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Antitermitic quinones from *Diospyros sylvatica*

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Abstract

Six quinones were isolated from the chloroform extract of the roots of *Diospyros sylvatica* and identified as 2-methyl-anthraquinone, plumbagin, diosindigo, diospyrin, isodiospyrin and microphyllone. The effect of the root extract on the orientation and survival of the subterranean termite, *Odontotermes obesus* was tested. In addition, four of these quinones were tested on the survival of the subterranean termite. In a direct-choice experiment, exposure to an extract-treated filter disc had a significantly repellent effect over the solvent-treated filter disc. The no-choice experiment revealed the toxic property of the extract as well as the tested quinones and showed high mortality of the *O. obesus* workers after 48 h on forced exposure. The major termiticidal components identified were plumbagin, isodiospyrin and microphyllone while diospyrin was not toxic to termites at the concentration tested. All the quinones are reported for the first time from *D. sylvatica*.

Keywords: *Diospyros sylvatica* Roxb; Ebenaceae; Root extract; Naphthoquinones; *Odontotermes obesus*; Repellent and toxic effects

1. Introduction

The persistent use of chemical termiticides is at present of environmental concern and has resulted in the need to search for plant-derived compounds as alternatives in termite control (Carter, 1976). Reports published decades ago had revealed that several wood species possess natural resistance to termite infestation but only a limited number of them had been examined (Wolcott, 1947; Sandermann et al., 1958). Termite-resistant woods are said to contain allelochemicals, such as quinones, flavonoids and terpenoids, that possess natural repellent and toxic properties (Scheffrahn, 1991).

The family Ebenaceae consists of only three genera, of these, the genus *Diospyros* is by far the largest, with 500 species (Willis, 1966). This genus is widespread in the tropics and the warm temperate regions of the world, of which 24 species are native to India. *Diospyros sylvatica*, also known locally as *gatha* is a moderate-sized tree distributed in the hills of Vizianagram and neighbouring Orissa state (Gamble, 1997). Chemical examination of *Ebenaceae* has been generally confined to the genus *Diospyros*. A number of *Diospyros* species are used in herbal medicine for the treatment of whooping cough, leprosy, dysentery, menstrual troubles, abdominal pains and as antibiotics (Watt and Breyer-Brandwijk, 1962). The wood of this genus has considerable economic importance as a source of hard wood timbers and also as edible-fruits (Irvine, 1961; Uphof, 1968). Chemical studies on a number of species have revealed that the stems and leaves of this genus have been reported to contain triterpenoids (Bhakuni et al., 1971) , while the roots are well known to contain naphthols and naphthoquinones. The antibacterial, antifungal and termite-resistant properties of *Diospyros* have all been attributed to the presence of naphthoquinones (Waterman and Mbi, 1979). In a brief study conducted on the resistance of various timbers of *Diospyros* species, the wood of *D. celebica* was found to be highly resistant to the subterranean termites,

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Reticulitermes lucifugus and *Reticulitermes flavipes* (Sandermann and Dietrichs, 1957). The naphthoquinones, 7-methyl-juglone and its dimer isodiospyrin isolated from the wood of *Diospyros virginiana* were reported to possess termiticidal activity against *R. flavipes* (Carter et al., 1978). Naphthoquinones and naphthalene derivatives have been isolated previously from this genus, but a survey of the literature revealed that triterpenes namely α-amyrin, lupeol and betulin were the only constituents reported from the bark of this plant (Ramachandra Row and Sankara Rao, 1966, 1968). This paper describes the extraction, isolation and characterization of six quinones **1**–**6**, among which three are termiticidal components (two naphthoquinones and a rare prenylated dimeric benzoquinone) and three related quinones from the roots of *D. sylvatica* which are reported for the first time from this plant.

2. Results and discussion

Shade dried roots of *D. sylvatica* were powdered in a Wiley mill and extracted with chloroform at room temperature for 24 h. The combined extracts were concentrated under reduced pressure to yield a dark brown gum. The extract was initially examined by thin-layer chromatography on silica gel and shown to contain several yellow and colourless compounds. On a preparative scale, lupeol, β-sitosterol and six quinones **1**–**6** (Fig. 1) were isolated from the chloroform extract of the roots by a sequence of chromatographic techniques followed by crystallization.

All compounds were characterized by their EI mass spectra, ^{1}H NMR spectra, IR data, and melting points and identified by a search in AntiBase (Laatsch, 2002). The faint yellow compound with the highest R_f value was not affected by sodium hydroxide and identified as 2-methyl-anthraquinone (**1**; Bani and Sunil, 1981) , due to its spectroscopic properties. Plumbagin (**2**; Thomson, 1971), diospyrin (**4**; Sidhu and Pardhasaradhi, 1967) and isodiospyrin (**5**; Fallas and Thomson, 1968) gave with base a blue-violet colouration which is characteristic for *peri*-hydroxyquinones. Both the dimers diospyrin (**4**) and isodiospyrin (**5**) appeared orange-brown in colour in visible light, but under UV light, diospyrin appeared orange-red, while isodiospyrin grew dark brown. Diosindigo A (**3**; Laatsch, 1980) is one of the two naturally occurring derivatives of Russig's Blue. It was easily identified by a direct comparison with an authentic sample (Laatsch, 1980). The pale yellow colour of the quinone with the highest polarity was again not changed by sodium hydroxide. The molecular formula was

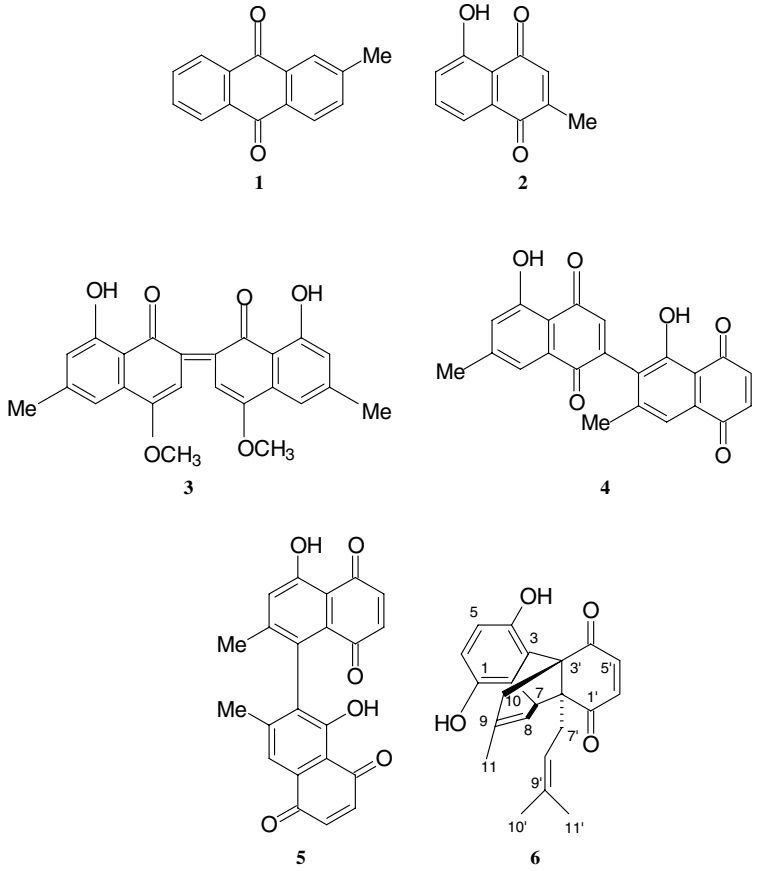

Fig. 1. Compounds **1**–**6** of *D. sylvatica* roots.

determined as $C_{22}H_{22}O_4$ by HR EIMS. The ^1H and ^{13}C NMR spectra showed the presence of two carbonyl groups, two trisubstituted double bonds, a *cis* di-substituted double bond, a 1,2,3,4-tetrasubstituted benzene, three methyl protons, two methylene protons, a methine and two quaternary carbons. These data proved the quinonoid microphyllone (**6**; Satoshi et al., 1995).

2.1. Direct choice test (repellent effect)

During a 60-min test period, *Odontotermes obesus* workers made equal contact to both the (blank control) untreated filter paper as well as the (solvent control) solvent-treated filter paper discs. In the case of blank control, the average number of workers present on both the blank control discs, gave no significant difference ($P > 0.05$, df = 5, paired comparisons *t*-test, Table 1). On exposure to the extract-treated filter paper discs at 100% (0.200 g/ml) as well as their dilutions at 50% (0.100 g/ml) and 10% (0.020 g/ml) concentrations, termites exhibited a significant avoidance behaviour. There was a significant increase in the number of termites in contact with the solvent control disc in comparison to the number of termites on the corresponding extract-treated disc ($P < 0.01$, df = 5, paired comparisons *t*-test, Table 1). The current study demonstrated that all three concentrations of the root extract were less preferred and avoided by the subterranean termite, *O. obesus*.

Previous reports have revealed that sensitivity of termites towards insecticides and repellents show species-specific differences (Smythe and Carter, 1970; Su and Scheffrahn, 1990; Su et al., 1995) and hence screening of the root extract on other termite species would be needed to ascertain the differences in repellent action but, this simple laboratory experiment (Smith, 1979; Sharma et al., 1994) was found to be useful to prove the repellent property of the tested root extract using the direct choice trials. Termite-repellent properties of several plant extractives have been recently reviewed, suggesting that allelochemicals such as terpenoids, quinones, flavonoids and fatty acids are the chemicals that are responsible for the natural repellent and toxic properties of termite-resistant wood species (Scheffrahn, 1991). Recently, the terpenoids like citral, eugenol and geraniol have been shown to possess greater toxic and repellent properties against termites with very low mammalian toxicity (Sharma et al., 1994; Cornelius et al., 1997). A study carried out on some commercially available formulations of botanical insecticides comprising of formulated cedarwood oil, formulated isoborneol, body oil and plant oil showed greater repellent effect (Blaske and Hertel, 2001) than that exhibited by this root extract, signifying that the terpenoids could possess greater repellent action via due to the volatile nature inducing vapour toxicity and thereby exerting an indirect repellent effect.

Table 1
Repellent effect of the root extract of *D. sylvatica* on *O. obesus* termites in a direct choice test

Experiment	Mean ± SD no. of termites in contact with the filter paper discs for 60 min	Difference in mortality
Blank control Blank control	5.0 ± 2.36 5.0 ± 2.36	No ($P > 0.05$, df = 5)
Blank control Solvent control	5.0 ± 2.36 4.0 ± 2.36	No ($P > 0.05$, df = 5)
Root extract 100% Solvent control	2.1 ± 1.33 10.0 ± 2.82	Yes ($P < 0.01$, df = 5)
Root extract 50% Solvent control	3.0 ± 1.89 10.0 ± 2.82	Yes ($P < 0.01$, df = 5)
Root extract 10% Solvent control	2.1 ± 1.33 12.0 ± 3.40	Yes ($P < 0.01$, df = 5)

Root extract 100%, 50% and 10% concentrations are equivalent to 0.200, 0.100 and 0.020 g/ml. Dilution of each stock solution was done using chloroform and as a solvent control. No. of counts in a 60-min period ($n = 12$). Each mean is based on 180 termites (six replicates × 30 termite workers per replicate, paired comparisons *t*-test was used for each experiment, df = 5).

2.2. No-choice test (toxic effect)

Forced direct exposure of *O. obesus* workers to 1% (0.010 g/ml), 5% (0.050 g/ml) and 10% (0.100 g/ml) concentrations of the root extract resulted in 98.3 ± 3.0%, 100.0 ± 0.0% and 100.0 ± 0.0% mortality after 48 h and was significantly higher than the solvent controls ($P < 0.05$, df = 6, Student's *t*-test, Table 2) clearly signifying contact toxicity as the rationale. Owing to the absence of volatile constituents in the root extract and the high mortality rate observed, this effect should hence be registered as a direct toxic character. Further, the percentage of dead termites in the starvation control in a test duration of 48 h was found to be as low as 1.66 ± 1.0%, clearly explaining that almost no mortality of the termite workers within a test duration of 48 h could rule out feeding deterrence or starvation to be the case and hence must be considered as a direct toxic effect (Arndt, 1968; Smythe and Carter, 1970; Bultman et al., 1979). The mean percentage of dead termites recorded in both the blank and the solvent controls were 3.33 ± 0.0% for a test duration of 48 h that increased to 74.1 ± 3.31% at the end of the week. This death of the termite workers may be explained as naturally occurring death or cannibalism (Cymorek and Pospischil, 1984).

The quinones tested at 0.010 g/ml, showed quite promising termiticidal activity on exposure of *O. obesus* workers for 24, 48 and 72 h and for up to a week. The highest percentage mortality rate of 75.0 ± 3.34% in 24 h

Table 2
Toxic effect of the root extract of *D. sylvatica* and the tested quinones on *O. obesus* workers over time (h) on forced direct exposure in a no-choice test

Experiment	% Mortality of termite workers recorded over time (h)			
	24 h	48 h	72 h	A week
Starvation control	n.m	1.66 ± 1.0	4.16 ± 0.19	8.1 ± 3.33
Blank control	n.m	$3.33 \pm 0.0^{*}$	$74.1 \pm 3.31^{*}$	$9.16 \pm 0.59^{*}$
Solvent control	n.m	3.33 ± 0.0	10.8 ± 0.59	74.1 ± 3.31
Root extract 10%	100.0 ± 0.0	$100.0 \pm 0.0^{**}$	–	–
Root extract 5%	63.3 ± 2.81	$100.0 \pm 0.0^{**}$	–	–
Root extract 1%	43.3 ± 1.94	$98.3 \pm 3.0^{**}$	–	–
Quinones				
Plumbagin	75.0 ± 3.34	$100.0 \pm 0.0^{**}$	–	–
Isodiospyrin	52.0 ± 2.30	$100.0 \pm 0.0^{**}$	–	–
Microphyllone	26.6 ± 1.26	$60.8 \pm 2.7^{**}$	$100.0 \pm 0.0^{**}$	–
Diospyrin	5.8 ± 0.31	$19.1 \pm 0.9^{***}$	$57.5 \pm 2.6^{**}$	$100.0 \pm 0.0^{**}$

$^{*}P > 0.05$, not significantly different, $^{**}P < 0.05$, $^{***}P < 0.5$, Student's *t*-test was used for each experiment, df = 6. n.m., no mortality observed. Each mean ± SD is based on 120 termite workers (four replicates, 30 insects per replicate). Root extract 10%, 5% and 1% concentrations are equivalent to 0.100, 0.050 and 0.010 g/ml. Dilution of each stock solution was carried out using chloroform and also as a solvent control. The quinones tested were studied at 0.010 g/ml.

was observed with plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) (**2**), while isodiospyrin (**5**) produced $52.0 \pm 2.30\%$ mortality in 24 h which is consistent with a previous report, that the poisonous and vesicant properties of many tropical *Diospyros* species may be attributed to the occurrence of the monomer plumbagin (Thomson, 1971). All the quinones showed difference in significance in various time intervals (h) between the solvent control and the treated groups with high percentage mortality ($P < 0.05$, df = 6, Student's *t*-test, Table 2).

Related quinones bearing the juglone moiety have previously shown to exhibit termiticidal activity. 7-Methyl-juglone and its 8-6′-dimer namely isodiospyrin isolated from the woods of *D. virginiana* were studied for their respective toxicities against *R. flavipes*. 7-Methyl-juglone showed to be toxic at 0.100 g in 24 h while the dimer was less toxic to termites (Carter et al., 1978). Naphthoquinones and other related quinonoid compounds are one of the major natural product classes with varied biological activities (Fournet et al., 1992; Sepúvelda-Boza and Cassels, 1996; Akendengue et al., 1999), the most interesting is their biological activity against parasitic protozoa namely *Leishmania*, *Trypanosoma* and *Plasmodium*. Three such quinones namely, hydrolapachol (2-hydroxy-1,4-naphthoquinone) was shown to have activity against *Plasmodium lophurae* (Hooker, 1936; Hudson, 1984). Plumbagin (5-hydroxy-2-methyl-1,4-naphthoquinone) and other related quinones have been active against *Leishmania* spp. (Fournet et al., 1992), while diospyrin was found to be active against *Leishmania donovani* (Ray et al., 1998). The quinones of *Diospyros* namely 7-methyl-juglone and plumbagin were shown to possess good antibacterial activity against various strains of bacteria (Khan and Timi, 1999), while the dimeric benzoquinone, microphyllone, was reported to possess antiallergic properties (Satoshi et al., 1995).

Our results reveal that the major termiticidal components identified were plumbagin (**2**), isodiospyrin (**5**) and microphyllone (**6**), while diospyrin was less toxic to *O. obesus* termites at the concentration tested. An earlier study had shown that the related quinones were toxic at slightly higher concentrations, but the present study did show promising termiticidal property at a much lower concentration which can be due to several factors. First of which, the active constituent present in the woods are highly stable and their distribution may widely vary within a tree, but the quinones when exposed to air are known to undergo light-induced reactions usually in the presence of a solvent thereby leading to the formation of artefacts or transformation products having either enhanced termite resistance or decreased termiticidal property. The other explanation offered is the species-specific differences in sensitivity of termite behaviour to repellents. Hence, the application of novel biocides in termite control programs would certainly require short- and long-term field studies to highlight their use as commercial termiticides. The investigated root extract is rich in naturally occurring quinones as the chemicals chiefly responsible for this repellent property and hence use of these plant-derived compounds as alternatives to termite control can be considered, as environmental safety can be assured for them.

3. Experimental

3.1. General experimental procedures

Melting points were recorded on a Cipla I-28 digital apparatus and are uncorrected. IR spectra: Perkin–

Elmer spectrometer in KBr pellet. EI MS spectra were recorded on a Finnigan MAT95 spectrometer (70 eV) and per fluorokerosene was used as reference substance in HREIMS. NMR spectra were measured on a Varian Unity 300 (300.145 MHz) spectrometer with TMS as internal standard and the chemical shifts reported in δ values relatively to TMS. Coupling constants (J) are given in [Hz]. Silica gel (Acme, 60–120 mesh) was used for column chromatography and Silica gel G was used for PTLC (preparative thin-layer chromatography). Spots on chromatograms were detected under UV light and by spraying with 5% H_2SO_4 in methanol or with 0.1 N NaOH, all given molecular formulae were determined by EI-HRMS.

3.2. Plant material

The roots of *D. sylvatica* were collected at the Vizianagaram hills, India in March 2002. The material was authenticated by Dr. S. Hara Sreeramulu, Taxonomist, Botany Department, Dr. V.S. Krishna College, Visakhapatnam. A voucher specimen (SG/DSR/02/105) has been deposited at the herbarium, Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam, India.

3.3. Extraction and isolation

Shade dried roots (1.8 kg) were powdered in a Wiley mill and extracted with chloroform (4×1.5 l) at room temperature for 24 h. The combined extracts were concentrated under reduced pressure to yield 34 g of a dark brown gum. A portion of the chloroform extract (25 g) was chromatographed over silica gel and successively eluted with petrol ether, petrol ether/chloroform, chloroform and chloroform/methanol (each fraction 250 ml). Elution using petrol ether:chloroform 9:1, afforded colourless needles that were identified as lupeol (80 mg) by its colour reaction (Liebermann–Burchardt reaction, pink colour) and m.p. 212°C. A yellow band was obtained on elution with petrol ether:chloroform 3:1, which on further purification by PTLC on silica gel in hexane:chloroform 4:1 afforded 2-methyl-anthraquinone (**1**) which crystallized from petrol ether:chloroform as faint yellow needles (16 mg, m.p. 175–176 °C, molecular formula $C_{15}H_{10}O_2$, R_f 0.7 in benzene:chloroform 1:1). The petrol ether:chloroform 1:1 eluate afforded plumbagin (**2**) that crystallized from petrol ether as orange-red needles (23 mg, m.p. 77°C, molecular formula $C_{13}H_7O_3$, R_f 0.53 in hexane:chloroform, 1:1). On elution with petrol ether:chloroform 1:3, a green oil was obtained and showed to be contaminated with a sterol. This fraction was separated into two components using PTLC on silica gel with benzene to yield trace amounts of diosindigo A (**3**) that crystallized from petrol ether/chloroform as deep blue needles (2 mg, sparingly soluble in chloroform, ethanol and other organic solvents; same R_f value as an authentic sample (Laatsch, 1980)), and colourless needles that were identical with β-sitosterol (30 mg, m.p, 136 °C, green colour with Liebermann–Burchardt reaction). On eluting the chromatogram with chloroform, a large yellowish orange band was obtained which consisted of at least three compounds. Purification of the mixture using PTLC on silica gel with chloroform:methanol 99:1 failed to produce any isolable compound and hence was discarded. An orange-red band eluted with chloroform showed to contain a mixture of two compounds on TLC. This fraction was subjected to PTLC on silica gel in chloroform:methanol 99:1. The fast moving zone afforded the less polar diospyrin (**4**) that crystallized from warm petrol ether as orange-red prisms (25 mg, m.p. 258°C, molecular formula $C_{22}H_{14}O_6$, R_f 0.27 in benzene), while the second slow moving polar eluate gave isodiospyrin (**5**) that crystallized from petrol ether as orange-red needles (32 mg, m.p. 228–230 °C, molecular formula $C_{22}H_{14}O_6$, R_f 0.18 in benzene). Further elution of the chromatogram using chloroform:methanol 99:1 gave a yellow solid, which on repeated crystallization using hexane:chloroform 3:1 yielded microphyllone (**6**) as bright yellow needles (30 mg, m.p. 167–168°C, molecular formula $C_{22}H_{22}O_4$, R_f 0.54 in chloroform:methanol 99:1).

3.4. Insects

The subterranean termites, *O. obesus* Rambur and *Microtermes obesi* Holmgren (workers) were selected from field-collected colonies in the Andhra University campus, Visakhapatnam, India and were maintained in segments of colonized logs, until used. The field-collected colonies were maintained at 25 ± 1°C and 80 ± 5% RH in plastic containers with moistened corrugated cardboard paper. The termite colonies were fed ad libitum with dried pine wood (*Pinus* spp., Pinaceae). The effect of the root extract on the orientation and survival of the termite, *O. obesus* was evaluated for repellent and toxic effect by the direct-choice and no-choice test, in addition the isolated quinones **2**, **4**–**6** were evaluated using a no-choice test (Blaske and Hertel, 2001).

3.5. Direct choice test (repellent effect)

Root extract of *D. sylvatica* was tested for termite-repellent property at three different concentrations of 100% (0.200 g/ml) from the stock solution, as well as their 2-fold 50% (0.100 g/ml) and 10-fold 10% (0.020 g/ml) dilutions. Dilution of each stock solution was generally done using chloroform. The filter paper discs were saturated with the root extract and also with the solvent (control), 24 h prior to testing. The filter paper discs were then placed in glass petri dishes that were left opened at room temperature so as to enable the solvent

to evaporate from the discs. The direct-choice test was carried out using an open glass petri dish of 5.5 cm diameter containing two moistened filter paper discs, each having 1.7 cm diameter (Gelman Sciences, Ann Arbor, MI) placed 1 cm apart in the centre of the dish. The test was performed using an extract-treated and one solvent-treated filter paper disc that was offered simultaneously to 30 workers of *O. obesus*. Six replicates for each test and control experiment were performed. Termite behaviour was monitored visually. For 60 min, the number of workers in contact with each disc was counted every 5 min ($n = 12$ counts). The mean numbers of termites on the test paper (extract-treated) and control-paper (solvent-treated) for each 60-min period was compared by a paired comparisons *t*-test ($P \geqslant 0.05$) (Kulkarni, 1999).

3.6. No-choice test (toxic effect)

The toxic effects of the root extract at 10% (0.100 g/ml), 5% (0.050 g/ml) and 1% (0.010 g/ml) dilutions were studied for 48 h against the survival time of *O. obesus* workers on forced direct exposure in a no-choice situation. A moistened, extract-treated cellulose filter paper (4.5 cm diameter; Gelman Sciences) was placed at the bottom of a covered plastic petri dish (5 cm diameter, 3.5 cm high). Thirty termite workers were placed and the mortality was recorded after 48 h. During the entire experiment, the termites were in direct, continuous contact with the extract-treated filter paper. Blank and solvent controls were performed with untreated and solvent-treated filter paper, respectively. Starvation control was also performed by placing 5 g of moistened sand at the bottom of the petri dish, thereby limiting the access of termites to only water. Each of the tests included four replicates. The toxic effect of the isolated quinones **2**, **4**–**6** at 0.010 g concentration was evaluated at 24, 48, 72 h and after a week on the survival time of *O. obesus* workers.

The percentage average mortality of the termite workers were calculated at various time intervals (h) and the difference in significance between the solvent control and the treated groups was compared by a Student's *t*-test ($P \geqslant 0.05$) (Kulkarni, 1999).

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