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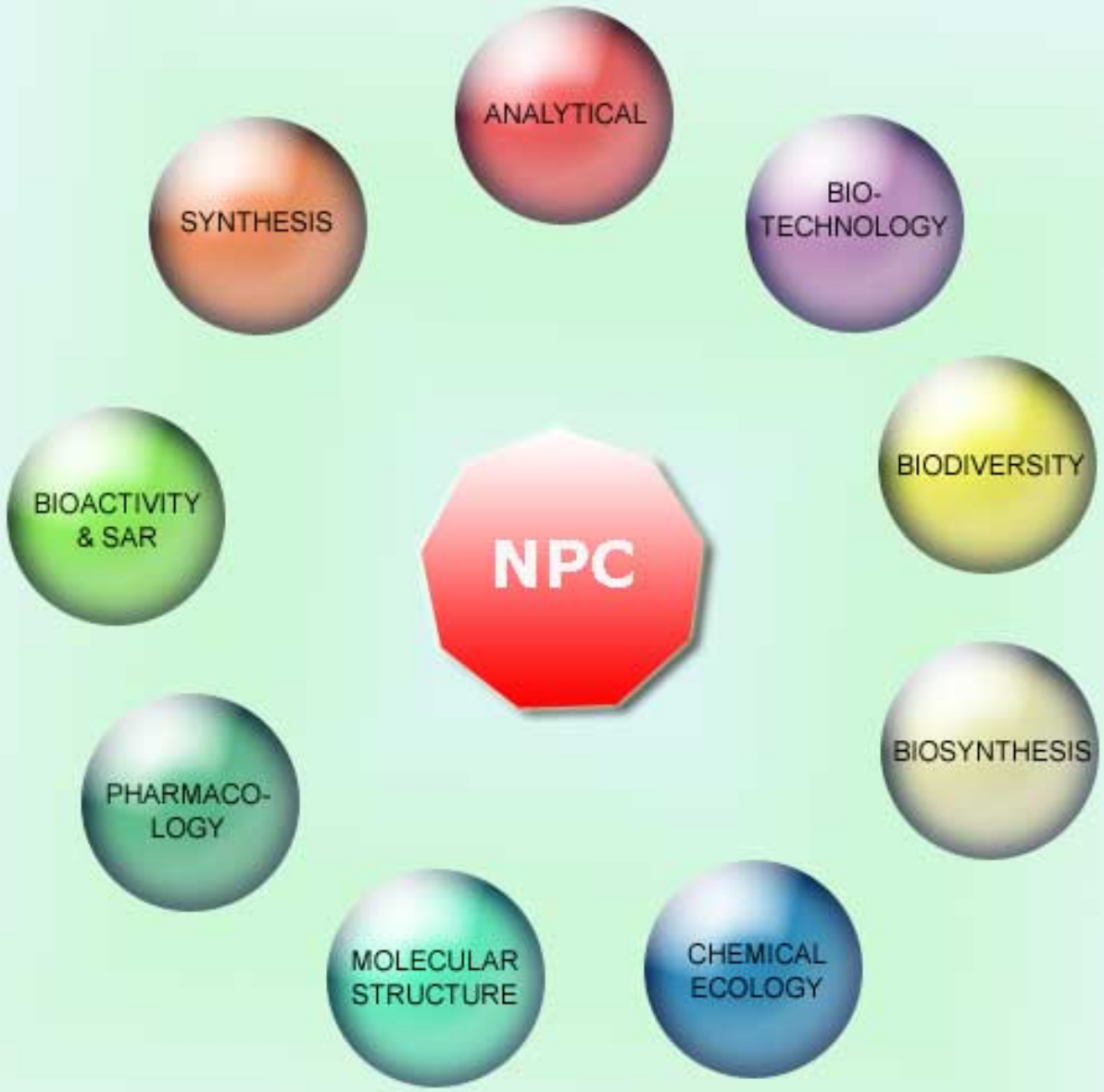

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Biological Activity of Essential Oils from *Aloysia polystachya* and *Aloysia citriodora* (Verbenaceae) against the Soybean Pest *Nezara viridula* (Hemiptera: Pentatomidae)

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The objective of the current study was to determine the chemical constituents, ovicidal activity, fumigant, contact toxicity and repellent effects of essential oils isolated by hydrodistillation from *Aloysia polystachya* and *A. citriodora* against eggs and second instar nymphs of *Nezara viridula*. The major components were carvone (83.5%) for *A. polystachya*, and citronellal (51.3%) and sabinene (22.9%) for *A. citriodora*. The ovicidal activity of both oils was tested by topical application at different concentrations ranging from 1.2 to 12.5 µg/egg; all concentrations had a toxic effect. Data probit analysis showed that the LC_{50} value for *A. polystachya* was 2.3 µg/egg and for *A. citriodora* 1.9 µg/egg. The fumigant activity was evaluated in an enclosed chamber. The toxicity increased with concentration from 11 to 176 µg/mL air, and with exposure times from 1 to 48 h. The LC_{50} values for *A. polystachya* and *A. citriodora* were 29.9 and 13.5 µg/mL air 24 h after treatment, respectively. To evaluate contact activity a glass-vial bioassay was used. The toxicity increased with concentration from 2.8 to 45 µg/cm^2 and with exposure time from 1 to 48 h. The LC_{50} for *A. polystachya* was 3.4 µg/cm^2 and for *A. citriodora* 8.1 µg/cm^2. The repellency bioassay demonstrated that both oils were active at the highest concentration (2.6 and 5.3 µg/mL air) and neutral at 1.3 µg/mL air. These results show that the essential oils from *Aloysia polystachya* and *A. citriodora* could be applicable to the management of populations of *Nezara viridula*.

Keywords: *Aloysia polystachya*, *Aloysia citriodora*, ovicidal activity, fumigant toxicity, contact toxicity, repellence, essential oils, southern green stink bug.

In Argentina, soybean [*Glycine max* (L.) Merrill] is a very important crop that has been expanding since its introduction 40 years ago. In the last season (2008/9), the area under cultivation to this crop was 16,600,000 ha with an estimated production of 13,755,000 tons [1]. The southern green stinkbug, *Nezara viridula* (L.), is a highly polyphagous pest that is widely distributed in the tropical and subtropical regions of the world. In Argentina, this pentatomid causes important economic damage to soybean crops from second instar nymphs to adults [2a]. Consequently, there can be premature fruit drop, delay in crop maturity and reduced seed quality and quantity [2b]. This pest also transmits plant pathogens [2c].

Control of this pest is based largely on the intensive use of chemical pesticides, including carbamates, organophosphates and some pyrethroids [3a]. In Argentina the most used insecticide is either endosulfan or mixtures of this product with pyrethroids. In the last few years, failures have been detected with this treatment in some productive areas [3b]. Traditionally, in these cases, the solution involved an increase in the doses used, and it is known that the massive use of this kind of insecticides not only increases production costs, but also can generate pentatomid resistant populations by selective pressure [3c]. Besides, as a result of increasing restrictions on the use of endosulfan in some countries, the most commonly used insecticide in soybean crops is the synthetic pyrethroid, deltamethrin. Although this is being used to control *N. viridula*, it is harmful to natural enemies, making it incompatible with integrated pest management (IPM) strategies for soybean and pulse crops [4a].

In order to avoid these problems, alternative tools of control are being investigated. They include biological control (classic, inundation and conservation), sterile male technique, trap crops, entomophatogenic fungi and botanical insecticides [4a-4e].

Essential oils are volatile, natural, complex compounds characterized by a strong odor and are formed by aromatic plants as secondary metabolites [5]. Traditionally, they have been used to control insects in the home or against stored product pests. Recent studies have shown that these natural products, being biodegradable and with low mammalian toxicity, possess insecticidal and repellent properties [6a-6c]. Major constituents from aromatic plants, mainly monoterpenes, are of special interest to industrial markets because of their potent biological activities, such as anti-bacterial, anti-fungal and anti-inflammatory activities, in addition to their toxicity to insects [5]. Besides, it has been demonstrated that essential oils has neurotoxic, cytotoxic, phototoxic and mutagenic effects in different organisms [7a,7b]. Because of the multiple sites of action at which the essential oil can act, the probability of developing a resistant population is very low.

Aloysia polystachya is an aromatic native plant which is widely distributed in subtropical regions of South America, mainly Paraguay and Argentina. This species is popularly known as "burrito", "poleo de castilla" or "poleo riojano". The decoction of leaves and flowers from this plant is widely used in folk medicine to treat gastrointestinal disorders, such as pain, nausea, vomiting, dyspepsia and gastritis [8a-8b]

Aloysia citriodora, popularly known as "cedrón", is an herbal species native to Chile and Argentina and used in folk medicine. The infusion's chemical composition reveals a large amount of polyphenolic compounds, and a powerful superoxide radical scavenging activity, as well as a moderate scavenging activity of hydroxyl radical and hypochlorous acid [9a-9b].

There is lack of information on the effectiveness of essential oils against *Nezara viridula*. In this study, essential oils from two Verbenaceae were tested for their biological activity as part of our search for effective and affordable natural products that can be used in the control of this soybean pest. Furthermore, the essential oils compositions were characterized.

The major component of the leaf essential oil of *A. polystachya* was carvone, and that of *A. citriodora* citronellal (Table 1). Previous work reported that the main compounds of the essential oil from fresh plants of *A. citriodora* were geranial (21.8%), neral (17.4%) and limonene (11.0%) [10]. However, it is known that significant intra-species variation exists in the chemical composition, depending on season, time and geographical origin [11a-11b].

Table 1: Constituents of the essential oils from leaves of *Aloysia polystachya* and *A. citriodora*.

Essential Oil	Compound	Retention index (min)	Composition %
A. polystachya	Limonene	8.5	16.5
	Carvone	14.7	83.5
A. citriodora	α-Pinene	7.0	2.3
	Sabinene	8.1	22.9
	p-Cymene	9.6	1.8
	Limonene	9.7	7.4
	Citronellal	13.3	51.3
	γ-Cedrene	20.4	2.3
	Cariophyllene	20.5	2.4
	α-Curcumene	22.1	9.6

The essential oil from *Lippia scaberrima* Sond. (Verbenaceae) contains limonene and carvone as its main components [12]. Similar results were reported in our study working with the oil from *A. polystachya*.

In topical toxicity bioassay, both oils exhibited potent, concentration – dependent ovicidal activity against *N. viridula*. The percentage inhibition of hatch (P.I.H) produced by *A. polystachya* was 15% at 1.2 µg/egg, 95% at 6.2 µg/egg and 97% at 12.5 µg/egg. With *A. citriodora* the P.I.H was 29% at 1.2 µg/egg, 89% at 6.2 µg/egg and 100% at 12.5 µg/egg. Significant differences were not observed between the dosages of these oils. The oils from *A. polystachya* and *A. citriodora* were toxic with LC_{50} values of 2.3 µg/egg (1.2 – 4.4) and 1.9 µg/egg (0.2 – 5.0), respectively. Many phytochemicals possess ovicidal activity against various insect species [6b, 13a]. The lipophilicity of the essential oil may allow the diffusion of the active metabolites through the corion affecting the insects' embryos. In previous studies with other hemiptera species, we reported that the apolar extract from *Schinus molle* also produces an ovicidal effect [13b].

The results of enclosed chamber tests showed that both essential oils had high toxicity against second instar nymphs of *N. viridula*. A strong difference was observed in mortality as oil concentration and exposure time was increased.

At the highest concentration (176 µg/mL air) the oil from *A. polystachya* proved to be able to induce more than 45% mortality after 1 h and achieved a level of 100% at 6 h, whereas the oil from *A. citriodora* caused more than 40% mortality after 6 h exposure and 100% after 12 h. At 88 µg/mL air, complete insect mortality was achieved after 6 h of exposure using *A. polystachya*, and with *A. citriodora* 97% mortality at

48 h. At 44 µg/mL air, both oils produced the same effect; at 48 h the percentage of mortality ranged between 94% and 100% for *A. citriodora* and *A. polystachya*, respectively. At 22 µg/mL air, both oils produced 87% mortality after 48 of treatment and, at the lowest concentration, more than 50%. The oils from *A. polystachya* and *A. citriodora* were toxic, with LC_{50} values of 29.9 µg/mL air (15.9 - 40.9) and 13.5 µg/mL air (9.6 – 17.5) 24 h after treatment, respectively. Essential oils from fruits and leaves of *Schinus areira* against second instar nymphs of *N. viridula* are less toxic than the oils used in this study [4e]. Oils from *A. polystachya* and *A. citriodora* have fumigant toxicity against *Rhizopertha dominica,* a grain stored pest [14a]. Likewise, the oil from *A. polystachya* also produces rapid kill against head lice, *Pediculus humanus capitis* [14b].

The lowest concentration of fumigant toxicity was used as the highest concentration to evaluate contact toxicity against second instar nymphs of *N. viridula*. The concentration of essential oil applied and times after treatment influenced the percentage mortality of the nymphs. At 45 µg/cm^2, the oil from *A. citriodora* was more toxic than that of *A. polystachya* 1 h after treatment. However, both oils showed the same response at 2 h (100% mortality). At 22.5 µg/cm^2, the oil of *A. polystachya* caused 100% mortality 2 h after exposure and of *A. citriodora* after 12 h. At 11.2 µg/cm^2, the oil of *A. polystachya* produced 100% mortality 6 h after exposure and of *A. citriodora* 95% mortality after 48 h. At 5.6 µg/cm^2, the percentage of mortality was 75% for *A. citriodora* and 80% for *A. polystachya*, 48 h after treatment. At the lowest concentration (2.8 µg/cm^2), the percentage of mortality ranged between 60 to 77% for *A. citriodora* and *A. polystachya*, respectively, 48 h after exposure. The LC_{50} value 24 h after treatment for *A. polystachya* was 3.4 µg/cm^2 (1.3 – 5.0) and for *A. citriodora* 8.1 µg/cm^2 (3.0 – 11.6).

Both essential oils present contact and fumigant toxicity and this could be indicating that the penetration of the biocidal compounds is via the tegument and respiratory system. The insecticidal activity of an essential oil could be attributed to either the major constituents of the oil, or to the synergic and/or antagonistic effects of all the components of the oil. However, the toxicological effect of a specific compound could vary with the pest-insect evaluated: citronellal produces fumigant activity against *Musca domestica* and *Oryzaephilus surinamensis*, but not *Tribolium castaneum*, *Sitophilus oryzae* and *Blatella germanica* [15].

Table 2: Repellent effect of essential oils from *Aloysia polystachya* and *A. citriodora* against second instar nymphs of *Nezara viridula*.

Essential oil	Doses (µg/mL)	R.P.	Z value	SL	A.P.	Z value	SL	Biological Activity
A. polystachya	5.3	0.9	5.6	**	0.1	-5.6	ns	Repellent
	2.6	0.7	2.3	*	0.3	-2.3	ns	Repellent
	1.3	0.6	0.8	ns	0.4	-0.8	ns	Neutral
A. citriodora	5.3	0.9	6.5	**	0.1	-6.5	ns	Repellent
	2.6	0.8	4.5	**	0.2	-4.5	ns	Repellent
	1.3	0.5	0.3	ns	0.5	-0.3	ns	Neutral

R.P: Repellency Proportion, A.P: Attractancy Proportion obtained 24 h after exposure; SL: Significant level; n = 5.

Because of the characteristics of the olfactometer, the highest concentration to evaluate repellency activity was half the minor concentration corresponding to fumigant toxicity 24 h after treatment. This was in order to prevent mortality of second instar nymphs during bioassay. Both oils showed repellent activity at 2.6 and 5.3 µg/mL air. At the lowest concentration (1.3 µg/mL air), neutral activity was observed (Table 2).

Volatile compounds can modify insect's behavior. Repellents are substances that act locally or at a distance, deterring an insect from flying to, landing on or climbing on the area where the product was applied [16a]. The results presented herein indicate that the essential oils from *A. polystachya* and *A. citriodora* have volatile compounds that generated an olfactory response on *N. viridula.* The repellency of some Verbenaceae essential oils has previously been evaluated against a number of stored-product insects. The ethanol and hexane extracts of *A. polystachya* produced a repellent effect against larvae and adults of *T. castaneum* [16b]. The essential oils of *A. citriodora* have a repellent activity against *Aedes aegypti* [16c]. Citronellal, the main component of *A. citriodora* oil, was previously reported as a mosquito repellent [16d], but no studies were undertaken using Pentatomidae bugs.

The observed biological activity demonstrates that essential oils are a source of active compounds which may potentially prove to be efficient insecticides and repellents. Furthermore, the essential oils evaluated in this study are used in folk medicine, and are thus considered to be less harmful to humans and the environment than the majority of conventional insecticides [17]. Consequently, the possibility of employing these natural products to control *Nezara viridula* may warrant further investigation.

Experimental

Plant material: *Aloysia polystachya* (Griseb.) Moldenke and *A. citriodora* Paláu were collected from Bahía Blanca, Buenos Aires, Argentina. Fresh leaf samples were placed in labelled bags, transported to

Universidad Nacional del Sur and identified at the Herbarium of the Departamento de Biología, Bioquímica y Farmacia, UNS (Herbarium Voucher Number: *A. polystachya* - BBB MGM 452; *A. citriodora* - BBB MGM 480). The fresh plant samples were subjected to hydro-distillation in a modified Clevenger type apparatus, as previously reported [18]. The essential oils obtained were dried over anhydrous sodium sulfate and refrigerated at 4°C. The chemical composition of each oil was determined by GC-MS. The compounds were identified by comparing their retention indices (Kovats Indices) with those of known compounds and also comparing their MS with those stored in the MS databases (NBS75K.L MS DATA). Relative percentage amounts were obtained directly from GC peak areas. GC-MS analyses were performed with a Hewlett-Packard 6890 chromatograph connected to a Hewlett-Packard 5972A mass spectrometer equipped with a capillary column (HP-5, 25 m x 0.25 mm, 0.25 μm film thickness). The carrier gas was helium with a flow rate of 1 mL/min. The GC oven temperature was held at 50ºC for 2 min, programmed at 5ºC/min to 200ºC, then held at this temperature for 15 min. MS were recorded at 70 eV. Mass range was from *m/z* 35 - 350 amu. The temperature of the injection block was 250ºC.

Obtaining and rearing Nezara viridula: To establish laboratory stock colonies, adults of *N. viridula* were collected in soybean fields in Rivera, Provincia de Buenos Aires, Argentina and reared, as described in previous work [19] under controlled conditions (27 ± 1ºC, 60 ± 10% HR and 14L:10D photoperiod). Egg masses were collected daily and placed in 9 cm diameter Petri dishes, lined with filter paper and maintained under the conditions previously described. Since first instar nymphs do not feed, they were maintained in the Petri dishes until the beginning of the second instar, when they were transferred to the respective diet until they were used for the bioassays.

Ovicidal activity: Eggs masses (>80 eggs, 5-6 days old) were topically applied on an opercule with 10 μL *n*-hexane essential oil solution or *n*-hexane (control) using a Hamilton microsyringe. The dosages evaluated were 1.2, 6.2 and 12.5 μg/egg. After treatment, the eggs masses were placed at the bottom of a Petri dish and covered with gauze cloth. The percentage of inhibition of hatch (PIH) was calculated from the formula PIH (%): [(C-T)/C]X100, where C is the control percentage hatch and T is the treated percentage hatch [13a]. Treated and control egg masses were held under the same conditions used for colony maintenance. The toxicity of the essential oils was based on the number of unhatched eggs 7 days after treatment. All treatments were replicated 5 times. Data were analyzed by Anova and DMS. Data probit analyses utilized Micro Probit 3.0 and LC_{50} and 95% confidence intervals were estimated. The LC_{50} values were considered significantly different if their 95% confidence intervals did not overlap.

Fumigant toxicity studies: The fumigant toxicity of the essential oils against second instar nymphs of *N. viridula* was evaluated in an enclosed chamber. The essential oils were dissolved in *n*-hexane. Filter papers (8.5 diameter, Whatman Nº1) were impregnated with 1 mL of the test compound solutions to provide dosages of 11, 22, 44, 88 and 176 μg/mL air. The papers were allowed to dry for 10 minutes before being placed on the bottom of a Petri dish (8.5 cm diameter x 2 cm high) and covered with a lid with a fine wire sieve attached over the central hole where 10 nymphs were released. Finally, each Petri dish was covered with another one and all were fitted together with an adhesive film. Each concentration and control was replicated 5 times. Mortality was recorded after 1, 2, 6, 12, 24 and 48 h from commencement of exposure. When neither leg nor antennal movements were observed, insects were considered dead. These symptoms were proved to ensure ultimate irrecoverable death in each period of exposure. Mortality data were subject to probit analysis using Micro Probit 3.0, and LC_{50} and 95% confidence intervals were estimated. The LC_{50} values were considered significantly different if their 95% confidence intervals did not overlap.

Contact toxicity studies: Procedures similar to those described by other authors were used to evaluate the contact activity of essential oils against second instar nymphs [20a-20b]. The interior surface of 14 mL glass vials was coated with an essential oil by pipetting 0.5 mL of either the appropriate *n*-hexane oil solution or *n*-hexane alone (control) into the vials. These were then rotated on a modified hot dog roller (heating element disconnected) until all the *n*-hexane had evaporated. Ten insects were introduced into the vials. The dosages evaluated were 2.8, 5.6, 11.2, 22.5 and 45 μg/cm^2. Each concentration and control was replicated 5 times. Mortality was determined after 1, 2, 6, 12, 24 and 48 h from commencement of exposure. The criterion for mortality was the inability of the insect to assume an upright posture within 5 s of being dislodged from the vial. The LC_{50} and 95% confidence intervals were calculated using Micro Probit 3.0.

Repellency bioassays: Behavioral responses of second instar nymphs were investigated with a simple olfactometer. The experimental area was formed by two hexagonal glass vials (A and B) (190 mL). Each had a metal lid with a central hole connected to a glass tube

(9 x 0.7 cm. diameter) fitted together to prevent vapors from escaping. Both glass tubes were connected by a central tube (3 x 0.7 cm diameter). Filter papers (5.5 cm diameter, Whatman Nº 1) were treated with 0.5 mL of either *n*-hexane solutions of the essential oil or solvent alone. The concentrations were 1.3, 2.6 and 5.3 µg/mL air. After solvent evaporation (10 min), treated papers were put at the bottom of vial A and untreated papers of vial B. In all cases, vapor stabilization was allowed for 1 hour. Ten insects were put in the central tube and after 24 h the number of insects in each glass vial was recorded. Each concentration and control was replicated 5 times.

A Repellency Proportion (RP) and an Attractancy Proportion (AP) were calculated as:

$$RP= NB/NT$$
$$AP= NA/NT$$

where: NB= number of insects on the untreated zone; NA= number of insects on the treated zone; and NT= number of total insects in each olfactometer. A statistical Z was calculated to standardize an originating proportion of a binomial distribution:

$$Z= n\,(RP-P_0) / \sqrt{nP_0\,(1-P_0)}$$
$$Z= n\,(AP-P_0) / \sqrt{nP_0\,(1-P_0)}$$

where: n= number of total insects used for each concentration; P_0 = expected proportion (P_0: 0.5). The Z value was compared with t $critic_{0.05}$ (t = 1.96, df = ∞; P < 0.05) or t $critic_{0.01}$ (t = 2.57; df = ∞; P < 0.01) [21].

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