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Color Alteration and Acaricidal Activity of Juglone Isolated from *Caesalpinia sappan* Heartwoods Against *Dermatophagoides* spp.

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Abstract Acaricidal effects of materials derived from *Caesalpinia sappan* heartwoods against *Dermatophagoides farinae* and *D. pteronyssinus* were assessed and compared with those evidenced by commercial benzyl benzoate and DEET. The observed responses varied according to dosage and mite species. The LD_{50} values of the methanol extracts derived from *C. sappan* heartwoods were 6.13 and 5.44 μg/cm^3 against *D. farinae* and *D. pteronyssinus*, respectively. Furthermore, the ethyl acetate fraction derived from the methanol extract was approximately 8.71 more toxic than DEET against *D. farinae*, and 4.73 times more toxic against *D. pteronyssinus*. The biologically active constituent from the ethyl acetate fraction of *C. sappan* heartwood extract was purified via silica gel chromatography and high-performance liquid chromatography. The structure of the acaricidal component was analyzed by GC-MS, ^{1}H-NMR, ^{13}C-NMR, ^{1}H-^{13}C COSY-NMR, and DEPT-NMR spectroscopy, and identified as juglone (5-hydroxy-1,4-naphthoquinone). Based on the LD_{50} values of juglone and its derivatives, the most toxic compound against *D. farinae* was juglone (0.076 μg/cm^3), followed by benzyl benzoate (9.143 μg/cm^3) and 2-methyl-1,4-naphthoquinone (40.0 μg/cm^3). These results indicate that the acaricidal activity of *C. sappan* heartwoods is likely to be the result of the effects of juglone. Additionally, juglone treatment was shown to effect a change in the color of the cuticles of house dust mites, from colorless-transparent to dark brownish-black. Accordingly, as a naturally occurring acaricidal agent, *C. sappan* heartwood-derived juglone should prove to be quite useful as a potential control agent, lead compound, and house dust mite indicator.

Key words: *Caesalpinia sappan*, *Dermatophagoides farinae*, *Dermatophagoides pteronyssinus*, indicator, juglone

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Changes in living environment, including an increase in the number of apartment households with centrally installed heating, space heating, tighter windows, and fitted carpets, have resulted in conditions that are increasingly conducive to the growth of dust mites [20]. The most important pyroglyphid mites are *Dermatophagoides pteronyssinus* (Trouessart) and *D. farinae* (Hughes), for the three following reasons: [20] their cosmopolitan occurrence and abundance; [2] they are one of the primary sources of a variety of potent allergens; [26] and their causal association with sudden infant death syndrome [2, 3, 9, 20, 26]. In the pursuit of the development of diagnostics and a therapeutic vaccine, a variety of important dust mite allergens have been explored, and many of these have now been confidently classified as major house dust mite antigens [3, 9, 26]. Populations of these mites have been controlled principally through the use of chemicals such as benzyl benzoate and *N,N*-diethyl-*m*-toluamide [20]. Although effective, the repeated use of such chemicals has been implicated in the widespread development of resistance, and has also been demonstrated to exert undesirable effects on non-target organisms, and therefore resulting in the imposition of significant environmental and human health concerns [8, 20, 26]. Additionally, the allergens associated with house dust mites are caused not only by the dust mites themselves, but also by their excrement, and with eggs of the mites [6]. It has become clear, especially after the work of Tovey *et al.* [7] that not only mites, but also their deposited feces, constitute relevant sources of allergens. Thus, both the mites themselves, and their allergenic byproducts, must necessarily be removed from the environments of asthmatics who are sensitized to these allergens. Accordingly, it appears obvious that an agent that could both indicate and kill mites would have clear advantages over agents that simply kill mites. Thus, there is a clear need for the development of new strategies for the selective control of house dust mites.

With a few exceptions, plant natural products tend to be rather slow-acting and of modest toxicity, and also tend to rapidly degrade in natural environments [15]. Moreover, the use of such naturally derived products may, in many cases, prove more economical than commercially available synthetic chemicals, thereby permitting their use in lower-income areas of the world [11]. Plant extracts, or their constituents, may then provide an alternative to the mite-control agents currently employed against house dust mites [12, 17, 18, 24]. As many of these compounds exert no adverse effects and evidence excellent biological activity, an approach based on plant natural products might result in the development of new classes of mite-control agents, which would be safer than the chemical products in current use [12, 16–18, 24]. The heartwood of *Caesalpinia sappan* has long been used in Oriental folk medicine to promote blood circulation, and is believed to exert a host of pharmacological effects, including analgesic, anti-inflammatory [14], antifungal [19], and antimicrobial effects [13]. However, relatively little work has been conducted regarding the acaricidal effects of *C. sappan* heartwoods against house dust mites, despite its many imputed pharmacological effects [13, 14, 19]. Therefore, in this study, we have evaluated the color-altering and acaricidal effects of the active component isolated from *C. sappan* heartwoods against house dust mites.

MATERIALS AND METHODS

Chemicals

Benzyl benzoate and *N,N*-diethyl-*m*-toluamide (DEET) were purchased from Aldrich (Milwaukee, WI, U.S.A.), and 2-hydroxy-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone were purchased from Fluka Chemical Corp. (Milwaukee, WI, U.S.A.). All other chemicals were of reagent grade. The methanol and water used for the HPLC mobile phase were of HPLC grade, and those used for other purposes were of analytical grade.

Isolation and Identification

The *C. sappan* heartwoods (7 kg), which belong to the family Leguminosae, were purchased from a local market in Chonju, Korea. The heartwood samples were ground in a blender, extracted twice with methanol (35 l) at room temperature for 2 days, and filtered (Toyo filter paper No. 2, Toyo Roshi, Japan). The combined filtrates were then concentrated *in vacuo* at 45°C, at a yield of 7.6% (532 g). The extract (532 g) was then sequentially partitioned into hexane (6.5 g), chloroform (25.2 g), ethyl acetate (402.1 g), butanol (52.7 g), and water-soluble (45.5 g) portions, for the subsequent bioassay. The organic solvent portions were concentrated to dryness with a rotary vacuum evaporator (EYELA Autojack NAJ-100, Tokyo, Japan) at 45°C, and the water portion was freeze-dried.

The ethyl acetate (402 g) portion was sequentially chromatographed on a silica gel column (Merck 70–230 mash, 700 g, 13.5×60 cm), and eluted successively with a stepwise gradient of ethyl acetate/methanol (0, 20, 50, and 100%) giving 10 fractions (E1-E5). The active E3 fraction (69.5 g) of the five fractions eluted from the ethyl acetate fraction evidenced a strong activity against *D. farinae* and *D. pteronyssinus* at a concentration of 80 μg/cm^3, as described below. The active E3 fraction was chromatographed on a silica gel column and eluted with ethyl acetate/methanol (3:1, v/v). The column fractions were analyzed by TLC (silica gel 60 F_{254}, ethyl acetate/methanol, 3:1, v/v), and the fractions evidencing similar TLC patterns were combined. The bioactive fraction (E31, 9.1 g) was rechromatographed on a silica gel column, and then eluted successively with ethyl acetate/methanol (2:1, v/v). For further separation of the biologically active E313 fraction (3.15 g), a Japanese Analytical Industry Recycling Preparative HPLC system (LC-908W-C60) was used, and the activities of the eluates were assessed. JAI GEL GS-310 and GS-220 columns, using water, were employed for HPLC, at a flow rate of 4.0 ml/min. Detection was conducted at a wavelength of 240 nm. This operation was consecutively repeated twice. Finally, a potent active principle (E3133, 439 mg) was isolated. The structural determination of this active isolate was predicated on the results of spectroscopic analysis. The ^{1}H and ^{13}C NMR spectra were recorded in methanol, with a Bruker AM-500 spectrometer (Rheinstetten, Germany) at 400 and 100 MHz, respectively. Ultraviolet spectra were obtained in methanol, using a Waters 490 spectrometer (Boston, MA, U.S.A.), and the mass spectra were obtained using a JEOL JMS-AX 302 spectrometer (Tokyo, Japan).

Dust Mites and Observation

Cultures of *D. farinae* and *D. pteronyssinus* were maintained for 5 years without exposure to any known acaricide. They were reared in plastic containers (15 cm×12 cm×6 cm) each containing 30 g of sterilized diet (fry feed No. 1/dried yeast, 1:1 by weight) at 25±1°C and 75% relative humidity in the dark. The fry feed (Miropa) was purchased from Korea Special Feed Meal Co. Ltd., Chonju, South Korea. The mites were observed with a video microscope (pictured by ICS-305B video microscope system (200×) Sometch, Seoul, Korea), and assessed for the following parameters: walking/active, slow movement, no movement/dead, and discoloration.

Bioassay

An impregnated fabric disk bioassay was employed in the determination of the acaricidal activities of the test samples. Differing quantities (80, 60, 40, 20, 10, 5, 2.0, 1.0, 0.5, 0.2, 0.1, 0.05, and 0.025 μg/cm^3) of each test material were dissolved in 100 μl of acetone, and then applied to disks constructed of black cotton fabric (0.5 g, 5 cm diameter:

700 mesh). The control fabric disks received only 20 μl of acetone. After the disks were dried under a fume hood (19°C) for 30 sec, each of the pieces was placed in the bottom of a Petri dish (5 cm diameter×1.2 cm). Then, 30 individuals of *D. farinae* (7–10 days old) and *D. pteronyssinus* (7–10 days old) were placed in each of the Petri dishes, and covered with a lid. The treated and control mites were maintained at 25±1°C and 75% relative humidity, in darkness. Mortality rates were determined 24 h after treatment, under a binocular microscope (20×). The mites were considered to be dead if the appendages did not move when prodded with a pin. All treatments were replicated three times. The LD_{50} values were calculated by probit analysis [4]. The percentage of mortality was determined and transformed into arcsine square-root values for analysis of variance (ANOVA). The treatment means were compared and separated by Scheffe's test, at a *P*-value of 0.05 (SAS Institute) [22].

RESULTS AND DISCUSSION

Acaricidal activities of various fractions obtained from the methanol fraction of *C. sappan* heartwoods against *D. farinae* and *D. pteronyssinus* were examined and compared with that of the commonly used compound, DEET, which was employed as a positive control for acaricidal activity (Table 1). When the five fractions obtained from the methanol extract of the *C. sappan* heartwoods were assayed according to the impregnated fabric disk method, the five fractions evidenced clear dose-response relationships for both species. The LD_{50} values of the hexane and ethyl acetate fractions obtained from the methanol extract of the *C. sappan* heartwoods were 25.64 and 4.23 μg/cm^3 against *D. farinae*, and 25.64 and 3.76 μg/cm^3 against *D. pteronyssinus*, respectively. In particular, the ethyl acetate fraction was approximately 8.71 times more toxic than DEET against *D. farinae*, and 4.73 times more toxic than DEET against *D. pteronyssinus*. However, no activity was observed on conjunction with the chloroform, butanol, or water fractions at 80 μg/cm^3. No mortality was observed in the untreated controls. This study is, to our knowledge, the first to describe the acaricidal activities of *C. sappan* heartwood-derived materials against *D. farinae* and *D. pteronyssinus*. Very little work has been conducted with regard to the management of arthropod pests in general, and this is certainly true of the house dust mite.

In this study, the ethyl acetate fraction obtained from the methanol extract of the *C. sappan* heartwoods exhibited relatively profound activity, and thus the isolation of the active component was pursued. The biologically active constituent from the ethyl acetate fraction evidencing the highest yield (73%) was purified by silica gel chromatography and HPLC. The isolation procedure used to purify the acaricidal constituent from *C. sappan* heartwoods is shown in Fig. 1. The bioassay-guided fractionation of the methanol extract ultimately yielded the active constituent, which was subsequently identified by spectroscopic analyses, including HPLC, GC-MS, and ^{13}C and ^{1}H NMR by direct comparison with an authentic reference compound. Furthermore, coupled with the results of ^{1}H-^{13}C COSY-NMR and DEPT-NMR spectral analyses, the molecular formula of the biologically active compound was found to be one of the compound

Table 1. Acaricidal activities of various fractions obtained from the methanol extract of *C. sappan* heartwoods and synthetic acaricide against *D. farinae* and *D. pteronyssinus*, using the impregnated fabric disk bioassay method.

Fraction[a]	Mite species	LD_{50} (μg/cm^3)	RT[b]
Methanol fraction	*D. farinae*	6.13	6.09
	D. pteronyssinus	5.44	3.29
Hexane fraction	*D. farinae*	25.64	1.44
	D. pteronyssinus	19.81	0.90
Chloroform fraction	*D. farinae*	-	-
	D. pteronyssinus	-	-
Ethyl acetate fraction	*D. farinae*	4.23	8.71
	D. pteronyssinus	3.76	4.73
Butanol fraction	*D. farinae*	-	-
	D. pteronyssinus	-	-
Water fraction	*D. farinae*	-	-
	D. pteronyssinus	-	-
DEET	*D. farinae*	36.84	1.0
	D. pteronyssinus	17.79	1.0

[a]Exposed for 24 h. [b]Relative toxicity=LD_{50} value of benzyl benzoate/LD_{50} value of each chemical.

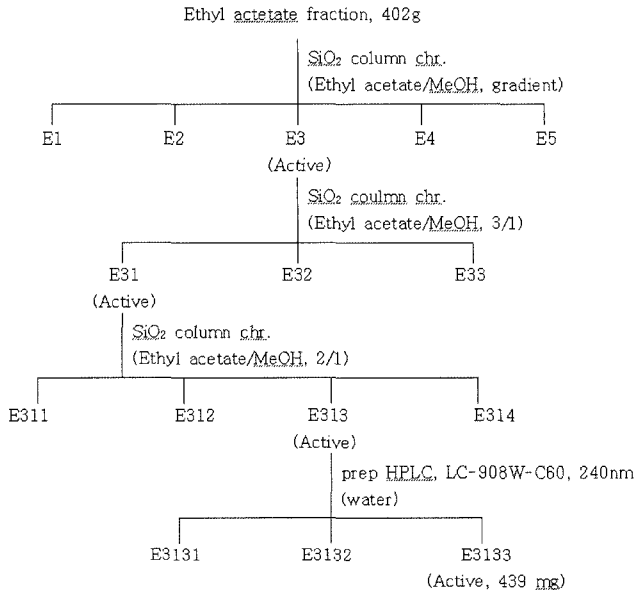

Fig. 1. Isolation procedure of the acaricidal constituent from *Caesalpinia sappan* heartwoods.

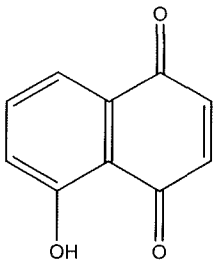

Fig. 2. Structure of juglone.

naphthoquinone derivatives isolated from the leaves of *Juglans regia* L. [1, 5]. The active constituent was identified as 5-hydroxy-1,4-naphthoquinone (juglone) (Fig. 2). This compound was identified on the basis of the following evidence: 5-hydroxy-1,4-naphthoquinone ($C_{10}H_6O_3$, MW, 174.15); EI-MS (70 eV) *m/z* (% relative intensity): M^+ 174, 146, 118, 92, 74, 63, 53; ^{1}H-NMR (CD_3OD, 400 MHz, δ ppm); 7.614–7.638 (2H, 3H), 7.266–7.284 (4H), 6.953 (8H, 9H), 11.834 (OH); ^{13}C-NMR (CD_3OD, 100 MHz, δ ppm); 190.059, 184.037, 161.235, 139.424, 138.492, 136.421, 131.595, 124.360, 119.023, 114.824. The spectroscopic data of 5-hydroxy-1,4-naphthoquinone was found to be the same as those for juglone isolated from walnut leaves [5].

Naphthoquinone compounds exhibit a variety of biological activities [20]. A number of 1,4-naphthoquinones, most notably juglone, display potent biological properties, including antimalarial activity, as well as antibacterial, antifungal, antiallergic, antiplatelet, antifeedant, and cytotoxic properties, along with antitumor effects [10, 23, 24]. However, juglone and its derivatives have not been associated previously with acaricidal effects. In this regard, the acaricidal effects against *D. farinae* and *D. pteronyssinus* exerted by juglone and its derivatives were compared with those of benzyl benzoate (Table 2). The observed responses varied in accordance with both compound and dosage. On the basis of the LD_{50} values, juglone was determined to be the compound that exhibited the most profound effects against *D. farinae* (0.076 μg/cm^3), followed by benzyl benzoate (9.14 μg/cm^3) and 2-methyl-1,4-naphthoquinone (40.06 μg/cm^3). Against *D. pteronyssinus*, juglone (0.062 μg/cm^3) evidenced the highest degree of toxicity, followed by benzyl benzoate (7.37 μg/cm^3) and 2-methyl-1,4-naphthoquinone (19.82 μg/cm^3). However, 2-hydroxy-1,4-naphthoquinone exhibited no detectable activity at 80 μg/cm^3. These results show that the acaricidal activity of the methanol extracts from the *C. sappan* heartwoods can be attributed principally to juglone, as the above compound was approximately 120 and 118 times more toxic to *D. farinae* and *D. pteronyssinus*, respectively, than benzyl benzoate.

Alterations in the color of house dust mites between the untreated group (A) and the groups treated (B) with each of the tested compounds were microscopically visualized and recorded (at 200× magnification) (Fig. 3). The untreated house dust mites were colorless and transparent. After dust mites were treated with juglone, however, the dust mites exhibited whole-body cuticle discoloration. This juglone-induced color alteration in the dust mites rendered the dust mites readily visible to the naked eye. In the dust mites treated with the other derivatives or with benzyl benzoate, however, the cuticles of the dust mites appeared identical to the untreated mites.

The allergens associated with house dust mites are caused not only by the dust mites themselves, but also by their excrement and eggs [4, 22]. Thus, treatment with common acaricides frequently results in a vicious circle, as the mere death of the mites does not render them particularly less allergenic, and it remains impossible to remove the attendant allergens from the house. These problems have underlined the need for the development of new strategies for the control of dust mite allergens. In this regard, our search for an agent that would serve to indicate dust mites is unique, in that our juglone-treatment technique also effects an alteration in the color of the dust mites, rendering them visible. Thus, the results of this study may force a new perspective on the roles of acaricides, as juglone was determined to both indicate the presence of dust mites and exert an acaricidal effect. Therefore, we are able to conclude that juglone harbors the ability to discolor the cuticles of dust mites, and also possesses excellent acaricidal properties.

Table 2. Acaricidal activities of juglone, its derivatives, and synthetic acaricide against *D. farinae* and *D. pteronyssinus*, using the impregnated fabric disk bioassay method.

Compound[a]	Mite species	LD_{50} (μg/cm^3)	95% Confidence limit	RT[b]
Juglone (5-hydroxy-1,4-naphthoquinone)	*D. farinae*	0.076	0.072–0.082	120.3
	D. pteronyssinus	0.062	0.058–0.065	118.9
2-Hydroxy-1,4-naphthoquinone	*D. farinae*	-	-	-
	D. pteronyssinus	-	-	-
2-Methyl-1,4-naphthoquinone	*D. farinae*	40.06	37.42–43.91	0.23
	D. pteronyssinus	19.82	17.21–21.22	0.37
Benzyl benzoate	*D. farinae*	9.14	8.79–9.31	1.0
	D. pteronyssinus	7.37	7.11–7.54	1.0

[a]Exposed for 24 h. [b]Relative toxicity=LD_{50} value of benzyl benzoate/LD_{50} value of each chemical.

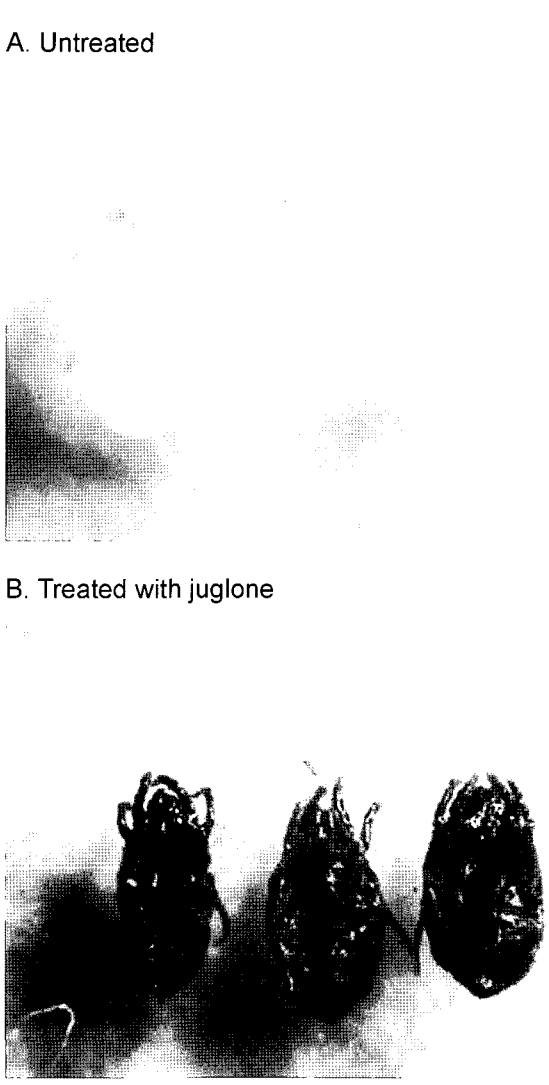

Fig. 3. Color alteration of dust mites by juglone.

In this study, we determined that the methanol extracts derived from *C. sappan* heartwoods exerted color-altering and acaricidal effects against the house dust mite species, *D. farinae* and *D. pteronyssinus*. Juglone, in particular, was found to evidence the highest degree of acaricidal activity among the constituents of these extracts, with an LD_{50} value of $<<0.05$ μg/cm^3. However, no similar results were associated with the juglone derivatives, 2-hydroxy-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone. According to these results, juglone appears to be the most promising of the compounds tested in this study for possible use against *D. farinae* and *D. pteronyssinus,* owing primarily to the low doses required to generate high mortality in a household environment. Interestingly, we noted that juglone treatment also resulted in a color change in the cuticles of dust mites, from colorless-transparent to dark brownish-black. This finding indicates that juglone may prove more useful than other compounds in regard to the actual removal of dust mite-associated allergens; thus, juglone appears to be a generally excellent candidate mite-control agent, lead compound, and house dust mite indicator. Its use in these pursuits should ultimately constitute a preventive agent against a large proportion of allergic diseases. The data reported in this study, however, are only the first step in our attempts to unravel the complex mechanisms inherent to the cuticle color-altering properties of this compound. Further research should be conducted regarding the safety issues of these compounds with regard to human health, as well as the mechanisms underlying the discoloration effect, and the development of formulations with improved acaricidal potency and stability.

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