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*A N I N T E R N A T I O N A L J O U R N A L*

# Brown Stink Bug Mortality by Seed Extracts of *Tephrosia Vogelii* Containing Deguelin and Tephrosin

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## ABSTRACT

*Extracts of the seeds of* Tephrosia vogelii *Hook. f. were studied in relation to its chemical composition and toxicity to the brown stink bug* Euschistus heros *(F.). The extracts were obtained in ethyl acetate and ethanol in the sequence according to the polar nature of the solvents. Extracts were sprayed in concentration of 1.0, 2.5, 5.0, 7.5 and 10% on third-instars nymphs and adults, and mortality was recorded. Presence two rotenoids in ethyl acetate was detected, with analyzed with gas chromatography-mass spectrometry (GC-MS). Crude fraction analyses confirmed the presence of these rotenoids (tephrosin - 2.71% in ethyl acetate and 3.66% in methanol; and deguelin - 10.46% in ethyl acetate and 1.22% in methanol) and three other rotenoids in small amounts. Eight days after applications, ethyl acetate caused more stink bugs mortality and on less time than ethanol extract, because great quantity of rotenoids, as polarity. Concentrations above to 1 and 2.5% of the ethyl acetate extracts caused mortality above 80% of the nymphs and adults of E. heros, respectively. Concentration were considered high, thus chemist analyzes demonstrated high rotenoids presence. In conclusion, seed T. vogelli extracts, rich in deguelin and tephrosin (3:1), cause mortality of E. heros, however, high concentration are necessary.*

**Key words:** *Euschistus heros*, *rotenoid*, botanical insecticide, *Glycine max*, soybeans.

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## INTRODUCTION

Complex of stink bugs causes extensive direct and indirect damage to soybean crops [1,2]. Chemical control of these species is indiscriminately used, and brown stink bug *Euschistus heros* (F.) insecticide resistance is typical in fields [3]. In recent years, besides resistance, early sprays on soybean fields of large spectrum pesticides eliminate natural enemies and causes secondary outbreaks and pest resurgence. Under these conditions, brow stink bug populations are increasing. After soybean harvest, these bugs dislocate to adjacent fields and became important pest of several horticultural crops including tomatoes, okra, persimmon etc.

In organic crops, the incidence of stink bugs is generally lower than conventional systems due to the biological emphasis on managing caterpillars and the used stink bugs eggs parasitoids [4]. Botanical insecticides could be used to control stink bugs in the borders, where infestation initiate. Botanical insecticides also may present a satisfactory cost benefit relationship when compared to pesticides [5].

In the past, rotenone was the primary agent used to control insects, and its insecticidal and repellent activities have been most studied in relation to stored-grain insects [6,7]. The genera that had most commonly used for extraction is *Derris* Lour. and *Lonchocarpus* Kunth (Fabaceae) [8]. Plants of the genus *Tephrosia* Pers. also have insecticidal properties due to the presence of rotenoids, particularly rotenone, deguelin and tephrosin [9]. Rotenoids are considered stomach poisons and they were particularly used against chewing insects [10,11].

In addition to their insecticidal effects, *T. candida* DC and *T. vogelii* Hook. f. should be like green manure crops [12]. This both utilization may be important for facilitating wide-range adoption by farmers, as in southern and eastern Africa [13]. There are two chemotypes of *T. vogelii* have identified in Africa, one which contains rotenoids and are suitable as insecticidal, and other, witch rotenoids are absent [13]. About 25% of *T. vogelii* cultivated in parts of Africa belongs to the chemotype without rotenoids [14], then plant contend knowledge is very important. Otherwise, proportion of the insecticidal rotenoids in *T. vogelii* plants have also seasonally affected (until 2.5 times of variation) [14], and latitude affect the rotenoids proportions, but it have not affect the rotenoids total [15].

Hence, we analyzed the rotenoid composition and contents of the introduction of *T. vogelii* cultivated under field conditions in Southern Brazil. Analysis of seed extracts obtained in ethyl acetate and ethanol solvents were achieved. We also tested extracts of *T. vogelii* on third-instar brown stink bug nymphs *E. heros* and adults due to the lack of: a) information about effects of rotenoids on seed-sucking insects and b) get options to manage the pest.

## MATERIAL AND METHODS

### Extract yields and analytical methods

Tests have performed at the Laboratory of Entomology, Department of Agronomy at Universidade Estadual de Londrina (UEL), Londrina, PR. The seeds were obtained from the Instituto Agronômico do Paraná (IAPAR) and grown at the school farm in Londrina, PR (23º23'S, 51º11'W), during the first trimester of 2010. A taxonomist identified plant samples and a voucher specimen was deposited at Herbarium FUEL, Londrina, PR (voucher specimen n: FUEL 49640) and retained for future reference. Seeds have crushed in a grinder (651.65 g) and extracted using a Soxhlet apparatus. Soxhlet extractions have conducted in two stages. The first stage utilized three liters of ethyl acetate. The residue of the first extraction was extracted with ethanol. Each

extraction lasted approximately 16 hours. The solvent was distilled by rotary evaporation under vacuum (Quimis®, Diadema, SP), and the remainder was evaporated by forced air at room temperature in a fume hood. After complete evaporation of solvents, each extract was placed in an amber glass bottle and refrigerated until use in bioassays. A sample of the ethyl acetate extract with the majority of its fixed oils present were analyzed by gas chromatography-mass spectrometry (GC-MS). A second extraction to isolate and identify the compounds employed Soxhlet extraction (hexane solvent) and 438 g of ground seeds. The oily extract (60.92 g) was purified over silica gel with hexane, dichloromethane and ethanol, and nearly all the fatty material was removed by hexane. The dichloromethane fraction (0.995 g) was submitted to column chromatography on a silica gel with hexane, dichloromethane and ethanol, alone or in mixtures in increasing order of polarity.

Quantification of rotenoids in ethyl acetate and methanol extracts have achieved on GC-MS equipment using standards dequelin and tephrosin. These compounds were isolated from *T. vogelii* seeds and the structural identification was defined with NMR$^{1}$H, $^{13}$C and mass spectra.

**Insects rearing and bioassays**

Colony insects were maintained in environmental chambers (25 ± 2.0ºC; 14L:10D; 70 ± 10% RH) and fed a mixture of seeds [i.e., soybean (*Glycine max* (L) Merrill), green bean pods (*Phaseolus vulgaris* L), peanut (*Arachis hypogaea* L.), sunflower (*Helianthus annuus* L)] and green fruits of privet (*Ligustrum lucidum* WT Aiton) in the laboratory. Stink bugs that began rearing were obtained from the Embrapa Soja (Londrina, PR) rearing facilities; the stink bugs at these facilities had previously been collected from their own fields (23°19' S, 51°12' W).

Nymphs and adults brown stink bug were sprayed with seed extracts. Third-instar nymphs and green bean pods (*P. vulgaris*), which served as a food source, were placed in plastic Petri dishes (9 cm in diameter). The treatments used were 1.0, 2.5, 5.0, 7.5 and 10% ethyl acetate and ethanol *T. vogelii* seed extracts and 1% commercial detergent, which served as an emulsifier. An additional control (just water) was used. The treatments were sprayed (200 µl per dish) on insects, using an airbrush (Model 147,493, Passehe®, Chicago, Illinois) coupled to a compressor-vacuum (Model: 089 - Cal, Fanem - Diapump ®, Guarulhos, SP), which was adjusted to a pressure of $10^{6}$ Pa. The dishes were kept in the same environmental chamber described above. The assays were conducted for eight days, and insect mortality was registered every other day when the pods were replaced.

Adults (7-20 days-old) were treated in a similar manner as the nymphs, with the exceptions that they were placed in plastic boxes (11x11x3 cm) and the spray volume was 400 µl per box. Mortality was evaluated 2, 4, 8 and 10 days after application of the extracts.

A completely randomized design was used for 5 replicates (each experimental unit consisted of 10 insects). The distribution mortality was assessed using the Hartley test and Shapiro-Wilk test ($p<0.05$), and the Kruskal-Wallis and Student-Newman-Keuls tests were used to compare the means ($p<0.05$) [BioEstat 5.0] [16].

## RESULTS

**Extract yields and analytical methods.** Ethyl acetate and ethanol extract yields from seeds of *T. vogelii* were 12.3% and 8.4% (w/w), respectively. Analysis of ethyl acetate extract revealed presence of two rotenoids corresponding to molecular ion peaks of

410 and 394 D (Fig 1). Further analysis of second extraction using hexane identified rotenoid structures as deguelin (I) (major constituent) and tephrosin (II), and analysis of crude fractions confirmed presence of these rotenoids as well as small quantities of three other rotenoids (III, IV and V) (Fig 2).

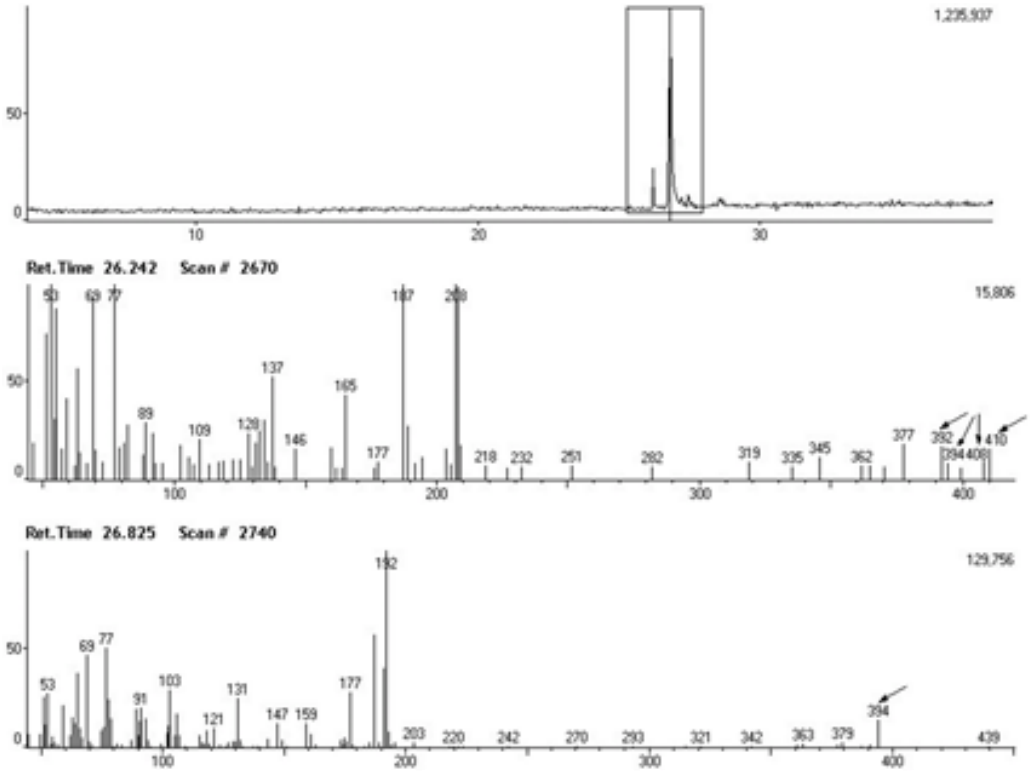


**Figure 1**. Analysis of rotenoids by gas chromatography-mass spectrometry (GC-MS).

Responses for the standarts rotenoids deguelin and tephrosin were linear (R2=0.9948 for tephrosine and R2=0.9919 for degueline) in the concentration range of in the concentration range of 0.05 to 2.5 mg/mL. The concentration of the rotenoids was achieved by a linearity plots in separated solvent crude extracts. It was found 2.71% in ethyl acetate and 3.66% in methanol for tephrosin and 10.46% in ethyl acetate and 1.22% in methanol for deguelin. These results agree with the polarities of rotenoids analyzed because deguelin is less polar compound showing major concentration in ethyl acetate, while tephrosin with an additional hydroxyl group show higher concentration in methanol.

After purification of major constituents by preparative thin layer chromatography, column chromatography and semi-preparative, high performance liquid chromatography, deguelin (M + 394, I) and tephrosin (M + 410, II) were identified (Fig 2). Structures were then defined by $^{1}H$ and $^{13}C$ deguelin [(NMR(400/100 MHz/$CDCl_3$) **$^{1}H$** – 1.32/1.38 (*s*, $(CH_3)_2$); 3.70/3.73 (*s*, $(OCH_3)_2$); 3.77 (*d* 4.0 Hz, $H_{12a}$); 4.11 (*d* 12.4 Hz, $H_{6eq}$); 4,56 (*dd* 3.2/12.4Hz, $H_{6ax}$); 4.84 (*m*, $H_{6a}$); 5.48 (*d* 10.0 Hz, $H_{5'}$); 6.38 (*s*, $H_4$); 6.38 (*d* 8.8 Hz, $H_{10}$); 6.57 (*d* 10.0 Hz, $H_{4'}$); 6.72 (*s*, $H_1$) and 7.67 (*d* 8.8 Hz, $H_{11}$) - **$^{13}C$** - 105,3 (1a); 111.7 (1); 144.14 (2); 149.8 (3); 101.2 (4); 147.7 (4a); 66.5 (6); 72.7 (6a); 158,0 (7a); 109.4 (8); 160.3 (9); 110.7) (10); 128.9 (11); 113.0 (11a); 189.4 (12); 44.6 (12a); 116.0 (4'); 128.8 (5'); 77.9 (6'); 28.4/28.7 (7'/8') and 56.1/56.7 $(OCH_3)_2$, and tephrosin (NMR(400/100 MHz/$CDCl_3$) **$^{1}H$** - 1.31/1.37 (*s*, $(CH_3)_2$); 3.65/3.74 (*s*, $(OCH_3)_2$); 4.32 (*s*, OH); 4.42 (*dd* 12.0/2.4 Hz, $H_{6ax}$); 4.49 (*dd* 1.2/2.4 Hz, $H_{6a}$); 4.55 (*dd* 12.0/2.4 Hz, $H_{6eq}$); 5.48 (*d* 10.0 Hz, $H_{5'}$); 6.39 (*d* 8.8 Hz, $H_{10}$); 6.41 (*s*,

$H_4$); 6.52 (*d* 10.0 Hz, $H_{4'}$); 6.49 (*s*, $H_1$) and 7.65 (*d* 8.8 Hz, $H_{11}$) - **$^{13}$C** 108.5 (1a); 111.7 (1); 148.3 (2); 148.3 or 151.0 (3); 101,0 (4); 148.3 or 150.9 (4a); 66.8 (6); 75.9 (6a); 156.5 (7a); 109.0 (8); 160.6 (9); 109.3 (10); 128.4 (11); 111.0 (11a); 191.3 (12); 67.4 (12a); 115.3 (4'); 128.7 (5'); 77.9 (6'); 26.1/26.4 (7'/8') and 55.7/56.2 $(OCH_3)_2$].

Rotenoids in minority are shown only in mass spectra of the column fractions corresponding to chromatogram peaks. Spectra indicates an isomer of deguelin (molecular ion peak of $M^+$ 394) [most likely rotenone (III)], an unsatured derivative with an extra hydroxil (molecular ion peak of at $M^+$ 408) [probably 6a, 12a-dehydrotoxicarol or vilosol (IV)], and another rotenoid (molecular ion peak of $M^+$ 392) with a skeleton of 6a, 12a-unsaturated compound [6a, 12a-desidrodeguelin or 6a,12a-desidrorotenone (V)] (Fig 2). All structural assignments for rotenoids 6a, 12a-saturated and 6a, 12a-unsaturated were consistent with fragmentation patterns observed by mass spectrometry, but only MS is unable to define if it has a dimethyl-chromene or isopropenyl-dihydrofuran system like extra ring at IV and V [17].

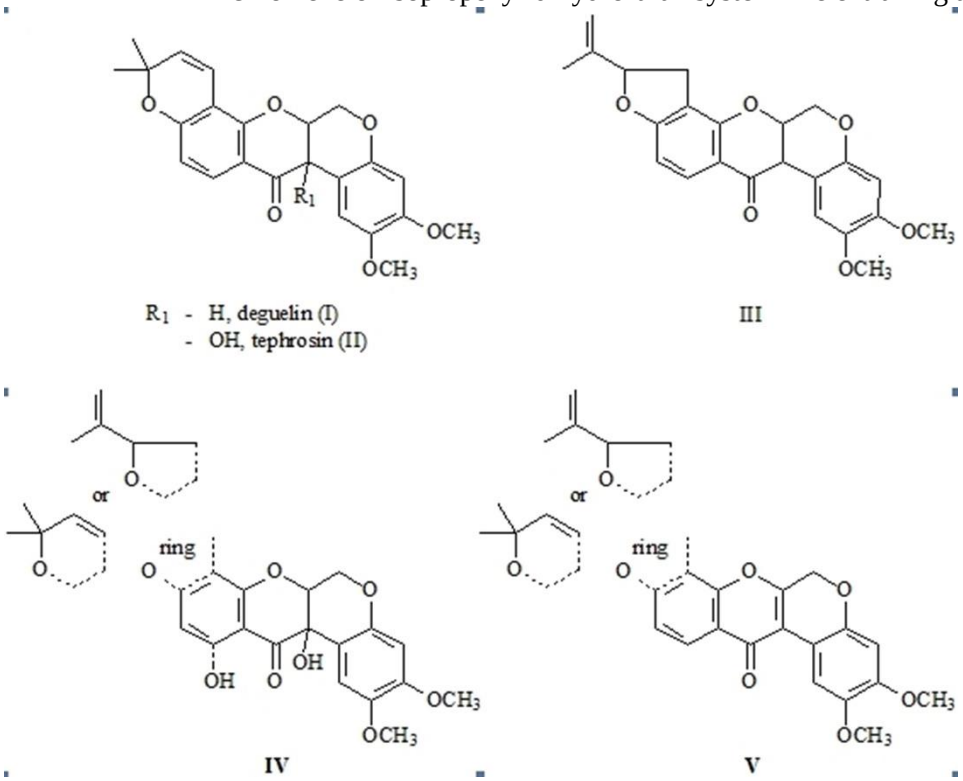


**Figure 2.** Rotenoids identified from the seeds of *Tephrosia vogelii.*

**Bioassays**

Mortality was dose dependent to nymphs and adults of *E. heros* treated with seed *T. vogelli* extracts. In nymphs' bioassays ethyl acetate extract dead quickly than ethanol extract. Eight days after sprayed, the great mortality of *E. heros* nymphs occurred as from concentration 1% ethyl acetate extract and 2.5% ethanol extract (approximately 80 and 90%, respectively) (Figure 3).

Both extracts cause slower and less mortality to adults *E. heros* compared to nymphs (Figure 3, Figure 4). For 80% adults' *E. heros* mortality are necessary concentration above 2.5% of ethyl acetate and 7.5% ethanol extract (Figure 4).

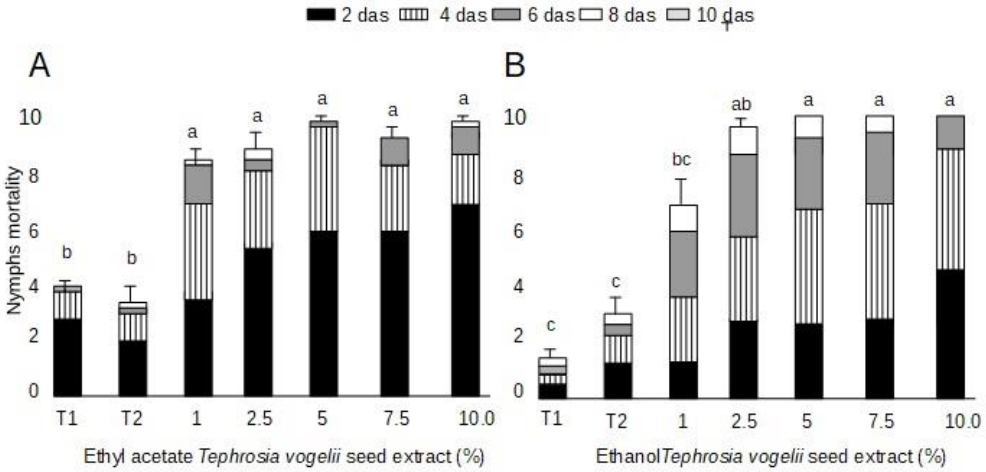


**Figure 3**. Mortality of *Euschistus heros* nymphs after spray ethyl acetate (A) and ethanol (B) *Tephrosia vogelii* seed extracts. Means followed by the same letter in a column do not differ by the Student-Newman-Keuls test ($p<0.05$). (N=5); das=days after spraying; T1=control (just water); T2=water + detergent 1.0%.

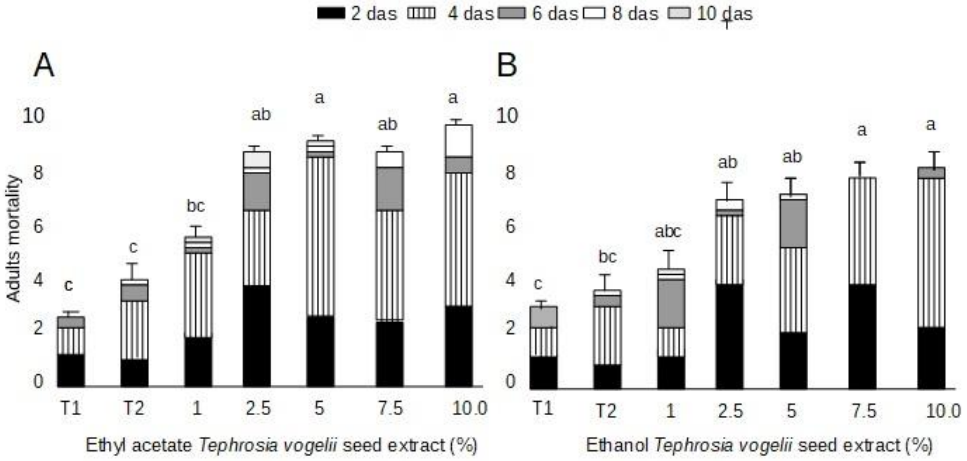


**Figure 4**. Mortality of *Euschistus heros* adults after spray ethyl acetate (A) and ethanol (B) *Tephrosia vogelii* seed extracts. Means followed by the same letter in a column do not differ by the Student-Newman-Keuls test ($p<0.05$). (N=5); das=days after spraying; T1=control (just water); T2=water + detergent 1.0%.

## DISCUSSION

Stink bug *E. heros* shown small susceptibility to seed extract *T. vogelii*, because concentration ethyl acetate extract above to 1% cause great mortality only in nymphs. Besides, ethyl acetate extract above 2.5% was necessary to kill adults of *E. heros.* Moreover, the extract were very rich rotenoids (deguelin and tephosin) contend. For exemple, 1% ethyl acetate *T. vogelli* seed extract has approximately 271 ppm of the tephrosin and 1046 ppm of the deguelin. Bruchids *Callosobruchus maculatus* (F) were very affected with 500 ppm of rotenone, deguelin and sarcobine [14], but not all insect dead with 72 hs evaluation, but approximately a half there were sick (with touch insects moved, but they not walked).

Ethanol extracts caused stink bug mortality, but lower levels and slower mortality than ethyl acetate extracts. These results are not surprising because ethyl acetate was used first in successive extraction. Therefore, ethanol extraction was used on residue of ethyl acetate extraction, so ethanol extracts were poor in rotenoids contend. In addition, ethyl acetate is a more suitable solvent to extract rotenoids due to its less-

polar nature leading to higher levels of deguelin, as shown before. Previous reports have also indicated the choice of solvent affects *T. vogelii* extract efficiency. While hexane extracts of *T. vogelii* were highly active against *Acanthoscelides obtectus* (Say) (Coleoptera: Bruchidae), *C. maculatus* and *C. chinensis* (L) (Coleoptera: Bruchidae), acetone and ethanol extracts yielded little or no effects [9]. We chose ethyl acetate because it is slightly more polar than hexane, which would permit extraction of all rotenoids, including more polar compounds. But, ethyl acetate didn't extract all rotenoids of *T. vogelii* seeds, because ethanol extract showed some effects.

In present study, the extract *T. vogelli* fit in Chemotype 1. Chemotype 1 should be contained deguelin, rotenone, sarcolobine, tephrosin and α-toxicarol. Nevertheless, Chemotype 2 did not contain rotenoids, but it's containing prenylated flavanones [13]. Rotenoid content yet should be varying with species and variety, phenological stage, cultivation region, and season [18, 15, 19, 14]. Deguelin and tephrosin were the main rotenoids found in our extracts; these yet were previously identified in species of genus *Tephrosia* included rotenoids find in minority [20]. Rotenoids know as stomach poison that must be ingested for activity [21]. However, the mortality of a non-chewing insect shown on brown stink bug, suggests that other types of action could occur, probably, contact toxicity. After 72 hs, *T. vogelli* extract and pure rotenoids caused *C. maculatus* mortality thought contact toxicity [14].

The principal rotenoids effects are most likely due to deguelin, because analysis showed almost 3 times deguelin than tephrosin. Predominance of deguelin relative to tephrosin in *T. vogelii* seeds has also been observed in leaves [13,14]. In *T. vogelli* plants, deguelin and rotenone are accumulated in photomixotrophic cells, while deguelin and tephrosin are produced mainly in heterotrophic cells [22]. Not all rotenoids are equally insect effective. Deguelin is more toxicity than tephrosin, and rotenone is more toxicity than deguelin ([14]).

Development formulation insecticide should be improved rotenoid effects on mortality of *E. heros*. Future studies may confirm if the eventual exhibited synergistic effect of other plant species associated with *T. vogelii* against Lepidopera species [23] also is effective to *E. heros*. The associate use, insecticide and green manure crops, should be reduce costs and enables even higth concentration rotenoid uses. As green manure crops, *T. candida* and *T. vogelii* improve soil quality by recycling phosphorous, fixing nitrogen and serving as an erosion-management strategy ([12]).

## CONCLUSIONS

In summary, ethyl acetate *T. vogelii* seed extracts caused mortality, in concentration above 1 and 2.5%, on nymph and adult *E. heros*, respectively. Ethyl acetate seed extracts of *T. vogelii* contained deguelin (the major constituent), tephrosin and three other minor rotenoids.

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