UNIVERSIDADE TECNOLÓGICA FEDERAL DO PARANÁ
PROGRAMA DE PÓS-GRADUAÇÃO EM AGRONOMIA

JUCELAINE HAAS

TOXICIDADE DE EXTRATOS VEGETAIS AO PERCEVEJO BRONZEADO DO EUCALIPTO *Thaumastocoris peregrinus* (HEMIPTERA: HETEROPTERA: THAUMASTOCORIDAE) E ORGANISMOS NÃO-ALVO

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PATO BRANCO
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Orientador:
Prof. Dr. Sérgio Miguel Mazaro
Co-orientadores:
Profa. Dra. Michele Potrich
Prof. Dr. Everton Ricardi Lozano

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por

JUCELAINE HAAS

Tese apresentada às oito horas e trinta min. do dia 27 de novembro de 2015 como requisito parcial para obtenção do título de DOUTORA EM AGRONOMIA, Linha de Pesquisa – Produção Vegetal, Programa de Pós-Graduação em Agronomia (Área de Concentração: Produção vegetal) da Universidade Tecnológica Federal do Paraná, Câmpus Pato Branco. A candidata foi arguida pela Banca Examinadora composta pelos membros abaixo designados. Após deliberação, a Banca Examinadora considerou o trabalho APROVADO.

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À minha filha Vitória,
cujos beijos, abraços e sorrisos são meus sapatinhos vermelhos mágicos;
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'Andy, how did you get up there?'

'I fell!'

Little Britain

RESUMO

Haas, Jucelaine. Toxicidade de Extratos Vegetais ao Percevejo Bronzeado do Eucalipto *Thaumastocoris peregrinus* (Hemiptera: Heteroptera: Thaumastocoridae) e Organismos Não-Alvo. 59 f. Tese (Doutorado em Agronomia) – Programa de Pós-Graduação em Agronomia (Área de Concentração: Produção vegetal), Universidade Tecnológica Federal do Paraná. Pato Branco, 2015.

Thaumastocoris peregrinus (Hemiptera: Thaumastocoridae) é um inseto originário da Austrália que está causando sérios danos à cultura do eucalipto ao redor do mundo. Ao alimentar-se da seiva das folhas, causa seu bronzeamento, podendo levar à desfolha. Medidas de controle estão sendo estudadas e a mais promissora é o parasitoide de ovos *Cleruchoides noackae* (Hymenoptera: Mymaridae). Produtos alternativos a base de compostos provenientes de plantas com potencial inseticida também poderiam ser uma ferramenta importante, e talvez serem utilizados concomitantemente com o parasitoide, visando um controle mais efetivo. Desta forma, o objetivo deste trabalho foi verificar a ação dos extratos aquosos de *Matricaria chamomilla*, *Echinodorus grandiflorus*, *Punica granatum*, *Maytenus ilicifolia* e *Origanum majorana* a 5% sobre *T. peregrinus*. Além disso, estudar sua possível toxicidade contra *C. noackae* e *Gallus domesticus* L., tendo em vista que estes compostos podem ter efeito negativo indesejado sobre organismos não alvo. Em uma primeira etapa, cromatografia líquida de alta eficiência (HPLC) foi utilizada para verificar os compostos fenólicos presentes nos extratos. Os extratos, então, foram testados sobre percevejo adultos, em confinamento (para verificar a ação inseticida) e teste de livre escolha (para verificar a ação repelente). Os três extratos que mostraram melhores resultados foram selecionados para os testes com os organismos não alvo. Com relação à *C. noackae*, testes pré e pós-parasitismo, de confinamento e de livre-escolha foram realizados para verificar se os extratos afetariam a escolha do hospedeiro pelas fêmeas ou o desenvolvimento das fases imaturas do parasitoide. Para verificar se os extratos seriam tóxicos a *G. domesticus*, estes foram adicionados à dieta de aves juvenis por cinco dias. Parâmetros como peso, consumo de alimento, quantificação de enzimas séricas e análise histopatológica foram realizados. Por meio das análises cromatográficas, foram detectados os ácidos gálico, ferúlico, cafeico, cumárico e vanílico. Os extratos levaram à mortalidade de 100% dos insetos em até 49% do tempo, quando comparados com a testemunha, mas *E. grandiflorus*, *Matricaria chamomilla* e *Maytenus ilicifolia* destacaram-se mostrando efeito repelente, sendo selecionados para a próxima etapa. Nenhum destes afetou a escolha do hospedeiro pela fêmea ou a emergência dos parasitoides, quando comparado com a testemunha. Além disso, os extratos não causaram alterações em *G. domesticus,* em nenhum dos parâmetros avaliados. Desta forma, verificou-se que *E. grandiflorus*, *Matricaria chamomilla* e *Maytenus ilicifolia* têm potencial para serem utilizados no controle de *T. peregrinus*, bem como mostraram-se seguros para *C. noackae* e *G. domesticus.*

Palavras-chave: Controle alternativo, Pragas florestais, Segurança, *Matricaria chamomilla*, *Echinodorus grandiflorus*, *Punica granatum*, *Maytenus ilicifolia, Origanum majorana, Cleruchoides noackae*

ABSTRACT

HAAS, Jucelaine. Toxicity of Plant Extracts to the Bronze Bug *Thaumastocoris peregrinus* (Hemiptera: Heteroptera: Thaumastocoridae) and Non-Target Organisms. 59 f. Tese (Doutorado em Agronomia) – Programa de Pós-Graduação em Agronomia (Área de Concentração: Produção vegetal), Federal University of Technology - Paraná. Pato Branco, 2015.

Thaumastocoris peregrinus (Hemiptera: Thaumastocoridae) is an insect from Australia which is causing severe damage to eucalyptus crops around the world. When feeding from the leaves sap, it causes bronzening, and in extreme cases, may lead to the tree death. Control methods have been studied and the most promising so far is the egg parasitoid *Cleruchoides noackae* (Hymenoptera: Mymaridae). Alternative products from plants with insecticidal properties could also be a viable option, and they might even be used concomitantly with *C. noackae*, aiming for a most effective control, but still safe for the environment. Thus, the objective of this work was to verify the action of 5% aqueous plant extracts of *Matricaria chamomilla*, *Echinodorus grandiflorus*, *Punica granatum*, *Maytenus ilicifolia* and *Origanum majorana* on *T. peregrinus.* In addition, we aimed to study the extracts potential toxicity to *C. noackae* and *Gallus domesticus* L., since the plant compounds might have negative effect upon the non-target organisms. At first, HPLC (High Performance Liquid Chromatography) was used to verify which phenolic compounds would be found in the plant extracts. These were tested on bronze bug adults, in confinement test (to verify the insecticidal action of the extracts) and free-choice test (to verify the repellency). The extracts that showed better results were selected for further tests with non-target organisms. Regarding *C. noackae*, pre-parasitism and post-parasitism, confinement and free-choice tests were performed to verify if the extracts would affect the host-choosing by the female or the development of the immature stages of the parasitoid. To verify if the extracts would be toxic to *G. domesticus*, the plant extracts were added to young birds feed for five days. Parameters such as weight gain, food intake, quantification of serum enzymes and histopathological analysis were carried out. HPLC analysis detected gallic, ferulic, vanillic, caffeic and cumaric acid in the extracts samples. All plant extracts tested reduced *T. peregrinus* survival, but *E. grandiflorus*, *Matricaria chamomilla Maytenus ilicifolia* had also a repellent effect, and were tested on the non-target organisms. None of these extracts affected neither the host choice by *C. noackae* nor adult emergency, when compared to the control group. In addition, the extracts did not cause alterations in any of the studied parameters. Thus, we verified that *E. grandiflorus*, *Matricaria chamomilla* and *Maytenus ilicifolia* have potential to be used to control *T. peregrinus* and are safe to *C. noackae* and *G. domesticus.*

Keywords: Alternative control, Forestry pests, Safety, *Matricaria chamomilla*, *Echinodorus grandiflorus*, *Punica granatum*, *Maytenus ilicifolia, Origanum majorana, Cleruchoides noackae*

LISTA DE TABELAS

SUMÁRIO

1 INTRODUÇÃO GERAL

De acordo com a SEAB (201), o plantio de eucalipto no Paraná representou 42% da área destinada ao setor floretal. Junto com o pinus, sua renda representou 5,7% da receita total do estado em 2014/2015. Queiroz (2009) relata que a intensificação do comércio, a partir da década de 90, levou a introdução de insetos-praga no território nacional, causando prejuízos e aumento nos custos da produção.

Entre as pragas exóticas recentemente introduzidas no território brasileiro de maior importância, destacam-se o psilídeo-de-concha, *Glycaspis brimblecombei* Moore (Hemiptera: Psyllidae) (WILCKEN 2003; BREDA et al., 2010), as vespas galhadoras, *Epichrysocharis burwelli* Schauff (Himenoptera: Eulophidae) (SANTANA; ANJOS, 2007) e *Leptocybe invasa* Fisher & LaSalle (Hymenoptera: Eulophidae) (WILCKEN, 2008a) e o percevejo bronzeado, *Thaumastocoris peregrinus* (Hemiptera: Thaumastocoridae) (WILCKEN et al., 2010; PEREIRA et al., 2013).

O percevejo bronzeado, como é conhecido, é um inseto fitófago, cuja presença no país foi primeiramente registrada em 2008, tanto em São Paulo quanto no Rio Grande do Sul (WILCKEN, 2010), posteriormente, em 2009 no Paraná (BARBOSA et al., 2010), Santa Catarina e Rio Grande do Sul (SAVARIS et al., 2011) e, em 2011 em Goiás (PEREIRA et al., 2013).

Thaumastocoris peregrinus provoca o prateamento das folhas pela sucção da seiva e posterior bronzeamento, seguido do secamento e queda das mesmas (WILCKEN, 2008b). Quando fortemente infestadas, as árvores exibem um avermelhamento das folhas. Dessa forma, há redução da capacidade fotossintética, afetando toda a cadeia produtiva. Conforme a infestação progride, a copa inteira fica amarelo-avermelhada e há desfolha parcial ou total, podendo levar a planta à morte (NOACK et al., 2009).

Países como a África do Sul, Chile e Brasil estão investindo no controle de *T. peregrinus* com a utilização do parasitoide de ovos *C. noackae*, único inimigo natural conhecido da praga na Austrália (IPEF, 2010). *C. noackae* é um parasitoide de ovos de percevejo e, com isso, controlam este inseto antes que este cause dano econômico à cultura (FAO, 2012; EMBRAPA, 2013).

Além do controle biológico, a utilização de plantas com atividade

inseticida também pode ser uma alternativa viável no controle de *T. peregrinus.* Zoubiri and Baaliouamer (2014) relatam que a utilização de produtos botânicos estão tendo uma importância cada vez maior devido às suas propriedades eco-toxicológicas e por serem uma fonte prolífica de compostos químicos bioativos.

No entanto, a utilização desses agentes de controle desperta preocupação sobre os possíveis efeitos negativos em outros organismos no ambiente. Ccertos compostos de plantas usados como inseticidas podem ser tóxicos para organismos considerados não-alvo (Isman, 2008). Nyahangare et al. (2012) complementam, relatando que riscos potenciais à saúde humana existem, principalmente durante a preparação e aplicação dos produtos a base de extratos vegetais.

No caso de *C. noackae*, não há estudos que avaliaram a toxicidade extratos vegetais sobre este parasitoide. Estes efeitos podem acontecer pela interferência dos extratos nos parâmetros biológicos ou no comportamento dos insetos não-alvo. Porém, esses efeitos podem ser evitados ou minimizados por meio de estudos de interação e estudos de época de aplicação dos agentes e época de liberação dos parasitoides. Tendo conhecimento da eficiência dos extratos vegetais, e sabendo da necessidade de, algumas vezes, serem empregados concomitantemente com *C. noackae*, é necessário o estudo desta interação em laboratório para poder estimar os resultados em campo e posteriormente testá-los neste ambiente.

Com relação à toxicidade de compostos das plantas inseticidas para vertebrados, um número restrito de pesquisas foi realizado (BELMAIN et al., 2001; BONINCONTRO et al., 2007; NYAHANGARE et al., 2012), sendo que nenhuma delas utilizou aves. Desta forma é importante conhecer e quantificar se um possível agente de controle apresenta efeitos colaterais para organismos não-alvo. Lucho et al. (2009) reforça a importância de testes serem realizados em aves, devido à pouca informação existente.

O Ministério da Agricultura, Pecuária e Abastecimento (Brasil, 2006), estabelece procedimentos para experimentos de segurança de agrotóxicos para vários organismos não-alvo, incluindo-se aves. Nardo et al. (1999) sugerem a utilização de frangos (*Gallus domesticus* L.) para este fim. *G. domesticus* é uma ave cosmopolita que pode entrar em contato com estes extratos de diversas maneiras,

sendo uma delas é ao se alimentar de insetos e grãos contaminados com os mesmos, ou ainda por contaminação direta quando da aplicação destes no campo. Além disso, este animal serve como modelo dos efeitos que podem vir a ocorrer com aves silvestres de diversas guildas eventualmente presentes na cultura do eucalipto e que entrem em contato com os extratos de planta inseticida. Desta forma, estudos de toxicologia que avaliem possíveis efeitos desse método alternativo de controle sobre aves são de grande importância.

Desta forma, o objetivo deste trabalho foi verificar a toxicidade de extratos vegetais sobre *T. peregrinus* e *C. noackae* e *G. domesticus*.

2 CAPÍTULO I –TOXICITY OF PLANT EXTRACTS ON *Thaumastocoris peregrinus* (CARPINTERO & DELLAPÉ) (HEMIPTERA: THAUMASTOCORIDAE)

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ABSTRACT

The eucalyptus bronze bug, *Thaumastocoris peregrinus*, is an exotic pest of eucalyptus crops that has spread worldwide. Thus, the objective of this study was to evaluate the toxicity of aqueous plant extracts at 5% of *Matricaria chamomilla*, *Echinodorus grandiflorus*, *Punica granatum*, *Maytenus ilicifolia* and *Origanum majorana* on *T. peregrinus*. For that, free and no free-choice tests were performed. Leaf disks, treated with 5% aqueous extract, were: (1) put inside a tube with *T. peregrinus* adults and their longevity evaluated. Each repetition was one leaf disk/tube (no-choice test); (2) for the free-choice test, one leaf disk of each treatment was put inside a petri dish, and offered to *T. peregrinus.* Faecal deposits on each leaf disk were quantified (free-choice test). In addition, High Performance Liquid Chromatography (HPLC) was carried out to verify phenolic compounds present in the plant extracts. All plant extracts lower the survival of *T. peregrinus* adults up to nearly 50%. Regarding the free-choice experiment, *T. peregrinus* fed with eucalyptus disk leaves containing *E. grandiflorus*, *Matricaria chamomilla* and *Maytenus ilicifolia* extracts produced less faecal deposits when compared to the other plant extracts and to the control group. In addition, HPLC detected gallic, ferulic, caffeic, coumaric, and vanillic acid in the extracts samples. These results suggest that these three plant extracts had a repellent effect on *T. peregrinus* adults, aside from reducing its survival, and the phenolic compounds may have contributed to these results.

Keywords: bronze bug, phenolic compounds, *Eucalyptus*.

1. INTRODUCTION

Thaumastocoris peregrinus (Carpintero and Dellapé) (Hemiptera: Thaumastocoridae), known as the bronze bug, is a small sap-feeding insect from Australia (Carpintero and Dellape, 2006; Noack et al., 2011; Nadel and Noack, 2012). It has spread globally and has become a major pest in various species of eucalyptus in Africa, South America, Europe and New Zealand (Martínez and Bianchi, 2010; Nadel et al., 2010; Wilcken et al., 2010; Laudonia and Sasso, 2012; Sopow et al., 2012).

This insect reaches no more than 4 mm when adult. It has a short life cycle (an average of 35 days) and the female can lay up to 60 eggs during her lifespan (Jacobs and Neser, 2005; Noack and Rose, 2007; Soliman et al., 2012). This high biotic potential enables *T. peregrinus* population to have several generations per year, and to spread rapidly to new areas (Nadel et al., 2015).

Chemical control has been proven to be effective in urban areas in Australia (Noack et al., 2009), although it raises issues about potential environmental problems. Biological control strategies are being studied to manage *T. peregrinus* population. The egg-parasitic wasp *Cleruchoides noackae* Lin and Hubner (Hymenoptera: Mymaridae) is the most promising so far, though there are not available published data to confirm its efficiency (Barbosa et al., 2010; Mascarin et al., 2012; Garcia et al., 2013; Santadino et al., 2013; Dias et al., 2014).

Plants with insecticidal activity could also be a viable alternative to control *T. peregrinus*. Botanicals are having renewed importance, due to their eco-toxicological proprieties and to being a source of bioactive compounds (Zoubiri and Baaliouamer, 2014). Besides, studies confirming their efficiency to control forest pests have been carried out (Kanat and Alma, 2004; Sharma et al., 2006); nevertheless, no information is available regarding *T. peregrinus.*

Therefore, the objective of this study was to evaluate the toxicity of the aqueous extracts of *Matricaria chamomilla* (Asteraceae), *Echinodorus grandiflorus* (Alismataceae), *Punica granatum* (Punicaceae), *Maytenus ilicifolia* (Celastraceae) and *Origanum majorana* (Lamiaceae) on *T. peregrinus* in laboratory.

2. MATERIAL AND METHODS

The bioassays and chemical analysis of the plant extracts components (High Performance Liquid Chromatography - HPLC) were performed at the Laboratory of Biological Control, and Central of Analysis of the Federal University of Technology - PR, in Dois Vizinhos and Pato Branco, respectively.

2.1 Insects

T. peregrinus eggs were obtained from a well-established colony kept at the Laboratory of Forest Entomology (Embrapa Florestas, Brazilian Corporation of Agricultura Research), reared on *Eucalyptus bentamii Maiden et Cambage (Myrtaceae) (23 ± 2ºC, 60 ± 10% RH and 12 hrs photoperiod)* as described by Beltramin (2014) and the experiments were run under the same conditions. Nymphs and adults of *T. peregrinus* were reared in branches of *Eucalyptus benthamii* Maiden et Cambage (Myrtaceae) (23 ± 2ºC, 60 ± 10% RH and 12 hrs photoperiod) and strips of towel paper were distributed along the leaves for oviposition. These strips were replaced daily in order to obtain eggs laid within a 24 hrs frame, which were used in the experiment.

2.2 Plant extracts

Leaves of *E. grandiflorus* and *P. granatum* (collected in Dois Vizinhos, Parana State, Brazil and voucher specimens deposited at the Herbarium of the Federal University of Technology – Paraná, UTFPR), *Maytenus ilicifolia, O. majorana* and flowers of *Matricaria chamomilla* (acquired from COOPERFLORA, Turvo, PR) were used to prepare the extracts.

Plant material was dried in oven (60ºC for 48 hrs) and ground in a Willy mill lab grinder. Each of the five plant extracts was prepared at a concentration of 5% w/v by adding 5 g of the plant powder to 100 mL of distilled water and the mixture was kept away from the light from 48 hrs. Filtration was performed with filter paper, shortly before the start of the experiment.

2.3 Toxicity (no-choice test)

Fully expanded leaves of *Eucalyptus dunni* were washed in sodium hypochlorite 2%, dried and immersed for 5 sec in the plant extracts. The control group was immersed in sterile distilled water. After that, the leaves were left to dry in a laminar flow cabinet. Circles of 2.4 cm in diameter were cut from the leaves with a circle cutter near the petiole and put inside sterile flat bottom glass tubes with hydrogel. One *T. peregrinus* adult (< 48 hrs) was placed on each disk leaf. To prevent scape, each tube was covered with voile. One tube was considered a repetition, and for each treatment, there were 12 repetitions.

The bioassay was kept in germination chamber (26 ± 2°C, 60 ± 10% RH and 12 hrs photoperiod) and *T. peregrinus* survival time was evaluated every six hours, for 144 hrs. The obtained data was submitted to one-way ANOVA followed by Scott-Knott Test (p <0.05) (Assistat 7.7®, Silva, 2014).

2.4 Repellence activity (free-choice test)

Leaf disks, one from each treatment, were prepared as described in item 2.3, and put inside petri dishes (150 x 20 mm) lined with filter paper dampened with water, randomly, at the same distance from each other. In the centre of each disk, 10 adults were placed. The dishes were closed and kept in germination chamber (26 ± 2°C, 60 ± 10% RH, 12 hrs photoperiod), and the number of faecal drops on each leaf disc was evaluated every 24 hrs for seven days (daily faecal drops (DFD) = total of faecal drops (TFD) – previous day total of faecal drops (PFD)).

Each dish was considered a repetition and 24 repetitions were used for this experiment. The experimental design was split-plot and the data was submitted to ANOVA followed by Tukey Test (p < 0.05) (Assistat 7.7®, Silva, 2014).

2.5 Qualitative Analysis of High Performance Liquid Chromatography (HPLC) Profiling

The qualitative analysis of extracts compounds was carried out using reversed-phase HPLC according to Francisco and Ressurreccion (2009) with slight

modifications. Each aqueous extract sample (10 µL) was injected in an HPLC equipment with photodiode array and fluorescence detection, reverse-phase column C18 (250 x 4.6 mm, 5 m). The mobile phase consisted of water/acetic acid (0.1%, v/v) (solvent A) and methanol/acetic acid (0.1% v/v) (solvent B), at 1 mL/min. The gradient conditions were as follows: 5-7% solvent B in 7 min, 17% of B in 75 min, 45% of B in 110 min, 70% of B in 117 min, 100% of B in 124 min and 5% of B in 129 min. The column was kept at 30ºC and the chromatograms processed with specific software.

Identification of unknown compounds was based on matching their retention times with those of pure standards and by the absorption spectra at the wavelengths 272, 313 e 360 nm of the ultraviolet region with photodiode detector. Quantification was determined with external standardization with identical standards of ferulic, gallic, vanillic, caffeic and coumaric acid in concentrations from 0.5 to 7.5 $\mu g.mL^{-1}$ (Table 1).

The detection limit (DOL) and quantification limit (QOL) values were obtained with the calibration curve equation according to ICH (1996).

3. RESULTS

3.1 No-choice and free-choice tests

All plant extracts affected *T. peregrinus* adults, reducing their survival, when compared to the control group (Table 2).

On the other hand, regarding faecal deposits in the free-choice bioassay, there was no statistical difference amongst the treatments at 24, 48, or 144 hours after starting the experiment. Nevertheless, *T. peregrinus* fed with eucalyptus disk leaves containing *E. grandiflorus*, *Matricaria chamomilla* and *Maytenus ilicifolia* extracts produced less faecal deposits when compared to the other plant extracts and the control group (Table 3). This result suggests that these plant extracts had a repellent effect on the insects.

3.2 High Performance Liquid Chromatography

The phenolic compounds, gallic, vanillic, caffeic, coumaric and ferulic acid were identified and quantified from the plant extracts (Table 4). The highest and lowest concentration of gallic acid was found on *P. granatum* (1.84 $mg.g^{-1}$) and *O. majorana* (0.72 $mg.g^{-1}$), respectively. Vanillic acid was found only in *Maytenus ilicifolia* (0.35 $mg.g^{-1}$). Caffeic acid was detected in both *Matricaria chamomilla* and *Maytenus ilicifolia* (0.72 $mg.g^{-1}$ and 0.06 $mg.g^{-1}$), respectively. Coumaric acid was only found in *E. grandiflorus* (0.01 $mg.g^{-1}$) . Ferullic acid was found in both *Matricaria chamomilla* (1.41 $mg.g^{-1}$) and *E. grandiflorus* (0.46 $mg.g^{-1}$).

4. DISCUSSION

The plant extracts from *P. granatum*, *O. majorana*, *Matricaria chamomilla*, *Maytenus ilicifolia* and *Echinodorus grandiflorus* reduced *T. peregrinus* adults survival. Although, the leaf disks treated with the last three extracts presented a reduced number of faecal drops, indicating a repellent/deterrent effect. The presence of phenolic compounds might have had an important role in this results.

Assays regarding the effect of plant extracts on heteropterans have been carried out with other plants, other forms of extraction and other insects (Carneiro et al., 2011; González et al., 2011; Krinski; Massaroli, 2014); but we did not found any research on the group Thaumastocoridae.

Phenols like coumaric and ferulic acid, found in the plant extracts used in our bioassays, may be found in the insoluble or cell wall, as reservoir for lignin biosynthesis, which by itself may be a defence mechanism (Lattanzio et al., 2006). In addition to toxic and deterrent action of phenolic compounds, oxidation of phenols to polymers, catalysed by polyphenol oxidase (PPO) and peroxidase (POD) is another potential defence mechanism in plants against herbivorous insects, which reduce digestibility, palatability, and nutritional value. Quinones formed by oxidation of phenols, for instance, bind covalently to leaf proteins and inhibit the protein digestion in herbivores (Bhonwong et al., 2009). Moreover, quinones also exhibit direct toxicity to insects (Duffey and Stout, 1996; Bhonwong et al., 2009). Alkylation of amino acids reduces the nutritional value of plant proteins for insects, which in turn negatively

affects the insect growth and development (Bhonwong et al., 2009).

Phenols also play an important role in cyclic reduction of reactive oxygen species (ROS) such as superoxide anion and hydroxide radicals, which in turn activate a cascade of reactions leading to the activation of defensive enzymes (Maffei et al., 2007).

Thus, the phenolic compounds present in *Matricaria chamomilla*, *E. grandiflorus*, *Maytenus ilicifolia*, *O. majorana* and *P. granatum* extracts may have played in important role in the obtained results of our experiments, both in repellent and toxic effect.

These results represent basic research, consequently they should be used to help select plants with insecticidal/repellent properties; and from there, obtain extracts by different extraction methods, detect the active compounds responsible for the action on the insects, and develop environmental-friendly insecticides.

5. ACKNOLEDGEMENTS

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Table 1 – Chromatographic parameters of phenolic compounds from the 5% (w/v) aqueous plant extracts analysed by HPLC.

Phenolic compound	R.T. (min)	UV band (nm)	Linear equation	R^2	LOD ($\mu g.mL^{-1}$)	LOQ ($\mu g.mL^{-1}$)
Gallic acid	8.6	272	Y= 0.313 X – 0.017	0.996	0.10	0.34
Vanillic acid	24.3	260, 280	Y = 0.263 X + 0.023	0.998	0.81	2.72
Caffeic acid	24.6	323	Y = 0.691 X – 0.001	0.997	0.03	0.11
Coumaric acid	28.7	309	Y = 0.540 X – 0.360	0.999	0.02	0.08
Ferulic acid	29.5	322	Y = 0.657 X + 0.007	0.999	0.10	0.36

R.T: retention time; LOD: detection limit; LOQ: quantification limit

Table 2 – Mean survival time, in hours (± SE), of *Thaumastocoris peregrinus* confined with *Eucalyptus dunni* leaves treated with 5% (w/v) aqueous plant extracts under laboratory conditions.

Treatment	Survival (hours)	Survival (days)
Control group	133.0 ± 7.41 a	5.5
Punica granatum	98.0 ± 7.91 b	4.0
Maytenus ilicifolia	89.0 ± 10.82 b	3.7
Echinodorus grandiflorus	77.0 ± 10.22 b	3.2
Origanum majorana	70.0 ± 8.90 b	2.9
Matricaria chamomilla	68.5 ± 6.66 b	2.8
F	7,1024**	

*Means followed by the same letter do not differ by Scott-Knott Test ($p < 0.05$).

Table 3 – Mean of *Thaumastocoris peregrinus* faecal drops (± SE) in *Eucalyptus dunni* disk leaves treated with 5% (w/v) aqueous plant extracts in a free-choice trial, under laboratory conditions.*

Treatments	Number of faecal drops over time (hrs)						
	24	48	72	96	120	144	168
Control group	2.87 ± 0.98 ns	1.04 ± 2.47 ns	22.88 ± 2.70 a	27.58 ± 3.49 a	29.54 ± 3.49 a	29.13 ± 3.36 ns	34.33 ± 4.01 a
Punica granatum	1.58 ± 0.41	6.63 ± 1.19	14.42 ± 1.81 ab	19.00 ± 1.613 ab	23.67 ± 1.56 ab	26.04 ± 1.54	30.87 ± 1,17 ab
Maytenus ilicifolia	2.00 ± 0.47	8.00 ± 1.51	11.08 ± 1.93 b	13.96 ± 2.25 b	18.17 ± 2.61 b	21.79 ± 2.44	27.66 ± 2.76 ab
Echinodorus grandiflorus	1.25 ± 0.52	8.08 ± 1.83	12.17 ± 2.56 b	16.04 ± 2.84 b	18.46 ± 3.14 b	22.63 ± 3.36	25.12 ± 3.42 ab
Origanum majorana	3.16 ± 0.64	12.00 ± 1.87	15.96 ± 2.22 ab	20.33 ± 2.40 ab	25.63 ± 3.16 ab	29.88 ± 3.36	33.97 ± 3,41 a
Matricaria chamomilla	1.87 ± 0.61	8.42 ± 1.93	12.42 ± 2.47 b	15.75 ± 2.90 b	19.67 ± 3.01 ab	21.97 ± 3.05	23.54 ± 3.16 b
Dms	10068						
F	1.9875*						

*Means followed by different letters in the same column differ significantly according to Tukey Test ($p < 0.05$); ns – not significant.

Table 4 – Relative concentrations of phenolic compounds obtained from 5% (w/v) aqueous plant extracts.

Extract	Concentration $mg.g^{-1}$				
	Gallic acid	Vanillic acid	Caffeic acid	Coumaric acid	Ferulic acid
Punica granatum	1.84	n.d	n.d	n.d	n.d
Maytenus ilicifolia	n.d	0.35	0.06	n.d	n.d
Echinodorus grandiflorus	0.30	n.d	n.d	0.01	0.46
Origanum majorana	0.72	n.d	n.d	n.d	n.d
Matricaria chamomilla	0.23	n.d	0.72	n.d	1.41

n.d. - not discovered.

3 CAPÍTULO II – TOXICITY OF PLANT EXTRACTS ON *Cleruchoides noackae* (HYMENOPTERA: MYMARIDAE)

Artigo científico a ser submetido à Revista Agricultural and Forest Entomology (formatado de acordo com esta revista) – Qualis Capes A2 em Ciências Agrárias I

Running title: Botanical insecticides on *Cleruchoides noackae*

ABSTRACT

1 *Cleruchoides noackae* (Hymenoptera: Mymaridae) is the most promising biological control agent against the bronze bug of eucalyptus. Nevertheless, plant extracts may also be a viable option in an integrated pest management program.

2 Thus, it is important to assess possible side effects that these extracts might have on the parasitoid. Therefore, this work aimed to verify the effect of 5% aqueous extracts of *Matricaria chamomilla*, *Maytenus ilicifolia* and *Echinodorus grandiflorus* on *C. noackae*.

3 Tests with eggs treated before being offered to the parasitoid female (pre-parasitism) and tests on which the eggs were treated one and seven days after the female had performed oviposition (post-parasitism) were carried out.

4 There was no statistical difference between the treatments and the control group regarding parasitoid emergence in none of the tests. Although, there was increase in egg viability and number of emerged parasitoids when comparing the eggs treated one and seven days after parasitism for all treatments.

5 In conclusion, none of the plant extracts caused any harmful effect on *C. noackae* under lab conditions, indicating they may be an environment-friendly option to control the bronze bug of eucalyptus.

Key words: Parasitoid, *Thaumastocoris peregrinus*, toxicity, aqueous extract, bronze bug, *Matricaria chamomilla*, *Maytenus ilicifolia*, *Echinodorus grandiflorus*

1. INTRODUCTION

The bronze bug *Thaumastocoris peregrinus* Carpintero & Dellapé (Hemiptera: Thaumastocoridae) is a eucalyptus pest originally from Australia and it is spreading very rapidly worldwide (Carpintero & Dellapé 2006; Wilcken et al. 2010; Nadel et al. 2012; Garcia et al., 2013). It is a sap-feeding insect that attacks eucalyptus leaves. Lightly infested trees may show little or no sign of damage. On the other hand, heavily infested ones display a reddening of the leaves, which are often shed. This may lead to eventual reduction in tree growth and yield (Wilcken et al., 2010).

Since *T. peregrinus* has become a serious problem in eucalyptus crops in recent years, little has been described about its management and control. In this regard, Noack et al. (2009) verified that the systemic insecticide imidacloprid [SilvaShield SL, 20% (AI)] was efficient at reducing its population in urban areas in Australia. But according to Barbosa et al. (2010), Laudonia & Sasso (2012), Nadel & Noack (2012) and Pereira et al. (2013), chemical control is too expensive to be used in large areas, aside from being environment-unfriendly.

An option to chemical control could be the employment of insecticidal plants, being low-cost and of easy access to producers. Thus, Haas et al. (not published) verified that *Echinodorus grandiflorus* (Alismataceae), *Matricaria chamomilla* (Asteraceae) and *Maytenus ilicifolia* (Celastraceae) caused reduction of longevity of *T. peregrinus* under lab conditions.

Additionally, it is agreed that a biological control program is a viable alternative to manage the bronze bug (Barbosa et al., 2010; Nadel et al., 2012; Santadino et al., 2013; Mutitu et al., 2013). General natural enemies such as predators and entomopathogenic fungi have been found attacking or infecting *T. peregrinus* and have been tested in bioassays to verify their potential (Barbosa et al., 2010; Mascarin et al., 2012; Souza et al., 2012; Garcia et al., 2013; Lorenceti, 2013; Santandino et al., 2013). Nonetheless, *Cleruchoides noackae* Lin and Huber (Hymenoptera: Mymaridae) is considered the most propitious biological control agent to be used in the field (Lin et al., 2007; Nadel et al., 2012).

C. noackae is a mymarid wasp, approximately 0.5 mm length and lace-like wing structure. It is a solitary parasitoid of *T. peregrinus* eggs retrieved from the

field in Australia and reared in the lab with the purpose of its future application (Lin et al., 2007). Since then, it has been exported to South Africa and Brazil and from the latter to Uruguay (Lawson, 2012; Botto, 2014).

Mymarid wasps are of great importance in pest management throughout the world (Pena et al., 2010; Sithanantham et al., 2013). However, no studies regarding the toxicity of plant extracts to this parasitoid have been published, regardless of their importance. Therefore, this work aimed to verify the effect of 5% aqueous extracts of *Matricaria chamomilla*, *Maytenus ilicifolia* and *Echinodorus grandiflorus* on *C. noackae*.

2. MATERIAL AND METHODS

The experiments were conducted at the Entomology Laboratory at Embrapa Florestas in Colombo, Brazil, where colonies of *T. peregrinus* and *C. noackae* have started to be maintained in 2009 and 2012, respectively.

2.1 Insects rearing conditions

Nymphs and adults of *T. peregrinus* were reared in branches of *Eucalyptus benthamii* Maiden et Cambage (Myrtaceae) (23 ± 2°C, 60 ± 10% RH and 12 hrs photoperiod) and strips of towel paper were distributed along the leaves for oviposition. These strips were replaced daily in order to obtain eggs laid within a 24 hrs frame, which were used in the experiment.

Cleruchoides noackae was maintained in plastic test tubes which lids were perforated and covered with a fine voile mesh to permit aeration. For their feeding, strips of filter paper (0.5 cm x 5 cm) soaked in a 50% honey solution were added inside the tubes. Daily, *T. peregrinus* eggs were given/replaced for the female oviposition. Both eggs and *C. noackae* adults were maintained in a germination chamber (22 ± 2°C, 60 ± 10% RH and 12 hrs photoperiod).

For all experiments, strips of towel paper containing 10 eggs of *T. peregrinus,* (< 24 hrs old) were used, as well as a couple of *C. noackae* emerged with less than 24 hrs. Aqueous extracts of *E. grandiflorus, Maytenus ilicifolia* and

Matricaria chamomilla at 5% were tested, being applied directly over each egg with a micropipette (2 µL/egg) and left to dry. Control group consisted of eggs treated with distilled water. Each treatment consisted of 20 replicates, each egg-card a replicate (adapted from Potrich et al., 2009).

2.2 Plant extract preparation

Leaves of *E. grandiflorus* (collected in Dois Vizinhos, Parana State, Brazil and voucher specimens deposited at the Herbarium of the Federal University of Technology – Paraná, UTFPR) and *Maytenus ilicifolia,* and flowers of *Matricaria chamomilla* (acquired from COOPERFLORA, Turvo, PR) were used to prepare the extracts. The plant material was dried in shadow for 48 hrs and then, ground in a Willy mill lab grinder. Each of the three plant extracts was prepared at a concentration of 5% w/v by adding 5 g of the plant powder to 100 mL of distilled water and the mixture was kept away from the light from 48 hrs. Filtration of the plant extracts was performed with filter paper, shortly before the start of the experiment.

2.3 Free-choice and no-choice tests (pre-parasitism treatment)

Free-choice and no-choice tests were conducted to evaluate the acceptance of the female to the eggs treated with the plant extracts.

No-choice (confinement) test: Strips of towel paper containing 10 *T. peregrinus* eggs (less than 24 hrs old) were treated with the aqueous extracts or distilled water, left to dry and then placed in a plastic tube with a couple *C. noackae* for 24 hrs. After that, the parasitoids were removed. The egg-cards were then kept inside the tubes for 20 days, when the biological parameters were evaluated (number of *T. peregrinus* nymphs to assess the number of unviable eggs; trapped nymphs and parasitoids in the eggshell; and emerged parasitoids). Each replicate consisted of one couple and one treated egg-card.

Free-choice test: One treated and one control egg-card were placed inside the tube with a couple of *C. noackae* for 24 hrs. Subsequent to the parasitoids removal, each egg-card was placed in an individualised plastic tube and the

evaluation was as described for the no-choice test. Each parameter was evaluated comparing each treatment with its respective control group.

2.4 Effect of aqueous extracts on immature stages of *C. noackae* (post-parasitism treatment)

Young *C. noackae* adults, one male and one female, were transferred to tubes containing honey solution and *T. peregrinus* eggs and then kept for 24 hrs. The exposed eggs were divided in two groups to be treated with the aqueous extracts; the first group was treated one day after parasitism, and the second group treated seven days after parasitism, to guarantee that the eggs would be treated in different immature phases of the parasitoid. The evaluation period and parameters were the same as described for the pre-parasitism tests.

2.5 Data analysis

For the pre-parasitism trials, the experimental design was completely randomised with four treatments and 20 replications. For the post-parasitism trial, a split-plot design was used. Data was submitted to Bayesian inference using Gibbs sampling and Poisson model. Results were compared with Student's *t* test using R software.

3. RESULTS

3.1 Free-choice and no-choice tests (pre-parasitism treatment)

3.1.1 No-choice (confinement) test

Only the eggs treated with *E. grandiflorus* presented lower viability when compared to the control group; none of the plant extracts affected the number

of emerged parasitoids (Table 1). On the other hand, eggs treated with *E. grandiflorus* and *Maytenus ilicifolia* presented more nymphs which were trapped in the eggs, unable to emerge completely, when compared to the control group (0.25 ± 0.11, 0.30 ± 0.12 and 0.05 ± 0.05, respectively).

3.1.2 Free-choice test

There was no statistical difference between the treatments regarding the number of trapped nymphs or emerged parasitoids. Nevertheless, there was a negative effect of *E. grandiflorus* on the eggs viability, when compared to the control group (1.95 ± 0.32 and 1.25 ± 0.25 of unviable eggs, respectively). In addition, the females did not show preference between the treated eggs and the control ones (Table 2).

3.1.3 Effect of aqueous extracts on immature stages of *C. noackae* (post-parasitism treatment)

None of the plant extracts affected the adult emergency, when compared to the control group, neither when applied after one day nor after seven days of parasitism. Nevertheless, all treatments had significantly higher egg viability (Table 3) and number of emerged parasitoid (Table 4) when the application occurred later in the insects development inside the eggs, in comparison to groups treated in the early phase.

4. DISCUSSION

These plant extracts have been selected due to their toxicity to *T. peregrinus*, *C. noackae* host, in our previous laboratory bioassays (Chapter I). The results observed makes evident that neither plant extract tested had negative effects on the parasitoids development, not influencing adult emergence.

Regarding the difference between days one and seven on the post-parasitism experiment, where there was a decrease in number of unviable eggs and

a higher number of adult parasitoids emerged when the eggs were treated seven days after parasitism. The difference might have been caused by the early manipulation of the eggs (treated after 24 hours of parasitism). Right after the eggshell is pierced, a scab is formed around the injury, protecting the embryo (Koppel et al., 2011). Thus, the puncture made by the parasitoid ovipositor would not have increased the permeability of the eggshell to the treatments in the batch treated after 24 hours of parasitism. Given that there is no published study regarding the development of *C. noackae*, we do not know what developmental phase the parasitoid was by the time of the applications.

Several plant extracts were toxic to *Trichogramma japonicum* (Hymenoptera: Trichogrammatidae). Plants like *Acorus calamus* (Acoraceae) caused up to 100% of mortality of the parasitoids (Barman et al., 2003). The concentrations used ranged from 25% to 100%, much higher than the 5% concentration used in our experiments. Certainly, high concentrations of plant extracts have no practical use in crop protection, since it is not economically viable and it would demand great quantities of plant material.

Other studies have been conducted regarding the safety of *Azadirachta indica* (Meliaceae) to parasitoids of Trichogrammatidae group, with the results varying with species and concentration (Hohmann et al., 2010; Mamoon-ur-Rashid et al., 2013; Singh et al., 2015).

Plant extracts efficiency as pest control method is due to secondary metabolites involved in plant defence against pathogens and herbivores. Amongst these compounds, plant phenols, flavonoids and tannins are some of the most common and widespread, playing a major role against insects (War et al., 2011). Some of their deleterious effects on insects are inhibition of protein digestion, toxicity, lesions in the midgut of insects and feeding deterrents (Simmonds, 2003; Bernards & Bastrup-Spohr, 2008; Bhonwong et al., 2009).

Plant compounds may negatively affect parasitoids in several ways, including reducing survivorship, clutch size and/or fecundity. Prejudicial impacts like these may occur either directly, when the developing parasitoid encounters the plant metabolite in the tissue of its host, or indirectly, due to compromised host size or quality (Ode, 2006). For instance, flavonoids had a negative effect on *Aphelinus mali* Hald (Hymenoptera: Aphelinidae), preventing more than 88% of adults from

emerging from mummified aphids (Ateyyat et al., 2012). It has also been demonstrated that the exposure of parasitoids to some phenolic compounds was associated with late pupation and adult emergency in *Alabagrus texanus* (Cresson) (Hymenoptera: Braconidae) (Rose et al., 2015). Therefore, the plants tested could have a negative effect on *C. noackae*.

Our results indicate that any of the tested plant extract could be used in an integrated pest management program and they would not be harmful to *C. noackae.* Nevertheless, these experiments were conducted under laboratory conditions, with high pressure caused by the plant extracts on the parasitoids. Under field conditions, biotic and abiotic factors could influence the outcome. Thus, further tests should be carried out to gather more information and validate the results obtained by our experiments.

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Table 1 – Biological parameters of *Thaumastocoris peregrinus* eggs parasitized by *Cleruchoides noackae* and treated with 5% plant extracts, in confinement (no-choice test) (22 ± 2ºC, 60 ± 10% RH and 12 hrs photoperiod).

Biological Parameter	***Parameter***	***Mean***	***Standard Deviation***	$P_{2.5\%}$	$P_{97.5\%}$	***p-value***
Unviable eggs	*Treatments*					
	Θ_1	5.95	0.55	4.9	7.07	
	Θ_2	6.25	0.56	5.21	7.39	
	Θ_3	5.90	0.54	4.89	7.02	
	Θ_4	6.75	0.58	5.67	7.93	
	Contrasts					
	$\Theta_1 - \Theta_2$	-0.30	0.78	-1.82	1.23	0.35 n.s.
	$\Theta_1 - \Theta_3$	0.05	0.78	-1.49	1.58	0.52
	$\Theta_2 - \Theta_3$	0.35	0.78	-1.15	1.90	0.67
	$\Theta_1 - \Theta_4$	-0.80	0.80	-2.35	0.75	0.16
	$\Theta_2 - \Theta_4$	-0.50	0.81	-2.08	1.08	0.27
	$\Theta_3 - \Theta_4$	-0.85	0.79	-2.42	0.68	0.14
Emerged parasitoids	*Treatments*					
	Θ_1	1.54	0.28	1.05	2.15	
	Θ_2	1.95	0.31	1.38	2.61	
	Θ_3	1.45	0.27	0.98	2.02	
	Θ_4	1.45	0.27	0.97	2.01	
	Contrasts					
	$\Theta_1 - \Theta_2$	-0.40	0.42	-1.24	0.41	0.17 n.s.
	$\Theta_1 - \Theta_3$	0.09	0.39	-0.67	0.86	0.60
	$\Theta_2 - \Theta_3$	0.50	0.41	-0.29	1.32	0.89
	$\Theta_1 - \Theta_4$	0.10	0.39	-0.65	0.87	0.60
	$\Theta_2 - \Theta_4$	0.50	0.41	-0.30	1.32	0.90
	$\Theta_3 - \Theta_4$	0.00	0.38	-0.74	0.75	0.50

$P_{2.5\%}$ and $P_{97.5\%}$ = Interval of 95% of credibility; Θ_1 = control, Θ_2 = *Matricaria chamomilla*, Θ_3 = *Echinodorus grandiflorus* and Θ_4 = *Maytenus ilicifolia*; n.s. non-significant at 5%.

Table 2 – Biological parameters of *Thaumastocoris peregrinus* eggs treated with 5% plant extracts pre-parasitism and parasitized by *Cleruchoides noackae*, in free-choice test, in the different treatments and contrasts (22 ± 2°C, 60 ± 10% RH and 12 hrs photoperiod).

Biological Parameter	***Parameter***	***Mean***	***Standard Deviation***	$P_{2.5\%}$	$P_{97.5\%}$	***p-valor***
Unviable eggs	***Tratamentos***					
	Θ_1	0.95	0.22	0.57	1.42	
	Θ_2	1.10	0.24	0.69	1.61	
	Contrast					
	$\Theta_1 - \Theta_2$	-0.15	0.32	-0.79	0.48	0.31 n.s.
Emerged parasitoids	***Tratamentos***					
	Θ_1	3.10	0.39	2.37	3.92	
	Θ_2	2.85	0.37	2.16	3.63	
	Contrast					
	$\Theta_1 - \Theta_2$	0.24	0.54	-0.81	1.30	0.67 n.s.
Unviable eggs	***Tratamentos***					
	Θ_1	1.25	0.25	0.81	1.78	
	Θ_3	1.95	0.32	1.37	2.62	
	Contrast					
	$\Theta_1 - \Theta_3$	-0.70	0.40	-1.50	0.09	0.04 *
Emerged parasitoids	***Tratamentos***					
	Θ_1	2.25	0.33	1.633	2.94	
	Θ_3	3.05	0.40	2.318	3.89	
	Contrast					
	$\Theta_1 - \Theta_3$	-0.81	0.52	-1.840	0.2132	0.06 n.s.
	Tratamentos					
	Θ_1	1.60	0.28	1.095	2.193	
	Θ_4	1.90	0.31	1.336	2.547	
	Contrast					
	$\Theta_1 - \Theta_4$	-0.30	0.42	-1.130	0.529	0.23 n.s.
Emerged parasitoids	***Tratamentos***					
	Θ_1	3.75	0.43	2.94	4.65	
	Θ_4	3.05	0.39	2.34	3.86	
	Contrast					
	$\Theta_1 - \Theta_4$	0.70	0.57	-0.43	1.87 m	0.89 n.s.

$P_{2.5\%}$ and $P_{97.5\%}$ = Interval of 95% of credibility; Θ_1 = control, Θ_2 = *Matricaria chamomilla*, Θ_3 = *Echinodorus grandiflorus* and Θ_4 = *Maytenus ilicifolia*; n.s. non-significant at 5%.

Table 3 – Unviability of *Thaumastocoris peregrinus* eggs parasitized by *Cleruchoides noackae* and treated with 5% plant extracts, after one and seven days of parasitism (22 ± 2ºC, 60 ± 10% RH and 12 hrs photoperiod).

Biological Parameter	***Parameter***	***Mean***	***Standard Deviation***	***$P_{2,5\%}$***	***$P_{97,5\%}$***	***p-value***
Unviable egss	*Treatments (one day after parasitism)*					
	Θ_1	4.10	0.45	3.27	5.03	
	Θ_2	3.69	0.43	2.91	4.59	
	Θ_3	5.15	0.51	4.20	6.19	
	Θ_4	4.59	0.48	3.71	5.59	
	Contrasts (one day after parasitism)					
	$\Theta_1 - \Theta_2$	0.41	0.62	-0.80	1.62	0.74 n.s.
	$\Theta_1 - \Theta_3$	-1.05	0.68	-2.40	0.29	0.06 n.s.
	$\Theta_2 - \Theta_3$	-1.46	0.66	-2.76	-0.16	0.01 *
	$\Theta_1 - \Theta_4$	-0.49	0.66	-1.81	0.80	0.23 n.s.
	$\Theta_2 - \Theta_4$	-0.90	0.65	-2.18	0.35	0.08 n.s.
	$\Theta_3 - \Theta_4$	0.55	0.70	-0.83	1.95	0.79 n.s.
	Treatments (seven days after parasitism)					
	Θ_{11}	2.05	0.32	1.46	2.75	
	Θ_{22}	2.45	0.35	1.81	3.17	
	Θ_{33}	3.15	0.41	2.41	4.02	
	Θ_{44}	2.45	0.35	1.82	3.19	
	Contrasts (seven days after parasitism)					
	$\Theta_{11} - \Theta_{22}$	-0.40	0.48	-1.35	0.55	0.20 n.s.
	$\Theta_{11} - \Theta_{33}$	-1.10	0.53	-2.16	-0.06	0.01 *
	$\Theta_{22} - \Theta_{33}$	-0.71	0.54	-1.77	0.34	0.09 n.s.
	$\Theta_{11} - \Theta_{44}$	-0.40	0.48	-1.35	0.55	0.20 n.s.
	$\Theta_{22} - \Theta_{44}$	-0.00	0.49	-0.98	0.96	0.50 n.s.
	$\Theta_{33} - \Theta_{44}$	0.70	0.54	-0.36	1.76	0.90 n.s.
	Contrasts of treatments between one day and seven days of treatment					
	$\Theta_1 - \Theta_{11}$	2.05	0.56	0.99	3.16	1.00 *
	$\Theta_2 - \Theta_{22}$	1.24	0.56	0.15	2.36	1.00 *
	$\Theta_3 - \Theta_{33}$	1.99	0.66	0.71	3.28	1.00 *
	$\Theta_4 - \Theta_{44}$	2.15	0.59	0.98	3.31	1.00 *

$P_{2.5\%}$ and $P_{97.5\%}$ = Interval of 95% of credibility; Θ_1 = control, Θ_2 = *Matricaria chamomilla*, Θ_3 = *Echinodorus grandiflorus* and Θ_4 = *Maytenus ilicifolia* (one day after parasitism); $\Theta_{1\,1}$ = control, $\Theta_{2\,2}$ = *Matricaria chamomilla*, $\Theta_{3\,3}$ = *Echinodorus grandiflorus* e $\Theta_{4\,4}$ = *Maytenus ilicifolia* (seven days after parasitism); n.s. non-significant at 5% and * significant at 5% of probability.

Table 4 – Emergence of *Cleruchoides noackae* adults from *Thaumastocoris peregrinus* eggs treated with 5% plant extracts, after one and seven days of parasitism (22 ± 2ºC, 60 ± 10% RH and 12 hrs photoperiod).

Biological Parameter	***Parameter***	***Mean***	***Standard Deviation***	$P_{2.5\%}$	$P_{97.5\%}$	***p-value***
Emerged parasitoids	*Treatments (one day after parasitism)*					
	Θ_1	2.55	0.35	1.91	3.28	
	Θ_2	1.90	0.31	1.34	2.55	
	Θ_3	2.25	0.34	1.64	2.96	
	Θ_4	2.50	0.36	1.85	3.26	
	Contrasts (one day after parasitism)					
	$\Theta_1 - \Theta_2$	0.65	0.47	-0.27	1.57	0.92 n.s.
	$\Theta_1 - \Theta_3$	0.30	0.50	-0.65	1.26	0.73
	$\Theta_2 - \Theta_3$	-0.35	0.46	-1.25	0.53	0.22
	$\Theta_1 - \Theta_4$	0.05	0.50	-0.93	1.05	0.54
	$\Theta_2 - \Theta_4$	-0.60	0.47	-1.54	0.33	0.10
	$\Theta_3 - \Theta_4$	-0.25	0.49	-1.20	0.70	0.30
	Treatments (seven days after parasitism)					
	Θ_{11}	4.00	0.46	3.15	4.97	
	Θ_{22}	3.85	0.44	3.03	4.75	
	Θ_{33}	3.94	0.45	3.12	4.89	
	Θ_{44}	3.80	0.44	2.30	4.71	
	Contrasts (seven days after parasitism)					
	$\Theta_{11} - \Theta_{22}$	0.15	0.63	-1.09	1.41	0.59 n.s.
	$\Theta_{11} - \Theta_{33}$	0.05	0.65	-1.24	1.32	0.53
	$\Theta_{22} - \Theta_{33}$	-0.10	0.62	-1.31	1.12	0.44
	$\Theta_{11} - \Theta_{44}$	0.20	0.64	-1.07	1.47	0.63
	$\Theta_{22} - \Theta_{44}$	0.05	0.62	-1.18	1.28	0.52
	$\Theta_{33} - \Theta_{44}$	0.14	0.63	-1.10	1.40	0.60
	Contrasts of treatments between one day and seven days of treatment					
	$\Theta_1 - \Theta_{11}$	-1.45	0.58	-2.61	-0.35	$5.60e^{-}03$ *
	$\Theta_2 - \Theta_{22}$	-1.95	0.54	-3.03	-0.91	$1.33e^{-}04$ *
	$\Theta_3 - \Theta_{33}$	-1.70	0.57	-2.81	-0.59	$9.33e^{-}04$ *
	$\Theta_4 - \Theta_{44}$	$-1.30e^{+}02$	4.02	-2.42	-0.21	$1.01e^{-}02$ *

$P_{2.5\%}$ and $P_{97.5\%}$ = Interval of 95% of credibility; Θ_1 = control, Θ_2 = *Matricaria chamomilla*, Θ_3 = *Echinodorus grandiflorus* and Θ_4 = *Maytenus ilicifolia* (one day after parasitism); Θ_{11} = control, Θ_{22} = *Matricaria chamomilla*, Θ_{33} = *Echinodorus grandiflorus* and Θ_{44} = *Maytenus ilicifolia* (seven days after parasitism); n.s. non-significant at 5% and * significant at 5% of probability.

4 CAPÍTULO III – TOXICITY ASSESSMENT OF INSECTICIDAL PLANTS TO CHICKEN

Artigo científico a ser submetido – Ecotoxicology and Environmental Research (formatado de acordo com esta revista) – Qualis Capes A2 em Ciências Agrárias I

ABSTRACT

Gallus domesticus L. is a cosmopolitan and easy-breeding species, which can be used as model for toxicity tests to birds. The objective of this work was to assess the safety of the aqueous extracts at 5% w/v of *Echinodorus grandiflorus* (Alismataceae), *Matricaria chamomilla* (Asteraceae) and *Maytenus ilicifolia* (Celastraceae), plants with insecticidal potential, to *G. domesticus* L. For five days, 48 chickens were fed with the regular diet associated with the aqueous extract (5,000 mg/kg of diet). The groups were: (1) *E. grandiflorus*; (2) *M. chamomilla*; (3) *M. ilicifolia* and (4) control group. They were observed for 12 days and then, sacrificed. Samples of selected organs were processed for histopathological investigations. No uncommon behaviour, aggressiveness or external lesions were verified. The food intake during the experiment was similar in all the groups and it significantly increased until the end of the experiment. In addition, there was no statistical difference between the treated groups and the control group regarding weight gain of the birds or liver enzymes. Histological examination of the small intestine, liver and kidney revealed no consistent alterations. These results strongly suggest that neither plant extract were toxic to *G. domesticus*.

Key-words: *Echinodorus grandiflorus*, *Matricaria chamomilla*, *Maytenus ilicifolia*, non-target organism, *Gallus domesticus*

1. INTRODUCTION

Plants contain a myriad of natural compounds, which exhibit important bioactive properties and may be used for several purposes, such as to treat ailments and act as plant protectors against both agroforestry pests and diseases.

There is ample evidence of the use of plants to treat illnesses historically (Kelly, 2009; Petrovska, 2012). Nowadays, this is the main therapeutic option for approximately 80% of the population around the word (World Health Organization, 2008).

Medicinal plants are also used to treat several moderate ailments in farm and domestic animals, both due to reduced financial involvement when compared to the traditional treatment and to the lesser risk for human consumption (Nwosu et al., 2004; 2008; 2011; Martínez & Luján, 2011; Pattanayak et al., 2013).

Unfortunately, many of those plants may present chemical compounds that have toxic potential. Therefore, the population may be jeopardizing their health instead of promoting it (Abruzzo, 2005).

Regarding agriculture, botanical compounds can suppress other plants such as weeds, due to its allopathic effect (Gella et al., 2013). These compounds can perform a very important role in both integrated disease (Itko et al., 2008; Rongai et al., 2012; Sundaramoorthy et al., 2014) and pest management (Roel, 2001; Seffrin et al., 2008; Dequech et al., 2009; Santos et al., 2010; Silva et al., 2010), reducing the application of pesticides, which may affect beneficial insects, cause groundwater contamination and outbreaks of secondary pests (Dubey et al., 2011; Khater, 2012). Botanicals are considered as an attractive alternative for pest management due to being reputedly harmless to the environment and to human health (Isman, 2006). One of the reasons is that they decompose quickly into harmless compounds within hours or days in the presence of sunlight (Khater, 2012). Botanicals are best suited for use in organic food production in industrialised countries and can play a greater role in the production and post-harvest protection of food in developing countries (Isman, 2006).

Among others, *Echinodorus grandiflorus* (Alismataceae), *Matricaria chamomilla* (Asteraceae) and *Maytenus ilicifolia* (Celastraceae) are plants commonly used to treat several kinds of illnesses from pain to attention-deficit hyperactivity disorder (Stasi et al., 2002; Niederhofer, 2009; Medeiros et al., 2013; Stoltz et al., 2014). Besides, their potential to be used in the production enhancement of both agriculture and farm animals is being investigated (Dias et al., 2005; Marques et al., 2010; Al-Kaisse & Khalel, 2011; Jakubcova et al., 2014). Currently, studies demonstrating the potential of these plants aqueous extracts to control pests are being carried out (Chapter I).

Despite the fact of being natural, botanicals should not be considered totally safe. Certain plant compounds are toxic for non-target organisms such as humans and other vertebrates (Isman, 2008; Nyahangare et al., 2012).

When applied on the field to control pests, plant extracts will interact with non-target organisms such as beneficial arthropods and vertebrates who are part of the agroecosystem, leading to unexpected effects. Regarding how birds could be exposed, they may eat seeds or insects covered with the plant extract, or even exposed directly during the extract application. In a world where protection of the environment and food security are major concerns, these possible negative impacts must be investigated thoroughly.

Therefore, the environmental protective agencies of several countries suggest toxicity tests should be carried out to verify the safety to the environment of any product which can be treated as chemical (Organisation for Economic Co-operation and Development, 2010; Environmental Protection Agency, 2012; Ministerio da Agricultura, Pecuaria e Abastecimento, 2012).

Since *Gallus domesticus* L. is a cosmopolitan and easy-breeding species which can be used as model for toxicity tests for birds, the objective of the present work is to assess the toxicity of *E. grandiflorus*, *M. chamomilla* and *M. ilicifolia* to *G. domesticus* L., as a non target organism.

2. MATERIAL AND METHODS

2.1 Preparation of plant material

Leaves of *E. grandiflorus* (collected in Dois Vizinhos, Parana State, Brazil and voucher specimens deposited at the Herbarium of the Federal University of Technology – Paraná, UTFPR) and *Maytenus ilicifolia,* and flowers of *Matricaria chamomilla* (acquired from COOPERFLORA, Turvo, PR) were used to prepare the extracts. Plant materials were dried in oven (60^{o}C) for 48 hours and ground in a grinder. Each of the three plant extracts was prepared at a concentration of 5% w/v by adding 5 g of the plant powder to 100 mL of distilled water. The mixture was kept away from light for 48 hrs. Filtration of the plant extracts was done with filter paper, shortly before the start of the experiment.

2.2 Experimental birds

The experimental procedures were approved by the Federal University of Technology - Paraná (UTFPR) Animal Care Committee, following the Ecological Effects Test Guidelines of the United States Environmental Protection Agency, EPA (OCSPP 850.2200).

An amount of 48 ten-day-old female Ross broiler chickens of similar body weight and in apparent good health were used for the experiment. At first, they were acclimated to the test facility and basal diet for three days (room temperature of 25 ± 5ºC and a 16-h photoperiod). The basal diet was formulated as described by Rostagno (2011). Tap water was offered *ad libitum* during the entire period of the experiment. The birds had either not been vaccinated or received antibiotics.

2.3 Birds evaluation

Each bird was weighted and a numbered ring was put around their right inferior limb. The birds were distributed randomly into four groups of 12 poults per floor pen at the Small Animals Research Station at UTFPR. For five days, the birds were fasted overnight and then, fed with the regular diet associated with the aqueous extract (5 ppm). This high dose is suggested by the Guideline OCSPP 850.2200 for substances expected to be of low toxicity, such as insecticidal plants.

The groups were: (1) *E. grandiflorus*; (2) *Matricaria chamomilla*; (3) *Maytenus ilicifolia* and (4) control group, to whom the diet was associated with distilled water. The birds were observed for 12 days for any clinical sign of alterations that could be associated with the treatment. These included: uncommon vocalisation, change in gait or coordination, aggressiveness, anorexia, gastrointestinal disorder, skin lesions, ruffled or denuding of feathers, weakness or decreased responsiveness to investigator presence. Body weight was determined on day 1, 5 and 12 of the experiment and the weight gain was determined. Feed consumption was recorded by the end of treatment administration (day 5 of experiment) and by the end of the experiment (day 12 of experiment).

All the birds were sacrificed at the end of the evaluation period and necropsied to observe possible pathological alterations. Samples (1 cm^3) of the small intestine, kidney and liver were collected from three birds per group, at random. The samples were fixed in Bouin's solution and embedded in paraffin blocks, sectioned at 7 µm and stained with Hematoxilin and Eosin (Behmer et al., 1976) in order to verify possible alterations in these tissues caused by the plant extracts in a qualitative analysis.

2.4 Activity of liver enzymes evaluation

Blood (5 mL) was collected from the external jugular vein of the birds as soon as they were sacrificed and then centrifuged at 5 000 rpm for five minutes to harvest the serum that was stored at -10^{o}C for 15 days. For the analysis, the samples were thawed and concentrations of alkaline phosphatase (ALP), aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined using GOLD ANALISA DIAGNÓSTICA commercial kits.

2.6 Data analysis

For most of the data, the experiment design was completely randomized with four treatments and 12 replications. For the analysis of the birds weight gain, it was used a split-plot design. Data was submitted to one-way ANOVA, followed by Tukey's test. The *p* value for statistical significance was defined as less than 5% (Assistat 7.7®, Silva, 2014).

3. RESULTS

3.1 Bird evaluation

The food intake during the experiment was similar to all the groups and it significantly increased until the end of the experiment. In addition, there was no statistical difference among the treatment groups and the control regarding weight gain of the birds (Table 1).

Table 1. Effect of 5% aqueous extract of *Echinodorus grandiflorus*, *Matricaria chamomilla* and *Maytenus ilicifolia* on feed consumption (g) on days 5 and 12 of the experiment and body weight gain (g) of *Gallus domesticus* (room temperature of 25 ± 5ºC and a 16-h photoperiod). Values were expressed as the group mean ±SE.

Group	feed consumption (mean) (g)		weight gain (mean) (g)
	5 (days)	12 (days)	
E. grandiflorus	201.8 ± 4.20 b	408.7 ± 9.46 a	698.0 ± 96.05^{ns}
M. chamomilla	206.4 ± 5.52 b	417.7 ± 11.69 a	687.2 ± 100.54
M. ilicifolia	192.1 ± 5.92 b	392.5 ± 14.85 a	692.8 ± 102.93
control group	184.7 ± 4.42 b	401.3 ± 10.97 a	667.4 ± 80.89

CV treatment (%) 9.64	CV time (%) 8.59	CV weight gain (%) 31.11

[a] p<0.05, significant difference of feed consumption from day 5 of the experiment in the respective group (Tukey's test)

There was 100% of bird survival during the experiment. No uncommon behaviour or aggressiveness was verified. The same can be stated for external lesions. Histological examination of the small intestine, liver and kidney revealed no alterations, indicating that none of the extracts caused damage to these tissues.

3.2 Activity of liver enzymes evaluation

There was no difference between the treatments and the control group regarding serum AST, ALT and ALP activities (Table 2).

Table 2. Effect of 5% aqueous extract of *Echinodorus grandiflorus*, *Matricaria chamomilla* and *Maytenus ilicifolia* on serum aspartate aminotransferase (AST), alanine aminotransferase (ALT) and alkaline phosfatase (ALP) in *Gallus domesticus.* Values were expressed as the group mean ±SE.

Group	AST (U/L)	ALT (U/L)	ALP (U/L)
E. grandiflorus	47.7±6.97[ns]	13.4±2.11[ns]	210.1±51.89[ns]
M. chamomilla	32.2±4.10	14.2±1.81	304.5±62.05
M. ilicifolia	47.7±9.53	19.3±1.56	313.9±57.85
control group	51.4±5.98	19.3±0.81	197.0±36.46
CV (%)	43.86	26.27	50.63

[ns] p<0.05, no significant difference of the serum enzymes between the treatments and control group.

4. DISCUSSION

These results imply that neither *E. grandiflorus*, *M. chamomilla* nor *M. ilicifolia* caused any physiological alteration that could lead to weight loss or incapacitate the birds either to feed or to get the nutrients necessary to build body mass. Also, no biochemical alteration was found that could be caused by degeneration of the hepatocytes due to the plant extracts added to the birds' diet. Associated with the results from the liver histopathology analysis, it denotes that the plant extracts caused no damage to the liver.

In an attempt to verify the calming effect of *M. chamomilla* on birds in order to use it as a stress modulator, the feed of immature quails (*Coturnix coturnix*) was enriched with this plant flowers powder. The results showed no alterations in the birds' performance, behaviour or in the physiological parameters studied (Marques et al., 2010). This can be explained by the interaction between the ingested plant secondary metabolites and the enzymes from the animal gastrointestinal tract during their metabolism. These compounds can undergo transformation and their effect may be modified or even nullified (Acamovic & Brooker, 2005).

Only rats have been used as model to verify *M. ilicifolia* and *E. grandiflorus* toxicity. About the former, Cunha-Laura et al. (2014) found that this plant hidroacetonic extract was not toxic to pregnant Winstar rats and did not interfere with the progress of embryo-fetal development. Montanari et al. (1998) also allege that the ethanol extract did not arrest male spermatogenesis. Regarding the latter, the crude extract of the plant did not have neither cytotoxic nor genotoxic activities (Silva et al., 2010). Also, the aqueous extract did not lead to detectable clinical signs of maternal toxicity in pregnant rats and no alterations in the reproductive performance (Brugiolo et al., 2010). Both authors suggest the presence of antioxidant substances in the extracts, such as saponins, contributed to these results.

Many plant secondary metabolites have been isolated, identified and grouped, i. e. alkaloids, amines, cyanogenic glycosides, terpenes, flavonoids, among others. These compounds may elicit beneficial or adverse effects in animals (Acamovic & Brooker, 2005). According to Bernhoft (2010), plants with potent bioactive compounds are often described as being both poisonous and medicinal, depending on the amount and the context of intake. Iason (2005) describes that, amongst the harmful effects, they can cause tissue damage, generate superoxide and other free radicals, inhibit digestibility and depress the activity of symbiotic microbial populations.

A large group of active compound classes are found in the plants aqueous extracts used in this experiment. In *Matricaria chamomilla*, phenolic compounds are the flower main constituents, such as chlorogenic acid, caffein acid, flavonols and coumarins (Gupta et al., 2010; Singh et al., 2011). In our studies (Chapter I) we verified the existence of galic acid and ferulic acid. *E. grandiflorus* also presents a high level of the same compounds (Lunardi et al., 2014). *Maytenus ilicifolia* also present high concentration of phenolic compounds, especially tannins, flavonoids and terpenoids (Mariot & Barbieri, 2007). The toxicity of a plant, high or low, can be inherent to its chemical composition, but it also can be due to biogeographic influence, part of the plant used in the extract, the solvent employed or even the variety (Hurst, 1942).

Liver and kidneys are the sensitive organs to the toxic action of plant metabolites (Hashemi et al. 2008; Abdegaldir et al. 2010; Pires Júnior et al., 2012; Padilla-Camberos et al., 2013; Cunha-Laura et al., 2014). Due to their high sensitivity to toxic agents, the study of different enzyme activities such as ALT, AST and ALP is of great importance in assessing liver damage. Necrosis or membrane damage releases enzymes into circulation and can be measured in serum (Hashemi et al., 2008). No lesions were found on the tissues of the liver and kidney, which certainly contributed to the normal levels of these enzymes when compared to the control group.

Other researchers studied the toxicological effect of medicinal plants to vertebrates, mostly to mammals (Montanari et al., 1998; Lopes et al., 2000; Abdelgadir et al., 2010; Lagu & Kayanja, 2013). Working with birds, Hashemi et al. (2008) verified that the aqueous extract of *Euphorbia hirta*, *Solanum torvum*, *Zinger officinale*, *Curcuma longa* and *Zinger zerumbet* were not toxic to broiler chicken in an oral acute toxicology experiment.

Studies to evaluate the toxicity of plants with well stablished use as insecticide to non-target organisms have been conducted. In this regard, Nnenna & Okey (2013) verified that the aqueous leaf extract of *Azadirachta indica* caused no toxic effect to broiler chicks, but serum liver enzymes were not quantified and there was no histopathological study to certify that there were no lesions in the liver or other tissues.

In a world constantly looking for sustainability and environmental-friendly, safer methods to control pests, the use of plant extracts is an economically viable option. And it has to be considered as such, being part of Integrated Pest Management (IPM) Programs. Nonetheless, it is pressing that plant extracts must not be taken lightly due to its ‘natural’ nature. Their possible harmful effects to non-target organisms should be verified.

It should be noted that the toxicological evaluation of a plant extract attempts to ascertain its possible collateral effects in order to ensure the safety of use (Barros et al., 2005). Since the dosage administrated to the animals was the higher advised by the United States Environmental Protection Agency, the results in this study strongly suggest that neither *E. grandiflorus*, *Matricaria chamomilla* nor *Maytenus ilicifolia* were toxic to *G. domesticus*.

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5 CONSIDERAÇÕES FINAIS

Os extratos aquosos a 5% de *Matricaria chamomilla*, *E. grandiflorus*, *Maytenus ilicifolia*, *Punica granatum* e *Origanum majorana* reduziram a longevidade de *T. peregrinus*. Mas os três primeiros também causaram repelência aos insetos adultos, sendo selecionados para testes em organismos não-alvo.

Os extratos selecionados não afetaram a emergência de *C. noackae* adultos, quando aplicados antes ou depois de as fêmeas terem parasitado os ovos de *T. peregrinus*.

Os mesmos extratos também não causaram efeitos negativos em *G. domesticus*. Não reduziram consumo de alimento ou ganho de peso, ou alteraram enzimas séricas.

Dada a importância de plantas inseticidas, seja na forma de extratos ou visando bioprospecção de compostos específicos, novos trabalhos deveriam ser realizados. Testes com os extratos vegetais *Matricaria chamomilla*, *E. grandiflorus*, *Maytenus ilicifolia*, *Punica granatum* e *Origanum majorana* sobre outros insetos-praga e/ou com outras formas de extração de compostos químicos e outros estágios de desenvolvimento; testes de avaliação de toxicidade sobre outros organismos não-alvo; e estudos para identificar e isolar os compostos químicos com maior potencial inseticida dessas plantas.

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