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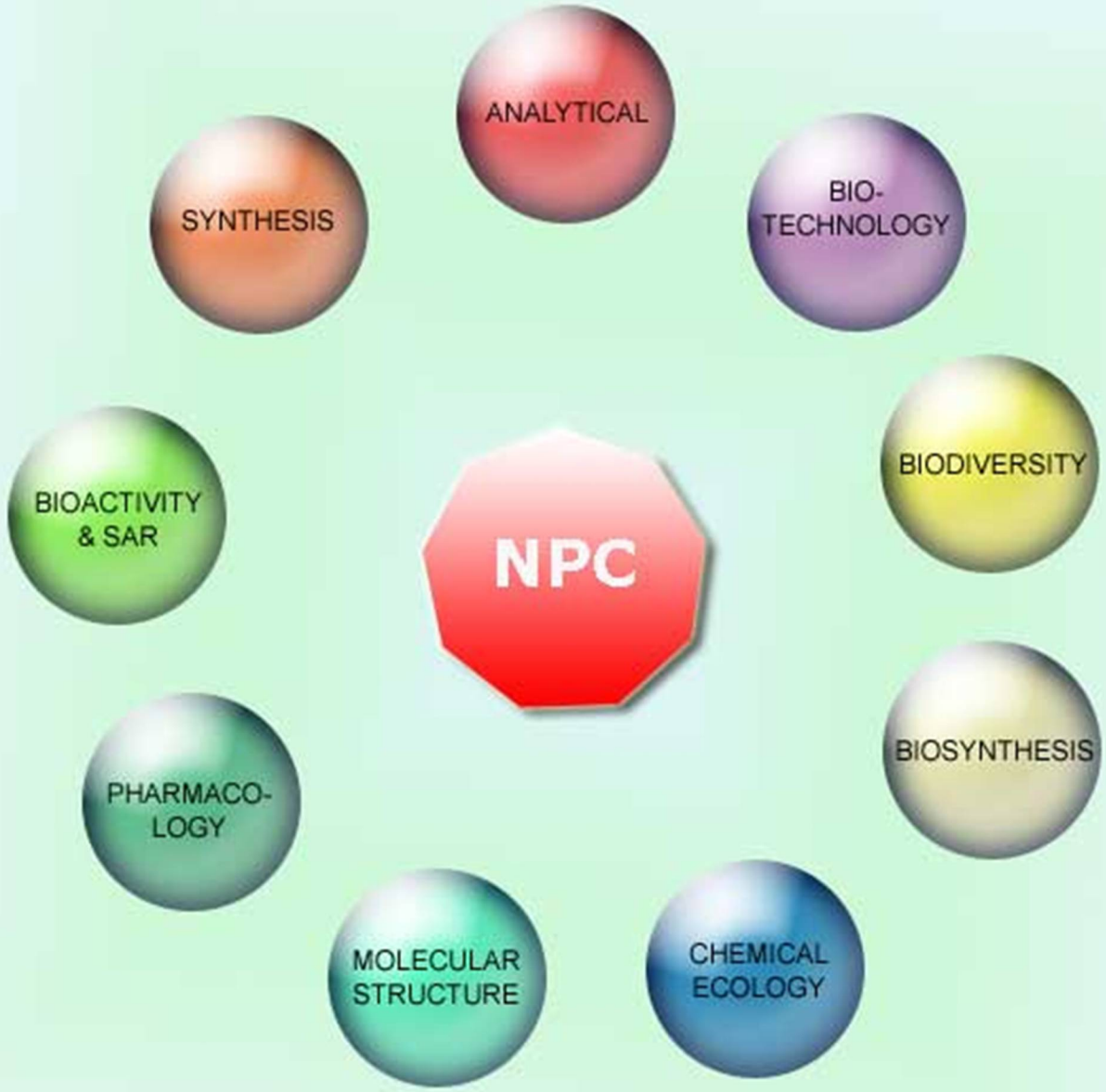

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Bioactivity of the Essential Oil of an Argentine Collection of *Acanthospermum hispidum* (Asteraceae)

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The essential oil of an Argentine collection of the annual herb *Acanthospermum hispidum* DC. (Asteraceae), which contains the sesquiterpenoids β-caryophyllene (35.2%), α-bisabolol (11.4%), and germacrene D (11.1%) as major constituents, displayed molluscicidal effects on an adult population of *Biomphalaria peregrina* Orbigny (LD_{50} 37.8 μg/mL) and produced alteration of the feeding and oviposition behavior of the polyphagous insect *Spodoptera frugiperda* Smith when incorporated in the larval diet at 250 μg/g. In addition, a selective antibacterial activity was observed against pathogenic strains of *Staphylococcus aureus* and *Enterococcus faecalis* (MICs 62.5 and 125 μg/mL), but was inactive at 125 μg/mL against three beneficial lactic acid bacteria. Synergistic interactions were further validated by FIC index determination of the combination of the antibiotics oxacillin and gentamicin and the essential oil on the four pathogenic strains evaluated.

Keywords: *Acanthospermum hispidum*, Essential oil, Molluscicidal activity, Antifeedant activity, Antibacterial activity, Synergistic interactions.

Acanthospermum hispidum DC. (Asteraceae, Heliantheae) is a herbaceous plant widespread in America, Africa, Australia, India, and Hawaii. Although *A. hispidum* is a pantropical species, it is considered to have an American origin. In Brazil, where the roots have been traditionally used to treat coughs and bronchitis, the plant is incorporated in a syrup produced by public health services to treat asthma. In Malaysia, the entire plant is mixed with castor oil and applied to the skin to treat scabies [1]. In Africa, it is used to treat vomiting, cephalgias, headaches, abdominal pains, convulsions, cough, eruptive fever, snake bites, jaundice, epilepsy, constipation, blennorrhoea, diarrhea, hepato-biliary disorders and malaria [2-4]. In the northwest of Argentina, infusions of the aerial parts of *A. hispidum* are employed in folk medicine as a diuretic, an abortive agent, and against infections [5,6], while the fresh leaves and flowers are traditionally used as an insect repellent. Laboratory tests showed that the CH_2Cl_2 extract of the aerial parts exhibited antiplasmodial and moderate cytotoxic activity [7], while the MeOH and aqueous extracts displayed antifeedant activity towards *Epilachna varivestis*, as well as bactericidal, antifungal, antiviral, anti-cancer, abortive and teratogenic effects [8-12].

A previous report on the composition of the essential oil (EO) of a Congo collection of *A. hispidum* indicated the presence of β-caryophyllene, α-humulene, bicyclogermacrene, germacrene D, α-bisabolol, carvacrol, nonanal and methyl carvacrol [13].

Antibacterial and antifungal effects have been reported for the EO of Indian collections of *A. hispidum* [14,15]. Nevertheless, this is the first report on the antibacterial activity of the EO of an Argentine collection of *A. hispidum* against pathogenic and non pathogenic bacteria, as well as on the synergistic interactions of the EO with commercial antibiotics. The toxic effects against the lepidopteran pest *Spodoptera frugiperda* and *Biomphalaria peregrina,* the snail vector of schistosomiasis were also evaluated.

Table 1: *Acanthospermum hispidum* essential oil composition.

Compounds	RT (min)	Area %	KI
Thymol methyl ether	38.78	0.3	1235
α-Copaene	47.68	3.5	1377
β-Cubebene	48.38	1.6	1388
β-Elemene	48.51	10.0	1391
(*E*)-β-Caryophylene	49.93	35.2	1419
α-Humulene (α-Caryophyllene)	51.60	9.7	1455
Germacrene D	52.82	11.1	1485
Bicyclogermacrene	53.49	9.7	1500
α-Muurolene	53.68	0.4	1500
β-Bisabolene	53.96	0.7	1505
Germacrene A	54.15	1.1	1509
β-Sesquiphellandrene	54.55	1.7	1522
δ-Cadinene	54.82	1.9	1523
Caryophyllene oxide	57.12	0.8	1583
α-Bisabolol	61.17	11.4	1686

RT: GC retention time. KI: Kovats index.

The EO of our collection contained the 15 components (GC-MS analysis) shown in Table 1. Identification of the volatiles was accomplished by computer matching with Wiley 275 and NIST05 libraries of mass spectra. GC retention times (RT), and Kovats indexes (KI) were compared with those reported in the literature [16] under the same chromatographic conditions. (*E*)-β-caryophyllene (35.2%), α-bisabolol (11.4%), and germacrene D (11.1%) were the major constituents of the EO of both a Congolese [13] and our Argentine collection.

(E)-β-Caryophyllene is a natural bicyclic sesquiterpene, rare in nature for having a cyclobutane ring. It is a plant volatile found in large amounts in the essential oils of many different spice and food plants, such as oregano (*Origanum vulgare* L.), cinnamon (*Cinnamomum* spp.) and black pepper (*Piper nigrum* L.). Because

of its weak aromatic taste, (*E*)-β-caryophyllene is commercially used as a food additive and in cosmetics.

Antifeedant and toxic activity of EO on S. frugiperda: Given the potential of volatile plant constituents as environmentally safe pesticides [17], we evaluated the effects of the EO on larvae of *S. frugiperda*, a pest that causes severe economic damage to various crops in Argentina. Diets treated with 250 µg of EO per g of diet, dissuaded 53.3 ± 0.25% (antifeedant index = AI%) larval feeding. The same dose produced 65.9% decrease in the larval growing rate (GR). The GR of treated larvae was 6.10 ± 1.79 mg/d while the control GR was 17.91 ± 3.63 mg/d. The diet consumption rate (CR) dropped by 49.3% (CR = 29.04 ± 5.69 mg/d for treated) compared with the control (CR = 57.28 ± 6.33 mg/d for control). When the EO was incorporated in the larval diet at 250 µg/g, oviposition capacity (determined by the number of eggs) of treated females decreased by 70% compared with control females.

Molluscicidal activity of EO on B. peregrina: Mortality rates of *B. peregrina* were registered after 24 h of exposure. The EO of *A. hispidum* affected the snail population (LC_{50} = 37.8 µg/mL, 95% CL 31.38, 47.03) when added to an experimental aquarium. It is important to point out that the WHO [18] reported that plant extracts showing LC_{50} values less than 100 µg/mL have some potential for the control of vector snails. *B. peregrina* is a vector of schistosomiasis, an endemic disease to about 75 countries throughout South America, Africa, and the Far East. The intermediate host, the mollusc, constitutes the weakest link in the cycle of transmission and thus is the logical point of attack to control the disease with molluscicidal agents that interrupt the parasite's life cycle and prevent human infection. Snails are currently controlled by the very toxic niclosamide, a synthetic molluscicide (LC_{50} = 0.1 µg/mL on *B. glabrata*) that is not only a strong contaminating product, but creates resistance on the target molluscs as well [19].

Higher plants, applied as crude aqueous suspensions are an effective and cheap sources of molluscicides, less likely to cause environmental contamination. Particularly, the essential oils of *Cymbopogon nervatus* (Gramineae) and *Boswellia papyrifera* (Burseraceae) produced molluscicidal effects (LC_{50} = 213.0 and 213.3 µg/mL, respectively), against the snail *B. pfeifferi* [20]. It is important to note that the EO of *A. hispidum* is around 6 fold more potent than the aforementioned oils.

Antibacterial activity of EO: Plant essential oils are known for their antibacterial effects [21] and they do not cause bacterial resistance, mainly because they consist of a mixture of many compounds [22]. Most of them affect the membrane integrity of bacteria and induce depletion of the intracellular ATP concentration [23]. Antibacterial doses of *A. hispidum* EO on two *S. aureus* (MICs 62.5 and 125 µg/mL) and one *E. faecalis* (MICs 125 µg/mL) strains (pathogenic) were innocuous to three beneficial lactic acid bacteria at 125 µg/mL (Table 2).

In addition, mixtures of EO and either GEN (gentamicin) or OXA (oxacillin) were stronger antibacterials (synergistic effects) than the EO and the ATBs alone on the four pathogenic strains evaluated (MICs of mixtures in Table 2). Quantification of the synergistic effects of mixtures of EO and ATBs was accomplished by determination of the FIC index. FICI was interpreted as follows. There is a synergistic effect if FICI $\leq$ 0.5; no interaction if 0.5 < FICI $\leq$ 4 and antagonism if FICI > 4 [24]. The smaller the FICI value, the stronger the synergism. As shown in Table 3, the most significant synergistic effect was caused by EO + OXA on *S. aureus*

Table 2: Antibacterial activity data.

Microorganisms	EO	GEN	EO + GEN		OXA	EO + OXA	
	MIC[a]	MIC	EO MIC	GEN MIC	MIC	EO MIC	OXA MIC
Staphylococcus aureus ATCC 6538 P	125	6	31.25	1.5	1.5	7.81	0.19
S. aureus F 7	62.5	>120	7.81	0.19	>120	31.25	1.5
Enterococcus faecalis ATCC 39212	125	6	31.25	1.5	6	31.25	1.5
E. faecalis F 208	>125	>120	15.62	0.75	60	15.62	0.75
Lactobacillus plantarum CE 105	-[b]	+[c]	-[d]		+[e]	-[d]	
L. plantarum CE 358	-	+	-		+	-	
L. paracasei CE 75	-	+	-		+	-	

[a] MIC data are expressed in µg/mL, [b] Grown at 125 µg/mL of EO,
[c] Inhibition at 6 µg/mL of GEN, [d] Grown at MIC doses of all mixtures,
[e] Inhibition at 1.5 µg/mL of OXA. Each experiment was carried out in triplicate.

Table 3: Fractional inhibitory concentration index (FICI) for the combinations between ATB and EO of *A. hispidum*.

Microorganisms	EO+GEN	EO+OXA
S. aureus ATCC 6538 P	0.5	0.2
S. aureus F 7	NC	0.5
E. faecalis ATCC 39212	0.5	0.5
E. faecalis F 208	NC	NC

NC= Not calculated.

ATCC 6538 P, as well as EO + GEN on *S. aureus* F 7 and *E. faecalis* F 208 that showed significant reduction of MICs values in the mixture (Table 2). The synergistic effects may occur *via* facilitation of entry of ATB into the cell due to disruption of the bacterial cell wall produced by the EO [25]. The forementioned results might explain the ethnomedical use of the plant to treat infections and support the incorporation of the EO of *A. hispidum* in formulations to control schistosomiasis and *S. frugiperda*. In addition, the EO could improve the performance of antibiotics in infectious processes at non toxic doses for lactobacilli strains.

Experimental

Plant material: Aerial parts of *A. hispidum* DC. (Asteraceae, Heliantheae) were collected during the flowering stage in April on the banks of Vipos river, Tucumán province, Argentina. A voucher specimen (LIL 604458) is on deposit at the Herbarium of Fundación Miguel Lillo, Tucumán, Argentina.

Extraction of essential oil: Fresh leaves and flowering tops (272.5 g) were cut into small pieces and subjected to hydrodistillation for 3 h, using a Clevenger-type apparatus. The oil was dried over anhydrous sodium sulfate and stored at 4°C.

Analysis of essential oil: GC and GC-MS analyses were carried out using a Hewlett-Packard 6890 Series II GC System and a Hewlett-Packard 5973 GC-MS, respectively, equipped with a HP-5MS capillary column (30 m x 0.25 mm, film thickness 0.25 µm). The initial temperature of the column was 50°C during 15 min. A temperature programming was applied from 50 to 100°C at a rate of 2°C/min, 100 to 160°C at 3°C/min, 160 to 280°C at 10°C/min, and finally 280°C for 15 min. The carrier gas was helium, and the injection mode split-less.

Test insects and artificial diet: Larvae from *S. frugiperda* Smith were obtained from our laboratory colonies. The colonies had not been previously exposed to insecticides. Larvae were fed on an artificial diet that consisted of a mixture of yeast (3 g), beans boiled and milled (250 g), wheat germ (12.5 g), agar-agar (12.5 g), ascorbic acid (1.5 g), methyl *p*-hydroxybenzoate (1.5 g),

formaldehyde (4 mL of a 38% water solution) and 500 mL distilled water [26].

Larval diet choice test: A portion of artificial diet was mixed with acetone, and after solvent removal *in vacuo*, this portion was employed as control diet. Another portion was impregnated with an acetone solution of EO (treatment) in order to leave 250 µg of oil per g of diet. After evaporation of the solvent, 120 mg of control and the same amount of treated diet were placed in a glass tube. Between the two diet portions, a larva was introduced into the tube. The larva was allowed to choose the diet, and, when 50% of control diet had been eaten (around 48 h after starting the bioassay), the remaining diets (control and treated) were weighed. The experiment was carried out in 20 replicates. Results of the choice test were then reported by the AI% = [1 – (T/C)] 100, where T and C represent the amounts eaten of control and treated diets, respectively [27].

Toxicity bioassay on S. frugiperda: A portion of the artificial diet was impregnated with acetone and, after solvent removal, this portion was employed as control diet. Another portion was impregnated with an acetone solution of EO in order to leave 250 µg of EO per g of diet. After evaporation of the solvent, control and treated diets were placed in test tubes (20 replicates for treated and 20 for control). Second instar larvae of homogeneous size and accurately weighted were placed in each tube and kept at 27°C until emergence of the 1st generation of adults. Fourteen days after the beginning of the experiment, the larval weight was determined again, in order to record the larval growing rate (GR). The weight of the diet provided to larvae was also determined in order to determine the consumption rate (CR). GR = (A – B)/t, is the larval weight increment per day (mean value) where A = Final larval weight, B = Initial larval weight, and t = Period of evaluation (t = 14 d). CR = D/t, is the larval diet consumed per day (mean value), where D = Weight of food eaten during the experimental period [28].

Statistical analysis: The results are reported as mean ± SEM. The differences in the mean values were evaluated by analysis of variance (ANOVA). The Tukey test was used for all pairwise multiple comparisons of groups. In all statistical analysis, $P > 0.05$ was considered not significant (Statistix 7.1, 2000).

Molluscicidal activity: *B. peregrina* Orbignyi adults were collected from small lakes in Tucumán province, Argentina and maintained in an aquarium with dechlorinated water, mineralized with 0.05 g/L of $Ca_3(PO_4)_2$ at 26 ± 2°C and pH 7.2 feeding on *Lactuca sativa* fresh leaves. Aquarium snails, of uniform shell diameter (7 mm) and age, were selected for the bioassay and maintained for 24 h without feeding before the experiment. A portion of EO (10 mg) was dissolved in 2 mL of MeOH and diluted with water in order to obtain concentrations of 100, 50 and 25 µg/mL (water-methanol rate 98:2). Solutions of EO (20 mL) were then poured into 100 mL flasks and 5 snails were then placed in each flask. Control experiments were performed placing 5 snails in a mixture of water and methanol (98:2). MeOH was not toxic to the snails at the concentrations tested. Each experiment was carried out in triplicate. After an exposure of 24 h, snails were placed in a Petri dish and the heartbeat detected upon microscopic observation. After the exposure period, snails that were alive were kept in fresh water and controlled for another 24 h in order to check movement and register mortality. Snails that survived were not subjected to further tests. A 10 µg/mL water solution of $CuSO_4$ was used as positive control [29]. Data were analyzed with the Finney computer program to determine the LD_{50} [30].

Antibacterial activity: Representative isolates of clinically relevant species, collected during clinical trials from hospital patients, were used in this study. The Gram-positive microorganisms were *Staphylococcus aureus* F 7 (methicillin-resistant), *Enterococcus faecalis* F 208 (ampicillin-resistant) and collection strains of *S. aureus* ATCC 6538 P and *E. faecalis* ATCC 39212. The non pathogenic strains, *Lactobacillus paracasei* ssp. *paracasei* CE 75 and *L. plantarum* CE 105, were isolated from regional cheeses, while *L. plantarum* CE 358 came from high mountain northwestern Argentine soils (Collection of Centro de Referencia de Lactobacilos, CERELA, Tucumán, Argentina). The antibacterial activity tests were conducted by the micro dilution method using Mueller-Hinton as culture medium in polystyrene microplates of 96 wells. The system DMSO-PEG 400 (1:1) was used as solvent for the fat-soluble products to improve their solubility in water [31]. The mixture DMSO-PEG 400 incorporated in the culture medium at 5% displayed no antibacterial effect and was used as control. The antibacterial action of EO was evaluated at doses ranging between 125 and 19.5 µg/mL, while antibiotics (ATBs) were tested between 120 and 0.09 µg/mL in order to determine the minimum inhibitory concentrations (MIC). After incorporation of the EO, microbroth dilution plates were inoculated with each micro organism to yield the appropriate bacterial density (10^5 CFU/mL) in a 100 µL final volume to be incubated for 24 h at 37°C. Synergistic effects between EO and ATBs were determined employing an *in vitro* methodology developed for commercial ATBs, the chequerboard MIC technique, often used to assess drug-drug interactions yielding the fractional inhibitory concentration index, FICI [24]. The FICI was calculated for each combination using the following formula: $FIC_{EO} + FIC_{ATB} = FICI$, where FIC_{EO} = MIC of EO in combination/MIC of EO alone, and FIC_{ATB} = MIC of ATB in combination/MIC of ATB alone.

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Review/Account

Natural Product Communications
2012
Volume 7, Number 2

Contents

Continued inside backcover