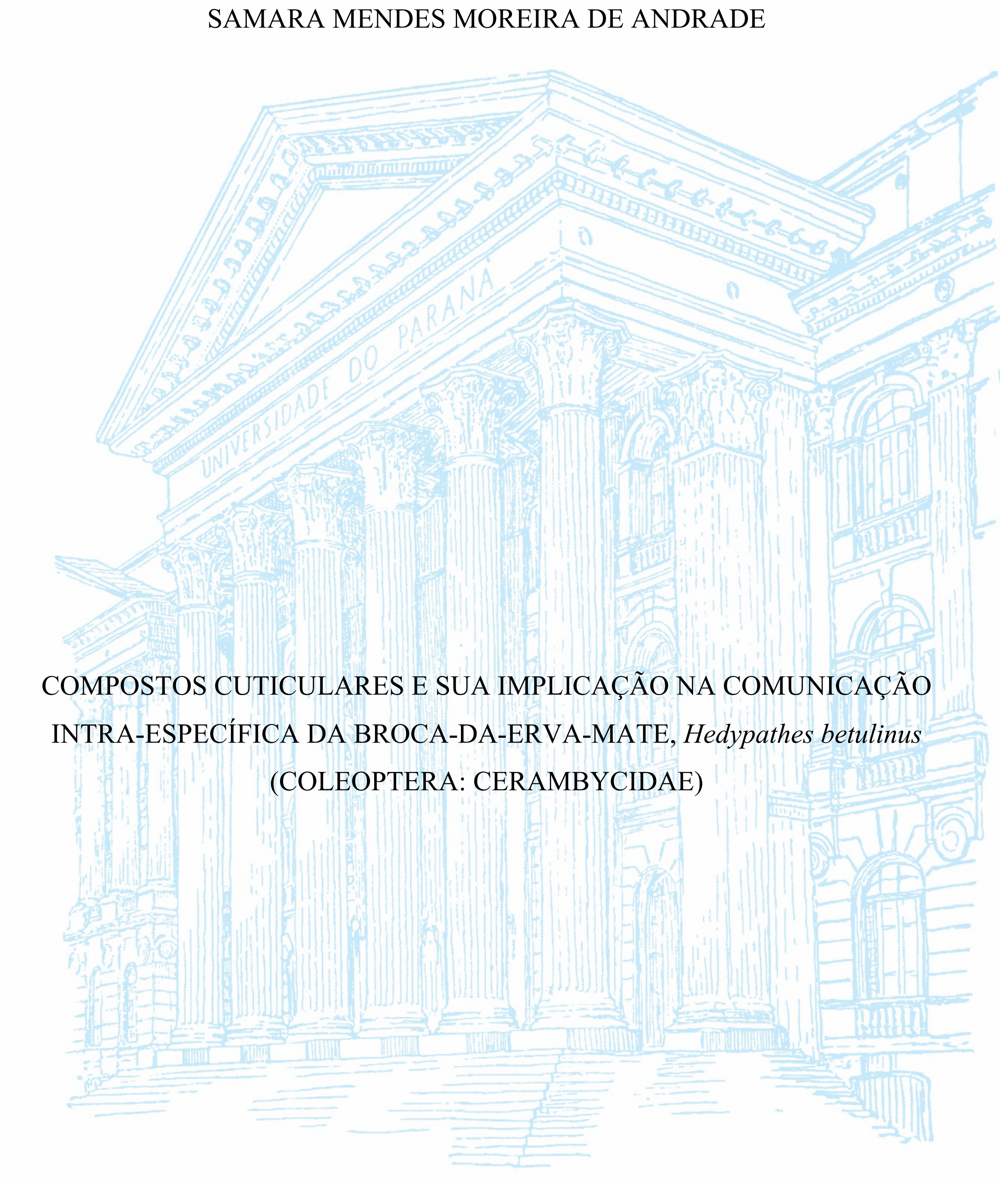
UNIVERSIDADE FEDERAL DO PARANÁ

SAMARA MENDES MOREIRA DE ANDRADE



COMPOSTOS CUTICULARES E SUA IMPLICAÇÃO NA COMUNICAÇÃO INTRA-ESPECÍFICA DA BROCA-DA-ERVA-MATE, *Hedypathes betulinus* (COLEOPTERA: CERAMBYCIDAE)

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Orientador: Prof. Dr. Paulo Henrique Gorgatti Zarbin
Coorientador: Dr. Jeremy Dean Allison

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Curitiba, 28 de Maio de 2018.

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RESUMO

O principal objetivo deste estudo foi avaliar o papel dos feromônios de contato das fêmeas no comportamento sexual de *Hedypathes betulinus*. Em laboratório, extratos cuticulares de *H. betulinus* foram manipulados de forma a fracionar as três diferentes classes de compostos presentes: fração H (alcanos saturados e metil ramificados), fração C (alquenos saturados) e fração E (compostos polares). Fêmeas de *H. betulinus* foram manipuladas para produzir três tratamentos, e sequencialmente apresentadas a machos: 1) morte por congelamento a -20°C; 2) extração de compostos epicuticulares; 3) reconstituição do extrato bruto, misturas individuais, binárias ou terciária de frações. Nossos resultados indicam que machos de *H. betulinus* dependem de pistas químicas para identificar fêmeas coespecíficas, os compostos químicos presentes na camada de cera epicuticular das fêmeas são indispensáveis no reconhecimento sexual desta espécie e os compostos necessários e suficientes para estimular a atividade de cópula em *H. betulinus* estão presentes na fração E. Análises de extratos epicuticulares brutos de indivíduos machos e fêmeas por cromatografia gasosa acoplada a espectrometria de massas (GC-EM) mostram diferenças nas concentrações dos compostos. Os únicos compostos macho-específicos encontrados foram (*E*)-geranilacetona e (*R*)-(*E*)-acetato do fuscumol, que são parte do feromônio sexual de longo alcance produzido por machos *H. betulinus*. (*R*)-(*E*)-acetato do fuscumol também foi encontrado em análises cromatográficas de frações E contendo extratos epicuticulares de cinco fêmeas. Esse composto apresenta concentrações muito diferentes em frações E de machos e fêmeas e bioensaios de acasalamento em laboratório mostram que o equivalente de fêmea do (*R*)-(*E*)-acetato do fuscumol sintético é suficiente para estimular a resposta de cópula em machos *H. betulinus* observada no bioensaio da fração E.

Palavras-chave: Besouros serra-pau. Reconhecimento sexual. Feromônio de contato.

ABSTRACT

The main goal of this study was to evaluate the role of female contact pheromones in the mating behaviour of *Hedypathes betulinus*. In laboratory, cuticular extracts of *H. betulinus* were fractionated in the three different classes of compounds present in it: Fraction H (saturated and methyl-branched alkanes), Fraction C (saturated alkenes) and Fraction E (more polar compounds). Females of *H. betulinus* were manipulated to produce three treatments, and sequentially presented to males: 1) death by freeze at -20°C; 2) extraction of epicuticular compounds; 3) reconstitution of the crude extract, individual, binary or tertiary blends of fractions. Our results indicate that male *H. betulinus* rely on chemical cues to identify conspecific females. The chemical compounds present on the epicuticular wax layer of females are required in mate recognition of this species. The necessary and sufficient compounds to stimulate mating activity in *H. betulinus* are present in the Fraction E. Analyses of male and female epicuticular crude extracts and fractions by gas chromatography along with mass spectrometry (GC-MS) showed diferences in concentrations of compounds. The only male-specific compounds were identified as (*E*)-geranilacetone and (*R*)-(*E*)-fuscumol acetate, which are part of the long-range sex-pheromone produced by males *H. betulinus*. (*R*)-(*E*)-fuscumol acetate is also present in chromatografic analyses of fractions E that contain epicuticular extracts of five females. This compound has very different concentrations in male and female Fractions E and mating bioassays in laboratory show that the female equivalent of this synthetic compound is sufficient to recover mating response in *H. betulinus* males observed in the fraction E bioassay.

Key-words: Sawyer beetles. Mate recognition. Contact pheromones.

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SUMMARY

1 INTRODUCTION

1.1 CHEMICAL COMMUNICATION OF INSECTS

Chemical compounds involved in the interactions and communication between insects and other organisms are called semiochemicals (LAW & REGNIER, 1971; NORDLUND & LEWIS, 1976; ZARBIN *et al.*, 2009). These chemical compounds are produced and released by one individual to another with the purpose of changing the behaviour of the receiver (NORDLUND & LEWIS, 1976; COOK *et al.*, 2007). Semiochemicals associated with interactions between organisms of different species are called allelochemicals, while pheromones are the semiochemicals involved in interactions between organisms of the same species (FIGURE 1) (BUTLER, 1970; HAPP, 1973; ZARBIN *et al.*, 2009).

Allelochemicals are defined according to the benefits they confer to the emitter and/or the receiver and include: 1) Allomones, when the emitter species benefits; 2) Kairomones, when the receiver benefits and; 3) Synomones, when both species benefit (emitter and receptor) (BROWN, 1968; BROWN *et al.*, 1970; WHITTAKER & FEENY, 1971; ZARBIN *et al.*, 2009; ALI *et al.*, 2015). Pheromones are classified based on the receptor species behavior they are associated with (KARLSON & LÜSCHER, 1959; NORDLUND & LEWIS, 1976; ZARBIN *et al.*, 2009; ALI *et al.*, 2015). For example, sex pheromones are involved in the attraction of individuals of the opposite sex for reproduction; aggregation pheromones are released to induce host detection and selection by a group of individuals to beat host resistance by mass attack, as a mechanism of defense against predators or mate selection; alarm pheromones are released to warn other individuals that there is a threat nearby; host marking pheromones increase the survivorship of insects of the same species by reducing competition between them; trail pheromones are very common in social insects and they are produced and released with the intention of leading the way to a recently discovered food source etc (BLUM & BRANDT, 1962; BLUM, 1969; BUTLER, 1970; ALI *et al.*, 2015; SARLES *et al.*, 2015).

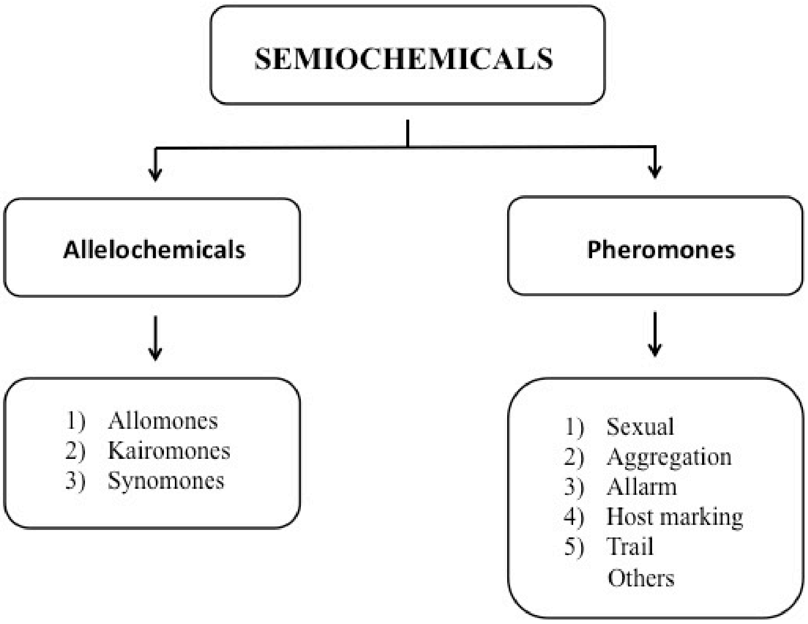

FIGURE 1: Classification of semichemicals (Adapted from Zarbin *et al.*, 2009).

1.2 CHEMICAL COMMUNICATION OF CERAMBYCIDS

Sex pheromones play an important role mediating mating behavior of Cerambycidae species and can be used for the development of detection and management strategies (ALLISON *et al.*, 2004; MILLAR *et al.,* 2009). Mate location in Cerambycidae generally involves pheromones that act over long and short distances (HANKS, 1999). Volatile attractant pheromones act over long distances and can be categorized in two different groups: sex pheromones and aggregation-sex pheromones (CARDÉ, 2014; HANKS & MILLAR, 2016). Sex pheromones are usually produced by females and attract only males, while aggregation-sex pheromones are produced by males and attract both sexes (HANKS & MILLAR, 2016). Species from the subfamilies Prioninae and Lepturinae are known to use sex pheromones, including (*3R,*5S)-3,5-dimethyldodecanoic acid (prionic acid) and 2,3-alkanediols from Prioninae species and *cis*-vaccenyl acetate from Lepturinae species (see HANKS & MILLAR, 2016).

Aggregation-sex pheromones are produced by species of Cerambycinae, Lamiinae and Spondylidinae and are more diverse than the sex pheromones (HANKS & MILLAR, 2016). In the Cerambycinae subfamily, aggregation-sex pheromones are usually hydroxyketones and 2,3-alkanediols, but unsaturated straight chain and methyl branched alcohols and aldehydes, terpenoids, pyrone, tetrahydropyran, and several cyclic terpenoid derivatives have also been identified (see HANKS & MILLAR, 2016).

Geranylacetone (E)-6,10-dimethyl-5,9-undecadien-2-one, the related alcohol (E)-6,10-dimethyl-5,9-undecadien-2-ol (known as fuscumol) and its acetate ester (E)-6,10-dimethyl-5,9-undecadien-2-yl acetate (fuscumol acetate) individually or in blends, and hydroxyethers and its corresponding compounds have been identified as sex-aggregation pheromones in the Lamiinae subfamily, while all known pheromones of Spondylidinae are of geranylacetone structural category (see HANKS & MILLAR, 2016). Fonseca *et al.* (2010) identified the sex pheromone of *H. betulinus* as a mixture of (*E)*-geranylacetone, (*E*)-fuscumol and (*E)*-fuscumol acetate. The sex-aggregation pheromone is produced and released by male *H. betulinus* and the terciary mixture of those compounds (alone and in combination with host plant volatiles) showed attractiveness to females in laboratory assays (FONSECA *et al.*, 2010).

Contact pheromones are also important in the mating recognition system of Cerambycidae (GINZEL *et al.*, 2006). Evidence exists for several species that males recognize females through contact chemoreception using the antennae (HANKS, 1999; ALLISON *et al.*, 2004). The cuticular wax layer of insects is composed of a mixture of long-chain alkanes, wax esters, fatty acids, alkenes, aldehydes and sterols (BLOMQUIST, 2010). Although their primary purpose is to maintain water balance and protect the insect from desiccation (BLOMQUIST & BAGNÈRES, 2010; GIBBS & RAJPUROHIT, 2010) they are also often involved in insect communication. Epicuticular hydrocarbons are involved in mate and colony recognition, chemical mimicry, dominance and fertility cues and primary pheromones. The organization of the hydrocarbons in the cuticular wax layer of insects is still unknown, but compounds of major importance in chemical communication are likely present in the most external layer of the cuticle (GINZEL *et al.*, 2003b; GINZEL, 2010). Three major groups of epicuticular hydrocarbons have been identified: 1) *n*-alkanes; 2) methyl branched compounds and; 3) unsaturated hydrocarbons (BLOMQUIST, 2010). Even though short-range sex pheromones are usually comprised of cuticular hydrocarbons, ketones and structurally complex lactones have also been identified as Cerambycidae contact sex pheromones (YASUI *et al.*, 2003; YASUI *et al.*, 2007). There are also studies that show that females of few Cerambycidae species can leave traces of cuticular compounds on bark or other substrates upon which they have walked for males to follow (WANG *et al.*, 2002).

1.3 MATE LOCATION AND MATING BEHAVIOUR OF CERAMBYCIDS

An improved understanding of the mating behaviour and mate location systems can be useful in the development of monitoring and control measures for Cerambycidae species (ALLISON *et al.*, 2004). In general, male cerambycids directly approach females and attempt to copulate with them without displaying any kind of precopulatory courtship behaviour (HANKS, 1999). In species that do not feed as adults, females are refractory to precopulation because of their short lifespan and limited oviposition chances (i.e. time spent in copula reduces oviposition). Conversely, species that feed as adults are more receptive to precopulatory courtship and the duration of copulation can vary among species (HANKS, 1999).

Hanks (1999) categorized Cerambycidae species according to the condition of the larval host plant at the time of colonization: (*i*) healthy host includes species that oviposit in vigorous plants; (*ii*) weakened host includes species that attack live or growing plants with defenses compromised by environmental stresses or attack of other insects; (*iii*) stressed host includes species that oviposit in severely stressed hosts that are likely to die due to the attack of other organisms or to intense drought and; (*iv*) dead host consists of species that only attacks hosts that are no longer alive. The duration of copulation in species with females that oviposit alone in healthy or weakened hosts is usually long, while the duration of copulation is usually brief in species that oviposit in stressed hosts (Hanks, 1999). In the latter case, the male follows the female while she searches for an oviposition site, repeating copulation and fending off conspecific males (Hanks, 1999). Due to diferences in reproductive behavior, some species require prolonged (i.e. *Chlorophorus varius*, *Dirphya nigricornis* and *Psacothea hilaris*), repeated (i.e. *Acalolepta luxuriosa* and *Xystrocera globosa*) and only a single copulation (i.e. *Oberea schaumii*, *Hylotrupes bajulus*, *Monochamus alternatus*, *Saperda inornata* and *Xylotrechus pyrrhoderus*) to realize their potential fecundity (HANKS, 1999).

Fonseca & Zarbin (2009) used laboratory assays to document the mating sequence of *H. betulinus*. The mating sequence of *H. betulinus* was described as (*i*) the female walks toward the male suggesting that chemical and/or visual cues produced by the male attracts the female; (*ii*) the female touches the male antennae with her antennae; (*iii*) immediately the male-female antennal contact is made the male mounts the female, rotates 180° and grasps the female pronotum or elytra with his forelegs and (*iv*) the male attempts to copulate with the female bending his abdomen towards the female and coupling male-female genitalia. Fonseca & Zarbin (2009) observed that males only tried to copulate with females after antennal contact was made, suggesting the presence of contact pheromones in *H. betulinus*. After mating, mate-guarding occurs (i.e. the male grasps the female pronotum or elytra and remains mounted on the female after copulation), preventing female copulation with other males and ensuring paternity (FONSECA & ZARBIN, 2009).

1.4 THE USE OF PHEROMONES IN INTEGRATED PEST MANAGEMENT

One of the most significant technological innovations in the management and control of insect pests involves the use of synthetic pheromones constructed based on actual insect pheromones, and other attractants already identified (PINTO-ZEVALLOS & ZARBIN, 2013). Pheromones are used in integrated pest management (IPM), which is based on observation and collection of data in order to apply as many management strategies as necessary to control pest insect populations (ALLISON *et al.*, 2004; ZARBIN *et al.*, 2009). Pheromone baited traps are mostly used to detect the presence, density and distribution of pest insect populations in a crop area and determinate whether it may be causing economical losses, with the goal of restricting the use of insecticides and reduce the economical and environmental costs of a crop (ZARBIN *et al.*, 2009; PINTO-ZEVALLOS & ZARBIN, 2013). However, pheromone baited traps can also be used as a method of control, reducing pest insect populations (ALLISON *et al.*, 2004; ZARBIN *et al.*, 2009; WITZGALL *et al.*, 2010; PINTO-ZEVALLOS & ZARBIN, 2013). For example, in mass-trapping or attract-and-kill techniques, synthetic pheromones are applied as an attractant to the pest insect with the intention of capturing the highest number of individuals as possible in a large-capacity trap or an insecticide-impregnated target, aiming to eliminate or decrease pest insect populations in the crop (ZARBIN *et al.*, 2009; WITZGALL *et al.*, 2010; PINTO-ZEVALLOS & ZARBIN, 2013). Another way of using pheromones as a control method is releasing high quantities of synthetic pheromones in the crop area where is necessary to control pest insect populations in order to decrease or avoid mate location, reducing mating chances and, consequently, the number of individuals of the next generation (ZARBIN *et al.*, 2009; PINTO-ZEVALLOS & ZARBIN, 2013).

There are many benefits in using semiochemicals as a method of control of pest insects in crop fields including the high selectivity of the chemical compounds, since it is effective in lower concentrations in comparison to the concentrations of the agrochemicals currently used, as well as low risk of contamination (COOK *et al.*, 2007; SUCKINLG, 2015; YEW & CHUNG, 2015). In addition, the use of semiochemicals prevent uncontrolled large scale application of agrochemicals in crops, which can lead to pest insects resistance (RAGUSO *et al.*, 2015).

1.5 STUDY SYSTEM

Maté, *Ilex paraguariensis* (St. Hilaire, 1920) (Aquifoliaceae), is native to South America and its distribution includes Brazil (States of Paraná, Santa Catarina, Rio Grande do Sul, Mato Grosso do Sul, São Paulo, Minas Gerais and Rio de Janeiro), Argentina (Provinces of Misiones, Tucumã and Corrientes) and Paraguay (area between Paraná and Paraguai rivers) (EMBRAPA, 2010). Maté is planted over 540.000 km^2, of which 450.000 km^2 occurs in Brazil, representing 83% of the native distribution of maté and 5% of the national territory of Brazil (OLIVEIRA & ROTA, 1983; EMBRAPA, 2010).

The state of Santa Catarina, the southern-central region of Paraná and in the north-central area of Rio Grande do Sul in Brazil are responsible for 99% of the comercial production of maté in Brazil (OLIVEIRA & ROTA, 1983; EMBRAPA, 2010). According to IBGE (2016), in 2016 the South of Brazil produced 346.769 tons of green leaves of maté, resulting in an income of R$507.654.000,00. Paraná is the biggest producer of maté in Brazil (86.4% of the national production), followed by Santa Catarina (8.3%) and Rio Grande do Sul (5.2%) (IBGE, 2016). *Ilex paraguariensis* is cultivated in approximatelly 180.000 rural properties distributed in 480 municipalities of Southern Brazil (EMBRAPA, 2010). Its harverst has an important economic and social impact in the region, being the main income source for small producers and generating at about 700.000 jobs, especially during harvest season (from June to August) (EMBRAPA, 2010).

Maté is widely consumed in Brazil, Argentina, Uruguay and Paraguay as chimarrão, tererê (both made from the green dried and crumbled leaves), maté cocido or tea (made from roasted leaves) (BASTOS *et al.*, 2007). Due to its high concentrations of caffeine, phenolic compounds and saponin, interest in the therapeutic uses of maté has increased recently (BASTOS *et al.*, 2007; EMBRAPA, 2010). The therapeutic effects of *I. paraguariensis* include antioxidant (FILIP *et al.*, 2000; BRACESCO *et al.*, 2003) and diuretic effects (GÖRGEN *et al.*, 2005), stimulant of thermogenesis (ANDERSEN & FOGH, 2001) and the central nervous system (see BASTOS *et al.*, 2007), improved digestion (GORZALCZANY *et al.*, 2001) and vasodilatation (STEIN *et al.*, 2005), and inhibition of hyperglicemia (LUNCEFORD & GUGLIUCCI, 2005). Maté leaves have also being explored for the development of numerous products including energy drinks and powders, weight loss supplements, cosmetics and treatments against insomnia (see BASTOS *et al.*, 2007).

FIGURE 2: a) *Ilex paraguariensis*; b) *Ilex paraguariensis* crop.

The control of pest insects is one of the biggest challenges to be overcome by the green maté producers (FONSECA & ZARBIN, 2009). Eighty-six arthropod species have been identified feeding on different parts of the plant, but *Ceroplastes grandis* (Hempel, 1900) (Homoptera: Coccidae), *Thelosia camina* (Schaus, 1920) (Lepidoptera: Eupterotidae)*,* *Hylesia* sp. (Lepidoptera: Eupterotidae), *Gyropsylla spegazziniana* (Lizer, 1917) (Homoptera: Psyliidae) and *Hedypathes betulinus* (Klug, 1825) (Coleoptera: Cerambycidae: Lamiinae) are considered the major pests of green maté (Iede & Machado, 1989). *Hedypathes betulinus* is considered the most important insect pest of green maté, resulting in severe damage to plants and significant economic losses (CASSANELLO, 1993; GUEDES *et al.*, 2000).

Economic losses associated to *H. betulinus* are mainly caused by the larval stage, which can destroy up to 60% of maté crops (CASSANELLO, 1993). Female *H. betulinus* oviposit only one egg per branch usually in openings in the trunk or in the base of the stalk of plants, but they can also oviposit in exposed roots or recently clipped parts of the plant (CASSANELLO, 1993; PENTEADO, 1995). The larvae feeds in the branches and twigs, building longitudinal subcortical galleries that block natural sap flow of the plant (CASSANELLO, 1993) (FIGURE 3c – p. 24). In addition, large quantities of sawdust are produced during larval activity, which protects the larvae, and can block sap flow of the entire plant if it reaches the roots (CASSANELLO, 1993). Plants infested by *H. betulinus* larvae usually have a reduced number of leaves, yellow leaves, broken branches and often a large amount of wood sawdust can be found in the base of the stalk of infested plants (CASSANELLO, 1993; GRIGOLETTI JÚNIOR *et. al*, 2000), making them easy to identify.

1.6 THE MATÉ BORER, *Hedypathes betulinus*

Hedypathes betulinus (Klug, 1825) (Coleoptera: Cerambycidae: Lamiinae) is also known as the maté borer or corintiano (CASSANELLO, 1993). Adult beetles are aproximatelly 25mm in length and are easily identified by their colour pattern, black colour with white bristles distributed mainly on the elytra, and the long antennae, characteristic of members of the Cerambycidae (IEDE & MACHADO, 1989; CASSANELLO, 1993) (FIGURE 3a). Sexual dimorphism is present in the scapes of the antennae and in the femurs of all three pairs of legs, which are larger in males than in females (CASSANELLO, 1993). Adults feed on the bark of branches and trunk of maté plants and females usually feed more than males (GUEDES *et. al*, 2000) (FIGURE 3b). Adult beetles are present in maté fields from September to March, with peak populations usually occurring in December (PENTEADO, 1995).

The egg is eliptical, has a smooth surface and a white-yellowish colour, and it takes approximatelly 12 days to the emergence of the larvae. The larvae is white, legless and takes approximately 278 days to complete development, consisting of eight to ten instars (CASSANELLO, 1993). The pre-pupal period consists of approximatelly 7 days and the pupal period starts when the larvae finishs building the pupal chamber and rests in a retracted and curved position. The pupal period lasts approximatelly 20 days until the emergence of the adult (CASSANELLO, 1993). In the laboratory, the life cycle of males takes approximatelly 423 compared to 490 days in females (CASSANELLO, 1993).

FIGURE 3: a) Male *Hedypathes betulinus*; b) Damage on *Ilex paraguariensis* caused by *Hedypathes betulinus* adults; c) Damage on *Ilex paraguariensis* caused by *Hedypathes betulinus* larvae.

1.7 CONTROL AND MONITORING METHODS OF *H. betulinus*

Insecticide application is prohibited in maté crops by law due to the lack of effective low toxicity compounds (SOARES & IEDE, 1997). Additionally, only adults would be susceptible to foliar applications as *H. betulinus* spends its larval and pupal period protected inside the branches and twigs of the host plant (SOARES & IEDE, 1997). Collection of adults by hand is widely practiced for the control of *H. betulinus* populations and it can be effective in small crop areas (SOARES & IEDE, 1997; FONSECA & ZARBIN, 2009). When removal of adults by hand is not practiced, populations of the maté borer can increase more than 3-fold in a year (SOARES & IEDE, 1997). However, this method of control is labor intensive and it has to be practiced multiple times during the adult flight period (SOARES & IEDE, 1997). Maté producers usually combine the active collection of adult *H. betulinus* with removal of infested trunks and branches to reduce the re-infestation of crops the following year (FONSECA & ZARBIN, 2009).

An alternative to the manual removal of adult *H. betulinus* is biological control. This managenment tactic uses natural enemies to control the population density of pest insects or weeds (BORGES, 2007). The main natural enemies of *H. betulinus* include an egg parasitoid *Eurytoma* sp. (Illinger, 1807) (Hymenoptera: Eurytomidae); the egg predators *Solenopsis* sp. (Westwood, 1840) and *Pheidole* sp. (Westwood, 1839) (Hymenoptera: Formicidae); the larval parasitoid *Labena* sp. (Cresson, 1864) (Hymenoptera: Ichneumonidae) (PAGLIOSA *et al.*, 1994; SOARES, 1998); and the adult predators *Alcaeorrhynchus grandis* (Dallas, 1851), *Brontocoris tabidus* (Signoret, 1852), *Tynacantha marginata* (Dallas, 1851) (Hemiptera: Pentatomidae), *Arilus* *carinatus* (Forster, 1771) and *Apiomerus* sp. (Hahn, 1831) (Hemiptera: Reduviidae) (SAINI *et al.*, 1993; SOARES *et al.*, 1995; DIAZ, 1997; SOARES & IEDE, 1997; SOARES, 1998; PENTEADO *et al.*, 2000). Studies have shown that *H. betulinus* is susceptible to attack by the nematode *Steinernema carpocapsae* (Weiser, 1955) (Rhabdita: Steinernematidae) (ALVES *et al.*, 2009) and the fungi *Beauveria baussiana* [(Bals. –Criv.) Vuill. 1912] (Hyphomycetes: Moniliales) and *Metarhizium anisopliae* [(Metschn.) Sorokīn 1883] (Hypocreales: Clavicipitaceae) (BORGES, 2007; LEITE *et al.*, 2011; SCHAPOVALOFF *et al.*, 2014). Alves *et al.* (2009) demonstrated that different concentrations of *S. carpocapsae* can result in a mortality rate of 78% of adult *H. betulinus*, while Borges (2007) reports that effective control of *H. betulinus* can be reached through two annual applications of *B. baussiana* on branches of infested maté plants. Leite *et al.* (2011) and Schapovaloff *et al.* (2014) reported a mortality rate of *H. betulinus* adults between 66 to 100% and 51 to 86% when treated with *B. baussiana* in laboratory assays, while applications of *M. anisopliae* under laboratory conditions resulted in adult *H. betulinus* mortality of 31% (LEITE *et al.*, 2011) and 81% (SCHAPOVALOFF *et al.*, 2014).

Additional cultural tactics used to control *H. betulinus* and other pest insects of *I. paraguariensis* include pruning, weeding and planting secondary vegetation between crop lines (SOARES & IEDE, 1997; BORGES *et al.*, 2003; LEITE *et al.*, 2006). These techniques eliminate potential shelters and facilitate the visualization of the pest insects on the crops, exposing them to solar radiation (promoting desiccation) and predators (SOARES & IEDE, 1997). In addition, it enhances the microclimate conditions and the efficiency of the biological control with entomopathogenic fungi (SOARES & IEDE, 1997; LEITE *et al.*, 2006). Reducing plant stress also leaves maté more resistant to pest attacks (SOARES & IEDE, 1997).

Monitoring programs for *H. betulinus* rely on visual inspection of *I. paraguariensis* trees, looking for signs of infestation. Adult *H. betulinus* are removed by hand and infested trunks and branches removed and burned (SOARES *et al.*, 1995; SOARES & IEDE, 1997; FONSECA & ZARBIN, 2009).

2 OBJECTIVES

2.1 GENERAL OBJECTIVE

The main goal of this study is to evaluate the role of female contact pheromones in the mating behaviour of *H. betulinus* in laboratory.

2.2 SPECIFIC OBJECTIVES

Specifically, we aim to:

1. Verify the presence of chemical compounds in the female cuticle able to stimulate the mating behaviour sequence in male *H. betulinus*.
2. Extract, isolate and identify the fractions of female *H. betulinus* cuticular extracts.
3. Verify *H. betulinus* male mating behaviour response to conspecific female cuticular fractions.

3 HYPOTHESES

1. Females of *H. betulinus* have cuticular compounds that are necessary for eliciting full mating behaviour sequence in conspecific males.
2. A subset of female *H. betulinus* cuticular fractions and/or compounds from these fractions induce complete mating behaviour sequence in conspecific males in laboratory bioassays.

4 MATERIAL AND METHODS

4.1 INSECT MAINTENANCE

Adult *H. betulinus* of unknown age were obtained from maté-tea crops of the Vier, Indústria e Comércio do Mate Ltda, in São Mateus do Sul - PR, Brazil from September, 2016 – April, 2017 and from November, 2017 – March, 2018. All insects were transfered to the Laboratório de Semioquímicos, Chemistry Department of the Federal University of Paraná (UFPR) where they were separated by sex [the scape of the antennae and the femur of all three pairs of legs are larger in males than in females (CASSANELLO, 1993)], and individually placed in round capped plastic containers Plaszom® (9.5x5.5cm) with holes on the side and cap to allow air circulation (FIGURE 4a). Fresh maté twigs (*I. paraguariensis*) of approximately 8 cm length were provided to all beetles twice a week. All insects were maintained under controlled conditions at 25±3°C and 50±10% RH under a 12:12 L:D cycle for at least 10 days prior to assay (FIGURE 4b).

FIGURE 4: a) Male *Hedypathes betulinus* in a capped plastic container with fresh maté twig for feeding; b) *Hedypathes betulinus* beetles kept individually under controlled conditions.

4.2 MATING BIOASSAYS TREATMENTS

Ten females of *H. betulinus* were individually kept under controled conditions (described above – p. 27) with free access to food for at least 10 days before being manipulated to generate the following treatments:

1. Females were killed by freezing at -20°C for one hour, warmed to room temperature for 15 minutes, and individually presented to a male in order to demonstrate that the chemical recognition cues were intact (i.e. males recognize and attempt copulation with dead females).
2. Female cadavers were individually placed in 30mL disposable scintillation vials containing 5mL of double distilled hexane for 10 minutes to obtain the cuticular extracts. The first rinse of each female was concentrated under gentle air flow to a final volume of 0.5mL, transferred to a 2mL clean glass vial and stored at -20°C. Females were then extracted a second time by placing them in a 200mL Pyrex® Sohxlet, at approximately 68°C, containing 400 mL of hexane for 12 to 24 hours. Each extracted female carcass was air dried overnight, to allow the complete evaporation of the solvent, and individually introduced to a male to demonstrate that no traces of chemical recognition signals remained on female cadavers (i.e. female cuticular hydrocarbons are required for mate recognition by males and had been removed by this methodology).
3. One female equivalent (FE) was reapplied drop wise to the elytra of the same female cadaver it was extracted from (= reconstituted females) and subsequently presented to a male.

Treatments had an interval of one to three days between them and different treatments were never performed in the same day.

4.3 *Hedypathes betulinus* MATING BIOASSAYS

Bioassays were conducted by placing a *H. betulinus* male, previously kept under laboratory conditions for at least 10 days, in a petri dish lined with filter paper (Qualy® 9 cm diameter). After five minutes an untreated (cuticular profile intact), treated or solvent washed *H. betulinus* female cadaver was presented to a conspecific male (FIGURE 5). Using tweezers, the female cadaver was slowly moved towards the conspecific male until contact was made between male and female antennae, simulating the beginning of the mating sequence behavior of this species (in this species the mating sequence begins when the female approaches the male and touches the male antennae with her antennae) (Fonseca & Zarbin, 2010). Each female was manipulated in order to produce the three different treatments [killed and cuticular profile intact, cuticular profile extracted and cuticular profile re-applied (p. 25)] and an individual male was only assayed with the same female. Each female was presented to two individual males.

Ten females and 19 males were used in the mating bioassay (one male died during the bioassay). Observations were terminated after the female cadaver touched the male antennae with her antennae three times or once males were observed to make antennal contact with the female (behaviour 1), mount (behaviour 2), rotate 180° on the top of the female (behaviour 3) and attempt copulation (behaviour 4) (males were always interrupted before copulation occurred). Whenever a male attempted to copulate with a female cadaver that was previously submitted to solvent extraction, the female cadaver was rinsed one more time and introduced to the same male. If males still attempted copulation with female cadavers that were submitted to two solvent extractions (24h of solvent extraction in the Soxhlet), males and females were excluded from bioassays (3 female cadavers and 6 males met this condition). Only rinsed female cadavers introduced to males that did not attempt copulation were used in the following treatment (reconstitution of the crude extract), in order to confirm that mating recognition signals had been successfully removed by solvent extraction. All bioassays were conducted from 04-24-March, 2017 and from 29-March to 01-April, 2018 between 14:00 and 18:00 hours. The response of males to females was scored as 1 when the behavior was observed, or 0 when the behavior was not observed.

FIGURE 5: *Hedypathes betulinus* mating bioassay.

4.4 EXTRACTION AND FRACTIONATION OF *H. betulinus* CUTICULAR COMPOUNDS

In January, 2017, 30 female *H. betulinus* were killed by freezing at -20°C for one hour and then placed in 30mL disposable scintilation vials containing 5mL of double distilled hexane for 10 minutes (n=30). Female extracts were then combined and mixed in a 250mL glass media bottle (Duran®) and stored at -20°C.

The extracts of the 30 female *H. betulinus* were transfered to a 250mL round bottom flask. The Duran® glass media bottle, in which the female extracts were stored, was rinsed with 3 aliquots of 2mL of double distilled hexane and added to the round bottom flask. The 250mL round bottom flask was placed onto a Buchii® rotovaporator with water bath set at 30°C under gentle vacuum and 110rpm until the organic extract was reduced to a volume of 2.1mL and then transfered to a 15mL disposable vial. The 250mL round bottom flask was rinsed with 3 aliquots of 2mL of double distilled hexane and transfered to the 15mL disposable vial containing the organic extract. The 15mL disposable vial was capped with a Teflon septa and stored at -20°C.

The female *H. betulinus* body wash extract was loaded on a column of 1.5g of oven dried, hexane wetted 10% $AgNO_3$ silica flash. The silica flash was rinsed with a bit of double distilled hexane and gradually elluted in order to get the following fractions:

1. 6mL of hexane (saturated hydrocarbons)
2. 6mL of cyclohexene (unsaturated hydrocarbons)
3. 6mL of ether (more polar compounds)

The final volume of each fraction was collected in 30mL disposable vials capped with Teflon® septa and stored at -20°C. Each fraction was diluted in double distilled hexane and divided in 0.5mL female equivalents (FE). The Hexane Fraction was diluted 1.6 times its final volume of 8mL, resulting in 25 FE. The Cyclohexene Fraction was diluted 2.1 times its final volume of 5.5mL, resulting in 23 FE. The Ether Fraction was diluted 7.8 times its final volume of 5.3mL, resulting in 82 FE. FE were placed in a 2mL disposable glass vials capped with Teflon® septa and stored in freezer at -20°C.

The fractionation of female extracts was repeated in January, 2018, but using extracts of 15 female *H. betulinus* to obtain more fractions to be used in the bioassays executed from January to April, 2018.

4.5 ANALYSES OF MALE AND FEMALE *H. betulinus* CUTICULAR EXTRACTS AND FRACTIONS

Thirteen males and 13 females were killed by freezing at -20°C for one hour and then individually placed in 30mL disposable scintilation vials containing 5mL of double-distilled hexane for 10 minutes in December, 2016. Crude extracts of males and females were concentrated to 0.5mL under gentle air flow and analysed by Gas Chromatography equipped with Mass Spectrometry (GC-MS) to determine if there were any quantitative or qualitative diferences in the chemical composition of the crude cuticle extracts of both sexes.

Extracts of five cohorts of five males each and extracts of five cohorts of five females each were fractionated as mentioned above (p. 30) in February-March, 2018. Each male and female fraction was concentrated to the final volume of 1mL under gentle air-flow. Male and female fractions were analysed and compared by Gas Chromatography equipped with Mass Spectrometry (GC-MS) to investigate if there were any quantitative or qualitative diferences in the chemical composition of the cuticle fractions of both sexes.

GC-MS analyses were performed on a Shimadzu® QP2010 Plus GC-MS equipped with flame ionization, eletronic pressure control, and operated in splitless mode. An Agilent® DB-5 (30m x 0,25mm x 0,25 µm) capillary collumn was used under the following analytical conditions: initial temperature of 100°C for 1 min increasing 7°C/min until 270°C, which was kept for 30 min. The chromatograms of crude extracts and fractions of males and females were compared for the presence of candidate female cuticular pheromone components and the mass spectra of male and female cuticular compounds were analysed and compared with the literature.

4.6 *Hedypathes betulinus* FRACTIONS BIOASSAYS

Bioassays of individual fractions and the binary and tertiary blends were conducted from 24-March to 25-May, 2017 and 23-January to 29-March, 2018. *Hedypathes betulinus* females were manipulated as described above to produce female cadavers with no traces of mating recognition signals on their cuticle (p. 27). Then, one FE of the proper fraction, or blends of fractions, was applied to the elytra of an *H. betulinus* female cadaver previously solvent extracted as described above (p. 27).

An individual *H. betulinus* male was placed in a petri dish lined with filter paper (Qualy® 9 diameter) and after five minutes, the female cadaver with the fraction(s) applied was introduced. In total, 121 *H. betulinus* males were presented to 72 female cadavers with individual fractions, binary blends or the tertiary blend of fractions applied to their elytra (TABLE 1). Males that displayed the mating behaviour sequence after making antennal contact with the female carcass that was previously submitted to solvent extraction in the Sohxlet for more than 24 hours were excluded from the analyses and female cadavers were discarded and not used (13 female cadavers and 26 males met this condition). Males that died between treatments were also excluded from the analysis (one male in the Fractions H+C bioassay). Whenever possible, the beetles were used just once, but beetles that were used more than once per bioassay had an interval of at least 72h between bioassays (23 males were used in more than one bioassay). Beetles were never used more than once per treatment.

TABLE 1: Number of *H. betulinus* males and female cadavers used in each fraction(s) bioassay. H = Hexane Fraction; C= Cyclohexene Fraction; E = Ether Fraction.

Individual Fractions	**Binary Blends of Fractions**	**Tertiary Blend of Fractions**
H (20 ♂, 10 ♀)	H+C (19 ♂, 10 ♀)	H+C+E (20 ♂, 10 ♀)
C (20 ♂, 10 ♀)	C+E (20 ♂, 10 ♀)	
E (22 ♂, 11 ♀)	H+E (22 ♂, 11 ♀)	

4.7 *Hedypathes betulinus* FRACTIONS QUANTIFICATION

Fractionantion of *H. betulinus* cuticular extracts of five cohorts of five females each and five cohorts of five males each were done and each fraction was analysed in GC-MS. Unidentified compounds that appeared to have different concentrations in male and female hexane, cyclohexene and ether fractions were quantified through co-injection with C14 syntethic standard (Sigma-Aldrich®) at 50ppm.

Shapiro Wilk Normality Test .05, Wilcoxon Signed Rank Test .05 and Two Sample T-Test .05 were performed in the software $R_{3.4.0}$ (The R Foundation for Statistical Computing 2017) to confirm the differences of quantities (ppm) of these compounds between male and female fractions.

4.8 *Hedypathes betulinus* FUSCUMOL ACETATE DETERMINATION AND QUANTIFICATION

The presence of 6,10-dimethyl-5,9-undecadien-2-yl acetate (fuscumol acetate) in *H. betulinus* male and female crude extracts was detected in GC-MS chromatogram analyses and its identification was done through comparison with the literature and co-injection with synthetic standards.

Quantification of fuscumol acetate present in the male and female Ether Fractions was done through co-injection of C14 internal standard (Sigma-Aldrich®) at 50ppm. Shapiro-Wilk Normality Test .05 and Wilcoxon Signed Rank Test .05 was performed in the software $R_{3.4.0}$ (The R Foundation for Statistical Computing 2017) to determine differences of quantities (ppm) of fuscumol acetate between male and female Ether Fractions.

The geometrical configuration of the double bond C-C of fuscumol acetate present in an Ether Fraction containing extracts of 15 *H. betulinus* females was performed through co-injection with syntethic standars of (*E*)- and (*Z*)-fuscumol acetate (6:4) at 50 ppm on a Shimadzu® TQP2014 GC-MS/MS operated in splitless mode. An Agilent® DB-5 (30m x 0,25mm x 0,25 µm) capillary collumn was used under the following analytical conditions: initial temperature of 100°C for 1 min increasing 7°C/min until 270°C, which was held for 30 min.

The determination of the stereochemistry of fuscumol acetate present in Ether Fractions of female *H. betulinus* was done through comparison with synthetic standards of (*R*)- and (*S*)-fuscumol acetate (1:1) at 100ppm. An aliquot of 2mL of an ether fraction containing extracts of 15 female *H. betulinus* was concentrated to 100µL under gentle air-flow prior to analyses. Syntethic standards of (*R*)- and (*S*)-fuscumol acetate and the concentrated aliquot of the ether fraction of 15 female *H. betulinus* were analysed with a Shimadzu® 2010 Gas Chromatograph equipped with a capillary β-DEX 325 column (30m x 0.25 mm x 0.25µm) operated in splitless mode under the following analytical conditions: isothermal of 100° C during 400 minutes.

4.9 SYNTHETIC FUSCUMOL ACETATE BIOASSAYS

Bioassays of the female equivalent (FE) of (*R/S*)-, (*R*)- and (*S*)-fuscumol acetate and the male equivalent (ME) of (*R*)-fuscumol acetate were performed from 19-March to 21-May, 2018. *Hedypathes betulinus* females were manipulated as described above in order to produce female cadavers without any chemical mating recognition clues on their cuticle (p. 27). After solvent extraction, one FE of synthetic (*R/S*)-, (*R*)- or (*S*)-fuscumol acetate or one ME of synthetic (*R*)-fuscumol acetate was applied to the elytra of each of 42 female cadavers. Threated female cadavers were then individually introduced to 81 *H. betulinus* males in petri dishes as described in the previous sub-section "*H. betulinus* Fractions Bioassays" (p. 31) (TABLE 2). Each treated female cadaver was introduced to two individual males (three males died between treatments and were excluded from the analyses: one male in the FE of (*R/S*)-fuscumol acetate bioassay; one male in the FE of (*R*)-fuscumol acetate bioassay; one male in the ME of (*R*)-fuscumol acetate bioassay). Male beetles were used only once per bioassay and never used more than once per treatment.

TABLE 2: Number of *H. betulinus* males and female cadavers used in each synthetic fuscumol acetate bioassay.

(*R*/*S*)-Fuscumol Acetate ♀	(*R*)-Fuscumol Acetate ♀	(*S*)-Fuscumol Acetate ♀	(*R*)-Fuscumol Acetate ♂
21 ♂, 11 ♀	21 ♂, 11 ♀	20 ♂, 10 ♀	19 ♂, 10 ♀

4.10 STATISTICAL ANALYSES

The sequence of behaviours was observed and scored as described above (p. 26). The Fisher Exact Test with an experiment-wise error response .05 was used to compare male *H. betulinus* copulation response to female cadavers with the individual fractions, binary or tertiary blends of fractions applied to the elytra to male copulation response to freeze-killed female cadavers. The Fisher Exact Test was also used to contrast all bioassays in pairs.

The Fisher Exact Test with an experiment-wise error response .05 was used to compare male *H. betulinus* copulation response to female cadavers with FE of synthetic (*R/S*)-, (*R*)- or (*S*)-fuscumol acetate or ME of synthetic (*R*)-fuscumol acetate applied to the elytra to male copulation response to freeze-killed female cadavers that were not solvent extracted. The Fisher Exact Test was also used to compare all synthetic fuscumol acetate bioassays, crude extract bioassay and ether fraction bioassay in pairs. All the analyses were done using the software $R_{3.4.0}$ (The R Foundation for Statistical Computing 2017).

5 RESULTS

5.1 *Hedypathes betulinus* MATING BIOASSAY

All *H. betulinus* males (n=19) assayed were observed to touch the freeze-killed female cadavers with their antennae (behaviour 1), mount (behaviour 2), rotate 180° on the top of the carcass (behaviour 3) and subsequently attempt copulation (behaviour 4).

These 19 males were then introduced to rinsed conspecific female cadavers in mating arenas. All males touched the rinsed female cadavers with their antennae but none mounted or attempted copulation with them, showing no positive response for the second, third and fourth mating behaviours were observed.

All 19 *H. betulinus* males made antennal contact with the female cadavers with the crude extract reapplied to the elytra. Of these, 15 males (78.947%) mounted the cadavers, rotated 180º and attempted copulation. Statistical analysis of the male copulation response to the freeze-killed female cadavers and the male copulation response to the crude extract treated female cadavers did not observe a difference in male response (*p*=0.105) (TABLE 3 – p. 40) (FIGURE 6 – p. 41).

5.2 BIOASSAYS OF FRACTIONS

Ten *H. betulinus* female cadavers were treated with the tertiary blend of one FE of each of the Ether Fraction (Fraction E), Cyclohexene Fraction (Fraction C) and Hexane Fraction (Fraction H) to assay a cohort of 20 conspecific males (i.e., one treated female cadaver was assayed to two individual males) (TABLE 1 – p. 33). All males touched the treated female carcasses with their antennae, eleven males mounted them (behaviour 2) and ten of these males assayed displayed behaviours 3 (180° rotation) and 4 (attempted copulation) after mounting the female cadavers. In total, ten positive responses were observed to the tertiary blend. A significant difference in male copulation response to untreated female cadavers and solvent extracted female cadavers treated with Fraction E, C and H was observed ($p = 4.359 \times 10^{-4}$) (TABLE 3) (FIGURE 6).

The mating response of cohorts of *H. betulinus* males (one cohort of 19 males, one cohort of 20 males and one cohort of 21 males) to female cadavers treated with a blend of one FE of each of the binary combinations (10 females treated with H+C, 10 females treated with E+C and 11 females treated with H+E) (one treated female carcass was presented to two individual males in each assay) (TABLE 1 – p. 33). Of the 20 *H. betulinus* males assayed to female cadavers treated with one FE of Fraction C and E, only nine males mounted, rotated 180° and attempted copulation with the female cadavers. Male copulation response to untreated female cadavers and to female cadavers treated with Fraction C and E was significantly different ($p = 1.453 \times 10^{-4}$) (TABLE 3) (FIGURE 6). Five of the 19 *H. betulinus* males assayed to ten female cadavers with one FE of the Fraction C and H applied to the elytra displayed behaviours 2, 3 and 4. Male copulation response to untreated female cadavers and female cadavers treated with Fraction C and H was significantly different ($p = 2.405 \times 10^{-6}$) (TABLE 3) (FIGURE 6). The highest number of male positive responses was observed to female cadavers treated with the binary blend of one FE of Fraction E and H. Sixteen of the 21 males assayed mounted, rotated 180° and attempted copulation with the 11 female cadavers treated with Fraction E and H. Male copulation response to untreated female cadavers and female cadavers treated with Fraction E and H was significantly different (*p* = 2.113×10^{-2}) (TABLE 3) (FIGURE 6).

Two cohorts of 20 *H. betulinus* males each and one cohort of 22 males were assayed with three separate cohorts of conspecific female cadavers with one female equivalent (FE) of each individual fraction (10 females treated with Fraction H, 10 females treated with Fraction C and 11 females treated with Fraction E, respectively) (one treated female cadaver was assayed to two individual males in each assay) (TABLE 1 – p. 33). Only two of 20 *H. betulinus* males displayed behaviours 2, 3 and 4 of the mating sequence to female cadavers treated with one FE of the Fraction C. Significantly fewer males exhibited a copulation response to untreated female cadavers than to female cadavers treated with Fraction C (*p* = 3.351×10^{-9}) (TABLE 3) (FIGURE 6). Of 20 *H. betulinus* males was introduced to 10 individual female cadavers treated with one FE of Fraction H, five mounted (behaviour 2), rotated 180° (behaviour 3) and attempted copulation (behaviour 4) with the female cadavers. Male copulation response to untreated female cadavers and female cadavers treated with Fraction H were significantly different (*p* = 7.708×10^{-7}) (TABLE 3) (FIGURE 6). The highest number of copulation attempts observed to the individual fractions was observed to females treated with Fraction E. Eleven of 22 males displayed behaviours 2, 3 and 4 to female cadavers treated with one FE of the Fraction E. Male copulation response to untreated female cadavers and female cadavers treated with Fraction E was significantly different (*p* = 1.839×10^{-4}) (TABLE 3) (FIGURE 6).

TABLE 3: Bioassay *p* values obtained through comparison between *Hedypathes betulinus* male copulation response to untreated freeze-killed conspecific female cadavers and male copulation response to female cadavers submitted to treatments (rinsed and sequentially reconstituted with the crude extract, individual fractions, binary or tertiary blends of fractions on the cuticle) using Fisher Exact Test (experimente wise-error = .05). Fraction C = Cyclohexene Fraction; Fraction E = Ether Fraction; Fraction H = Hexane Fraction; * = significant values.

Bioassays	***p* values**
Fraction E	1.839×10^{-4}*
Fraction H	7.708×10^{-7}*
Fraction C	3.351×10^{-9}*
Fractions E+H	2.113×10^{-2}*
Fractions H+C	2.405×10^{-6}*
Fractions E+C	1.453×10^{-4}*
Fractions E+H+C	4.359×10^{-4}*
Crude Extract	0.105

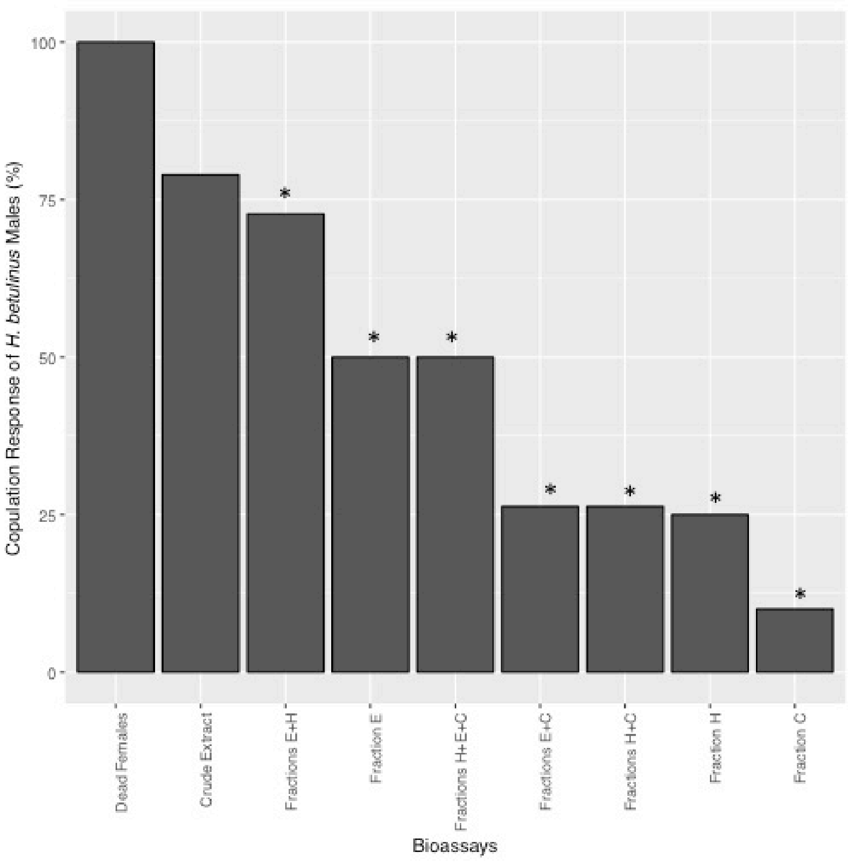

FIGURE 6: The percentage of male *Hedypathes betulinus* that attempted copulation with freeze killed females with the cuticular profile intact (Dead Females), female cadavers submitted to reconstitution of the crude extract on the cuticle (Crude Extract Bioassay), and with female cadavers treated with individual fractions, binary and tertiary blends of fractions. Fraction C = Cyclohexene Fraction; Fraction E = Ether Fraction; Fraction H = Hexane Fraction; * = significant *p* values.

5.3 COMPARISON OF FRACTIONS

The male copulation response rate to female cadavers treated with individual fractions and binary and tertiary blends were contrasted. The rate of male copulation response to female cadavers treated with the crude extract and the individual fractions, binary and tertiary blends of fractions were calculated to determine the minimum number of fractions necessary and sufficient to recover the male copulation activity observed to the crude extract. Twenty-two paired contrasts of bioassays were done in total, of which 10 resulted in significant p values (TABLE 4).

A significant difference in the male copulatin response to females treated with the crude extract and females treated with Fraction H (p = 1.231x10^{-3}) and C (p = 1.664x10^{-5}), the binary blends of Fractions H+C (p = 2.918x10^{-4}) and E+C (p = 0.048) bioassays (TABLE 4). When the copulation response of males to females treated with Fraction E was contrasted with the response of males to females treated with Fraction C a significant difference was observed (p = 7.410x10^{-3}) (TABLE 4). When the copulation response of males to females treated with Fraction H was compared to the response of males to females treated with the binary blend of Fractions E+H a significant difference was observed (p = 4.804x10^{-3}) (TABLE 4). When the individual Fraction C bioassay was contrasted with the binary blends of Fractions E+H (p = 3.725x10^{-2}), E+C (p = 3.095x10^{-2}) and the tertiary blend of Fractions E+H+C (p = 1.381x10^{-2}) significant diferences in male copulation responses were observed (TABLE 4).

TABLE 4: Paired contrasts of the male copulation response rate of all bioassays to the male copulation response rate of crude extract and individual fractions bioassays using Fisher Exact Test (experiment wise-error = .05). Fraction C = Cyclohexene Fraction; Fraction E = Ether Fraction; Fraction H = Hexane Fraction; * = significant *p* values.

Bioassays	**Crude Extract**	**Fraction E**	**Fraction H**	**Fraction C**
Fraction E	0.102	---	---	---
Fraction H	1.231×10^{-3}*	0.121	---	---
Fraction C	1.664×10^{-5}*	7.410×10^{-3}*	0.407	---
Fractions E+H	0.726	0.215	4.804×10^{-3}*	4.482×10^{-5}*
Fractions H+C	2.918×10^{-3}*	0.199	1	0.235
Fractions E+C	4.804×10^{-2}*	0.767	0.320	3.095×10^{-2}*
Fractions E+H+C	9.584×10^{-2}*	1	0.190	1.381×10^{-2}*

5.4 ANALYSES OF MALE AND FEMALE *H. betulinus* CUTICULAR EXTRACTS AND FRACTIONS

Gas chromatography analysis of crude extracts of male and female *H. betulinus* showed that male extracts presented two sex-specific compounds (FIGURE 7). Comparison of the mass spectra of these compounds with literature revealed that compound #1 is (*E*)-geranilacetone (FIGURE 8) and compound #2 is (*E*)-(*R*)-fuscumol acetate (FIGURE 9).

No sex-specific compounds were observed in gas chromatography analysis of male and female *H. betulinus* hexane (Fraction H), cyclohexene (Fraction C) and ether (Fraction E) fractions. Quantitative differences in male and female fractions were observed for some compounds. The concentrations of two unidentified compounds from Fraction H (compounds U and V), Fraction C (compounds X and W) and Fraction E (compounds Y and Z) of male and female *H. betulinus* were compared (FIGURE 10, 11 and 12).

Significant difference was not observed in the concentrations of the compound U present in male (79.85 ppm) and female (69.83 ppm) hexane fractions [*p*=0.6905, comparison of unidentified compound U (ppm) in male and female Hexane Fractions (Wilcox Test .05)] (FIGURE 10). However, a siginificant difference in the concentrations of the compound V present in female (123.82 ppm) and male (210.89 ppm) hexane fractions [p=3.945x10^{-3}, comparison of unidentified compound V (ppm) in male and female Hexane Fractions (T-test .05)] (FIGURE 10).

Concentrations of compounds present in the Fraction C of males and females *H. betulinus* were not statistically different. The concentration of 28.84 ppm for compound X was determined on female cyclohexene fractions, while the concentration of 72.55 ppm on male cyclohexene fractions [*p*=0.1289 comparison of non- identified compound X (ppm) in male and female cyclohexene fractions (T-test .05)]. The concentration of 38.82 ppm of compound W was determined on female cyclohexene fractions, while the concentration of 109.94 ppm on male cyclohexene fractions [p=9.524x10^{-2} comparison of non-identified compound W (ppm) in male and female Cyclohexene Fractions (Wilcox test .05)] (FIGURE 11).

Concentrations of compounds present in the Fraction E of males and females *H. betulinus* were statistically different. The Fraction E of females *H. betulinus* present the concentration of 2.18 ppm of compound Y, whereas the Fraction E of conspecific males present the concentration of 6.23 ppm of this compound [p=7.937x10^{-3} comparison of unidentified compound Y (ppm) in male and female Ether Fractions (Wilcox test .05)]. The Fraction E of females *H. betulinus* present the concentration of 4.36 ppm of compound Z, whereas the Fraction E of conspecific males present the concentration of 10.68 ppm of this compound [p=3.661x10^{-2} comparison of unidentified compound Z (ppm) in male and female Ether Fractions (T-test .05)] (FIGURE 12).

Comparison of chromatograms of the Ether Fraction of male and female *H. betulinus* identified one compound present in very different concentrations in male and female fractions (FIGURE 12). This compound was identified as (*R*)-(*E*)-fuscumol acetate by comparison of the mass-spectra with the literature (FIGURE 13) and co-injection with synthetic standards (FIGURE 14); (FIGURE 15). Quantification of male and female Ether Fractions show that one FE of the female Ether Faction (86.5µL) present an average of 0.147 ppm of this compound, while one ME of the male Ether Fraction (86.5µL) has an average of 29 ppm [p = 1.587x10^{-2}, comparison of fuscumol acetate concentration (ppm) in male and female Ether Fractions (Wilcox test .05)].

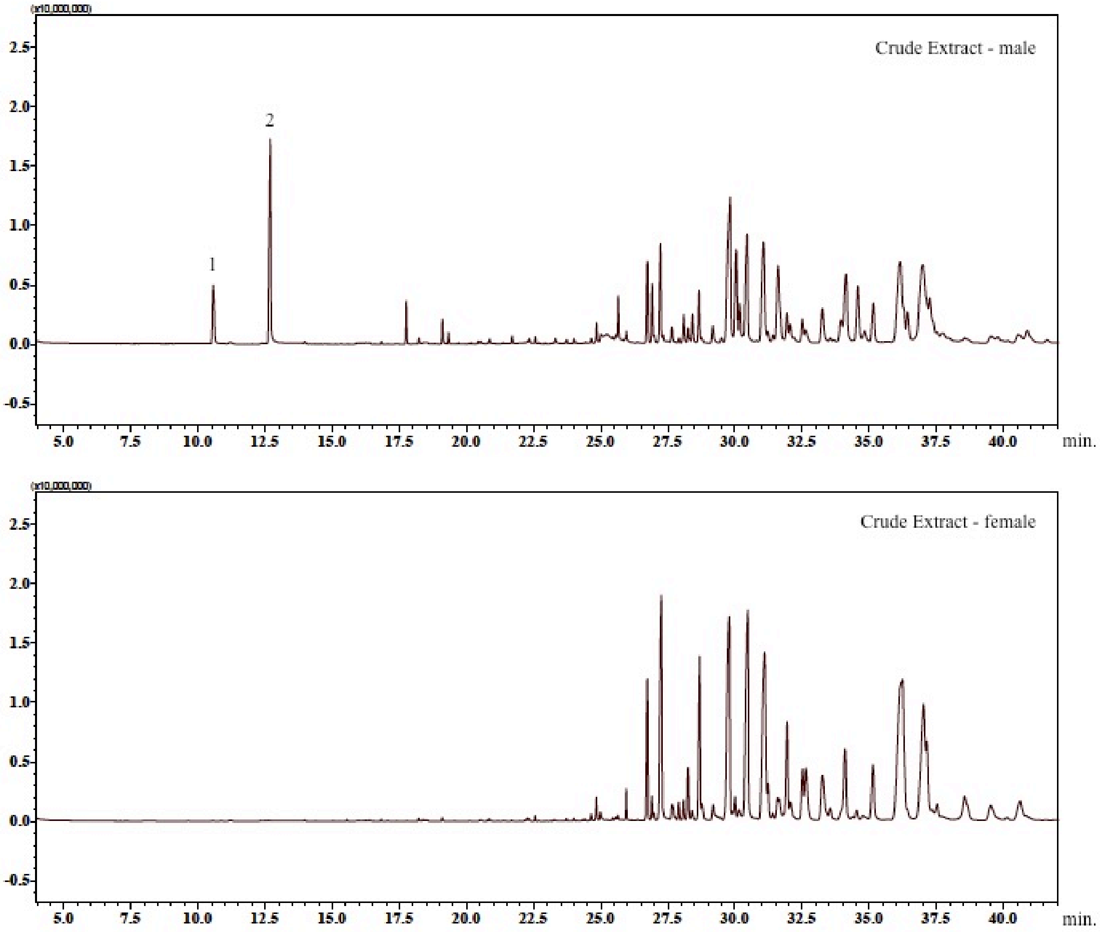

FIGURE 7: Chromatograms of *Hedypathes betulinus* individual male and female cuticular extracts. 1 = (*E*)-geranilacetone; 2 = (*E*)-(*R*)-fuscumol acetate.

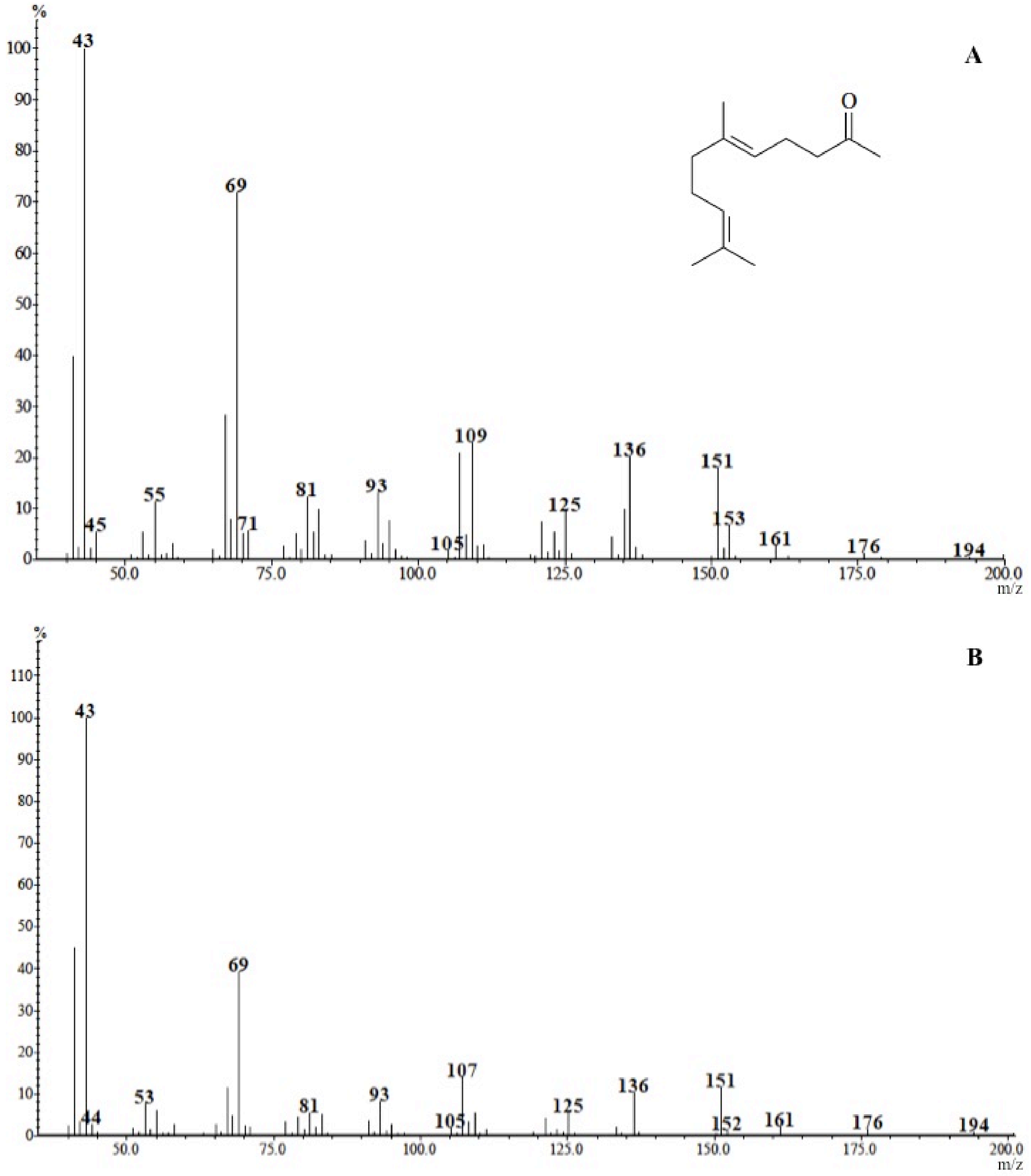

FIGURE 8: Comparison of the mass spectra of geranilacetone present in the ether fraction of *Hedypathes betulinus* males (a) and the mass spectra of (*E*)-geranilacetone produced by males of *Hedypathes betulinus* in Fonseca *et al.* (2010) (b).

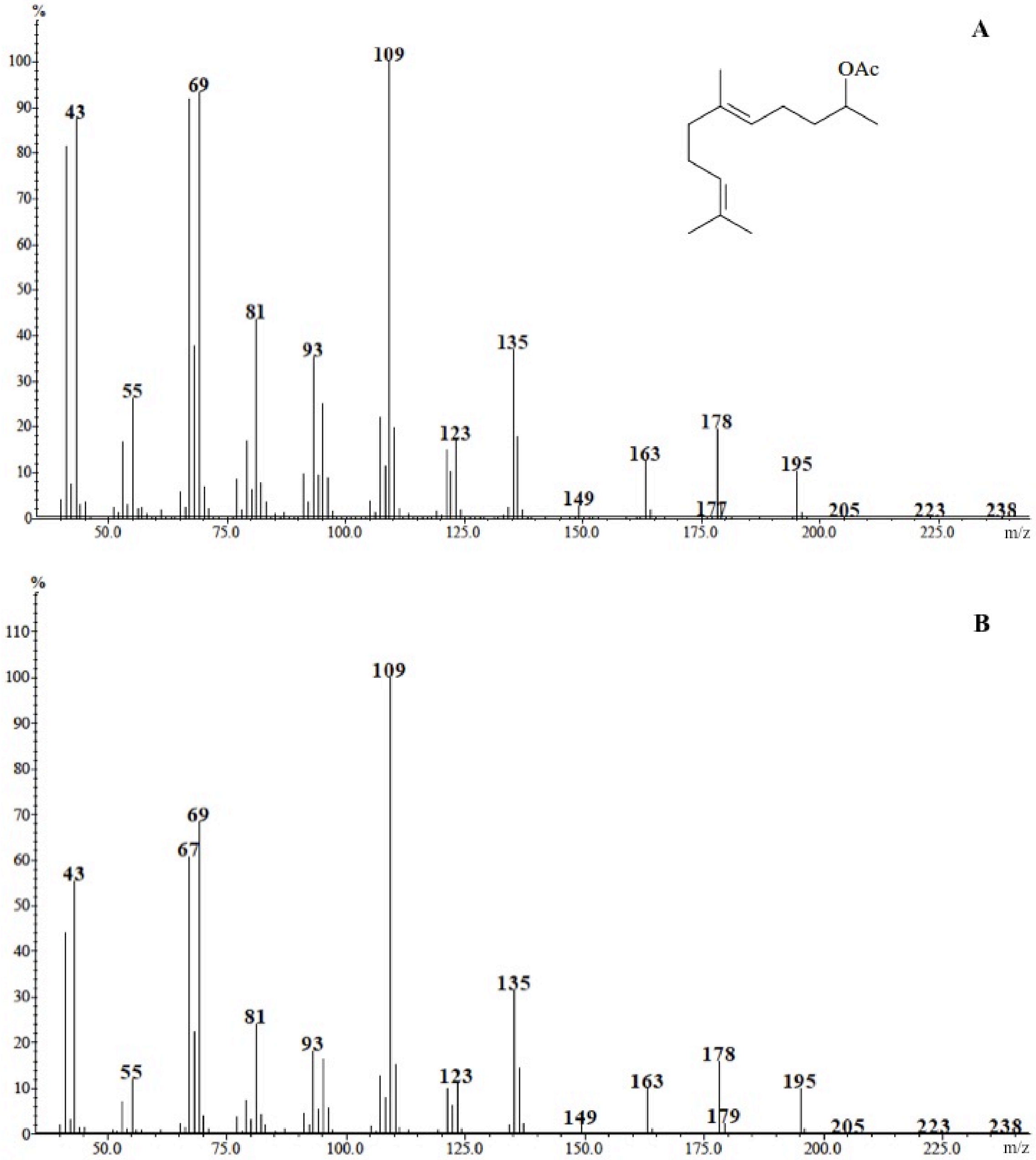

FIGURE 9: Comparison of the mass spectra of fuscumol acetate present in the ether fraction of *Hedypathes betulinus* males (a) and the mass spectra of (*E*)-(*R*)-fuscumol acetate produced by males of *Hedypathes betulinus* in Fonseca *et al.* (2010) (b).

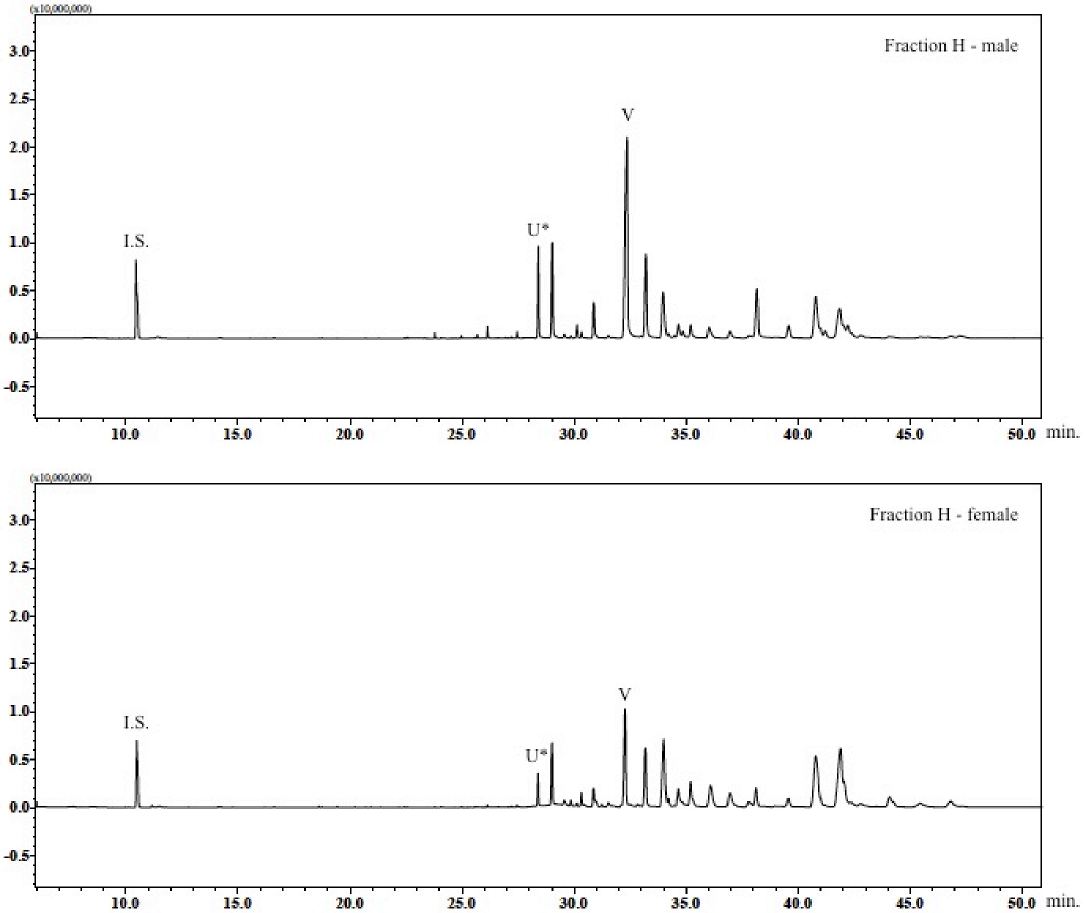

FIGURE 10: Chromatograms of *Hedypathes betulinus* male and female hexane fractions with the internal standard C14 (I.S.). Letters represent unidentified compounds. Fraction H = Hexane Fraction; * = significant difference of concentration among male and female fractions.

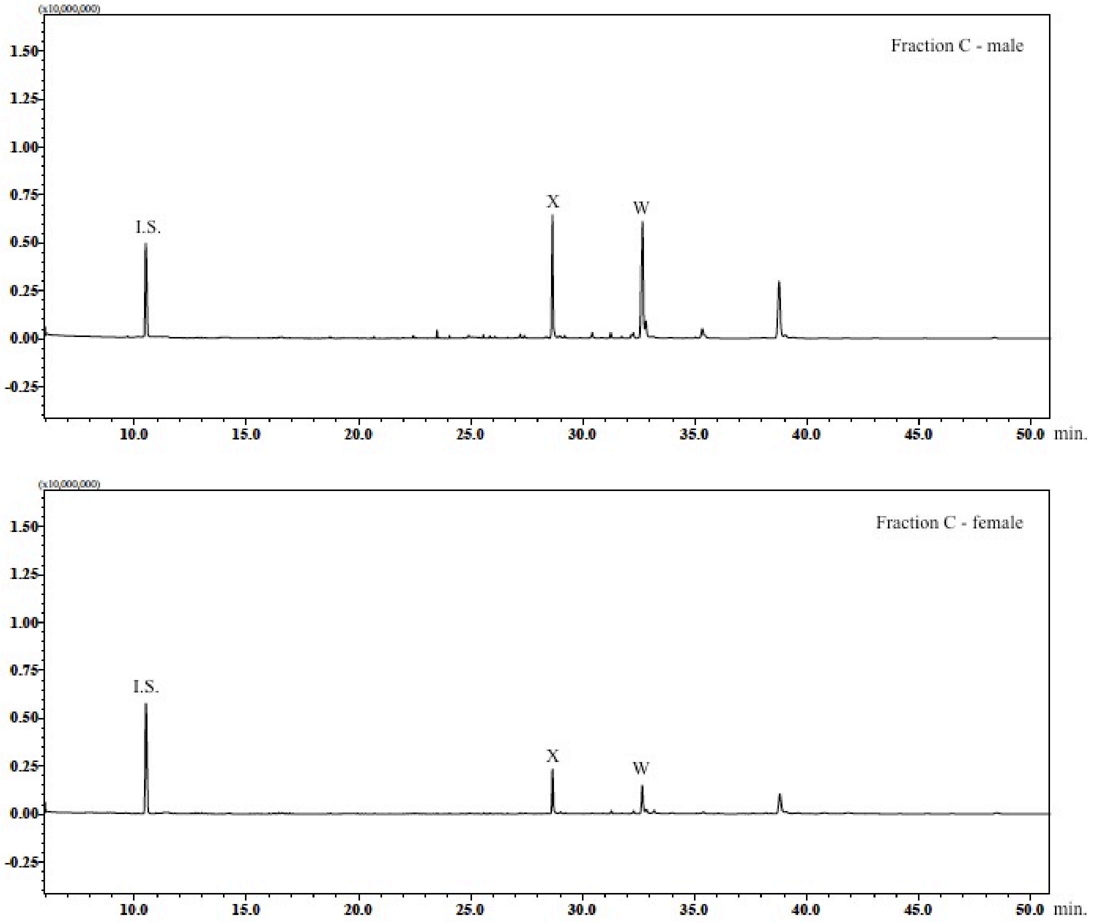

FIGURE 11: Chromatograms of *Hedypathes betulinus* male and female cyclohexene fractions with the internal standard C14 (I.S.). Letters represent unidentified compounds. Fraction C = Cyclohexene Fraction.

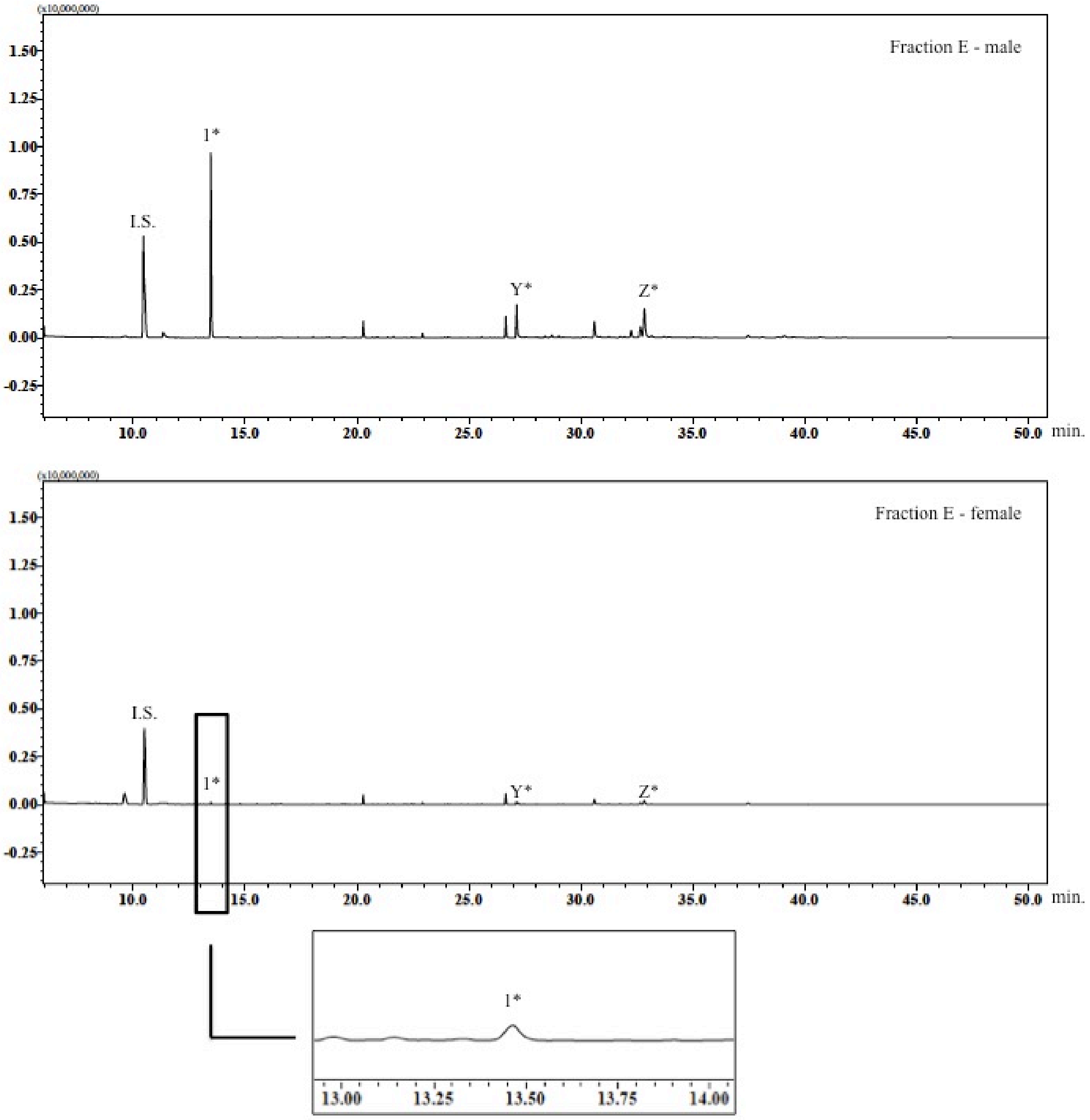

FIGURE 12: Chromatograms of *Hedypathes betulinus* male and female ether fractions with the internal standard C14 (I.S.). Letters represent unidentified compounds. 1 = Fuscumol acetate; Fraction C = Ether Fraction; * = significant difference of concentration among male and female fractions.

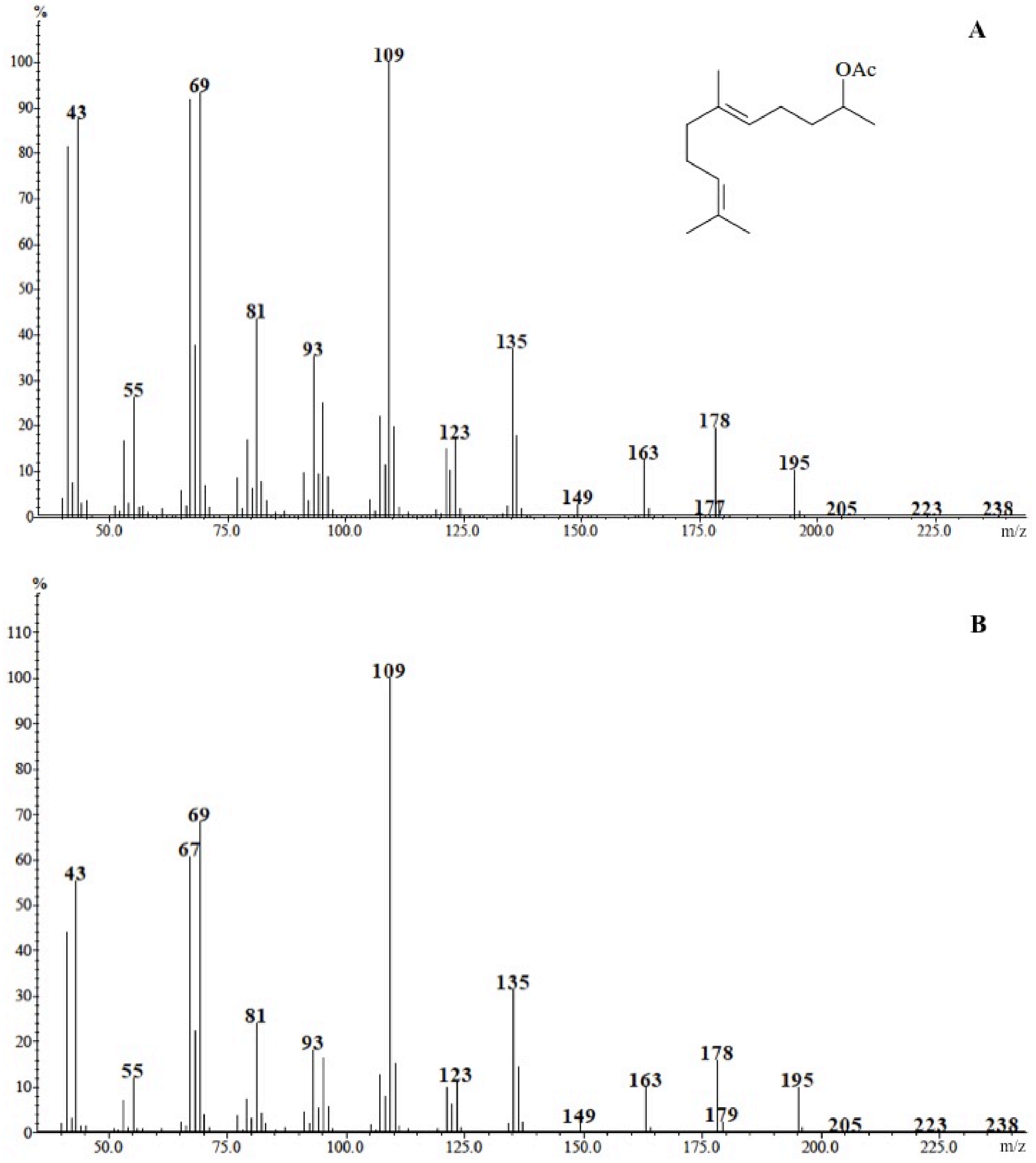

FIGURE 13: Comparison of the mass spectra of fuscumol acetate present in the ether fraction of *Hedypathes betulinus* females (a) and the mass spectra of (*E*)-(*R*)-fuscumol acetate produced by males of *Hedypathes betulinus* in Fonseca *et al.* (2010) (b).

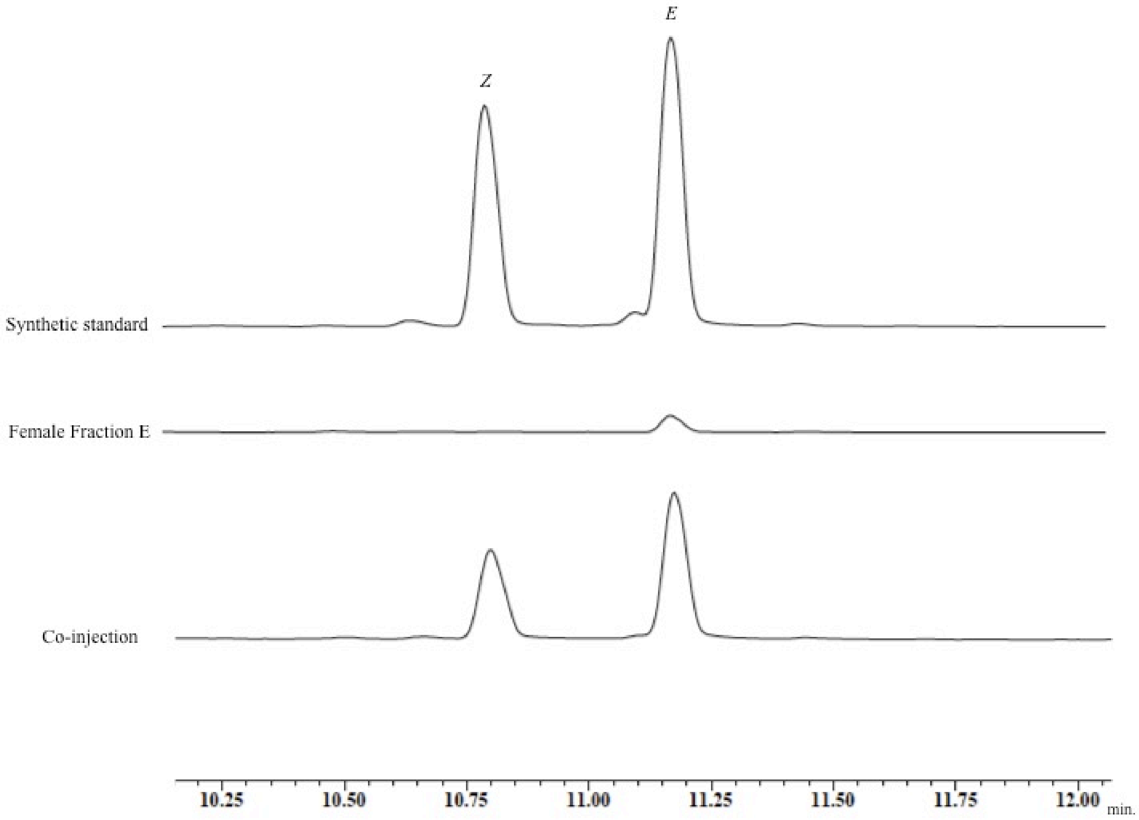

FIGURE 14: Comparison of the chromatograms of fuscumol acetate synthetic standard (synthetic standard), fuscumol acetate present in the ether fraction of *Hedypathes betulinus* females (Female Fraction E) and the co-injection of fuscumol acetate synthetic standard and fuscumol acetate present in the ether fraction of females (co-injection). *Z* = (*Z*)-fuscumol acetate; *E* = (*E*)-fuscumol acetate.

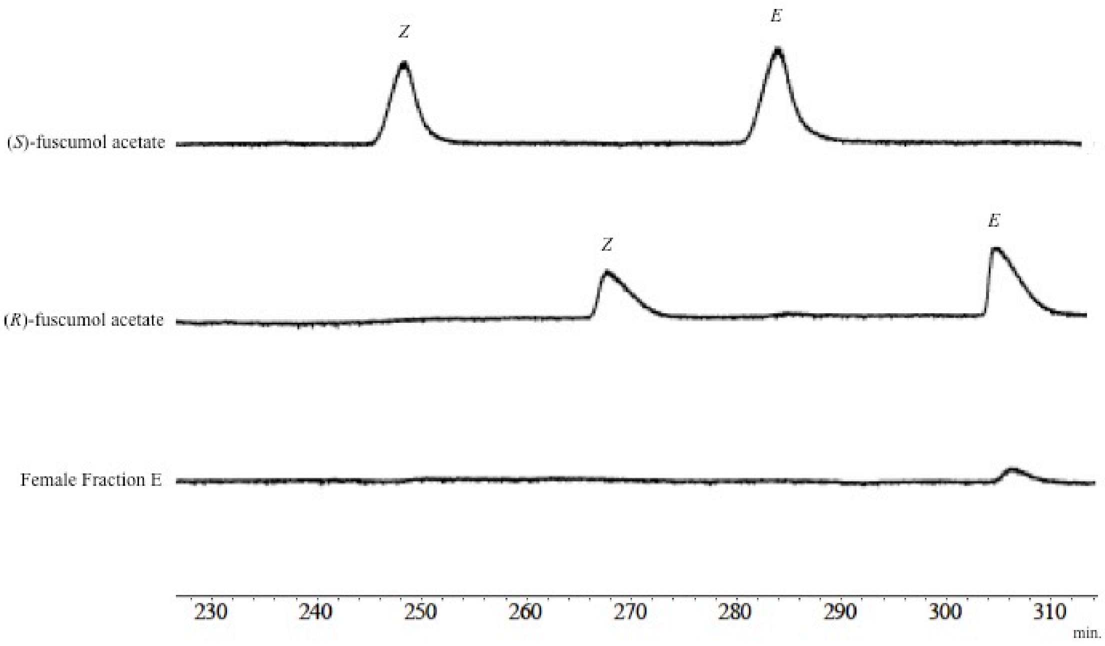

FIGURE 15: Comparison of the chromatograms of (*R*)- and (*S*)-fuscumol acetate synthetic standards and natural fuscumol acetate present in the ether fraction of *Hedypathes betulinus* females (Female Fraction E). *Z* = (*Z*)-fuscumol acetate; *E* = (*E*)-fuscumol acetate.

5.5 SYNTHETIC FUSCUMOL ACETATE BIOASSAYS

Twenty-one *H. betulinus* males were assayed with 11 female cadavers treated with one FE of synthetic (*R/S*)-fuscumol acetate each (TABLE 2 – p. 36). All 21 males made antennal contact with the treated female cadavers, but only four males mounted, rotated 180° and attempted copulation with treated female cadavers (p = 4.700x10^{-8}, comparison of the male copulation response to untreated female cadavers and female cadavers treated with the FE of synthetic (*R/S*)-fuscumol acetate) (TABLE 5) (FIGURE 16).

Eleven *H. betulinus* female carcasses treated with one FE of (*R*)-fuscumol acetate were introduced to 21 conspecific males (TABLE 2 – p. 36). Five of the 14 males assayed mounted, rotated 180° and attempted copulation with the 11 female cadavers treated with FE (*R*)-fuscumol acetate [p = 2.444x10^{-7}, comparison of the male copulation response to untreated female cadavers and female cadavers treated with the FE of synthetic (*R*)-fuscumol acetate] (TABLE 5) (FIGURE 16).

Twenty *H. betulinus* males were assayed to 10 conspecific female cadavers treated with one FE of (*S*)-fuscumol acetate (TABLE 2 – p. 36). All 20 males made antennal contact with the treated female cadavers, but only three males mounted, rotated 180° and attempted copulation with treated female cadavers (p = 2.569x10^{-8}, comparison of the male copulation response to untreated female cadavers and female cadavers treated with the FE of synthetic (*S*)-fuscumol acetate) (TABLE 5) (FIGURE 16).

Ten *H. betulinus* female carcasses treated with one ME of (*R*)-fuscumol acetate were introduced to 19 conspecific males (TABLE 2 – p. 36). One of the 19 males assayed mounted, rotated 180° and attempted copulation with the 10 female cadavers treated with one ME (*R*)-fuscumol acetate [p = 3.390x10^{-5}, comparison of the male copulation response to untreated female cadavers and female cadavers treated with the ME of synthetic (*R*)-fuscumol acetate] (TABLE 5) (FIGURE 16).

TABLE 5: Bioassay *p* values obtained through comparison between *Hedypathes betulinus* male copulation response to untreated freeze-killed conspecific female cadavers and male copulation response to female cadavers submitted to treatments (rinsed and sequentially reconstituted with one female equivalente (FE) of the synthetic (*R/S*)-, (*R*)- or (*S*)-fuscumol acetate or one male equivalente (ME) of the synthetic (*R*)-fuscumol acetate on the cuticle) using Fisher Exact Test (experiment wise-error = .05). * = significant values.

Bioassays	***p* values**
FE (*R/S*)-Fuscumol Acetate	$4.700\text{x}10^{-8}$*
FE (*R*)-Fuscumol Acetate	$2.444\text{x}10^{-7}$*
FE (*S*)-Fuscumol Acetate	$2.569\text{x}10^{-8}$*
ME (*R*)-Fuscumol Acetate	$1.131\text{x}10^{-9}$*

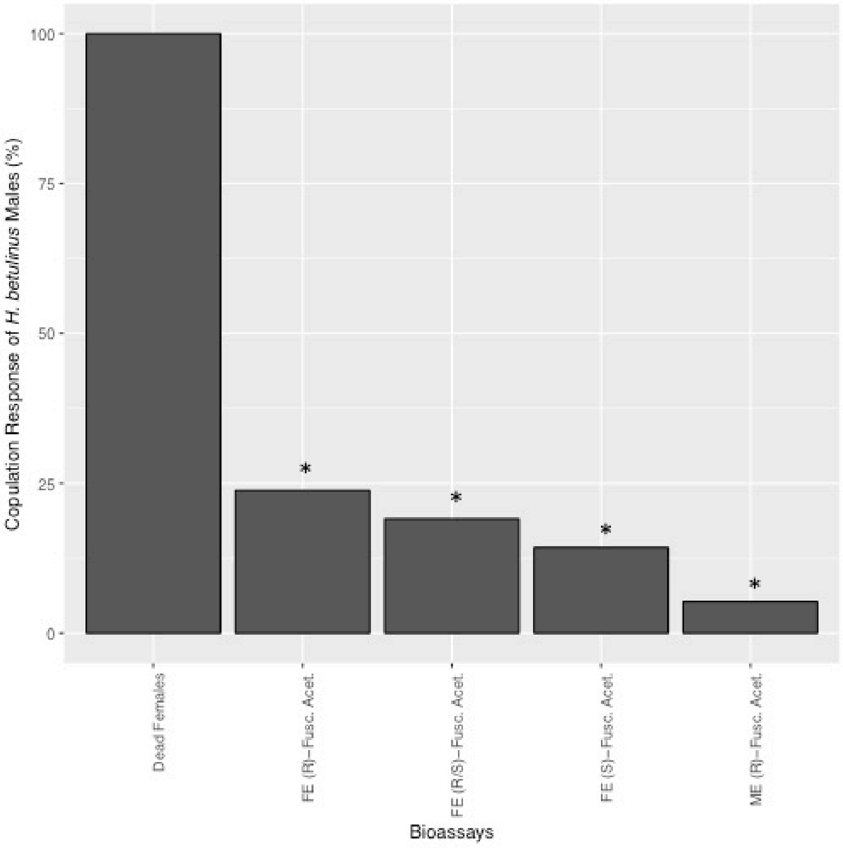

FIGURE 16: The percentage of male *Hedypathes betulinus* that attempted copulation with freeze-killed females with the cuticular profile intact (Dead Females) and with solvent-extracted female cadavers treated with individual ether fraction and synthetic (*R,S*)-, (*R*)- or (*S*)-fuscumol acetate. FE (R)-Fusc. Acet. = female equivalent of synthetic (*R*)-fuscumol acetate; FE (R/S)-Fusc. Acet. = female equivalent of synthetic (*R/S*)-Fuscumol Acetate; FE (S)-Fusc. Acet. = female equivalent of synthetic (*S*)-fuscumol acetate; ME (R)-Fusc. Acet. = male equivalent of synthetic (*R*)-fuscumol acetate; * = significant *p* values.

5.6 COMPARISON OF SYNTHETIC FUSCUMOL ACETATE BIOASSAYS

In total, 14 paired contrasts of bioassays were done, resulting in six significant *p*-values were (TABLE 6).

Significant *p*-values were obtained when the crude extract bioassay was contrasted with the FE (*R/S*)-fuscumol acetate ($p = 3.449\text{x}10^{-4}$), the FE of (*R*)-fuscumol acetate ($p = 1.231\text{x}10^{-3}$), the FE (*S*)-fuscumol acetate ($p = 8.750\text{x}10^{-5}$) and the ME of (*R*)-fuscumol acetate ($p = 6.709\text{x}10^{-6}$) bioassays; and when the female Fraction E bioassay was contrasted with the FE of (*S*)-fuscumol acetate ($p = 2.320\text{x}10^{-2}$) and the ME of (*R*)-fuscumol acetate ($p = 1.995\text{x}10^{-3}$) bioassays (TABLE 6).

TABLE 6: Paired contrasts of the male copulation response rate of all synthetic fuscumol acetate bioassays to the male copulation response rate of female crude extract and female ether fraction bioassays using Fisher Exact Test (experiment wise-error = .05). FE Crude Extract = female crude extract; FE Fraction E = female ether fraction; FE (R)-Fusc. Acet. = female equivalent of synthetic (*R*)-fuscumol acetate; FE (R/S)-Fusc. Acet. = female equivalent of synthetic (*R/S*)-Fuscumol Acetate; FE (S)-Fusc. Acet. = female equivalent of synthetic (*S*)-fuscumol acetate; ME (R)-Fusc. Acet. = male equivalent of synthetic (*R*)-fuscumol acetate; * = significant *p* values.

Bioassays	**F Crude Extract**	**F Fraction E**	**FE (*R*)-Fusc. Acet.**	**FE (*S*)-Fusc. Acet.**	**FE (*R/S*)-Fusc. Acet.**
FE (*R*)-Fusc. Acet.	$1.231\text{x}10^{-3}$*	0.115	---	---	---
FE (*S*)-Fusc. Acet.	$8.750\text{x}10^{-5}$*	$2.320\text{x}10^{-2}$*	0.696	---	---
FE (*R/S*)-Fusc. Acet.	$3.449\text{x}10^{-4}$*	$5.461\text{x}10^{-2}$	1	1	---
ME (*R*)-Fusc. Acet.	$6.709\text{x}10^{-6}$*	$1.995\text{x}10^{-3}$*	0.185	0.604	0.345

6 DISCUSSION

Observations of male mating behaviour when introduced to freeze-killed female cadavers with the cuticular profile intact (dead females) confirmed the mating sequence reported by Fonseca & Zarbin (2009). I observed that copulation only occurred after a male made antennal contact with a female. Males could get close to a female within a few millimeters of a female and not attempt to copulate with her if antennal contact was not made. After the male touched the female carcass with its antennae, the male immediately mounted the female cadaver and started licking the surface of the female elytra with its palpi. Similar behaviours have been observed in other species of cerambycids, like *Rytidodera simulans* (White) (MOHD SABRI & ABDULLAH, 2016), *Anoplophora malasiaca* (Thomson, 1865) (FUKAYA *et al.*, 1999; YASUI *et al.*, 2003), *Monochamus galloprovincialis* (Olivier, 1795) (IBEAS *et al.*, 2009), *Psacothea hilaris* (Pascoe, 1857) (FUKAYA & HONDA, 1992) and *Oemona hirta* (Fabricius, 1775) (WANG & DAVIS, 2005). This behaviour has been proposed to be involved in chemical recognition of female contact sex pheromones by males (IWABUCHI, 1999; MILLAR & HANKS, 2017) and to have a calming effect on females (CROOK *et al.*, 2004; MOHD SABRI & ABDULLAH, 2016; MILLAR & HANKS, 2017).

In addition, preliminary assays were performed by introducing a glass rod treated with the crude extract of one female carcass to two individual males. Both males assayed with the female extract treated glass rod attempted to copulate with it (FIGURE 17), which strongly suggest that males rely on chemical rather than behavioural or visual stimuli to identify conspecific females.

Mate recognition cues have been partially restored by re-applying the cuticular compounds on the elytra of rinsed female cadavers since no difference was observed in male mating behaviour responses to female cadavers reconstituted with their own crude extract and to freeze-killed females that were not solvent extracted. Even though the extraction and reapplication process might have changed the natural structure of the wax layer of females epicuticle (GINZEL *et al.*, 2003b; BARBOUR *et al.*, 2007), those changes did not affect the mating recognition of *H. betulinus* males. This finding also demonstrates that some specific compound or combinations of compounds on the epicuticular wax layer of female *H. betulinus* are necessary for mate recognition in *H. betulinus*.

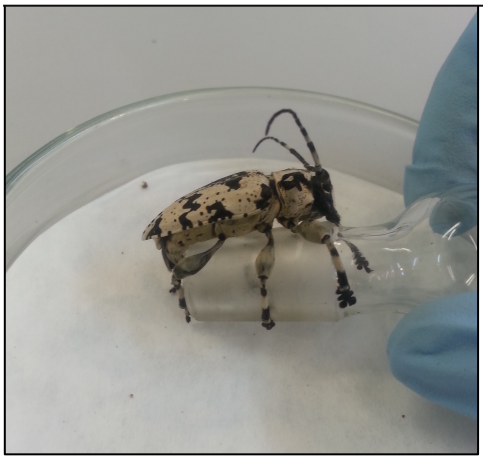

FIGURE 17: Male *Hedypathes betulinus* attempting to copulate with a glass rod treated with the cuticular extract of a conspecific female.

The chemical composition of the crude extract of *H. betulinus* females might be different than the final three fractions eluted from the crude extract. There is a possibility that some chemical compounds present in the epicuticular wax layer of *H. betulinus* females that might have some importance in the mating recognition system of this species have been lost or altered during the fractionation process. Only saturated and unsaturated hydrocarbons, and other more polar compounds present in the body washes of females that have chemical affinity to the polarity degree of the solvents used would be eluted from the column of $AgNO_3$ impregnated silica flash. (see GINZEL, 2010).

Even though many studies have proven that chemical compounds present in the body surface of females are important for mate recognition of many Cerambycidae species (KIM *et al.*, 1993; GINZEL *et al.*, 2003b; GINZEL & HANKS, 2003, 2005; BARBOUR *et al.*, 2007; MOHD SABRI & ABDULLAH, 2016), only few components of active cuticular sex-pheromones have been identified. Millar & Hanks (2017) suggest that this might be related to the structure of cuticular lipids, which are very complex, and the difficulty in purifying and obtaining individual standards for tests in bioassays.

We determined that the more polar compounds of the females cuticle (Fraction E) are sufficient and necessary to stimulate the mating sequence in male *H. betulinus*, but the interaction of polar compounds with saturated and unsaturated hydrocarbons might be important for the mating recognition in this species. Our results show that individual Fraction C and H alone were not sufficient to stimulate male mating behaviour response comparable to the crude extract of females, but these fractions elicit male mating behaviour response comparable to the crude extract whenever combined with Fraction E in binary (Fractions E+H) or ternary (Fractions E+H+C) blends.

Fukaya *et al.* (1999, 2000) reported that the female contact sex pheromone of *Anoplophora malasiaca* (Thomson, 1865) consists of saturated hydrocarbons and more polar compounds. Four methyl-branched alkanes appeared to be the most important in eliciting full mating response from males (9-methylheptacosane, 9-methylnonacosane, 15-methylhentriacontane and 15-methyltritriacontane) when mixed with the more polar fractions. Additional active components of the more polar fraction were identified as a blend of heptacosan-10-one, (*Z*)-18-heptacosen-10-one, (18*Z*,21*Z*)-heptacosa-18,21-dien-10-one and (18*Z*,21*Z*,24*Z*)-heptacosa-18,21,24-trien-10-one (YASUI *et al.*, 2003). Males appeared to be refractory to heptacosan-12-one, a fifth compound present in this fraction (YASUI *et al.*, 2003). Subsequent analyses of the more polar fraction of *A. malasiaca* females cuticle executed by Yasui *et al.* (2007) identified three complex bicycling lactones as being part of the contact pheromone of this species. The female contact sex pheromone of *A. malasiaca* is considered the most complex contact pheromone of Cerambycidae reported in the literature until now (MILLAR & HANKS, 2017).

Although the individual Fraction H had to be combined with other fractions to elicit male behaviour copulation response similar to female crude extract in *H. betulinus*, there are many reports in the literature that show that saturated alkanes are necessary and sufficient to elicit high mating behaviour response on males of other cerambycids. Branched chain alkanes are necessary for mating recognition in many other insect species and the majority of branched chain alkanes present in the insect cuticule are methyl-branched alkanes (MILLAR, 2010). Ginzel *et al.* (2003b) studied the cuticular chemical profile of female *Xylothrecus colonus* and reported that the contact sex pheromone of this species is a mixture of n-pentacosane, 9-methylpentacosane, and 3-methylpentacosane. In *Neoclytus acuminatus acuminatus* (Fabricius, 1775), the female contact sex pheromone consists of a mixture of 7-methylpentacosane, 7-methylheptacosane, and 9-methylheptacosane (LACEY *et al.*, 2008; HUGHES *et al.*, 2015). Silk *et al.* (2011) determined that the contact sex pheromone of *Tetropium fuscum* (Fabricius, 1787) and *Tetropium cinnamopterum* (Fall,

1900) is a mixture of (*S*)-11-methylheptacosane and (*Z*)-9-heptacosene. Solvent-washed female carcasses treated with (*S*)-11-methylheptacosane eluted a higher male copulation response rate than the (*R*)-enantiomer, while a mixture of (*S*)-11-methylheptacosane and (*Z*)-9-heptacosene was necessary to stimulate copulation response of male *Tetropium cinnamopterum* (SILK *et al.*, 2011). However, Bello *et al.* (2015) investigated the specific rotation of the synthetic (*S*)-11-methylheptacosane reported in Silk *et al.* (2011) and found out that it is consistent with the specific rotation of the (*R*)-enantiomer, suggesting that the stereochemical identification of these compounds might be incorrect.

Unsaturated hydrocarbons are important for mate recognition in Cerambycidae, even though our results showed that the cyclohexene fraction had to be combined with other fractions to stimulate male copulation response comparable to female crude extract in *H. betulinus*. Ginzel *et al.* (2006) reported that (*Z*)-9-nonacosene is only present in cuticular extracts of female *Megacyllene caryae* (Gahan, 1908) and it is an important component of the contact sex pheromone of this species. (*Z*)-9-nonacosene is a homolog of (*Z*)-9-pentacosene, which was described by Ginzel *et al.* (2003a) as the contact sex pheromone of *Megacyllene robiniae* (Forster, 1771). The structural similarity of the female contact sex pheromone of *M. caryae* and *M. robiniae* is expected due to their close phylogenetic relationship (GINZEL *et al.*, 2006). A series of methodological studies executed by Fukaya & Honda (1992; 1995) and Fukaya *et al.* (1996) with the yellow-spotted longicorn, *Psacothea hilaris* (Pascoe, 1857), indicate that different cuticular compounds were responsible for eliciting different steps of the mating sequence in males of this species. It was observed that the initial male orientation response was elicited by compound(s) present on the females prothorax, while compound(s) distributed over the entire body surface of females were responsible for eliciting abdominal bending and genital coupling by males (FUKAYA & HONDA, 1992, 1995; FUKAYA *et al.*, 1996). Even though the authors observed that the methyl-branched alkene (*Z*)-methylpentatriacont-8-ene does not induce the male orientation behavioural step after antennal contact with females, it still elicited copulation in *P. hilaris* males, although at lower levels than the females crude extract, indicating that other compounds might be involved (FUKAYA *et al.*, 1996). Zhang *et al.* (2003) determined that (*Z*)-9-tricosene, (*Z*)-9-pentacosene, (*Z*)-7-pentacosene, (*Z*)-9-heptacosene and (*Z*)-7-heptacosene are considerably more abundant in female cuticular extracts of *Anoplophora glabripennis* (Motschulsky, 1854) than in male cuticular extracts. A blend of these five alkenes elicited mating response in *A. glabripennis* males comparable to the crude extract of females (ZHANG *et al.* 2003).

Gas chromatography analyses of cuticular profiles of some Cerambycidae species have shown few diferences between males and females despite the fact that males can clearly differentiate other males from females after antennal contact is made (HANKS *et al.*, 1996; MILLAR & HANKS, 2017). Analyses of gas chromatograms of cuticular extracts of male and female *H. betulinus* apparently have quantitative differences among them and two sex-specific compounds were identified on male cuticular extracts. Gas chromatographic analysis and mass spectra of these two compounds reveal that compound 1 is (*E*)-6,10-dimethyl-5,9-undecadien-2-one (geranylacetone) and compound 2 is (*R*)-(-)-(*E*)-6,10-dimethyl-5,9-undecadien-2-yl acetate (fuscumol acetate). These compounds have been previously identified as being part of the long-range sex-pheromone of *H. betulinus* (FONSECA *et al.*, 2010; VIDAL *et al.*, 2010). The combination of crude extracts of a cohort of five males and crude extracts of a cohort of five females and the fractionation of these male and female extracts with hexane, cyclohexene and ether permit clearer examination of the cuticular profile of male and female *H. betulinus* due to the higher concentration of compounds and partition of crude extracts in different fractions.

The largest difference in concentrations between males and females in Fraction E was observed for (*E*)-(*R*)-fuscumol acetate. (*E*)-(*R*)-Fuscumol acetate might also be produced by females of this species in very low concentrations, so it is not detected in gas chromatografic analysis of individual females crude extracts. Another possible explanation is that the (*E*)-(*R*)-fuscumol acetate might be transferred from males to females during copulation, once adult females are collected from the fields and transferred to laboratory to be used in bioassays.

Males of *H. betulinus* respond to differences in concentration of fuscumol acetate and the *R* enantiomer of fuscumol acetate alone or in combination with its *S* enantiomer [(*R/S*)-fuscumol acetate bioassay] is sufficient and necessary to elicit male copulation response rate comparable to the individual female fraction E [the fraction in which the natural (*R*)-(*E*)-fuscumol acetate was identified]. According to a recent study developed by Bello *et al.* (2015), in which the absolute configuration of 36 methyl-branched hydrocarbons from 20 species of 9 insect orders was determined [including the Cerambycidae species: *Brothylus gemmulatus* (LeConte, 1859), *Xylotrechus colonus* (Fabricius, 1775), *Monochamus clamator* (LeConte, 1852) and *M. titillator* (Fabricius, 1775)], all were present in the (*R*)-configuration. Although, there are a few exceptions, like males of *Tetropium fuscum* which can discriminate the (*R*)- and (*S*)-enantiomers of 11-methylheptacosane (the female contact sex pheromone of this species), and only perform the mating sequence in response to the (*S*)-configuration, while a blend of (*S*)-11-methylheptacosane and (*Z*)-9-pentacosene was required to elicit the full mating sequence for male *T. cinnamopterum* (SILK *et al.*, 2011). Another example is the study of Fukaya *et al.* (1997), which shows that even though the (*Z*)-enantiomer of 21-methylpentatriacont-8-ene elicited stronger mating responses on males of *Psacothea hilaris* than its (*E*)-enantiomer, males of this species do not distinguish between (*R*)- and (*S*)-enantiomerical forms of this compound.

Other compounds of *H. betulinus* male and female Fraction E that present different concentrations between sexes might be important to stimulate mating behaviour in *H. betulinus* males and could increase the male copulation response rate if combined with the (*R*)-(*E*)-fuscumol acetate. For example, the compounds Y and Z were not identified, but their mass spectra suggest that the compound Y might be a Δ-lactone, due to the fragmentation peak 99, and the compound Z might be a cetone, according to the GC-MS library (FIGURE 18).

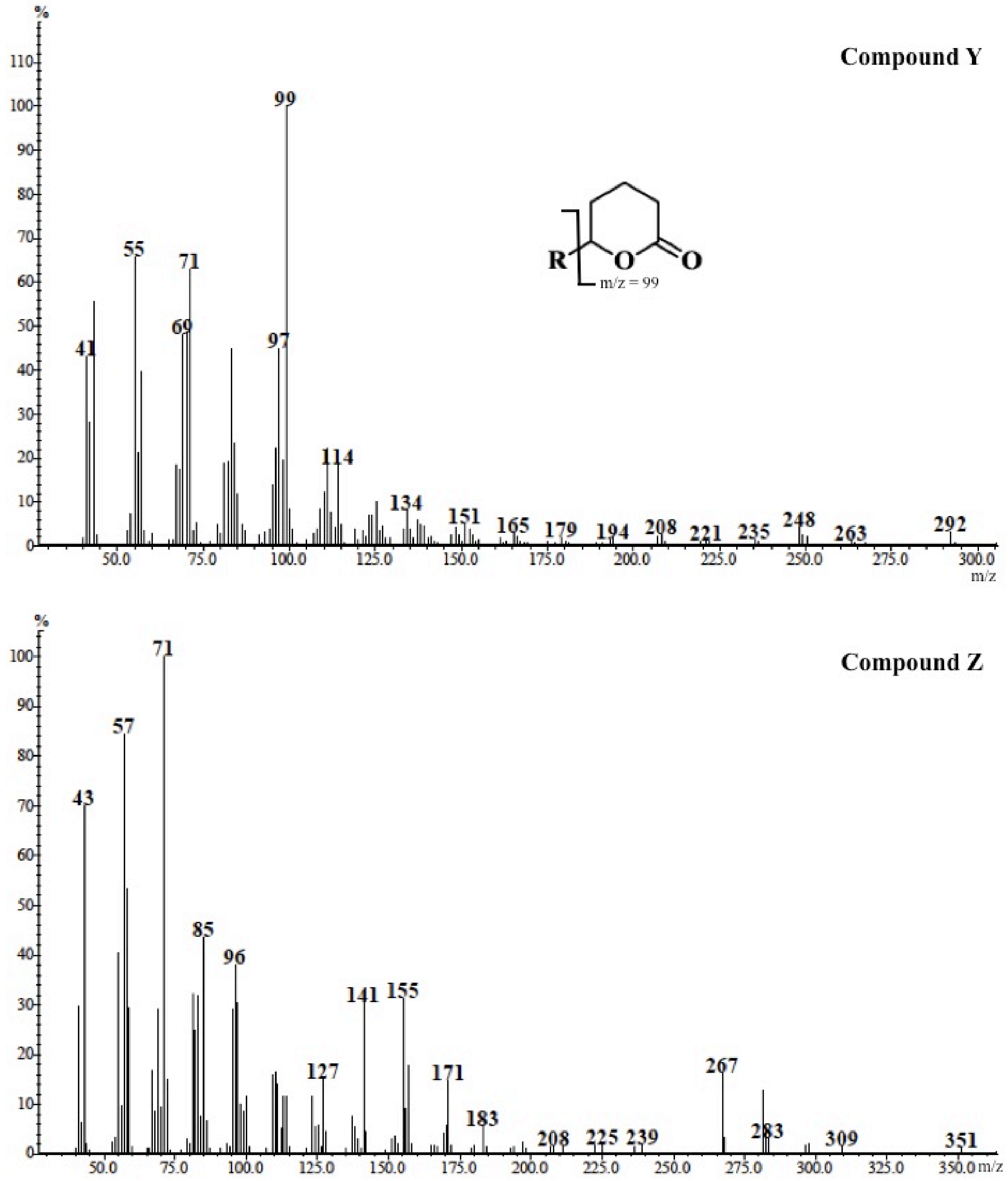

FIGURE 18: Mass spectra of unidentified compounds Y and Z present in the ether fraction of male and female *Hedypathes betulinus* (FIGURE 12 – p. 51).

Management tactics and control programs using the contact pheromones of pest species are not well developed. In recent years there has been an increase in the number of studies of the physiology, function and action mechanisms of pheromone binding proteins (PBPs) in insect antennae (VOGT *et al.*, 1999; PENG & LEAL, 2001; ISHIDA *et al.*, 2002; KRIEGER & ROSS, 2005; REISENMAN *et al.*, 2016). Advance in these and other fields may result in novel applications of short-range sex pheromones of cerambycids and other pest insects in the development and optimizations of monitoring and control tactics.

The antennae of insects are importante sensory organs and respond to stimuli from multiple modalities including sound, smell and touch (LI *et al.*, 2011). Numerous antennal sensilla are present covering the surface of all antennal segments, which conain sensory receptors involved in mechanical or chemical reception (ALTNER & PRILLINGER, 1980; ZACHARUK, 1980). The chemosensilla have gustatory and olfactory receptors that detect chemical stimuli, presenting high concentration of proteins surrounding the dentrites of gustatory and olfactory neurons (LI *et al.*, 2011). These proteins act as carriers in the transport of hydrophobic molecules across the aqueous sensillar lymph to reach the olfactory receptors (LI et al., 2011; LEAL, 2013). The odorant binding proteins (OBPs) and the chemosensory proteins (CSPs) are the most studied olfactory proteins in insects (see PELOSI *et al.*, 2005). OBPs have been extensively studied in different insect orders like, Cerambycidae (PENG & LEAL, 2001; HU *et al.*, 2016), Hymenoptera (KRIEGER & ROSS, 2005), Hemiptera (VOGT *et al.*, 1999), Isoptera (ISHIDA *et al.*, 2002) etc. PBPs are basically OBPs that are capable of binding pheromone molecules that enter the sensilla pores, transporting them through the sensilla aqueous compartment to the olfactory receptors, thus the insect can localize and identify conspecifics for reproduction (TEGONI *et al.*, 2004). Future studies are needed to improve our understading about the action mechanisms of the PBPs in the mating recognition system of pest insects aiming to develop new and upgrade existing management and control strategies involving the manipulation of this insect mate recognition system.

7 CONCLUSIONS

- Males *H. betulinus* rely on compounds present in the epicuticular wax layer of females for mate recognition
- The chemical compounds present on the epicuticular wax layer of females are required in mate recognition of *H. betulinus*.
- The ether fraction [individually (fraction E bioassay), combined with fraction H (fraction E+H bioassay), or combined with fractions C and H (fraction E+C+H bioassay)] is necessary and sufficient to recover male copulation response to freeze-killed female cadavers treated with the crude extract of epicuticular wax layer in this species (crude extract bioassay), and the necessary and sufficient compounds to recover mating activity in *H. betulinus* are present in this fraction.
- (*E*)-geranilacetone and (*R*)-(*E*)-fuscumol acetate are only present in gas chromatografic analyses of epicuticular crude extract of males.
- Gas chromatographic analyses of epictuticular crude extracts of male and female *H. betulinus* show diferences in concentrations of some compounds present in the epicuticular wax layer of both sexes.
- (*R*)-*(E)*-Fuscumol acetate is present in gas chromatografic analyses of female ether fractions that were obtained from extracts of at least five females.
- (*R*)-*(E)*-Fuscumol acetate is present in very different concentrations in *H. betulinus* male and female ether fractions (Fraction E), but the female equivalent of its racemic mixture and its R enantiomer are necessary and sufficient to recover male copulation response to freeze-killed female cadavers treated with the individual ether fraction (fraction E bioassay) in this species.

8 FINAL CONSIDERATION

Future studies should investigate if the presence of other compounds that have different concentrations in male and female *H. betulinus* ether fractions can increase mating response rate in males observed to (*R*)-fuscumol acetate.

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