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Acaricidal Activity and Function of Mite Indicator Using Plumbagin and Its Derivatives Isolated from *Diospyros kaki* Thunb. Roots (Ebenaceae)

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Acaricidal effects of materials derived from *Diospyros kaki* roots against *Dermatophagoides farinae* and *D. pteronyssinus* were assessed using impregnated fabric disk bioassay and compared with that of the commercial benzyl benzoate. The observed responses varied according to dosage and mite species. The LD_{50} values of the chloroform extract of *Diospyros kaki* roots were 1.66 and 0.96 μg/cm^2 against *D. farinae* and *D. pteronyssinus*. The chloroform extract of *Diospyros kaki* roots was approximately 15.2 more toxic than benzyl benzoate against *D. farinae*, and 7.6 times more toxic against *D. pteronyssinus*. Purification of the biologically active constituent from *D. kaki* roots was done by using 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 spectra, and identified as plumbagin. The acaricidal activity of plumbagin and its derivatives (naphthazarin, dichlon, 2,3-dibromo-1,4-naphthoquinone, and 2-bromo-1,4-naphthoquinone) was examined. On the basis of LD_{50} values, the most toxic compound against *D. farinae* was naphthazarin (0.011 μg/cm^2) followed by plumbagin (0.019 μg/cm^2), 2-bromo-1,4-naphthoquinone (0.079 μg/cm^2), dichlon (0.422 μg/cm^2), and benzyl benzoate (9.14 μg/cm^2). Additionally, the skin color of the dust mites was changed from colorless-transparent to dark brown-black by the treatment of plumbagin. Similar results have been exhibited in its derivatives (naphthazarin, dichlon, and 2-bromo-1,4-naphthoquinone). In contrast, little or no discoloration was observed for benzyl benzoate. From this point of view, plumbagin and its derivatives can be very useful for the potential control agents, lead compounds, and indicator of house dust mites.

Keywords: Acaricide, *Diospyros kaki* Thunb., plumbagin, discoloration, mite indicator

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Changes in living environment such as a rise in the number of apartment households with centrally installed heating, space heating, tighter windows, and fitted carpets have improved conditions for the growth of house dust mites [25]. The most important pyroglyphid mites are *Dermatophagoides pteronyssinus* (Trouessart) and *Dermatophagoides farinae* (Hughes), for the following three reasons: their cosmopolitan occurrence and abundance; they are a major source of multiple potent allergens; and their causal association with sudden infant death syndrome [10, 25]. Toward the development of diagnostics and a therapeutic vaccine, important dust mite allergens have been explored and now classified as major house dust mite antigens [10]. Control of these mite populations has been principally through the use of chemicals such as benzyl benzoate and *N,N*-diethyl-*m*-toluamide [25]. Although effective, their repeated use has sometimes resulted in the widespread development of resistance, has undesirable effects on non-target organisms, and has fostered environmental and human health concerns [19, 25]. Additionally, the allergens of house dust mites are not only caused by the dust mite themselves but also by their excrements, involving the mite eggs [11]. It is now clear from the work of Helson [10] that not only mites, but also their deposited feces are important sources of allergens and thus, for effective treatment, must be removed from the environment of asthmatics who are sensitized to these allergens [9]. Accordingly, it is obvious that the agent that could both kill mites and act as a mite indicator would have advantages over agents that only kill mites. For these reason, we have highlighted the need for the development of new strategies for selective control of house dust mites.

Plant natural products tend to be rather slow-acting, of modest toxicity, and rapidly degraded in the environment. Moreover, their uses may be more economical than the commercially available synthetic chemicals for use in low-income areas of the world [12]. Therefore, plant extracts or their constituents may provide an alternative to currently used mite-control agents against dust mites [3, 20]. Since

many of them are largely free from adverse effects and have excellent biological actions [1, 4, 5], they could lead to the development of new classes of possibly safer mite-control agents [14, 21]. *Diospyros kaki* is the species of persimmon tree that grows in Korea [13, 22]. Persimmon contains many medicinally bioactive compounds, such as carotenoids, tannins, flavonoids, terpenoids, steroids, naphthoquinones, sugars, amino acids, minerals, and lipids [24]. Chemical studies on a number of species have revealed that the stems and leaves of this genus have been reported to contain triterpenoids [2], whereas the roots are well known to contain naphthols and naphthoquinones [7, 32]. Although several reports have indicated that *Diospyros* species contain naphthoquinones as antibacterial, antifungal, cytotoxical, antifeedant, insecticidal, antimalarial, anti-inflammatory, and antitumor activities [7, 8, 15, 27, 30, 32], the acaricidal activity of naphthoquinones derived from the *D. kaki* roots has not been investigated. Furthermore, house dust mites are not possible to see easily, because they are very small and transparent. Accordingly, we are making every effort to develop a mite indicator that can easily distinguish the dust mites with the naked eyes. From this point of view, we evaluated the function of a mite indicator and its acaricidal effect using the active component isolated from *D. kaki* roots against the house dust mite.

MATERIALS AND METHODS

Chemicals and Plant Materials

Benzyl benzoate was purchased from Aldrich (Milwaukee, WI, U.S.A.). Naphthazarin (5,8-dihydroxy-1,4-naphthoquinone), dichlon (2,3-dichloro-1,4-naphthoquinone), 2-bromo-1,4-naphthoquinone, and 2,3-dibromo-1,4-naphthoquinone were supplied by Sigma (St. Louis, MO, 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. The roots of *D. kaki* Thunb. were collected during Spring 2005 in Chonbuk Province, Korea [17].

Isolation and Identification

The samples (5 kg) were homogenized with a grinder after washing and then extracted three times with chloroform (3×10 l) at room temperature for 2 days. The extract was applied to filtration through filter paper (Toyo filter paper No. 2; Toyo Roshi, Tokyo, Japan) *in vacuo*. The combined filtrate was then concentrated *in vacuo* at 45°C using a rotary vacuum evaporator (Model N-3NW; EYELA, Japan). The chloroform extract of *D. kaki* roots showed a strong acaricidal activity against *D. farinae* and *D. pteronyssinus* (Table 1). To purify the active compound of the chloroform extract derived from *D. kaki* roots, the extract (30 g) was chromatographed on a silica gel column (Merck 70-230 mash, 600 g, 5.5 i.d.×68 cm) and successively eluted with a gradient of hexane:chloroform (30:70 to 0:100, v/v), giving the six fractions (C1–C6). The active C1 (5.5 g) fraction was rechromatographed on a silica gel column and successively eluted with hexane:chloroform (30:70, v/v). The column fractions were analyzed by TLC and fractions with similar TLC patterns were pooled. In this step, six fractions (C11–C16) were obtained and bioassayed at 80 μg/cm^2. The active C11 fraction (3 g) was purified by Prep. HPLC (Recycling Preparative HPLC; Japan Analytical Industry Co., Ltd, Japan) for separation of the biologically active constituent. The first column was Jaigel GS Series Column (GS310 50 cm+GS310 30 cm×2, 21.5 mm i.d.× 500 mm L; Japan Analytical Industry Co., Ltd, Japan), using hexane:chloroform:isopropanol (30:70:2) at a flow rate of 5 ml/min and detection at 268 nm. In this step, five fractions (C111–C115) were obtained and bioassayed at 80 μg/cm^2. The active C114 fraction (2 g) was further chromatographed on a Jaigel W Series Column (W-253 50 cm+W-252 50 cm, 20.0 mm i.d.×500 mm L; Japan Analytical Industry Co., Ltd, Japan) under the same condition. Finally, the potent active principle (C1144, 800 mg) was isolated. The structure of the active isolate was determined by instrumental analysis. ^{1}H and ^{13}C NMR spectra were recorded in deuteriochloroform with a JNM-LA 400F7 spectrometer, at 400 and 100 MHz (TMS as an internal standard), respectively, and chemical shifts are given in δ (parts per million). Unambiguous ^{1}H and ^{13}C NMR chemical shifts were obtained using a ^{1}H-^{13}H DEPT spectrum as well as a ^{1}H-^{13}H COSY spectrum. UV spectra were obtained in chloroform with a Jasco V-550 spectrometer.

Gas Chromatography-Mass Spectrometry (GC-MS)

The chloroform extract of *D. kaki* roots was analyzed on a gas chromatograph (6890, Agilent)-mass spectrometer (5973IV, Agilent) (GC-MS). The GC column was a 30 m×0.25 mm i.d. DB-5 (0.25 mm film) fused silica capillary column (J&W Scientific, Folsom, CA, U.S.A.). The GC conditions were as follows: injector temperature, 260°C; column temperature, isothermal at 70°C for 3 min, then programmed to rise to 300°C at 10°C/min and be held at this temperature for 5 min; ion source temperature, 230°C.

Table 1. Acaricidal activity of the chloroform extract derived from *D. kaki* roots and synthetic acaricide against *D. farinae* and *D. pteronyssinus*, using the impregnated fabric disk bioassay method.[a]

Plant extracts	Mite species	LD_{50} (μg/cm^2)	95% Confidence limit	RT[b]
Chloroform extract	*D. farinae*	1.66	1.24–1.92	15.2
	D. pteronyssinus	0.96	0.57–1.26	7.6
Benzyl benzoate	*D. farinae*	9.14	9.13–9.14	1.0
	D. pteronyssinus	7.35	7.35–7.36	1.0

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

Helium was used as the carrier gas at the rate of 0.8 ml/min. The effluent of the GC column was introduced directly into the source of the MS. Spectra were obtained in the EI mode with 70 eV ionization energy. The sector mass analyzer was set to scan from 50 to 600 amu for 2 s. Compounds were identified by comparison with retention times, and the mass spectra were obtained with the authentic standards on the GC-MS system used for analysis. When an authentic sample was not available, the identification was carried out by comparison of mass spectra obtained experimentally with those in the mass spectra library (*The Wiley Registry of Mass Spectral Data*, 6th Ed.).

Dust Mites and Observation

Cultures of *D. farinae* and *D. pteronyssinus* were maintained without exposure to any known acaricide. They were reared in plastic containers (15 cm×12 cm×6 cm) 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. Mites were observed with a video microscope (pictured by ICS-305B video microscope system (200×) Sometch, Seoul, Korea) for the following parameters: walking/active, slow movement, no movement/dead, and discoloration.

Bioassay

An impregnated fabric disk bioassay was used for the acaricidal activity of test samples. Various amounts (80, 60, 40, 20, 10, 5, 2.0, 1.0, 0.5, 0.2, 0.1, 0.05, 0.025, 0.01, and 0.005 μg/cm^2) of each test material dissolved in 100 μl of acetone were applied to disks of black cotton fabric (0.5 g, 5 cm diameter: 700 mesh). Control fabric disks received only 20 μl of acetone. After the disks were dried in a fume hood (19°C) for 30 s, each piece 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 Petri dish and covered with a lid. Treated and control mites were held at 25±1°C and 75% relative humidity in the dark. Mortalities were determined 24 h after treatment under a binocular microscope (20×). Mites were considered to be dead if appendages did not move when prodded with a pin. All treatments were replicated three times. LD_{50} values were calculated by probit analysis [28]. The percentage mortality was determined and transformed to arcsine square-root values for analysis of variance (ANOVA). Treatment means were compared and separated by Scheffe's test at P=0.05 (SAS Institute) [28].

Results and Discussion

Acaricidal effects of materials derived from *Diospyros kaki* roots against *Dermatophagoides farinae* and *D. pteronyssinus* were examined using impregnated fabric disk bioassay and compared with that of the commonly used benzyl benzoate, which served as the positive control for the acaricidal activity (Table 1). The observed responses varied according to dosage and mite species. The LD_{50} values of the chloroform extract of *Diospyros kaki* roots were 1.66 and 0.96 μg/cm^2 against *D. farinae* and *D. pteronyssinus*. Furthermore, the chloroform extract of *Diospyros kaki* roots was approximately 15.2 more toxic than benzyl benzoate (as positive control) against *D. farinae*, and 7.6 times more toxic against *D. pteronyssinus*. There was no mortality in the untreated controls. This study is the first to report the acaricidal function of a *D. kaki* root-derived extract against *D. farinae* and *D. pteronyssinus*. Very little work has been done with respect to managing arthropod pests including house dust mite. Owing to the potent activity of the chloroform extract derived from *D. kaki* roots, the isolation of the active component was pursued. The isolation procedure used to purify the acaricidal constituent from the chloroform extract of *D. kaki* roots is shown in Fig. 1. Bioassay-guided fractionation of the chloroform extract afforded an active constituent identified by spectroscopic analyses, including HPLC, GC-MS, EI-MS, ^{13}C and ^{1}H NMR, by the direct comparison with an authentic reference compound. Furthermore, in conjugation with the analysis of the ^{1}H-^{13}C COSY NMR and DEPT NMR spectra, the molecular formula of this biologically active compound was deduced to be one of the compound naphthoquinone derivatives isolated from the roots of certain species of *Plumbago* plants [6]. In this regard, the biologically active constituent was characterized as plumbagin. This compound was identified on the basis of the following evidence. Plumbagin as a yellow orange microcrystalline solid ($C_{11}H_8O_3$, MW: 188.18); EI-MS (70 eV) *m/z* (%

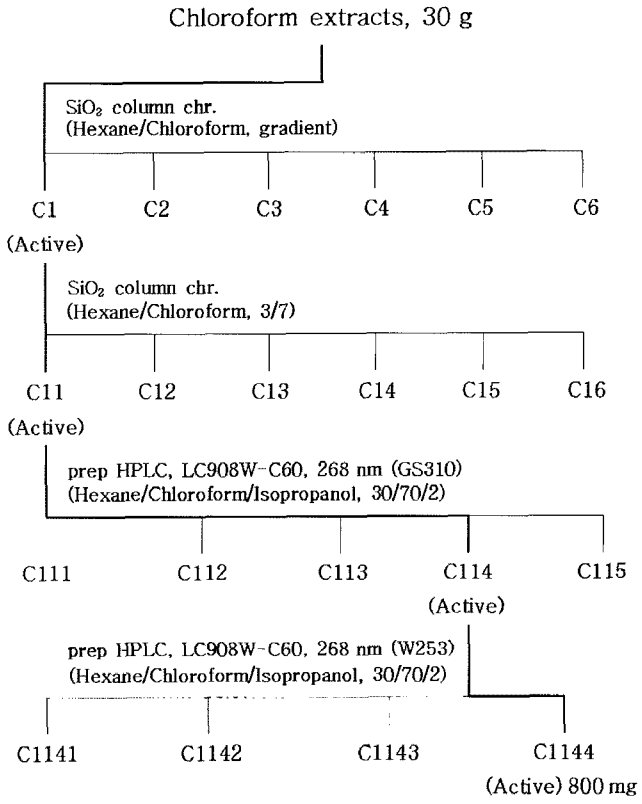

Fig. 1. Isolation procedure for the acaricidal constituent of the chloroform extract from *Diospyros kaki* roots.

Table 2. Volatile compounds derived from the chloroform extract of *D. kaki* roots, identified by GC-MS mass spectrometry.

Peak number	Compound	Mass spectral data[a]	Retention time (min)	Relative (%)
1	Plumbagin	63, 92, 120, 131, 160, 173, 188	13.66	31.2
2	2-Thiazolamine	51, 78, 106, 134, 176	13.72	26.7
3	1,2-Benzenediol	53, 69, 95, 134, 151, 166	14.17	1.9
4	Isoquinoline	51, 77, 106, 134, 162, 175, 190	14.5	12.6
5	Phenol	77, 106, 134, 173, 193, 205, 220	15.66	8.6

[a]Major fragmentation ions, base peak (listed first) and other ions in decreasing order of relative abundance.

relative intensity) M^+ 188 (100, base peak), 173 (18), 160 (19), 131 (16), 120 (12), 92 (9), 63 (4); ^{1}H NMR (CD_3OD, 400 MHz); δ 7.570–7.588 (1H, *d*), 7.224–7.248 (1H, *d*), 7.608–7.623 (1H, *d*), 6.793–6.797 (1H, *d*), 2.186–2.190 (3H, *d*); ^{13}C NMR (CD_3OD, 100 MHz); δ 190.03, 184.54, 160.96, 149.44, 135.94, 135.28, 131.89, 124.02, 119.16, 114.98, 16.54.

The substances identified by GC-MS in the chloroform extract of *D. kaki* roots are presented in Table 2. Analysis led to identification of 5 components from the chloroform extract of *D. kaki* roots. The main constituents were plumbagin (relative percent=31.2%), 2-thiazolamine (26.7%), 1,2-benzenediol (1.9%), isoquinoline (12.6%), and phenol (8.6%). Together, plumbagin, 2-thiazolamine, and isoquinoline made up 70.5% of the chloroform extract of *D. kaki* roots. This study is the first to report the data of *D. kaki* root-derived constituents. In previous studies, Lajubutu *et al.* [16] and Mallavadhani *et al.* [23] reported that the main constituents of *Diospyros* species were α-amyrin, 2-methyl-anthraquinone, astragalin, betulinic acid, chitranone, diosindigo, diospyrin, diosquinone, isodiospyrin, microphyllone, naphthol, plumbagin, β-sitosterol, scopoletol, and trihydroxy-triterpenoid acid. These results indicate that the differential constituents are influenced by extrinsic and intrinsic factors, such as the extracted methods, the plant species, parts of the plant, and the geographical location of where the plants were grown [31].

The quinonoid structure is associated with a wide range of biological activities and variously substituted naphthoquinones [33]. The naphthoquinone plumbagin pharmacophore is known to impart pronounced biological effects in its derivatives (naphthazarin, dichlon, 2-bromo-1,4-naphthoquinone, and 2,3-dibromo-1,4-naphthoquinone), leading to antitumor, antiproliferative, antimycobacterial, antiplatelet, anti-imflammatory, antiallergic, antimalarial, antifungal, antiviral, and antileishmanial activities [30]. However, the acaricidal activity has not been previously reported for plumbagin and its derivatives. In this regard, the acaricidal activities of plumbagin and its derivatives were compared with that of benzyl benzoate against *D. farinae* and *D. pteronyssinus* (Table 3, Fig. 2). On the basis of LD_{50} values, the most toxic compound against *D. farinae* was naphthazarin (0.011 μg/cm^2) followed by plumbagin (0.019 μg/cm^2), 2-bromo-1,4-naphthoquinone (0.077 μg/cm^2), dichlon (0.422 μg/cm^2), and benzyl benzoate (9.143 μg/cm^2). Against *D. pteronyssinus*, naphthazarin (0.015 μg/cm^2) showed the most toxic effect followed by plumbagin (0.021 μg/cm^2), 2-bromo-1,4-naphthoquinone (0.072 μg/cm^2), dichlon (0.415 μg/cm^2), and benzyl benzoate (7.358 μg/cm^2). However, no activity was observed for

Table 3. Acaricidal activities of plumbagin, its derivatives, and synthetic acaricide against *D. farinae* and *D. pteronyssinus*, using the impregnated fabric disk bioassay method.[a]

Compound	Mite species	LD_{50} (μg/cm^2)	95% Confidence limit	RT[b]
Plumbagin	*D. farinae*	0.019	0.015–0.021	481
	D. pteronyssinus	0.021	0.017–0.024	350
Naphthazarin	*D. farinae*	0.011	0.008–0.015	831
	D. pteronyssinus	0.015	0.012–0.017	491
Dichlon	*D. farinae*	0.422	0.419–0.0425	21.7
	D. pteronyssinus	0.415	0.0410–0.419	17.7
2-Bromo-1,4-naphthoquinone	*D. farinae*	0.077	0.074–0.081	118.7
	D. pteronyssinus	0.072	0.068–0.076	102.2
2,3-Dibromo-1,4-naphthoquinone	*D. farinae*	-	-	-
	D. pteronyssinus	-	-	-
Benzyl benzoate	*D. farinae*	9.143	9.139–9.148	1.0
	D. pteronyssinus	7.358	7.352–7.363	1.0

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

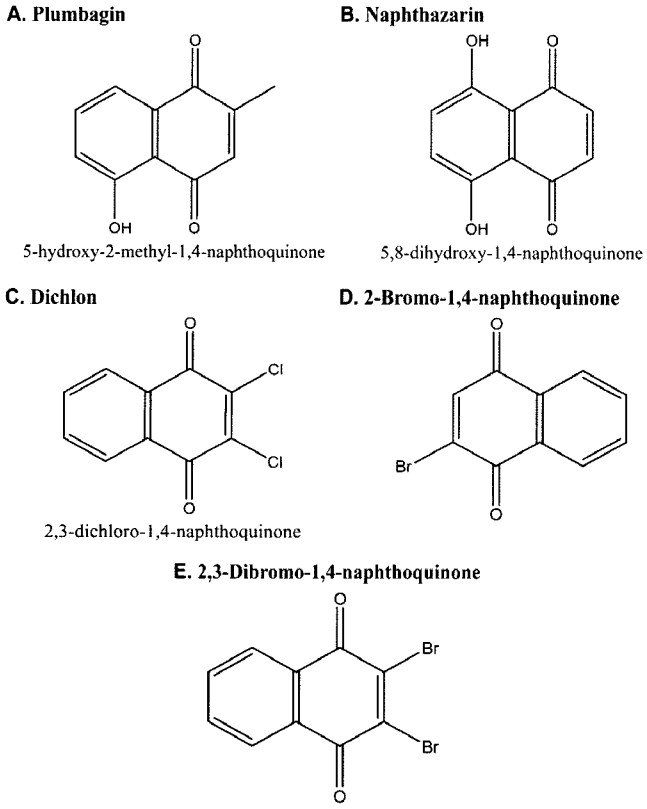

Fig. 2. Structure of plumbagin and its derivatives.

2,3-dibromo-1,4-naphthoquinone at 80 μg/cm^2. These results indicate that the acaricidal activity of the chloroform extracts from *D. kaki* roots can be mostly attributed to plumbagin, because the above compound was more toxic (about 481 and 350 times) than that of benzyl benzoate against *D. farinae* and *D. pteronyssinus*, respectively. Additionally, naphthazarin was about 831 and 490 times more toxic than benzyl benzoate against *D. farinae* and *D. pteronyssinus*, respectively. In this case, the differential susceptibility of these compounds to *D. farinae* and *D. pteronyssinus* might be attributable to differences in biological factors (*e.g.*, body size, body weight, external structure, *etc.*) and biochemical characteristics such as detoxifying enzyme activity (*e.g.*, mixed-function oxidases, hydrolases, glutathione S-transferases, *etc.*), although the exact mechanisms involved in this phenomenon remains unknown [18, 24].

The morphological changes of dust mites between untreated and treated with all compounds were studied through a microscope (200×) (Fig. 3). On the untreated dust mites with all compounds, house dust mites were shown to be colorless and transparent. After treatment, dust mites with plumbagin and its derivatives (naphthazarin, dichlon, and 2-bromo-1,4-naphthoquinone), the skin coloration of dust mites exhibited various colors in the whole body. The color change of dust mites by the treatment of plumbagin and its derivatives (naphthazarin, dichlon, and 2-bromo-1,4-naphthoquinone) renders it easy to distinguish the dust mites with the naked eye. After dust mites were treated with benzyl benzoate, however, the skin coloration of dust mites showed much the same as untreated ones.

The allergens of house dust mites are not only caused by the dust mite themselves but also by their excrements, involving the mite eggs [11]. Accordingly, the treatments of the common acaricides often cause a vicious circle, because it is actually impossible to remove allergens in the house. These problems have highlighted the need for the development of new strategies for the control of dust mite allergens. For this reason, we need a new concept for the acaricides involving in both the indicator of dust mites and acaricidal activity. In this regard, our research for an indicator of dust mites is a unique work in the world, owing to a color alteration of dust mites by plumbagin and its derivatives (naphthazarin, dichlon, and 2-bromo-1,4-naphthoquinone). To the best of our knowledge, we could suggest plumbagin and its derivatives (naphthazarin, dichlon, and 2-bromo-1,4-naphthoquinone), having the ability for discoloration of dust mites and excellent acaricidal activity.

This discoloration is likely to be related the phenolic metabolism in the plant defense reaction. In phenolic metabolism, polyphenol oxidase catalyzes two basic reactions: hydroxylation to the *o*-position adjacent to an existing hydroxyl group of the phenolic substrate (monophenol oxidase activity) and oxidation of diphenol to quinonoids (diphenol oxidase activity) [24]. Both reactions utilize molecular oxygen as a cosubstrate. Diphenol oxidases have received much attention owing to their high catalytic rate and their association with the formation of quinonoids, which lead to production of the dark-brown pigment melanin [24, 29]. In addition, tyrosinase is mainly responsible for melanin biosynthesis (melanogenesis) in animals and enzymatic browning (melanosis) in plants [13]. Namely, the similar mode of action may be happening again in a mite body by the treatment of quinonoid derivatives. Because polyphenol oxidase exists in both insects and mites as a propolyphenol oxidase form, it is thought to confer disease resistance in insects [24]. The enzyme is also believed to be involved in both immunity and self-recognition [24].

In this study, we have found the color change of dust mites and the acaricidal effect by the chloroform extracts from *D. kaki* roots against *D. farinae* and *D. pteronyssinus*. In particular, plumbagin had the highest acaricidal activity with a LD_{50} value $\ll$0.5 μg/cm^2. Moreover, similar results have been exhibited with its derivatives (naphthazarin, dichlon, and 2-bromo-1,4-naphthoquinone). In contrast, no activity was observed for 2,3-dibromo-1,4- naphthoquinone. Additionally, plumbagin and its derivatives were about 100–800 times more toxic than benzyl benzoate against *D. farinae*. From this point of view, plumbagin and its

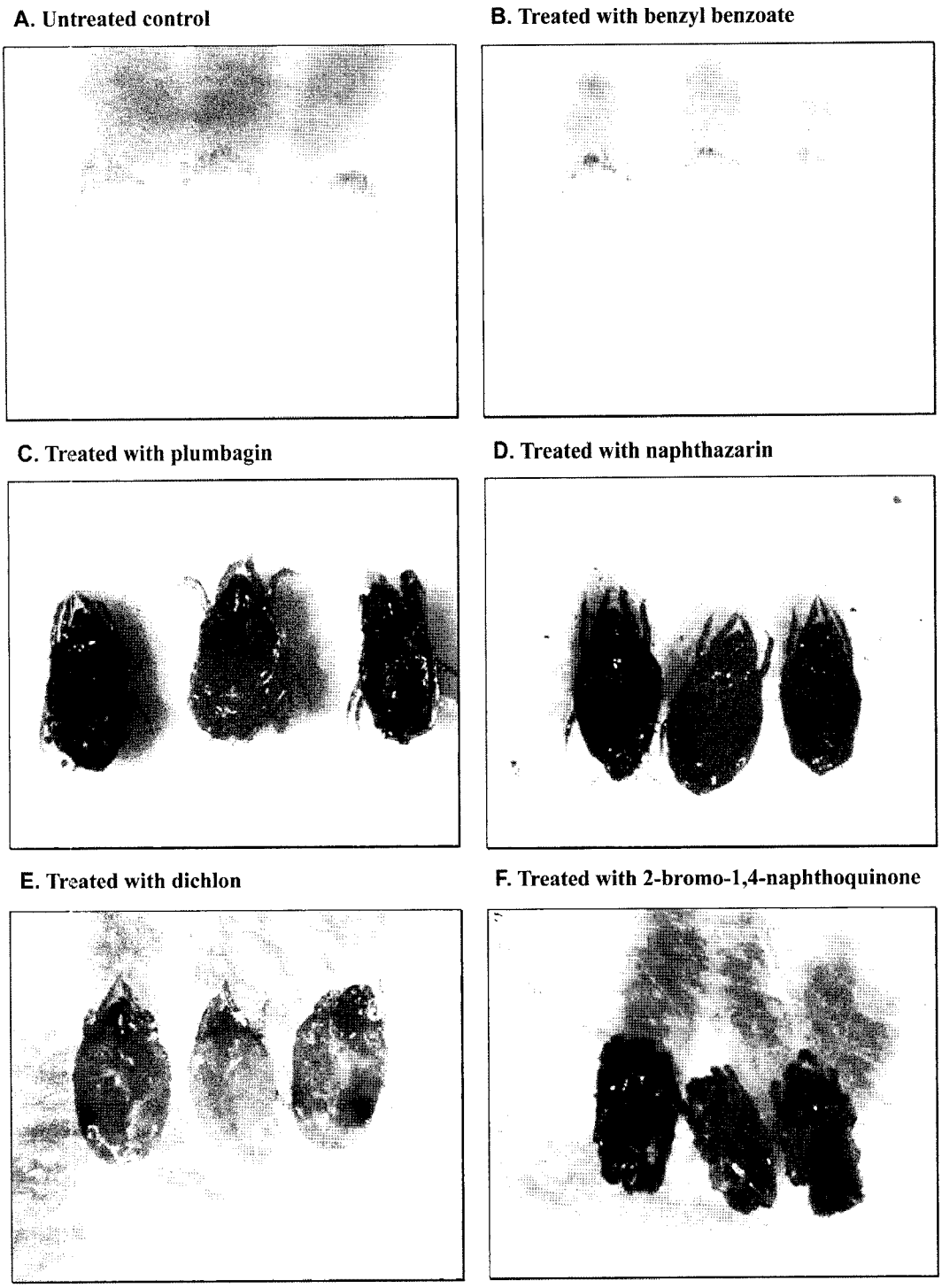

Fig. 3. Morphological changes of dust mites by plumbagin and its derivatives.

derivatives are the most promising for the possible use against *D. farinae* and *D. pteronyssinus* owing to the low doses required to produce a high dust mite mortality in the household. In a previous study, the oral LD_{50} value of plumbagin for rats was reported as 65 mg/kg, indicating high acute toxicity to mammals [26]. However, no reports of occupational exposure to plumbagin during its production or processing were found in the available literature. For this reason, plumbagin has been used as an anticancer agent for many years. Furthermore, plumbagin and its derivatives were shown to have a high capability as mite indicators. However, little or no discoloration was observed for benzyl benzoate.

In conclusion, these results indicate that plumbagin and its derivatives can be very useful in removing allergens, as potential mite control agents, lead compounds, indicator

of house dust mites, and in prevention of allergic disease. Our current data are just a first step in unraveling the complex mechanisms of discoloration of the dust mites by this compound. Further research should be done on the safety of these compounds for human health, discoloration mechanisms, and formulations for improving the acaricidal potency and stability.

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