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Insecticidal activities of a *Diospyros kaki* root-isolated constituent and its derivatives against *Nilaparvata lugens* and *Laodelphax striatellus*

Ju-Hyun Jeon [a], Yeon-Kook Kim [a], Sang-Guei Lee [a,b], Geon-Hwi Lee [a,c], Hoi-Seon Lee [a,*]

[a] *Department of Bioenvironmental Chemistry and Institute of Agricultural Science & Technology, College of Agriculture & Life Science, Chonbuk National University, Jeonju 561-756, Republic of Korea*
[b] *Applied Entomology Division, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Republic of Korea*
[c] *Reclaimed Land Agriculture Research Division, National Institute of Crop Science, RDA, Iksan 570-080, Republic of Korea*

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ABSTRACT

Diospyros kaki root-derived materials were examined for insecticidal properties against *Nilaparvata lugens* and *Laodelphax striatellus*. Based on the LD_{50} values, the chloroform fraction of *D. kaki* extracts showed the most activity against *N. lugens* (3.78 μg/female) and *L. striatellus* (7.32 μg/female). The active constituent of the chloroform fraction was isolated by various chromatographic methods and was identified as 5-hydroxy-2-methyl-1,4-naphthoquinone by spectroscopic analyses. To establish the structure–activity relationships, the insecticidal effects of 5-hydroxy-2-methyl-1,4-naphthoquinone and its derivatives against *N. lugens* and *L. striatellus* were determined using micro-topical application bioassays. On the basis of LD_{50} values, 5-hydroxy-1,4-naphthoquinone was the most effective against *N. lugens* (0.072 μg/female) and *L. striatellus* (0.183 μg/female). 2-Bromo-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, and 5-hydroxy-2-methyl-1,4-naphthoquinone also had potent insecticidal activities against *N. lugens* and *L. striatellus*. In contrast, no insecticidal activity was observed with 2-methoxy-1,4-naphthoquinone or 2-methyl-1,4-naphthoquinone. These results indicate that the functional group (bromo- and hydroxyl-) at the C-2 position of the 1,4-naphthoquinone skeleton and the change in position of the hydroxyl group play important roles in insecticidal activity. Therefore, naturally occurring *D. kaki* root-derived 5-hydroxy-2-methyl-1,4-naphthoquinone and its derivatives may be suitable as insecticides.

Introduction

Nilaparvata lugens Stål, the brown planthopper and *Laodelphax striatellus* Fallén, the small brown planthopper, are major rice insect pests. Both species damage rice plants directly by sucking phloem sap and indirectly by transmitting viral diseases, including grassy stunt, wilted stunt, and rice stripe viruses, resulting in grain quality decline and serious yield loss (Zhang et al., 2008; Senthil-Nathan et al., 2009; Lee et al., 2010). *N. lugens* cannot over-winter in Korea but migrates from China and Vietnam every year (Jung and Im, 2005; Dang et al., 2010). The control of *N. lugens* and *L. striatellus* is dependent mainly on synthetic insecticides, such as buprofezin, fipronil, and imidacloprid (Dang et al., 2010). Although synthetic insecticides were useful for controlling planthoppers, their repeated use causes resistance, residual toxicity, and environmental pollution (Senthil-Nathan et al., 2009). Therefore, the development of planthopper control alternatives is needed. Plant-derived extracts and their bioactive constituents have been suggested as alternatives to the most commonly used insecticides because many plants demonstrate insecticidal activities against insect pests (Ahn et al., 1998; Isman et al., 2001; Tak et al., 2006; Kim et al., 2010; Lee et al., 2010). Because of this, many studies have focused on essential oils, plant extracts, or phytochemicals as new sources of pest control agents or lead compounds (Park et al., 2000, 2011).

The persimmon tree (*Diospyros kaki* L.) is used in traditional medicine to treat apoplexy, arteriosclerosis, cough, and diarrhea (Kim et al., 2009). *Diospyros* species contain many medicinal phytochemicals, including amino acids, carotenoids, coumarin, flavonoids, lipids, minerals, naphthoquinones, steroids, sugars, and tannins (Lee and Lee, 2008). Many studies have addressed the antibacterial, antifeedant, antifungal, anti-dust miticidal, antimalarial, and cytotoxical activities of *D. kaki* root-derived materials (Ganapaty et al., 2004; Gujar, 1990; Kuk et al., 2002; Lee and Lee, 2008). However, the insecticidal activities of naphthoquinones derived from the *D. kaki* roots have not been investigated. In our study, the constituent of *D. kaki* roots active against *N. lugens* and *L. striatellus* was isolated by various chromatographic methods and was characterized by spectroscopic analyses. The structure–activity relationships of 1,4-

* Corresponding author at: Department of Bioenvironmental Chemistry, College of Agriculture and Life Sciences, Chonbuk National University, Jeonju 561-756, Republic of Korea. Tel.: +82 63 270 2544; fax: +82 63 270 2550.
E-mail address: hoiseon@chonbuk.ac.kr (H.-S. Lee).

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naphthoquinone derivatives for insecticidal activities against *N. lugens* and *L. striatellus* are also discussed.

Materials and methods

Chemicals and plant material

2-Bromo-1,4-naphthoquinone, 2-hydroxy-1,4-naphthoquinone, 5-hydroxy-1,4-naphthoquinone, 2-methoxy-1,4-naphthoquinone, and 2-methyl-1,4-naphthoquinone were supplied by Sigma-Aldrich (St. Louis, MO). Imidacloprid, which was used as a positive control, was obtained from the Department of Agricultural Biology, National Institute of Agricultural Science and Technology, RDA, Suwon, Korea. All chemicals were of reagent grade and commercially available. *D. kaki* roots were collected in Chonbuk Province, Korea (Lee, 2006).

Plant preparation

D. kaki roots (5 kg) were homogenized in a grinder after washing and then extracted with methanol (10 l) twice at room temperature for 2 days. The methanol extract of *D. kaki* roots was filtered through filter paper (Toyo filter paper No. 2; Toyo Roshi, Tokyo, Japan) under vacuum. The combined filtrate was then concentrated under vacuum at 45 °C using a rotary vacuum evaporator (EYELA autojack NAJ-100, Japan). The methanol extract (700 g) was sequentially divided into hexane (27 g), chloroform (161 g), ethyl acetate (113 g), butanol (182 g) and water-soluble (203 g) fractions for the bioassay. The solvent fractions were concentrated using rotary vacuum evaporation at 45 °C and the water-soluble fraction was freeze-dried.

Isolation and identification

To isolate the active compounds of the chloroform fraction derived from *D. kaki* roots, the chloroform fraction (10 g) was separated by silica gel column chromatography (Merck 70-230 mesh, 600 g, 10 i.d. × 50 cm, Rahway, NJ, USA). It was continuously eluted using a stepwise gradient of hexane/chloroform (30:70, 20:80, 10:90 and 100, v/v) to give six fractions (C1–C6). The active C2 fraction (2.03 g) was further separated by silica gel column chromatography and was successively eluted with hexane/chloroform (30:70, v/v). The column fractions were analyzed via TLC and the fractions with similar patterns were pooled. Six fractions (C21–C26) were obtained and used in the bioassays against planthoppers.

The active C21 fraction (1.37 g) was isolated by HPLC (LC-908W-C60, JAI, Tokyo, Japan). The first separation was performed on a JAI 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 (30:70, v/v) at a flow rate of 5 ml/min and detection at 268 nm. In this step, five fractions (C211–C215) were obtained and bioassayed. The active C214 fraction (895 mg) was subjected to further chromatography using a JAI W Series Column (W253 50 cm + W252 50 cm, 20.0 mm i.d. × 500 mm L, Japan Analytical industry Co., Ltd., Japan) using hexane/chloroform/isopropanol (30:70:2, v/v) at a flow rate of 3.5 ml/min and detection at 268 nm. Finally, the potent active C2143 principle (430 mg) was isolated. The structure of the active compound was determined by instrumental analysis. IR spectra were recorded on a Spectrum GX (Perkin Elmer, CA, USA) infrared spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded in deuteriochloroform (with TMS as an internal standard) using a JNM-ECA600 spectrometer at 600 and 150 MHz, respectively, with the chemical shifts expressed in δ (ppm). UV spectra were obtained in chloroform using a Waters 490 spectrometer (Boston, MA, USA) with EI-MS spectra obtained using a JEOL JMS-DX 30 spectrometer (JEOL, Tokyo, Japan).

Insect strains

The bioassays examined two species of planthoppers. Susceptible strains of *N. lugens* and *L. striatellus* were obtained from the National Academy of Agricultural Science, RDA, Suwon, Korea, and reared on rice plant (*Oryza sativa* L.) seedlings (7–10 days after germination) in acrylic cages. The planthoppers were maintained in the laboratory at 25 ± 1 °C, with a light:dark photoperiod of 16:8 h, and 65–75% relative humidity (RH) without any exposure to insecticides.

Bioassay

The active compound isolated from *D. kaki* roots and the derivatives of 5-hydroxy-2-methyl-1,4-naphthoquinone were tested for their insecticidal activities against *N. lugens* and *L. striatellus* using a micro-topical application technique. Various concentrations of each test compound were dissolved in acetone and applied to 30 macropterous female adults (3 to 5 days old). Test insects were lightly anesthetized with carbon dioxide and a 0.2 μl (0.1 μl for *L. striatellus*) droplet of each compound was applied topically to the middle-abdomen of each insect using a hand microapplicator (Burkard Manufacturing Co., Ltd., Rickmansworth, UK). Control planthoppers (*N. lugens* and *L. striatellus* female adults) were treated with 0.1 μl of acetone only. The treated planthoppers were kept on rice seedling in a glass tube (3 cm diameter × 20 cm high) at 25 ± 1 °C, 65–75% relative humidity, with a light:dark photoperiod of 16:8 h. They were observed for mortality 48 h after treatment. Three replicates were performed.

Statistical analysis

Mortality was transformed to arcsine square-root values for analysis of variance (ANOVA). The LD_{50} (lethal dosage needed to kill 50% of adult *N. lugens* and *L. striatellus*) values were calculated using a standard probit analysis (SAS Institute, 1999). Means (±SE) of untransformed data are reported.

Results and discussion

Isolation and identification

Tables 1 and 2 show the insecticidal activities of *D. kaki* root components against *N. lugens* and *L. striatellus*. When the methanol extracts of *D. kaki* roots were examined for toxicity against *N. lugens* and

Table 1
Insecticidal properties of various fractions obtained from the methanol extract of *D. kaki* roots against *N. lugens* and *L. striatellus*, using micro-topical application bioassay.[a]

Fraction	Planthoppers	LD_{50} values (μg/female)[b]	Slope ± SE	95% CL[c]
Methanol extract	*N. lugens*	10.351	3.5 ± 0.81	9.9835–11.6337
	L. striatellus	18.693	2.4 ± 1.12	17.3212–19.3938
Hexane fraction	*N. lugens*	–[d]	–	–
	L. striatellus	–	–	–
Chloroform fraction	*N. lugens*	3.782	5.5 ± 2.27	3.0284–4.1123
	L. striatellus	7.321	3.1 ± 1.72	6.7303–7.8821
Ethyl acetate fraction	*N. lugens*	83.155	1.7 ± 0.65	81.7684–84.6633
	L. striatellus	98.273	2.3 ± 0.96	97.2207–99.8912
Butanol fraction	*N. lugens*	–	–	–
	L. striatellus	–	–	–
Water fraction	*N. lugens*	–	–	–
	L. striatellus	–	–	–
Imidacloprid	*N. lugens*	0.021	1.7 ± 0.35	0.0186–0.0231
	L. striatellus	0.078	1.4 ± 0.22	0.0702–0.0810

[a] LD_{50} values (48 h mortality) calculated by probit analysis.
[b] Different concentrations (250, 125, 62.5, 31.2, 15.6, 7.8, 3.9, 1.95 and 0.98 μg/female) of fraction were used to determine LD_{50}.
[c] CL, confident limit.
[d] No activity.

Table 2
Insecticidal activity of the twenty subsequent fractions from the chloroform fraction obtained from *D. kaki* roots against *N. lugens* and *L. striatellus*.[a]

Fraction	Concentration (μg/female)	Mortality (%)[b]	
		N. lugens	*L. striatellus*
C1	50	17 (±2.3)	4 (±0.5)
C2	50	100 (±0.0)	100 (±0.0)
C3	50	21 (±1.5)	0
C4	50	0	0
C5	50	0	0
C6	50	0	0
C21	30	100 (±0.0)	100 (±0.0)
C22	30	33 (±2.2)	14 (±1.2)
C23	30	3 (±0.9)	0
C24	30	0	0
C25	30	0	0
C26	30	0	0
C211	10	0	0
C212	10	0	0
C213	10	9 (±1.1)	0
C214	10	100 (±0.0)	100 (±0.0)
C215	10	21 (±1.8)	13 (±2.2)
C2141	1	0	0
C2142	1	36 (±2.4)	21 (±2.9)
C2143	1	100 (±0.0)	100 (±0.0)

[a] The chemical solutions were applied by topical application and the mortalities were measured 48 h after treatment.
[b] Each value represents the mean ± standard deviation with three replicates.

L. striatellus, significant differences were observed. The LD_{50} values of methanol extracts were 10.35 and 18.69 μg/female against *N. lugens* and *L. striatellus*, respectively. The chloroform fraction derived from the methanol extract showed the highest potential activity against *N. lugens* (3.78 μg/female) and *L. striatellus* (7.32 μg/female). However, weak or no insecticidal activities were observed from the hexane, ethyl acetate, butanol, and water soluble fractions.

Due to the strong insecticidal activities of the chloroform fraction, isolation of the active compound was conducted by various chromatographic methods. Structural determination of the isolated compound was made by spectroscopic analyses, including UV, electron impact mass spectrometry (EI-MS), infrared spectra, ^{1}H NMR, and ^{13}C NMR, and by direct comparison with authentic reference compound. The active compound was identified as 5-hydroxy-2-methyl-1,4-naphthoquinone ($C_{11}H_8O_3$, MW, 188): EI-MS (70 eV) *m/z* (% relative intensity): M^+ 188 (100, base peak), 173 (18), 160 (19), 131 (16), 120 (12), 92 (9), 63 (4). Infrared absorption bands indicated the presence of a hydroxyl group (–OH) at 3324 cm^{-1}, a carbonyl group (C═O) at 1665 and 1641 cm^{-1}, an aromatic carbon–carbon double bond (C═C) at 1614 cm^{-1}, and an aromatic carbon-hydrogen bond (C–H) at 759 cm^{-1}. ^{1}H NMR (600 MHz, $CDCl_3$): δ 2.18–2.19 (3H, d, J = 1.6 Hz), 6.79–6.80 (1H, d, J = 1.6 Hz), 7.22–7.25 (1H, m, J = 9.6 Hz), 7.57–7.59 (1H, d, J = 7.2 Hz), 7.61–7.62 (1H, d, J = 6.0 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ 16.5, 114.9, 119.2, 124.1, 131.9, 135.9, 149.4, 161.0, 184.5, 190.0. The spectroscopic data of 5-hydroxy-2-methyl-1,4-naphthoquinone matched previously reported naphthoquinones (Lee, 2006; Shin et al., 2007; Lee and Lee, 2008; Bothiraja et al., 2011). Shin et al. (2007) reported that yields of 5-hydroxy-2-methyl-1,4-naphthoquinone from *Nepenthes ventricosa*×*maxima*, *N. thorelii*, *Plumbago capensis*, and *P. scandens* extracts were 0.51, 0.092, 0.15 and 0.26%, respectively. In our study, the yield of 5-hydroxy-2-methyl-1,4-naphthoquinone from *D. kaki* extract was 0.98%.

Insecticidal activity

The insecticidal activities of 5-hydroxy-2-methyl-1,4-naphthoquinone and its derivatives against *N. lugens* and *L. striatellus* were examined by comparing LD_{50} values in micro-topical application bioassays (Table 3). Based on 48 h LD_{50} values against *N. lugens*, the most potent insecticidal activity was observed in 5-hydroxy-1,4-naphthoquinone (0.072 μg/female), followed by 2-bromo-1,4-naphthoquinone (0.093 μg/female), 2-hydroxy-1,4-naphthoquinone (0.103 μg/female), and 5-hydroxy-2-methyl-1,4-naphthoquinone (0.120 μg/female). The 48 h LD_{50} value of imidacloprid against *N. lugens* was 0.021 μg/female. Based on 48 h LD_{50} values against *L. striatellus*, the most potent insecticidal activity was observed in 5-hydroxy-1,4-naphthoquinone (0.183 μg/female), followed by 2-bromo-1,4-naphthoquinone (0.282 μg/female), 2-hydroxy-1,4-naphthoquinone (0.285 μg/female), and 5-hydroxy-2-methyl-1,4-naphthoquinone (0.314 μg/female). No activity was observed for 2-methoxy-1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone against *N. lugens* or *L. striatellus*. According to the LD_{50} values, *N. lugens* was more susceptible than *L. striatellus* to 5-hydroxy-2-methyl-1,4-naphthoquinone and its derivatives. Earlier studies have demonstrated that even closely related species can show widely different susceptibilities to the same plant extracts (Akhtar and Isman, 2004; Jeong et al., 2009). The results of these studies suggest that responses to the insecticidal materials are species-specific.

Plant extracts, essential oils, and plant secondary metabolites may be alternatives to synthetic insecticides due to their complex mixtures of anthraquinones, monoterpenoids, naphthoquinones, phenols, and related substances (Bakkali et al., 2008; Kim et al., 2010). Naphthoquinones and heterocyclic derivatives are a group of phytochemicals with widely recognized pharmaceutical activities, such as antibacterial, anticancer, antifungal, anti-inflammatory, antiplatelet, and antitumor activities (Kim et al., 2009). Furthermore, 5-hydroxy-2-methyl-1,4-

Table 3
LD_{50} values of isolated compound and its derivatives against *N. lugens* and *L. striatellus*.[a]

Compound	Planthoppers	LD_{50} values (μg/female)[b]	Slope ± SE	95% CL[c]
5-Hydroxy-2-methyl-1,4-naphthoquinone	*N. lugens*	0.120	3.9 ± 0.35	0.1081–0.1323
	L. striatellus	0.314	3.1 ± 0.28	0.3037–0.3311
2-Bromo-1,4-naphthoquinone	*N. lugens*	0.093	1.3 ± 0.41	0.0742–0.1035
	L. striatellus	0.282	1.7 ± 0.26	0.2733–0.2971
2-Hydroxy-1,4-naphthoquinone	*N. lugens*	0.103	1.5 ± 0.23	0.0866–0.1209
	L. striatellus	0.285	1.9 ± 0.38	0.2773–0.2938
2-Methoxy-1,4-naphthoquinone	*N. lugens*	–[d]	–	–
	L. striatellus	–	–	–
2-Methyl-1,4-naphthoquinone	*N. lugens*	–	–	–
	L. striatellus	–	–	–
5-Hydroxy-1,4-naphthoquinone	*N. lugens*	0.072	1.7 ± 0.24	0.0591–0.0974
	L. striatellus	0.183	2.1 ± 0.37	0.1215–0.2083
Imidacloprid	*N. lugens*	0.021	1.7 ± 0.35	0.0186–0.0231
	L. striatellus	0.078	1.4 ± 0.22	0.0702–0.0810

[a] LD_{50} values (48 h mortality) calculated by probit analysis.
[b] Different concentrations (1, 0.5, 0.25, 0.125, 0.0625 and 0.0312 μg/female) were used to determine LD_{50}.
[c] CL, confident limit.
[d] No activity.

naphthoquinone isolated from *D. kaki* roots killed the domestic mites, *Dermatophagoides farinae* and *D. pteronyssinus* (Lee and Lee, 2008). Thiboldeaux et al. (1998) reported that 5-hydroxy-1,4-naphthoquinone killed the Saturniid moth larvae, *Actias luna* and *Callosamia promethea*. However, no work has been done regarding the control of rice insect pests using 5-hydroxy-2-methyl-1,4-naphthoquinone and its derivatives despite their biological activities. In this study, 5-hydroxy-2-methyl-1,4-naphthoquinone and its derivatives revealed potent insecticidal activities against adult *N. lugens* and *L. striatellus* females. These results indicate that 5-hydroxy-2-methyl-1,4-naphthoquinone and its derivatives may be useful as a rice insect-control insecticides or as a lead compound.

Structure–activity relationships

When evaluating our results, we focused on the importance of two structural features for insecticidal activities: (1) the functional groups (bromo-, hydroxy-, methyl-, and methoxy-); and (2) the position of the hydroxyl group. The structure–activity relationships between the active compound and insecticidal activity against *N. lugens* and *L. striatellus* were evaluated by comparing the LD_{50} values (Table 3, Fig. 1). 2-Bromo-1,4-naphthoquinone (which has a bromo group at the C-2 position) and 2-hydroxy-1,4-naphthoquinone (which has a hydroxyl group at the C-2 position) exhibited the highest insecticidal activities against *N. lugens* and *L. striatellus*. 2-Methoxy-1,4-naphthoquinone (which has a methoxy group at the C-2 position) and 2-methyl-1,4-naphthoquinone (which has a methyl group at the C-2 position) had no insecticidal activity against *N. lugens* and *L. striatellus*. Changing the position of the hydroxyl group in 1,4-naphthoquinone skeleton changes insecticidal activities against *N. lugens* and *L. striatellus*. 5-Hydroxy-1,4-naphthoquinone (which has a \hydroxyl group in the C-5 position) had higher activity than 2-hydroxy-1,4-naphthoquinone. Interestingly, the structure–activity relationships between 5-hydroxy-1,4-naphthoquinone and 5-hydroxy-2-methyl-1,4-naphthoquinone exhibited that the introduction of a hydroxyl group into C-5 position of 2-methyl-1,4-naphthoquinone produced a moderate increase in the insecticidal activities against *N. lugens* and *L. striatellus*. In a similar study, Unelius et al. (2006) reported that the insecticidal effects of the benzoic acid derivatives against the pine weevil, *Hylobius abietis*, are related to the positions of the substituents and to the various functional groups present on the aromatic ring. According to our results, the introduction of different functional groups into the C-2 position in 1,4-naphthoquinone skeleton and changing the position of hydroxyl group increases the insecticidal activities against *N. lugens* and *L. striatellus*.

In conclusion, these results indicate that *D. kaki* root-isolated 5-hydroxy-2-methyl-1,4-naphthoquinone and some derivatives may be used as alternative insecticides for the control of some rice insect pests. Further research should be conducted to address safety issues of the plant-derived materials for human health, their mode of action, and effective formulations to improve insecticidal potency and stability.

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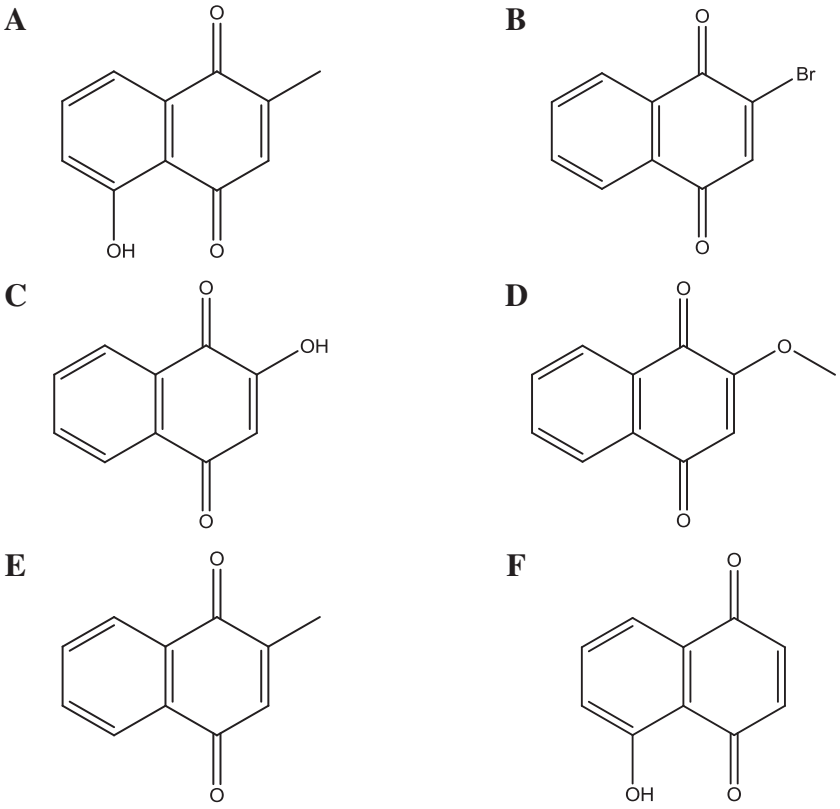

Fig. 1. Chemical structures of isolated compound and its derivatives. (A) 5-Hydroxy-2-methyl-1,4-naphthoquinone. (B) 2-Bromo-1,4-naphthoquinone. (C) 2-Hydroxy-1,4-naphthoquinone. (D) 2-Methoxy-1,4-naphthoquinone. (E) 2-Methyl-1,4-naphthoquinone. (F) 5-Hydroxy-1,4-naphthoquinone.

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