Artigo

TOWARDS NEW BOTANICAL PESTICIDES: THE TOXIC EFFECT OF *Eremanthus goyazensis* (Asteraceae) LEAVES ESSENTIAL OIL AGAINST *Brevipalpus phoenicis* (Acari: Tenuipalpidae)[#]

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This work reports the chemical characterization of *Eremanthus goyzensis* essential oil and its toxic effect over *Brevipalpus phoenicis*. The essential oil displayed a major composition of sesquiterpenes (61.87%) including *trans*-caryophillene (26.81%) and germacrene-D (13.31%). The fumigation test indicated a promising bioactivity over adult *B. phoenicis* individuals at 24 h (2.03 μL/L of air) and 48 h (1.08 μL/L of air) of exposition. A brief discussion of essential oils composition and their singular role on the toxic effect over *B. phoenicis* is provided here. Our results may contribute to a new and profitable use of a species of Brazilian flora on agribusiness.

Keywords: Asteraceae; red and black flat mite; botanical pesticide.

INTRODUCTION

To date, the agribusiness pest control has been done most commonly through spraying synthetic acaricides on crop fields. Despite its wide employment, such chemicals are known for their drawbacks e.g., high prices, environmental and health injuries, and their capabilities of inducing Acari resistance.[1]

Brevipalpus phoenicis (Geijskes, 1939) (Acari: Tenuipalpidae) is a cosmopolitan false-spider-mite which has various plants as hosts.[2,3] Under Brazilian geoclimate conditions the specie plays a pivotal role of the transmission of *citrus leprosis virus* (CiLV), a RNA virus responsible for citrus leprosies, mainly on sweet orange trees.[4,5] The disease negatively impacts Brazilian citrus agribusiness by an annual estimation of 100 million dollar expense in *Brevipalpus* spp. control.[6] Before vanishing from Florida crop fields, the disease had almost shut down the citrus industry in USA.[7] There are reports that there is a northward movement of the virus, which could endanger citrus crop fields of North America.[8]

Currently *B. phoenicis* suffers a selective pressure caused by the most commonly used acaricides on citrus crop protection such as dicofol,[9,10] hexythiazox,[11] and propargite.[12] In addition to this pressure, *B. phoenicis* shows parthenogenetic development leading to a quick promotion of the appearance of an evolved and chemical-resistant type of mite.

Eremanthus goyazensis (Gardner) is an Asteraceae specie endemic of quartzite rupestrian fields. This ecosystem emerges at the top of Brazilian savannas ("Cerrado") located in central Brazil within the states of Minas Gerais, São Paulo, Bahia, and Goiás.[13] At this habitat the specie is known as "candeia", among some other species of the genus.

The current literature registers for the genus *Eremanthus* a chemical volatile fraction featured by monoterpenes, such as β-pinene, β-mircene and a majority of sesquiterpenes highlighting a-bisabolol, β-caryophyllene and germacrene-D.[14,15] In leaves, α-bisabolol is detected in high concentrations. The *Eremanthus* spp. wood logging takes place at the habitat often and the leaves are dismissed along the process, thus it may be commercially interesting to explore this potential highly valued by-product.

This genus is circumscribed in Lychnophorinae sub-tribe (Vernonieae). The subtribe had its chemistry recently reviewed,[16] where a variety of sesquiterpenes lactones (SLTs) majority is described. The common phytochemical investigations among Lychnophorinae species from late 1970's[17-21] used to be proceed focusing on the analysis of the medium polarity fractions of leaves extracts, which only afforded non-volatile compounds isolation. Such a goal-oriented phytochemical workflow may explain why the volatile fractions of Lychnophorinea species were somehow dismissed.

Regarding all the damages and risks of the *B. phoenicis* upon citrus crops as well as the agribusiness needs of novel, safe and efficient ways to control crop pest we investigated toxic effects of *E. goyzensis* leaves essential oil over *B. phoenicis* adult individuals. The *Eremanthus* species were used in order to move forward on the phytochemical characterization of endangered Asteraceae species.

EXPERIMENTAL

Botanic material

Aerial parts (leaves) of *Eremanthus goyazensis* were harvested in the morning during the 2011 summer at the limits of Ouro Preto - MG, Brazil. The plant was identified by Dr. G. H. B. de Souza, and a voucher (code number - NPL372) was kept at Herbarium UEC in University of Campinas – Brazil. Harvesting was done under the allowance of CNPq (010143/2011-4).

Essential oil and chemical analysis by GC-MS

The fresh plant material (690.0 g of *E. goyazensis* leaves) was placed inside a two liter Florence flask which was connected to a

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Clevenger apparatus modified for hydrodistillation. The entire process elapsed 4 h of distillation under controlled temperature (100 °C) and the yield was calculated based on the weight of the fresh leaves. In the end, a slight difference between the still water and essential oil densities allowed the separation of 2 distillated layers. The organic layer, formed by essential oil, was collected, dried by allowing its contact with anhydrous Na_2SO_4 and maintained under a N_2 micro-ambient inside amber vials at -20 °C. All the storage conditions were kept until the start time of the chromatographic analysis and fumigant effect essays.

Fingerprinting and semi-quantitative measurements by GC-MS

The previously obtained essential oil was chemically analyzed through a Shimadzu QP2010 GC-MS system. An electron ionization mass spectrometry (EI-MS) detector has operated under a settled 70 eV ionization energy, source temperature of 250 °C, scanning ratio of 0.50 scan/s for a method with total ion chromatogram (T.I.C.) analyzer mode switched on, within a mass weight frame corresponding to the interval of 40 up to 500 *m/z* mass charge ratio. Chromatographic resolution was guaranteed by the use of a split injector (240 °C) operating at a ratio of 1/10 injection quantity towards the column. One DB-5MS (30 m x 0.25 mm x 0.25 um) column was employed and Helium was used as a carrier gas (flow: 1.33 mL/min.; settled pressure: 81.5 kPa). Programmed heating (3 °C/min) was used to elute the compounds out of the column orderly and clearly. Starting temperature was at 60 °C and finishing temperature was 240 °C.

The chemical fingerprint was acquired by the GC-MS data processing. The mass spectra for each and every major signal of essential oil's chromatogram was compared, using GC-MS labsolution® software, with the spectra of 3 commercial data Banks (NIST 62, Wiley 7 and FFNSC 1.3). In parallel, the chromatographic relative retention index (R.R.I.) was calculated using data of the injection of a homologous set of *n*-alkanes (C_9-C_{22}) on Kováts equation.[22] Mass spectra similarities values higher than 95% combined with RRIs values higher than 99% were considered "perfect match" at the identification process.

For semi-quantitative analysis, the data generated at GC-MS were used by the software in a way to know the peak area by integration of each signal.

***B. phoenicis* treatments**

All spider mites were maintained in captive breeding system at the lab (T = 25 ± 1 °C; R.H. = 70 ± 10% and daylight period = 12 h). Populations of *B. phoenicis* were allowed to breed on acaricide-free fruits of *Citrus sinensis* L. Osbeck, var. Pera-rio. Every fruit was harvested, washed, dried and half of their surfaces covered with paraffin, while the other half was bordered with glue (Tanglefoot®) and left raw in order to hold the spider mite infestations.

A simulation of Citrus scab was made by brushing a mixture of sand, plaster, wheat flour and distilled water (4:1:1:3) over all the fruits' surface right before the placement of the spider mites at the arena, in order to create the physical conditions of the disease in the experiment, which make *B. phoenicis* colonization favored.[6,23,24]

After the placement of 50 spider mites in each arena, the fruits were kept on a polystyrene plaque in a temperature controlled room settled for the same conditions used in a captive breeding system in the lab. According to the needs observed along the experiment, the fruits were renewed.

Fumigant effect of *Eremanthus goyazensis* essential oil

Bioassays were performed in a temperature controlled room settled for the same conditions used in a captive breeding system at the lab, and following a factorial arrangement 6 x 3 (factor 1 = 6 concentrations of essential oil; factor 2 = 3 exposure times). A completely randomized design (CRD) was used and executed in 4 replicates. Spider mites were exposed to the following doses of *E. goyazensis* leaves essential oil: 0; 0.5; 1.0; 1.5; 2.0 and 2.5 µL of oil/ L of air. The mortality rate was accessed after 24, 48 and 72 h of exposure.

In order to observe the fumigant effect of *E. goyazensis* leaves essential oil on *B. phoenicis* mites, 0.5 L plastic bags adapted to work as fumigation chambers were used. Inside each bag a wad of wet cotton was placed and upon their surfaces were 4 discs of *Citrus sinensis* peel (Ø = 3 cm; one disc = 1 repetition) containing 10 adults, which totaled 40 spider mites per chamber.

Afterwards, all the different concentrations described previously were reached at the chambers. Tiny pieces (5 x 2 cm) of absorbent paper were placed at the boundaries of the chamber. The dosage of the essential oils on the paper surfaces was done with a micropipette (Acura-Socorex®). The procedure was adapted from literature.[25] Spider mite colonies after 24, 48 and 72 h of exposure were accessed to evaluate the mortality. Mites were considered dead if after a slight touch of a thin brush on their back they did not move for a distance equal to their body size.

***B. phoenicis* mortality statistics**

All data from fumigant effect essay were analysed with a one--way analysis of variance (ANOVA); for analysis, the original data (x) were transformed to $(x + 0.5)^{1/2}$. The means were compared using the Tukey test.[26] Statistical analysis was performed using the PROC GLIMMIX procedure in the SAS software package.[27] For lethal doses (LD_{50}) measurements, a Probit analysis was done through a 4.3.0 version of Stat Plus software.

RESULTS AND DISCUSSION

The essential oil hydrodistillated from *E. goyazensis* leaves displayed a light-green color and the process had a yield of 0.15% v/m. A trustful and reliable identification by GC-MS fingerprinting identified a majority of 12 different terpenoids (Figure 1). Hydrocarbons skeletons of sesquiterpenes are 61.87% of the T.I.C. area and within this group there is a majority of *trans*-caryophillene 26.81% (Table 1).

Caryophyllene-like sesquiterpenes are common to occur in many species of Lychnophorinea subtribe (Vernonieae: Asteraceae). The majority of the chemical reports are from phytochemical studies of non-polar extracts that were carried out 40 years ago[18-21,28] but also from a few recent essential oil chemical investigations *exempli gratia*: *Eremanthus erythropappus*.[14,15] Time-scale shifts of caryophillene levels have been reported,[29-31] which raises up the importance of knowledge of chemical ecology in the issue of bioprospecting.

LD_{50} values for *E. goyazensis* leaves essential oils mortality over *B. phoenicis* were measured as well as all their confidence intervals (Table 2). The data displayed a high toxicity of the oil over the mite even at the slighter exposition time-frame. The following longer exposure time, 48 h, showed that a higher toxicity decreases the LD_{50} values down to 1.08 µL/L of air. In sequence, 72 h of exposure time was toxic enough to kill the entire spider mite population at the lower dose (0.5 µL/L air) of essential oil that made the LD_{50} measure not feasible. In general, mortality increased as the exposure period increased (Figure 2).

Combined analysis of mortality, time of exposure and doses showed that at the total time of exposure equal to 24 h, just the highest dose was significantly different ($F = 5.42$; $df = 5$; $P < 0.05$) for the control in means of toxicity (Table 3). An average of 6.75 individuals' mortality was observed. At the same point of view, 48 h time

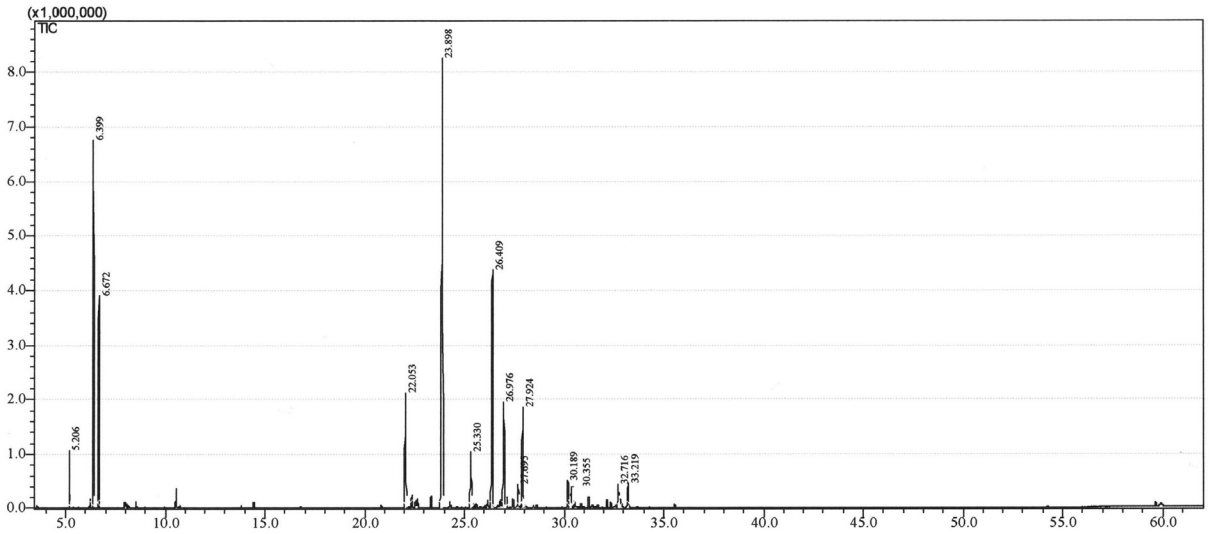

Figure 1. *Total ion chromatogram of E. goyazensis leaves essential oil. Acquired in GC-MS QP2010 Shimadzu (70 eV)*

Table 1. Relative percentage of *Eremanthus goyazensis* leaves essential oil compounds

Name	R.R.I.	R_t	% TIC
α-pinene	935	5.20	1.53
β-pinene	985	6.40	11.34
β-mircene	990	6.67	6.58
α-copaene	1375	22.05	6.02
trans-cariophyllene	1425	23.98	26.81
α-humulene	1450	25.33	3.05
germacrene D	1475	26.41	13.31
Bicyclogermacrene	1487	26.97	6.03
γ-cadinene	1508	27.69	1.21
δ-cadinene	1514	27.92	5.44
spathulenol	1574	30.19	1.58
cariophyllene oxide	1577	30.35	1.15
unknown	1639	32.71	1.28
unknown	1651	33.22	1.57
Monoterpenes hydrocarbons	-	-	19.45
Sesquiterpenes hydrocarbons	-	-	61.87
Oxygenated sesquiterpenes	-	-	2.73
Identified (%)	-	-	84.05

Relative retention indexes (R.R.I.) were determined according Kováts equation. Retentions time (R_t) were plotted in this table at the same scale displayed in chromatogram. Percentage of total ion chromatogram (%TIC) represents each single compound`s percentage of the sum of all ions recorded at mass spectrometer during the analysis

of exposure showed relevant toxicity ($F = 3.74$; $df = 5$; $P < 0.05$) at the highest dose, as well. On the other hand 72 h time of exposure all doses were significantly ($F = 6.84$; $df = 5$; $P < 0.05$) toxic. The interaction between concentrations and exposure times was not significant ($F = 4.27$; $df = 10$; $P > 0.05$) in this assay (Table 3), which means that regardless of the tested range of the variable "exposure time", the effects of different doses are similar.

Literature reports a fumigant effect of *Xylopia sericea* (Annonaceae) leaves essential oil over *Tetranychus urticae* (Acari: Tetranychidae) at a LD_{50} of 2.04 μL/L of air, however these essential

Table 2. LC_{50} mean values of *Eremanthus goyazensis* leaves essential oil to *Brevipalpus phoenicis* in fumigant assay

Exposure times	n	LC_{50} (95% CL) [1]	Slope ± SE	X^2
24 h	40	2.03 (1.60-2.45)	1.27 ± 0.48	1.82
48 h	40	1.08 (0.52-1.64)	1.35 ± 0.24	2.17

[1] LC_{50} values are expressed as μL/L air of pure essential oil with their 95% confidence limits (CL)

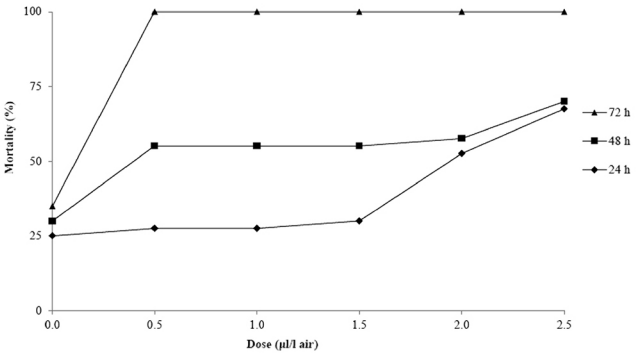

Figure 2. *Mortality of B. phoenicis exposed to three periods of time to E. goyazensis leaves essential oil*

Table 3. Averages (±SE) of mortality of *B. phoenicis* at 3 different exposure time-frame to *E. goyazensis* leaves essential oils

Concentration [1]	24 h [2]	48 h [2]	72 h [2]
0.0	2.50 ± 0.65 b A	3.00 ± 0.41 b A	3.50 ± 1.22 b A
0.5	3.00 ± 0.71 ab A	5.50 ± 0.96 ab A	10.0 ± 0.00 a A
1.0	2.75 ± 0.63 ab A	4.50 ± 0.96 ab A	9.50 ± 0.29 a A
1.5	2.75 ± 0.25 ab A	5.50 ± 0.87 ab A	9.25 ± 0.48 a A
2.0	5.25 ± 1.44 ab A	5.75 ± 1.65 ab A	9.25 ± 0.25 a A
2.5	6.75 ± 0.63 a A	7.00 ± 0.82 a A	10.0 ± 0.00 a A

[1] Concentrations expressed as μL/L air of pure essential oil. [2] Means followed by the same lower case letter in the column or upper case letter in the row do not differ by Tukey test ($P \geq 0.05$); CV – coefficient of variation = 21.42%

oils are markedly a cubenol (57.43%) and α-epi-muurolol (26.09%) rich essential oil.[32] Such previous investigation of Cerrados' species toxicity over Acari species does not support the result of this present study, which found a *trans*-caryophyllene (23.98%) rich essential oil of *Eremanthus goyzensis* (Vernonieae: Asteraceae) leaves that is toxic over *Brevipalpus phoenicis*. Nevertheless, our recent investigation of *Lychnophora ericoides* (Vernonieae: Asteraceae) leaves essential oil fumigant effect over the already mentioned *Tetranychus urticae* has shown that a monoterpenes rich oil has a LD_{50} of 8.01 μL/L of air.[33]

Regardless of one's specific chemical features, e.g., caryophillene discussed above, the fumigant toxic effect of essential oils over Acari species are overall more likely linked to the complex mixture of terpenoids that are frequently found in such oils. A recent report of *Piper* spp. essential oils toxicity over *Rhipicephalus* sp. (Acari: Ixodidae) larvae (immersion test; LC_{50} of 2.33 μL/mL of solution for *P. mikanianum*) showed that those oils with a chemical profile of phenylpropanoids have higher activity compared to those with chemical profile of terpenoids.[34] On the other hand, *Protium bahianum* fruits and leaves essential oils are free of phenylpropanoids, have high levels of pinene (34%) and are toxic to *T. urticae*.[35] Noteworthy is the presence of this monoterpene (pinene) in *E. goyazensis* essential oils composition presented is this current investigation. Other species of the genus *Protium* such as *P. heptaphyllum* have been reported[36] to have a terpene rich essential oil a-terpinene (47.6%), 9-epi-caryophillene (21.4%) and 14-hydroxy-9-*epi*- caryophillene (16.7%), that is toxic to *T. urticae*.

CONCLUSION

Encouraged by literature data briefly discussed above and based in our data presented herein this study, we might conclude that *Eremanthus goyzensis* leaves essential oil are very toxic to adult *Brevipalpus phoenicis* individuals. Field dilutions of the *E. goyazensis* oils could feature a possible "resistance-free" approach to stop the northward movement of *B. phoenicis* in order to avoid endangering orange tree fields.

The scenario of Cerrados' devastation and the wood logging of *Eremanthus* spp. should be an opportunity for this preliminary study to turn this endemic specie into a potential source of a botanical pesticide. Such a new valuation of *Eremanthus* spp. would be a contribution towards its conservation, as well as another proof of the biome importance on Agrochemicals discovering.

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