selected plants as potential acaricides against nymphs of *Ixodes ricinus*. To reach this overall aim, toxicity was evaluated in a laboratory setting by different bioassays to determine optimal concentration and exposure time, and potential active components were determined by GC-MS analyses.

**Specific aims for each paper**

The aim of *Paper I* was to evaluate the toxic activity of *p*-menthane-3,8-diol (PMD), the principal active component of the EO of the lemon eucalyptus (*Corymbia citriodora*. Hook) and to evaluate five bioassay methods, four to test oil toxicity and one for measuring the duration of toxic effect.

The aim of *Paper II* was to investigate the toxicity of two naturally occurring, commercially available and inexpensive EOs -ylang-ylang oil (YYO) from *Cananga odorata* and star anise oil (SAO) from *Illicium verum*. Toxic activity was measured in terms of optimal LC and LT.

The aim of *Paper III* and *IV* was to investigate the use of medical or economically important plants of Libya as repellents of local harmful arthropods including Acari such as scorpions and spiders. I chose five plant species *Artemisia herba-alba*, *Juniperus phoenicea*, *Origanum majorana*, *Rosmarinus officinalis* and *Salvadora persica* with the aim of extracting their essential oils and evaluating their toxic activity, including LC and LT, and investigating the active components of EOs by analysing them with (GC–MS).
Materials used in the study

Tick collection and preservation.
Unfed nymphs of *I. ricinus* were collected in a woodland area 6-8 km south of Uppsala city in east-central Sweden by dragging a 1 m² light-coloured flannel cloth over the ground vegetation (Mejlon and Jaenson 1993). The cloth was inspected at every 10 m steps, at which time all nymphs adhering to the cloth were collected. Nymphs were maintained at 85-95% relative humidity (RH) and ≈ 4°C in complete darkness for 2 months. Before testing, the nymphs were allowed to adapt to the test environment (21-23°, 85-95% RH) for 24 hours.

Plant Material.
Plant materials were collected as wild grown material in the Green Mountain (Al- Jabal Al-Akhder), a heavily forested fertile upland area in north-eastern Libya. The collection consisted of five plants divided between two research works (papers III and IV) as shown in Table 1. Plant leaves were stored in polyethylene bags in a freezer at -20 °C before steam distillation within 30 days.

Table 1. The Latin and local name, the EOs yielded from 100mg of plant material.

<table>
<thead>
<tr>
<th>No.</th>
<th>Latin name Common</th>
<th>Libyan name</th>
<th>Part used/ 100g weight</th>
<th>EO yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Artemisia herba-alba Asso</td>
<td>Sheih</td>
<td>Dry Leaves and flowers</td>
<td>2.54g</td>
</tr>
<tr>
<td>2</td>
<td>Juniperus phoenicea L.</td>
<td>Araar</td>
<td>Fresh leaves</td>
<td>2.00g</td>
</tr>
<tr>
<td>3</td>
<td>Origanum majorana L.</td>
<td>Bardagosh</td>
<td>Fresh Leaves and stem</td>
<td>3.02g</td>
</tr>
<tr>
<td>4</td>
<td>Rosmarinus officinalis L.</td>
<td>Ikleel</td>
<td>Fresh leaves</td>
<td>2.05g</td>
</tr>
<tr>
<td>5</td>
<td>Salvadora persica L.</td>
<td>Miswak</td>
<td>Fresh leaves</td>
<td>0.07g</td>
</tr>
</tbody>
</table>
Identification of volatile compounds from oils extracted by steam distillation with gas chromatography–mass spectrometry (GC-MS) analysis.

Five EOs were extracted by steam distillation of (fresh and dried) plant leaves and stems (Table 1). About 100g of each plant was subjected to steam distillation using a Clevenger-type apparatus for 3-4 hours until total recovery of oil. The distillate was collected and extracted with 100 ml of n-pentane (99%) and dehydrated with MgSO₄, after which the mixture was filtered. The EOs dried oils were weighed and stored in tightly closed glass vials at -20°C in the darkness until further investigations. The oils were analysed by gas chromatography coupled with a mass spectrometer (GC–MS) at the Department of Chemistry at the Royal Institute of Technology (KTH, Stockholm) and the
Department of Zoology at Stockholm University. Volatiles were separated by GC-MS using the Hewlett Packard GC 6890N (Agilent Technologies Inc., USA) equipped with a DB-5 column (30 m length, 0.25 mm internal diameter and 0.25 µm stationary phase film thicknesses). MSD Productivity ChemStation (v.02.01.1177, Agilent Technologies) was used to identify all compounds by comparing their mass spectra and retention indexes with those from three sources - the NIST-2008 MS library, published retention indic values and available authentic standards (in the MSD software library). The quantitative compositions of the EOs were reported as a relative percentage of the total peak area.

**Commercial essential oil (EOs)**

Three oils were commercially available, bought from (www.crearome.se), and used in papers I and II:

First, essential oil extracted from *Corymbia citriodora* (Figure 8.B), having PMD (*p*-menthane-3,8-diol) (Figure 8.A) as the main active component, was tested. The oil is normally obtained by steam distillation of the leaves. The weight of the oil is 0.92 kg/l and PMD constitutes approximately 50% w/w of the oil extracted from *C. citriodora*.

![Figure 8. Paper I. A) p-Menthane-3,8-diol (PMD). B) Corymbia citriodora (Hook.) K. D. Hill & L. A. S. Johanson, (Myrtaceae, Dicotyledonae).](image)

**Star anise oil** was obtained by steam distillation of the fruits and seeds of *Illicium verum* (Figure 12.B), the Chinese star anise, which is grown almost exclusively in southern China and Japan. The dried fruits may contain 5–8% of EO, consisting predominantly (85–90%) of anethole, i.e., *trans*-1-methoxy-4-(prop-1-enyl) benzene (Figure 9.b) Phellandrene, safrole, 1,8-cineole (Figure 9.c) *α*-terpineol, estragole, limonene, linalool, methyl-chavicol, para-
anisaldehyde and trepinen-4-ol (Figure 9.a) also present in star anise oil are known to be toxic to certain arthropods (Duke and Powles 2008).

![Figure 9. a. Terpinen-4-ol, b. cis- and trans-1-methoxy-4-(prop-1-enyl) benzene, c. 1,8-cineole.](image)

**Ylang-ylang oil** is oil from *Cananga odorata* (Figure 11.A) cultivated in Madagascar, and obtained by steam distillation of the flowers. The main constituents of the oil are linalool (Figure 10.a) β-caryophyllene (Figure 10.b) γ-muurolene (Figure 10.c) and (E,E)α-farnesene (Figure 10.d) (Baratta et al. 1998). Benzyl acetate, linalool, p-cresyl methyl ether and methyl benzoate give the oil its characteristic odour (Manner and Elevitch 2006).

![Figure 5. Principle compounds found in EOs used in this study. a. linalool, b. β-caryophyllene, c. γ-muurolene, d. (E,E)α-farnesene.](image)

![Figure 6. Plants used in paper II. A) Cananga odorata (Lam.) Hook. f. Thomson, (Annonaceae, Magnoliopsida). B) Illicium verum Hook.f., (Schisandraceae, Magnoliopsida).](image)
Method development

In the first study (Paper I), five bioassay methods (A–E) for measuring contact toxicity were evaluated to find the optimal application method, which was then used in the remaining studies (Paper II, III and IV).

A. **Open filter paper method** - a bioassay design based on the one described in (WHO 1996), where the nymphs come in contact with the natural substance on filter paper. The test is conducted with a graduated series of dosages applied to filter paper (Whatman no. 1). The filter papers were placed at the bottom of plastic cups (122 cm³ volume), where the solvent evaporated completely before introducing tick nymphs to the cups, which covered by fine-meshed cloth secured with rubber bands around the top to prevent the ticks’ escape. Each cup was put separately into a closed plastic container with wet tissue paper at the bottom to maintain high humidity.

B. **Limited exposure time method** - a method designed by myself to estimate the duration of the toxic activity. In this method we started with the same procedures as described in method A, except that after different exposure periods the nymphs were moved from impregnated filter papers to unimpregnated filter papers and kept in fresh air, the mortality was then checked every 30 min for 4.5–5 h and at 24, 48 and 72 h, to find out when the effect of the substance declined.

C. **Folded filter paper method** - based on a method described by Al-Rajhy (Al-Rajhy et al. 2003), where the filter papers were folded into “envelopes” using metal clips. The nymphs were put into each envelope and the envelopes were put, separately into Petri dishes (17 cm²), which were kept in one larger Petri dish (308 cm²). Thus, nymphs were subjected simultaneously to each concentration, and the humidity level was maintained constant by using a wet tissue paper at the bottom of the biggest Petri dish, with a glass cover on the top. Any dead nymphs were recorded at 4, 6, 12 and 24 h.
D. **Direct contact with non-absorbing surface** - to test the toxicity of the oil on a non-absorbing surface and to find the duration of toxic activity of the PMD-containing oil. Here we used cylindrical glasses that were prepared by diluting the oil in 1 ml acetone, then covering the whole area of the cylindrical glass with it. Complete evaporation of the acetone took place in a fume hood for 1 h. A fourth jar, treated with only acetone, was used as a control. Nymphs were then placed in each cylinder; the top was covered with fine meshed cloth. The number of dead nymphs were recorded after 60 min. To find the duration of toxic activity of the PMD-containing oil, new nymphs were introduce to the same oil-treated cylinders after 24 and 48 h and the procedure was repeated.

E. **Topical application method** - for each oil concentration each nymph was covered completely with the oil for 1 minute by pipetting dosage of oil on the nymphs of *I. ricinus*. The oil was diluted in 98% isopropanol (1, 2-propanediol), and by measuring the nymphal surface area we calculated the oil dosage. The mean surface area of an *I. ricinus* nymph, 0.0815 cm$^2$, was estimated from ten nymphs which were selected randomly and whose surface area was calculated using ImageJ program measurement (http://rsb.info.nih.gov/ij). For each oil concentration, each nymph was covered completely with the solution for 1 min. The nymphs were then dried with filter paper, and kept at 21°C and 85% RH in 50 ml Falcon vials. Dead nymphs were recorded every 30 min.

In the first paper, we used all five methods to determine which gave the most reliable results. Four methods measured the toxicity of the oil (A, C, D and E), and the fifth, B, estimated the duration of the toxic effect. From the results of paper I and from comparison between methods we chose only two methods for paper II (A and B), and only method (A) for papers III and IV.
Summary of papers

Summary of Paper I

The oil of *Corymbia citriodora*, lemon eucalyptus, was evaluated for its toxicity against nymphs of *Ixodes ricinus* (Acari: Ixodidae). The main arthropod-repellent compound in the oil is *para-menthane-3,8-diol* (PMD). Five methods were tested for the utility as contact toxicity bioassays to find the optimal application method. Mortality rates (number of dead nymphs) were counted at 30 min intervals during the first 5 h after the start of exposure and at longer intervals thereafter.

The nymph mortality rate increased with increasing concentration of PMD and duration of exposure. A comparison of the results from methods A, C, D and E at one fixed concentration (0.1 mg PMD/cm²) is shown in Figure 12. Methods A, C and E each gave a LC₅₀ of 0.035–0.037 mg PMD/cm², and a LC₉₅ of 0.095–0.0975 mg PMD/cm² at 4 h of ET. In terms of lethal time, the three methods gave an LT₅₀ of 2.1–2.8 h and a LT₉₅ of 3.9–4.2 h at 0.1 mg PMD/cm². Which means the three method gave almost the same results. Only method D gave an acute toxicity due to using a non-absorbing surface which kept the effect of the substance used more concentrated to the ticks, and thus do not resemble the general methods of acaricides application in which the substances usually are applied to cloth or skin, and both considered as absorbing surfaces to the EOs.

To determine the duration of PMD toxicity, concentrations of 0.002, 0.01, 0.1 mg PMD/cm² were tested. Mortality was recorded at each concentration after 1 h, after which new ticks were added to the test chamber. The results indicated that PMD retained lethal activity for 24 h but had disappeared completely by 48 h.

The overall results obtained from the different bioassays indicate potentially high toxicity of PMD on *I. ricinus* and suggest that this substance could be useful for tick control. And also suggest that method A as the optimal application since the EO was allowed to evaporate more than method C or E.
Figure 12. Results of five different methods used to assay the toxic effects of *C. citriodora* EOs against tick nymphs (Paper I). Toxicity was assayed at a fixed concentration, 0.1 mg/cm² of PMD. The LT₅₀ range was 2.1-2.8 h and LT₉₅ range was 3.9-4.2 h for methods A, C and E.

Summary of paper II

Ylang-ylang oil (YYO) from *Cananga odorata* and Star anise oil (SAO) from *Illicium verum* are both known to possess bacteriostatic and germicidal properties (Duke and Powles 2008; Kim et al. 2007). The insecticidal property of YYO has also been tested on some human ectoparasites; it has a significant and wide spectrum of acaricidal activity (Hink and Duffey 1990). We evaluated the toxicity of these oils against *I. ricinus* nymphs, including maximal LCs by testing four concentrations of each oil: 0.05, 0.1, 0.2, and 0.4 μl/cm². Testing was done by using a filter-paper method that I had previously evaluated and found to be reliable (method A, Paper I). Mortality rates were obtained by counting dead nymphs at 30-min intervals during the first 5 h of exposure and then at 24, 48 and 72 h.

For YYO, mortality increased with increasing oil concentration and time of exposure, this is especially obvious for 0.4 μl YYO/cm², for which mortality reached 95% in just 4.4h. Even with half this dose (0.2 μl YYO/cm²), 95% mortality was reached within 4.5h, which is considered an acceptable outcome (Piesman 1993). At lower concentrations of YYO, such as 0.05 and 0.1 μl/cm², the acaricidal effect was present but weak. In fact, a significant repellency effect was observed only at a concentration of 0.2 μl YYO/cm². The two highest concentrations of YYO (0.2, 0.4 μl/cm²) gave maximum LC of 50 and 95% mortality after 4.5 h exposure. Mortality of 95% was obtained after 24 h with
the next highest dose (0.1 μl/cm²), whereas LC₉₅ required 3 days with the lowest concentration of YYO (0.05 μl/cm²). The lethal effect time (LT) was correlated with the duration of exposure, with a significant effect at 0.4 μl YYO/cm² after 3 h’ (LT₅₀ = 3.2 h, LT₉₅ = 4.3 h) (Figure 13).

In contrast, only the highest concentration of SAO, 0.4 μl SAO/cm², showed significant increasing mortality with the time of exposure. This reached LT₅₀ after 10 h and LT₉₅ after 24 h. However, with the lower concentration (0.2 μl/cm²) 50% mortality was reached after 24 h and 100% at 72 h. At the lowest concentration of SAO (0.1 μl/cm²), there was 67% mortality after 48 h (Figure 14). With SAO the acaricidal activity was not as strong, but remained within the framework of what can be considered acceptable (Piesman 1993) in terms of LT (LT₅₀ = 14 h, LT₉₅ = 24 h at 0.4 μl SAO/cm²).

The study indicates that YYO at 0.2 and 0.4 μl/cm² and SAO at 0.4 μl/cm² exhibit strong acaricidal properties against nymphs of I. ricinus. These results suggest that both YYO and SAO should be considered as potentially useful substances in the control of ticks.

Figure 13. Mortality (%) of I. ricinus nymphs exposed to different concentrations of YYO (µl/cm²) and different ETs (hours).
Summary of Paper III

In Libya, many plants have medicinal or economic importance and have had a deep impact on the life and culture of the Libyan people. For example, *Salvadora persica*, commonly known as Miswak, and Libyan Rosemary leaves, *Rosmarinus officinalis*, have a long history of use in local traditional medicine. In addition, EOs from these plants are widely available from the flavour and fragrance industries, which gives a possible fast track to commercialization of acaricides based on these oils. These plant species and their EOs were investigated for their possible acaricidal and repellent effects. Miswak and Rosemary leaves were collected from Libya and their EOs extracted by steam distillation. The resulting oils were then tested on *Ixodes ricinus* nymphs using a “open filter paper method” (method A, Paper I). The chemical composition of the oils was also analysed using GC-MS.

*R. officinalis* EO diluted in acetone to 0.5 µl/cm² and 1µl/cm², exhibited 20% and 100% mortality, respectively, after about 5 h of ET. A total of 50% and 95% of *I. ricinus* nymphs were killed by direct contact with the oil when exposed to 1µl/cm² *R. officinalis* oil, giving estimated LC values of 0.7µl/cm² (LC₅₀) and 0.95 µl/cm² (LC₉₅). The LC₃₀ for the lower concentration of oil (0.5µl/cm²) was reached before the end of the first 24 hours of ET, as tick mortality at 24 hours was 60% (Figure 16). For *S. persica* leaf EO, a concentration of 1µl/cm² was found to have a significant repellency effect against *I. ricinus* nymphs. A 95% repellency was observed at this concentration of *S. persica* for up to 1.5 hours exposure. This gradually reduced to 50% repellency at an estimated 5.45 hours. No significant mortality was recorded at this concentration.
dose of *S. persica* oil even after 24 hours exposure. GC-MS analysis of the EOs from both plants showed that the main components of both oils were monoterpenes, particularly 1,8-cineol, α-pinene, and β-pinene. However, these components were in markedly different proportions in the two oils. Although both oils were rich in 1,8 cineol (20.8% for *S. persica* and 24.07% for *R. officinalis*) (Figure 15.b.), *R. officinalis* was richer in α-pinene (13.03% vs 5.9% in *S. persica*) (Figure 15.c.), while *S. persica* was richer in β-pinene (16.8% vs 2.45% in *R. officinalis*) (Figure 15.d.). The main component (2E)-hexenal (32.7%), (Figure 15.a.) that was present in *S. persica* but absent in *R. officinalis* oil was found to have a significantly repellent effects on the adult brown plant hopper *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) (Zhou et al. 2003).

We conclude that both of the oils used in this study show effects against *I. ricinus* ticks. However, *S. persica* oil appears to have a stronger and more long-lasting repellency effect than *R. officinalis* oil, while only *R. officinalis* oil had a toxic effect against *I. ricinus* nymphs. Thus, both EOs show potential merits as alternative approaches for *I. ricinus* tick control.

![Figure 15. The main components found in EOs used in paper □; a. (2E)-Hexenal (32.7%) only in *S. persica* oil, b. 1,8-cineol (20.8% for *S. persica* and 24.07% for *R. officinalis*), c. α-pinene (5.9% for *S. persica* and 13.03% for *R. officinalis*), d. β-pinene (16.8% for *S. persica* and 2.45% for *R. officinalis*).](image)

![Figure 16. Mortality of *I. ricinus* nymphs exposed to *R. officinalis* oil over 24 hours. *I. ricinus* nymphs were exposed to 0 (control), 0.5 and 1.0 μl/cm² of *R. officinalis* oil. Nymph mortality was recorded every 0.5 h over a 5 h period and then again at 24 h.](image)
**Summary of Paper IV**

EOs were extracted from leaves of four plants native to Libya, Libyan white wormwood (*Artemisia herba-alba* Asso), marjoram (*Origanum majorana* L.) and Ar-aar (*Juniperus phoenicea* L.). Oils were tested for their effects on *Ixodes ricinus* L. tick nymphs (Acari: Ixodidae), which were evaluated using a bioassay “open filter paper method”. Two dilutions were tested for each of the three EOs, 0.5 µl/cm² and 1.0 µl/cm². The toxic effect was calculated in terms of LC₅₀, LC₉₅ and LT₅₀, LT₉₅, corresponding to the concentrations and times required to kill 50% and 95% of the nymphs, respectively. Mortality rates for five hours were obtained by counting the surviving nymphs every 30 minutes for the first five hours and then every 24 h for 3 days.

100% mortality was produced with the higher concentration EOs (1µl/cm²) of *A. herba-alba* and *J. phoenicea* within the first 2 hours of exposure. On other hand, 100%, mortality with *O. majorana* was not reached until the third day (72h). However, the LC₅₀ of mortality reached within the first 24h of ET at 0.5µl/cm² of *O. majorana*, produced 60% tick’s mortality at 24h (Figure 18). In contrast at 0.5µl/cm² concentration of *A. herba-alba* LT₅₀=1.7h, LT₉₅ = 2.75h and at 1µl/cm² LT₅₀=1.3h, LT₉₅ =1.9h (Figure 19). No significant difference between two dosages, both gave almost the same significant toxicity at nearly same time range. Chemical composition of the EOs was elucidated by GC-MS analyses and the results indicate that thujone (Figure 17.b.), 1,8-cineol (Figure 15.b.), β-thujanone and camphor (Figure 17.a.) were the most abundant components in *A. herba alba*, while the major components of *J. phoenicea* were α-terpinyl acetate (Figure 17.e.), α–pinene (Figure 15.c.) and germacene D (Figure 17.c.). Finally, terpinen-4-ol (Figure 9.a.), linalool (Figure 10.a.) and α-terpineol (Figure 17.d.) were the main components in *O. majorana* oil. The results suggest that that *A. herba* and *J. phoenicea*, are especially promising candidates for alternative approaches for *I. ricinus* tick control, either alone or in combination with other oils.

![Figure 17. The main active components found in the oils used in paper IV](image1)

**Figure 17.** The main active components found in the oils used in paper IV. **a.** camphor, **b.** thujone, **c.** germacene D, **d.** α-terpineol, **e.** α-terpinyl acetate.
Figure 18. Comparison between % mortality of *I. ricinus* exposed to the three oils in paper IV at fixed concentration 1 µl/cm²

Figure 19. Comparison between % mortality of *I. ricinus* exposed to the three oils in paper IV at fixed concentration 0.5 µl/cm²
Concluding remarks

These studies identified a number of natural compounds that are potentially useful for the control of ticks. These include the oil of *Corymbia citriodora*, (Paper I), Ylang-ylang oil from *Cananga odorata* and star anise oil from *Illicium verum* (Paper II), oil of *Salvadora persica* and *Rosmarinus officinalis* (Paper III), and oils from three plants from Libya, Libyan white wormwood (*Artemisia herba alba*), marjoram (*Origanum majorana*) and Ar-aar (*Juniperus phoenicea*) (Paper IV).

The most potent oils appears to be those from *A. herba alba*, *J. phoenicea*, *R. officinalis*, *C. citriodora* and ylang-ylang, followed by Star anise and *O. majorana*. In a fact, *A. herba alba*, *J. phoenicea*, *R. officinalis* yielded 100% mortality within 2–5 hours at 1µl/cm², and even at a lower dose 0.5 µl/cm² nearly all nymphs were dead within 24 hours (Paper III, IV). GC-MS analysis also indicates that most of these oils tend to be rich in monoterpeneoids. This suggests it may be possible to screen natural compounds directly by looking for the presence of these oils before undertaking the labour and time intensive work of bioassays.

However, all of them have significant acaricidal effects by accepted standards, as well as some repellency effect. The only one that doesn’t have a toxic effect was *S. persica* but this still has a repellency effect at the dose we used. Thus, all the EOs used in this study show a broad spectrum of effects against *I. ricinus* nymphs. Even the oils that gave a weak result (as Star anise or the *O. majorana* oil) could be more effective if used at a higher concentration and/or over longer periods. The oils could also potentially be used in combination to produce stronger, broader, faster and longer-lasting effects.

It appears that some chemical constituents of these oils such as α-pinene, β-pinene, 1,8-cineol, camphor, linalool, terpinene-4-ol, α-terpinol, β-caryophyllene and β-thujanone, found to have an acetylcholines-terase activity in arthropods and interfere with the nervous system in invertebrates, Among other things, this has the advantage of being a target site not shared with mammals (Jankowska et al. 2017). This make them a good potential alternative for controlling ticks and tick-born diseases.
Svensk sammanfattning

Fästingar är det gemensamma namnet för de små spindeldjur i ordningen Ixodida som, tillsammans med andra kvalster, utgör subklassen Acari. Fästingar är en grupp av ektoparasiter som uteslutande livnär sig på blod från främst däggdjur och fåglar, men även från reptiler och groddjur. Fästingar återfinns över hela världen och de är de viktigaste vektorerna av sjukdomsframkallande organismer för människor och djur på norra halvklotet.

Fästingar och fästingburna sjukdomar har under de senaste åren fått stor uppmärksamhet på grund av de medicinska effekter som har påverkat folkhälsan negativt. Fästingar påverkar människors och djurs hälsa i hela världen och är orsaken till betydande eko-ekonomiska förluster. Utvecklingen av en effektiv och miljömässigt säker kontroll har därför blivit högt prioriterad. Allmänhetens oro för miljöpåverkan och för säkerheten hos kemiska substanser driver forskningen mot alternativa, hållbara metoder för fästingkontroll, som t.ex. växtbaserade acaricider.


Det finns två huvudsakliga sätt att kontrollera fästingar: Kemisk kontroll genom användandet av syntetiska substanser och kemikalier extraherade från växter men även feromoner och biologisk kontroll, som är miljömässigt säkrare men dyrare och svårare att utveckla. Syftet med mitt avhandlingsarbete är att utvärdera potentialen hos utvalda växter som källor till effektiva och
billiga akaricider, d.v.s. medel för att bekämpa fästingar. Många naturliga substanser har en relativt låg toxicitet för däggdjur och sönderdelas snabbt i miljön, egenskaper som gör dem till attraktiva alternativ till syntetiska akaricider. 

Denna studie undersöker och utvärderar alternativa naturliga kemiska föreningar från extrakt av flera olika växter för kontroll av fästingar och därigenom fästingburna sjukdomar. Ett annat mål i denna avhandling var att bestämma toxicitet mot fästingar av dessa föreningar i laboratoriet, samt att hitta de optimala applikationsmetoderna och de optimala doserna för att kontrollera fästingar, tillsammans med en uppskattning av varaktigheten av den toxiska aktiviteten.

I artikel I utvärderade vi olja från *Corymbia citriodora* som bekämpningsmedel av nymfer av *Ixodes ricinus* genom att bestämma toxiciteten hos para-mentan-3,8-diol (PMD), den viktigaste arthropodavstötande föreningen i oljan. Ämnet testades mot nymfer av *I. ricinus* genom att använda fem olika metoder. De övergripande resultat som uppnåtts hittills från de olika bioanalyserna indikerar en potentiellt hög toxicitet hos PMD på *I. ricinus* och ger vid handen att detta ämne kan vara användbart för fästingkontroll.

I artikel II identifierar vi att Ylang-ylang olja (YYO) och Star Anis olja (SAO) uppvisar starka akaricida egenskaper mot nymfer av *I. ricinus* och föreslår att även dessa två oljor bör utvärderas som potentiellt användbara vid kontroll av fästingar. Många tidigare studier har dokumenterat toxiciteten och akaricidegenskaperna hos de kemiska komponenterna i YYO och SAO mot många olika arthropoder.


potential som naturliga akaricider mot *I. ricinus*. Under de senaste två decennierna tyder många undersökningar på att vissa kemiska beståndsdelar i dessa oljor påverkar nervsystemet hos leddjur, men inte hos däggdjur, vilket är fördelaktigt.

Faktum är att de flesta essentiella oljorna är relativt ogiftiga för däggdjur och fiskar i toxikologiska tester och de uppfyller sålunda kriterierna för bekämpningsmedel med reducerad risk. Detta gör dem till ett bra alternativ för att kontrollera fästingar och fästingburna sjukdomar.
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