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ARTICLE · JANUARY 2013

DOI: 10.4103/2229-5119.110348

Khellin: A furanochromone with toxicity against *Oncopeltus fasciatus* (Hemiptera) and *Aedes aegypti* (Diptera)

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ABSTRACT

Natural products isolated from plants may be an alternative source of larvicidal and insecticide activity. Khellin is a natural furanochromone isolated from fruits of *Ammi visnaga* (L.) Lam. (*Umbelliferae* family), which grows extensively in the Mediterranean region. This substance shows several types of biological activity, such as *in vitro* cytotoxicity, antispasmodic action, and phototherapeutic potential. Dengue is a tropical disease caused by an arbovirus transmitted by *Aedes aegypti*; the milkweed bug, *Oncopeltus fasciatus*, is a phytophagous Hemiptera and a Phytomonas vector. Our main goal was to evaluate the toxicity of khellin in relation to the nymphs of *O. fasciatus* (Hemiptera) and larvae of *A. aegypti* (Diptera). To the best of our knowledge, this is the first report concerning furanochromone bioactivity against insect vectors for human disease.

Keywords: *Ammi visnaga* (L.), bioactivity, culicidae, hemiptera, larvicidal, natural products

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Access this article online

Website: www.jnatpharm.org

DOI: 10.4103/2229-5119.110348

Quick Response Code:

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INTRODUCTION

Plants are a rich source of pharmacologically active substances and some of them show larvicidal and insecticide activity[1,2] with regard to controlling undesirable insects in agricultural crops,[3] homes, and gardens that have public health implications.[4,5] Khellin [Figure 1] is a furanochromone isolated from *Ammi visnaga* (Lam.) Lamarck, a plant in the *Umbelliferae* (Apiaceae) family that grows extensively in the Mediterranean region. In Europe, the plant has often been referred to as the toothpick herb or bishop's weed, while in Turkey it is known as "disotu", "kilir," and "hiltan".[6]

A. visnaga has been used as a diuretic and to treat urinary tract pain caused by urethral stones. Pharmacological studies have confirmed that furanochromone derivatives, especially khellin, visnagin, and pyranocoumarin fractions, are responsible for the antispasmodic action of this plant.[6] A butanolic extract of *A. visnaga* has demonstrated antioxidant activity, while the essential oil has exhibited antibacterial activity.[7,8] Studies using khellin have shown several types of biological activity, such as *in vitro* cytotoxicity[9] and bioactivity for vitiligo treatment.[10,11]

Dengue is an epidemic and neglected disease that accounts annually for several million cases and deaths worldwide.[12] Dengue fever is transmitted by the mosquito *Aedes aegypti* Linnaeus, 1762 (Diptera: Culicidae), and is one of the most rapidly spreading insect-borne diseases. Worldwide, more than 2.5 billion people are at risk of infection by the dengue vector, *A. aegypti*.[12] In Brazil, up to October 2010, 936,260 cases had been notified, with 592 dengue-related deaths.[12]

A. aegypti is a cosmopolitan mosquito that can be found in tropical and subtropical

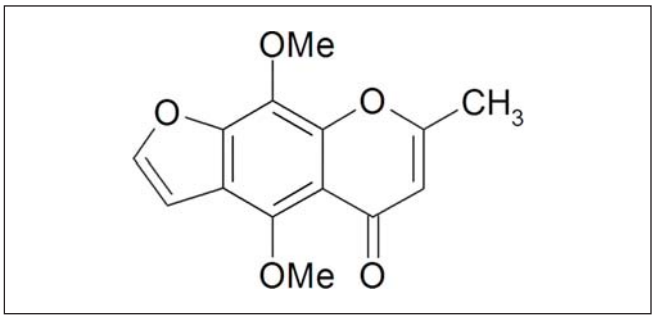

Figure 1: Chemical Structure of khellin (4, 9-dimethoxy-7-methylfuro[3, 2-g] chromen-5-one)

regions.[13,14] Several viral serotypes produce human infection.[15] Studies have revealed that *A. aegypti* larvae present widespread resistance to the organophosphate temephos throughout Brazil.[16] Resistance of adult *A. aegypti* to the currently used pyrethroids such as cypermethrin and deltamethrin has also been reported.[17] In view of this information and the fact that development of a multi-serotype vaccine is still a distant goal,[12] the only means of reducing infection rates is to control the insect vector. Therefore, new approaches are urgently needed.

The milkweed bug, *Oncopeltus fasciatus* Dallas, 1852 (Hemiptera: Lygaidae), occurs over a wide geographical range extending from Massachusetts westwards over the greater part of the United States and southwards to Mexico and Brazil.[18] This Hemiptera is a phytophagous insect usually found on *Asclepias curassavica* (popular name "oficial de sala") and is a Phytomonas vector.[18,19] It provides a convenient model for testing the effects of substances on development and mortality.[19] Milkweed bugs can be cultivated in dry sunflower seeds (*Helianthus annuus*), present a short cycle, require little space, and are large enough to be easily observed.[20] In their natural environment, they feed on the seeds of milkweed plants by piercing the seed pod. They also will feed on nectar and on the plant's juices. Since they are not a much of pollinator, they have been considered to be pests that pierce leaves and facilitate pathogen penetration.[21] The present study evaluated the toxicity of khellin on nymphs of the Hemiptera *O. fasciatus* and larvae of the Diptera *A. aegypti*.

MATERIALS AND METHODS

General Experimental

^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ and DMSO/benzene with TMS as internal standard on a Bruker AC 200 L instrument operating in 200 and 50.32 MHz, respectively. UV sprectra was recorded in a Shimadzu UV-1208 UV-Vis Spectrophotometer using $CHCl_3$. Si gel for column chromatography were silica gel 60 (0.2–0.5 mm) (Macherey –Nagel). Solvents used were from Merck and Pronalab with "pró-analysis" and "pure" degree of purity.

Plant Material

The plant material was collected near Hatay, Turkey, and was identified by the botanist Vagif Hatemov, at Mustafa Kemal University, Hatay. A voucher specimen has been deposited in the Herbarium of the Biology Department, Mustafa Kemal University.

Extraction and Isolation

The dried, ripe fruits (100 g) were extracted with methanol (2 L) in a Soxhlet extractor for 30 h. The solvent was evaporated and the residue (8 g) was dissolved in EtOH–water. This solution was extracted with hexane (5 ´ 200 mL) and chloroform (5 ´ 200 mL). The chloroform extract (1,2 g) was subject to column chromatography over Si gel (0.2–0.5 mm Merck; 100 g) and eluented with mixture of hexane, hexane-dichloromethane, dichloromethane-acetone, 50 ml fractions being collected as follows: fractions 1–6 (hexane), 7–17 (hexane-dichloromethane, 7:3), 18–38 (hexane-dichloromethane, 5:5), 39–50 (hexane-dichloromethane, 3:7), 51–61 (dichloromethane), 62–85 (dichloromethane-acetone, 9:1), 86–100 (dichloromethane-acetone, 5:5),101–115 (dichloromethane-acetone, 3:7), 115–130 (acetone). Fractions 62–85 were combined and compound 1 (560 mg) obtained by crystallization with methanol.

Khellin (1) crystallized from MeOH, m.p. 154–155°C, UV max nm: 275.322. ^{1}H-NMR(200 MHz; $CDCl_3$): δ, ppm 7.65 (d, *J*= 2 Hz, H-2); 7.03 (d, *J*= 2 Hz, H-3); 6.06 (s, H-6); 4.19 (s, OCH_3); 4.06 (s,OCH_3); 2.4 (s, CH_3). ^{13}C-NMR ($CDCl_3$): δ= ppm 178.2 (C5); 163.9 (C13); 148.8 (C11); 147.3 (C9); 147.1 (C4); 145.5 (C3); 129.9 (C10); 119.4 (C7); 113.7 (C11); 110.8 (C6); 105.2 (C2); 62.3 (C9 –OCH_3); 61.8 (C4 –OCH_3); 20.1 (C7 –CH_3).[6]

Biological Assays

O. fasciatus (Hemiptera: Lygaidae)

The *O. fasciatus* specimens used in this study were taken from a longstanding colony that has been reared and maintained in the Vector Insect Laboratory, Severino Sombra University, RJ. The food used was sunflower seeds; water was provided; and the insects were maintained in an incubator (BOD) at 24.5 ± 1°C and 68 ± 10% RH.[22]

Bioassays were performed in the Vector Insect Laboratory, Severino Sombra University. The treatment on *O. fasciatus* consisted of applications to groups of 20 fifth-stage nymphs in triplicate, with three replications. There were two controls: a control group without khellin or solvent and a testimony control, with the addition of chloroform and solvent dilution. The insects were previously deprived of water and food (sunflower

seeds) for 24 h before treatment. Khellin was dissolved with acetone and then diluted in 0.15 M NaCl (1:4), to concentrations of 0.5 to 300 mg/mL. The treatment consisted of applications of the substance to the abdomen of the insects (µL) at concentrations of 1, 10 and 100 µg/nymph. After the treatments, the treated insects and control groups were kept at 24.5 ± 1°C and 68 ± 10% relative humidity (RH) with a normal diet (sunflower seeds and water) and were observed for 30 days, regarding development and mortality.

A. aegypti (Diptera: Culicidae)

A. aegypti eggs were obtained from the Vector Support and Research Venter (Núcleo de Apoio e Pesquisa de Vetores, NapVE), Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro. The bioassays were performed in the Diptera Laboratory, Instituto Oswaldo Cruz, FIOCRUZ. All the experiments were carried out on third-instar (L3) larvae. The treatment on *A. aegypti* consisted of applications of the substance at final concentrations of 10 and 50 µg/mL, in glass containers (4.0 cm diameter ´ 4.5 cm high) containing dechlorinated water (10 mL).[23] After 1 hour of treatment, the medium was added to a diet of fish meal (Alcon Guppy) at 0.3 mg/larvae, for the mosquitoes to develop.[24] Ten third-stage (L3) larvae were used per group: test, control (without khellin or solvent), and testimony control (solvent containing the dilution of the substance). Larval viability, pupae, emergence, and mortality of immature stages were evaluated. In both treatments, the experiments were performed in triplicate with three replicates. After treatment, the insects were kept on a normal diet in climate-controlled chambers (BOD) at 27± 1°C and 70± 10% RH, with 12 h photoperiods, and were observed for 35 days with regards to development and mortality.

Statistical Analyses

The results were analyzed using the Tukey test with a significance level of 5%, analysis of variance (ANOVA)[25] and the χ^2 test with a significance level of $P \leq 0.01$. Standard deviations were calculated using the averages from the experiments. The statistical analysis for calculating LDs was the trimmed Spearman-Karber analysis (1978).[26]

RESULTS AND DISCUSSION

Topical treatment on *O. fasciatus* using khellin resulted in 40–50% ($P < 0.001$) nymph mortality at 10 and 100 µg/nymph, and 50–80% ($P < 0.001$) adult mortality (1, 10 and 100 µg/nymph) [Table 1A], as well as a 22% reduction in adult longevity (100 µg/nymph) [Table 1A]. The control groups did not show significant mortality, and the result was that 31–55% of adults lived for up to 35 days after the treatment [Table 1A]. The time of *O. fasciatus* molting was not significantly different in the khellin treatments. The insects treated with khellin presented egg-laying of 85 to 100 eggs ($P < 0.01$) at 10 and 100 µg/nymphs, while the controls presented 118 to 200 eggs. The treated group showed 90% to 100% inhibition of egg hatching from normal oviposition at concentrations of 10 and 100 µg/ nymphs, respectively [Table 1B].

A. aegypti larvae (L3) treated with khellin showed a shorter larval stage period (1–5 days *vs.* 3–9 and 4–7 days for the controls) ($P < 0.001$) and a shorter L3-adult stage period (2–10 days *vs.* 4–11 and 5–11 days for the controls), at 50 µg/mL [Table 2A]. These time intervals from larval stage to pupae stage did not show any significant differences in relation to the time intervals for the control groups [Table 2A]. Nevertheless, *A. aegypti* larvae (L3) treated with khellin solutions showed viability of 53% (50 µg/mL) ($P < 0.1$) and 70% (10 µg/mL) in the larval stage, but 100% emergence (pupae to adults) [Table 2B]. The khellin treatment showed 50% larval toxicity at 50 µg/mL ($P < 0.1$) [Table 2C] and LC50 of 50 µg/mL.

Table 1: Duration of development, moulting, mortality, longevity (A) and egg laying and viability (B) *O. fasciatus* with khellin furanocromone **topical treatment**

Treatment A	Period days		Ecdysis		Nymph mortality	Adults mortality	Adults longevity (35 days)
	X ± SD	VI	X ± SD	%	%	%	%
Control	10.3 ± 5.9 a	2-17	20 ± 1.0 a	100	0	20	55
acetone	2.6 ± 1.9 b***	1-7	13 ± 1.0 b	100	35	38	31
1µg	4.1 ± 2.4 b	3-10	16 ± 2,0 b	100	20	50***	30
10µg	5 ± 2.9 b	3-10	11 ± 1.0 b	92	40	55***	31
100 µg	5.1 ± 3.6 b	3-17	10 ± 0.5 b	100	50***	80***	9***

B	eggs/female	eggs X ± SD	Eggs viability (F2) %
Control	50	200 ± 2.0a	55
acetone	19.7	118 ± 12b**	66
1µg	50	150 ± 2.0b	43
10µg	17	85 ± 5.0c*	0
100 µg	25	100 ± 30b	10

Treatment with khellin furanocromone on *O. fasciatus* at 1, 10, 100 µg/nymph concentrations. Mean values and standard deviation (X ± SD), percentage (%) and range of variation (VI). Experiment conducted with 20 fifth instar nymphs of each group in triplicate and three repetitions. Values followed by the same letter (a=a, b=b, c=c) have no significant differences P> 0.05 for the Tukey test. Significance levels are represented by as *** P <0.001, ** P = <0.01, * P <0.1 vs. control acetone (testimony).

Table 2: Duration of development **(A),** viability **(B) and** mortality **(C)** *Aedes aegypti* larvae treated L3 in the middle of farming **with** khellin furanocromone

Treatment	Larval (days)		Pupal (days)		L3 – adult (days)	
A	X ± SD	VI	X ± SD	VI	X ± SD	VI
Control	5.9±1.3 a	(3-9)	2.3±0.9 a	(1-4)	9±1a	(4-11)
Acetone	5.4±0.8 a	(4-7)	2.6±0.9 a	(1-4)	9±1a	(5-11)
10µg/mL	4.3±2 c**	(1-8)	3±0.7 a	(2-4)	7±4c*	(3-11)
50µg/mL	2.7±1.6 b***	(1-5)	3±0.8 a	(1-4)	4.9±3.6b***	(2-10)

B	L3-L4		L4-Pupae		Pupae		L3 – adult	
	X ± SD	%	X ± SD	%	X ± SD	%	X ± SD	%
Control	10±0a	100	10±0a	100	10±0a	100	10±0a	100
Acetone	10±0a	100	10±0a	100	10±0a	100	10±0a	100
10µg/mL	10±0a	70	7±1.7a	100	7±1.7a	100	7±1.7a	70
50µg/mL	10±0a	53	5±3b*	93,7	5 ±2.6b*	100	5 ±2.6b*	50

C	L3			L4			Pupae
	X ± SD	VI	%	X ± SD	VI	%	
Control	0	0	0	0	0	0	0
Acetone	0	0	0	0	0	0	0
10µg/mL	3±1,7a	(3-3)	30	0	0	0	0
50µg/mL	4,6±3b*	(1-1)	46.6	0.3±0.5	(0-3)	3.3	0

Experiments with 10 larvae (L3) of *A. aegypti*, for each test group and control, in triplicate, with three repetitions (n = 30). Mean and standard deviation (X ±SD). Range of Variation (VI). Values followed by the same letter (a=a, b=b, c=c) have no significant differences. Levels of significance by the Tukey test, represented as *** P <0.001, ** P = <0.01, * P <0.1 vs. acetone control (testimony)

Mortality was not observed in the acetone control solution (without khellin) and untreated solution (control) [Table 2C].

Based on these findings, khellin appears to have potential as a substance acting against the milkweed bug and dengue vector. The present results showed 50% toxicity in relation to *O. fasciatus*, and this mortality rate was not correlated with any reduced in the molting cycle. However, the same substance has been found to cause 99.5% inhibition of larval growth on *Spodoptera littoralis*.[27] In relation to pests of stored products, coumarin has shown insecticide activity at LD50 of 2.72–39.71 mg/g.[28] The toxicity of khellin was demonstrated by the fact that only 10% of the milkweed bugs' eggs hatched. The toxic effect of khellin on the larvae of *A. aegypti* corroborates the toxic effect on *O. fasciatus* produced by treatment with coumarin. Coumarin (3, 6, 8-tribromo-7-hydroxy-4-methyl-chromen-2-one) has been found to be a potent larvicidal agent against L4 larvae of *A. aegypti* at LC50 = 2.23 ppm. It has been shown to produce 100% larval mortality at 25 ppm against these mosquitoes.[29] The same activity has been demonstrated by 4-hydroxycoumarin derivatives, with a strong larvicidal effect at LC50 values of 8.23 ppm against *A. aegypti*.[30]

CONCLUSION

Further experiments should be conducted in order to demonstrate the possible effects of khellin on the internal and external morphology of mosquitoes and phytophagous insects. Our findings are consonant with the hypothesis that this substance may be used for insect control, thereby reducing insect vector and pest populations.

ACKNOWLEDGEMENT

This research was supported by grants from Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), Fundação Nacional de Desenvolvimento do Ensino Superior Particular (FUNADESP) and Fundação para o Desenvolvimento Científico e Tecnológico (FIOTEC); with thanks to Dr. Nildimar Honorio Rocha (IOC/FIOCRUZ) for the eggs of *A. aegypti*. Financial support was also received from FCT-Portugal (CEQUIMED-PEst-OE/SAU/UI4040/2011) FEDER, POCI, PTDC/SAU-FCF/100930/2008-COMPETE, QREN, EU/FEDER, University of Porto.

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Cite this article as: Maleck M, Carvalho dos Santos FC, Serdeiro MT, Guimarães AÉ, Ferreira B, Gunaydin K, *et al*. Khellin: A furanochromone with toxicity against *Oncopeltus fasciatus* (Hemiptera) and *Aedes aegypti* (Diptera). J Nat Pharm 2013;4:32-6.

Source of Support: This research was supported by grants from Fundação de Amparo a Pesquisa do Estado do Rio de Janeiro (FAPERJ), Fundação Nacional de Desenvolvimento do Ensino Superior Particular (FUNADESP), Fundação para o Desenvolvimento Científico e Tecnológico (FIOTEC). and FCT-Portugal (CEQUIMED-PEst-OE/SAU/UI4040/2011) FEDER, POCI, PTDC/SAU-FCF/100930/2008-COMPETE, QREN, EU/FEDER, University of Porto. **Conflict of Interest:** None declared.