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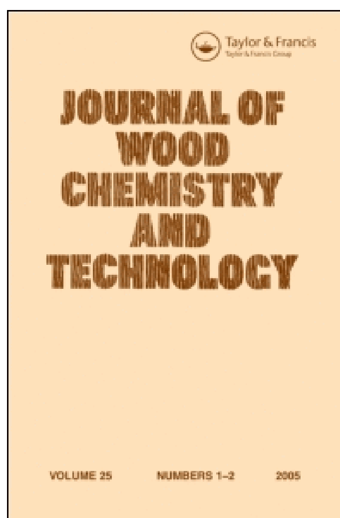

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Selective Solvent Extraction of the Bark of *Rhizophora apiculata* as an Anti-Termite Agent against *Coptotermes gestroi*

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Selective Solvent Extraction of the Bark of *Rhizophora apiculata* as an Anti-Termite Agent against *Coptotermes gestroi*

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Abstract: The anti-termite action of the bark of mangrove plant *Rhizophora apiculata* and four other species against the attack of the subterranean termite *Coptotermes gestroi* was investigated. The bark powder was extracted and partitioned into four main extracts—petroleum ether (PE), chloroform (CE), ethyl acetate (EE), and *n*-butanol (BE). Termite bioassay on different extracts was carried out. The total phenol content (TPC) of the extracts was determined by the Folin-Ciocalteu method and expressed as gallic acid equivalent (GAE). Butylated hydroxytoluene (BHT) and Butylated hydroxyanisole (BHA) were used as a positive control in the free-radical-scavenging activity test. It was believed that both the toxicity and antioxidant properties of extractives imparted natural resistance to the bark of *R. apiculata*. Considering wood weight loss, EE appeared to be the most potent inhibitor. The EE was then fractionated into three fractions (F1, F2, F3) by means of thin layer chromatography. F2 exhibited the lowest mean percentage weight loss. Through chemical and spectral analyses, we attribute the presence of bioactive constituents in the EE to a mixture of aromatic carboxylic acids or phenolics.

Keywords: Anti-termite, bark extracts, *Coptotermes gestroi*, phenolics, *Rhizophora apiculata*

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INTRODUCTION

Wood is a three-dimensional polymeric composite whose biological and technical properties are mainly determined by the chemical composition of the cell wall. Wood cell walls are made up primarily of cellulose, hemicelluloses (polyoses), and lignin.[1] In addition to the cell wall polymers, the properties of wood are strongly influenced by extractives, accessory compounds extractable by solvents of different polarity. These compounds encompass phenolics, terpenes, carbohydrates, fats, waxes, and others.[2,3]

The moisture content of timber is crucial with respect to destructive microbial and insecticidal confrontations.[4] Wood with water content of 12% or below, as typically found within buildings, is not susceptible to biological threats. Wood moisture of 12–18% enables attack by insects (beetle, wood borer, termite, etc.). Infestation by fungi (molds, staining fungi) is also possible when the moisture content rises (sporadically) above 18%.

A number of methods have been devised to reduce degradation of wood by biotic and abiotic factors via appropriate chemical treatments. It may be necessary to use inorganic compounds or synthetic pesticides to preserve the woods and lengthen their application life.[5]

Although the preservative chromated copper arsenate (CCA) is highly effective in protecting wood against a wide variety of wood-destroying organisms, the perceived environmental hazards of the disposal of such metals may limit the future use of it. Indeed, the availability of CCA-treated lumber has been greatly reduced in many European countries, the United States, and Japan.[4]

R. apiculata (bakau minyak) is the most important species of commercial mangrove timber in the Asia-Pacific region.[6] The chemical defenses in the Rhizophoraceae are largely carbon based (e.g., polyphenolics), although some species also contain tropane alkaloids. This family has long been exploited for the high proanthocyanidin (condensed tannin) and flavonoid contents of its wood, bark, and leaves. These are known to deter feeding by insects and other herbivores.

The concept of using insect antifeedants (feeding deterrents) gained strength in the 1970s and 1980s with the demonstration of the potent feeding deterrent effect of azadirachtin and neem seed extracts to a large number of pest species.[7] The discovery and demonstration of plant natural products as insect antifeedants has been unquestionably successful. In addition to the neem triterpenoids, extensive work has been performed on clerodane diterpenes from the Lamiaceae[8] and sesquiterpene lactones from the Asteraceae.[9]

Recent work has found that a non-biocidal antioxidant repelled and caused some mortality with two species of termites, and the authors suggested that both the toxicity and inherent antioxidant properties of phenolic extractives were important in natural termite resistance of certain heartwoods.[10] Extractives from woods and barks having termite resistant activity have been under investigation for many years and have provided some promise as low hazard

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termite control agents.[11,12] Extracts from wood and bark are complex natural products. Generally, the inner bark and sapwood are rich in nutrients such as sucrose and glycosides. Heartwood and outer bark, by contrast, are typically deficient in these nutrients, but are rich in secondary metabolites that function to protect the living tissues against biological attack. It contains many chemical constituents in varying concentrations; most residues are rich in cellulose and lignin, but also contain waxes, cutin, suberin, resins, terpenoids, tannins, and other phenolics.[13]

To date, there is no documentation about the termiticidal effects of bark extracts derived from mangrove plants, especially from *R. apiculata* that has ever been studied so far. Therefore, our objectives in this study were to evaluate the resistance of the barks of five species of plants to termite biodeterioration and also determination of bioefficacy of extracts of *R. apiculata* bark, a waste material of charcoal industry, against the subterranean termite, *C. gestroi*, chosen as one of the most destructive species in Malaysia.

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MATERIALS AND METHODS

Sources of Woods and Barks

Rubber *Hevea brasiliensis (Willd. ex Adr. Juss.) Muell. Arg* and pine *Pinus* sp. woods were obtained from the Forest Research Institute Malaysia (FRIM), Malaysia. *Acacia mangium Willd.* and mahogany *Swietenia macrophylla King* were collected from Nibong Tebal Sawmill Sdn. Bhd., Penang and in the vicinity of the School of Pharmaceutical Sciences, USM, respectively. *R. apiculata* was obtained from Matang Mangrove Forest Reserve, Perak, Malaysia.

Source of Test Termite

A colony of subterranean termite *C. gestroi* was found at the grass land near to the Lecture Hall G and H, USM, Penang, Malaysia. The method of collecting termites was adopted from Pearce.[14]

Chemicals

Methanol and chloroform were purchased from R&M Chemical, U.K.; ethyl acetate and *n*-butanol from Fisher Chemicals, U.K.; petroleum ether (60–80°C) from Surechem, England; hexane from Merck, Germany; 95% ethanol from ChemAR, Systerm and sulfuric acid from Mallinckrodt Baker, Mexico. Ferric chloride and sodium hydroxide were obtained from R&M Chemical, U.K. and

ChemAR, Systerm, respectively. Folin Ciocalteu reagent, sodium carbonate, gallic acid, 1, 1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxyanisole (BHA), and butylated hydroxytoluene (BHT) were purchased from Sigma (Sigma Aldrich GmbH, Sternheim, Germany). Silica gel 60 (230–400 mesh ASTM) was purchased from Fluka, Switzerland, whereas TLC plate was obtained from Merck, Germany.

Determination of pH

The EE was evaporated until dryness under reduced pressure. Then, distilled water was added prior pH determination (Basic, Denver Instrument).

Ferric Chloride ($FeCl_3$) Test

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The presence of phenols and moderately acidic groups in EE extracts were detected by Ferric chloride assay.[15,16] A positive test is indicated by the formation of black-colored complexes.

Ultraviolet (UV) and Visible Spectral Analysis

UV-visible spectra of the EE extracts were recorded on a Jasco V-530 UV/VIS spectrophotometer (200–400 nm, 1000 nm/min). Alkali was added to EE extracts and their spectra recorded as described previously. Addition of alkali to aromatic acids causes spectra to shift toward shorter wavelengths (hypsochromic shifts).[15]

PREPARATION OF TEST BARKS

Wood barks were whittled away from their logs, cleaned and chopped into an approximate size of (2.0×2.0) cm^2, which were further dried for one week at about 50°C in oven. The barks were finally transferred into a desiccator and cooled to ambient temperature for about half a day before being weighed out on a four figure balance (AND, ER-180A).

Termite Collection

A plastic container, containing a roll of tissue paper as bait, was partially buried under the soil surface near to the nest. After the termites become trapped, a dampened filter paper was used to attract and collect them away from the debris by tapping them off gently into a new tray containing a dampened filter paper

as well. Dead or injured termites were removed with a moistened, fine paint-brush. The termites were identified using keys for soldier identification.[14,17,18] Trapped termites were subjected for testing within 24 hours to ensure against the risk of death in termites. In order to permit a correlation of the test results, termites from the same colony were used throughout these studies.

Description of Bioassay

Termite Bioassay on Different Bark Species (1st Round)

Termite bioassay was carried out according to a no-choice test procedure (ASTM D 3345-74, 1980).[19] A clean glass (6 cm diameter × 11 cm) was packed with approximate 200 g of screened, washed, and heat-sterilized sand, and was moistened with distilled water to maintain relative humidity near saturation. Percentage saturation and water to be added to sand, respectively, were computed using Eqs. (1) and (2):

$$\%\,\text{saturation} = \frac{\text{Weight of water until saturation point}}{\text{Oven} - \text{dried weight of sand}} \times 100 \tag{1}$$

$$\%\,\text{water to be added} = \%\,\text{saturation} - 7.0\% \tag{2}$$

For this bioassay, saturation point was reached at approximate 40 ml of water.

Thus, 26 ml of distilled water was added to the container containing 200 g of heat-sterilized sand to stand over night. To prevent leaching of chemical substances from the bark, a small piece of flat stone was used to raise the bark above the surface of the sand. In six replications, approximately 1 g termites were used per container bark. The subterranean termites were selected so that about 90% were workers before replacing the container top loosely.

In addition, six containers with heat-sterilized sand, distilled water, and termites (approximately 1 g) but without test barks were used to test the validity of the test.

All containers were placed in a dark cabinet[11] at 26 ± 1°C for 6 weeks and their conditions were recorded weekly. These included the presence of tunneling and the position of termites in the containers throughout the whole experiment. Distilled water was added to all containers when the moisture content of the sand dropped below two percentage points of the original moisture content.

At the end of the test, barks were removed from the containers, carefully brushed clean with running tap water, oven-dried, and reweighed to determine the percentage of weight losses.

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Descriptive statistics of percentage weight losses were computed.

Termite Bioassay on Bark Extract Treated Wood (2nd Round)

Termite bioassay was carried out according to the no-choice test procedure described earlier. Test materials to be placed in individual containers were wood blocks treated with 500 ppm of the bark extracts and untreated wood blocks served as control. Additional control wood blocks treated with each solvent used in the extraction process were included to account for possible effects of trace amounts of these solvents. Assessments were based on counting of wood weight loss, recording the amount of tunneling present, the behavioral responses of the termites toward the test materials, and approximate termite mortality. All the measurements were carried out in six replicates and the values were averaged. In addition, each tested block was examined and visually ranked using ASTM rating system as listed in Table 1.

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Termite Bioassay on Different Fractions Impregnated Wood Blocks (3rd Round)

Termite bioassay using the impregnated test blocks (F1, F2, and F3; 500 ppm each) was also carried out according to ASTM D 3345-74 standard method, in order to monitor sudden activity changes due to the chromatographic fractionation step. Untreated and ethyl acetate solvent treated wood blocks were used as control and added control, respectively. Assessments were based on the procedures described in termite bioassay on different bark species. All the experiments were performed in six replicates and the values were averaged.

Table 1. Mean weight losses and approximate mortality after six weeks bioactivity of barks exposure to termite attack (1st round)

Code	Scientific name	Weight loss[a]		Approximate mortality
		g	%	
S	*Swietenia macrophylla*	0.0708 ± 0.0093	5.03 ± 0.66	Moderate
R	*Rhizophora apiculata*	0.1260 ± 0.0260	9.71 ± 2.10	Moderate
H	*Hevea brasilienisis*	0.8220 ± 0.0618	77.45 ± 10.04	Slight
A	*Acacia mangium*	0.6330 ± 0.0569	55.25 ± 5.20	Slight
P	*Pinus* sp.	0.8336 ± 0.0415	73.48 ± 5.44	Slight

[a]Values are means ± standard deviations from six replications. S, mahogany; R, mangrove; H, rubber; A, acacia and P, pine.

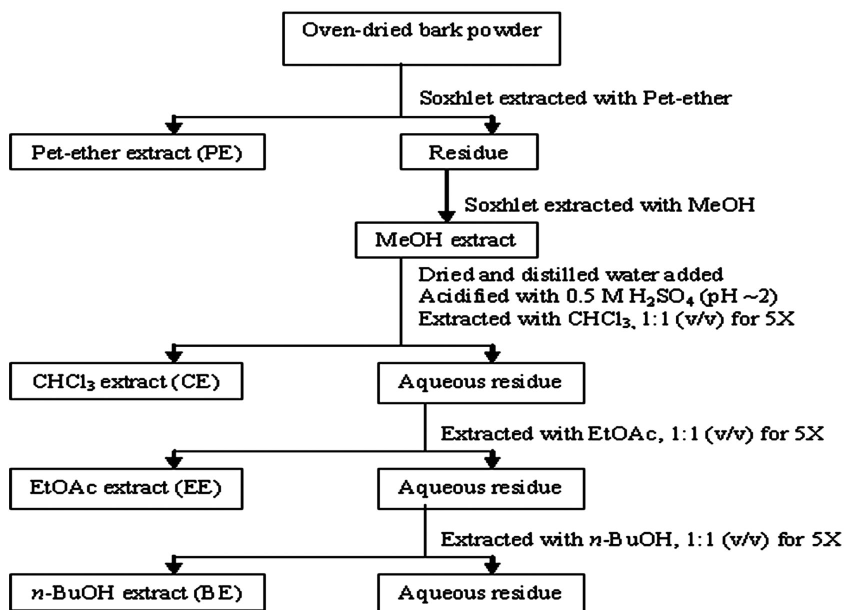

Figure 1. Extraction scheme for preparation of various extracts from the bark of *R. apiculata.*

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Extraction and Wood Impregnation of Bark Powder Extracts

Logs of *R. apiculata* were debarked; bark was cleaned and cut into pieces (8 mm × 8 mm); dried at 50°C for one week, and ground by mechanical grinder (Retsch GmbH) to 250 mesh size. Bark was extracted by a modified method of Sakushima et al.[20] (Figure 1). Ground bark (620 g) was extracted sequentially in a Soxhlet extractor first with petroleum ether (6–80°C) and then with methanol for 6 hours, respectively. The extraction process was repeated several times to get enough extract in order to carry out chemical and biological testing. Each extract was then concentrated under reduced pressure in a rotary evaporator below 50°C. Yield of extract is given by the ratio of mass of the extract to mass of ground bark. The former extract (PE) gave an orange-yellow wax (0.96%), while the latter extract (methanol) gave a dark chocolate gum (4.11%).

The dried methanol extract was further dissolved in distilled water, acidified with 0.5 M H_2SO_4 (pH ∼2.6), and filtered to remove any solid materials. Liquid-liquid extraction was carried out in a separatory funnel as per the scheme in Figure 1. First, the content was extracted with (100 × 5) ml of chloroform in a 1:1 ratio by volume, and successively with (100 × 5) ml of ethyl acetate and (100 × 5) ml of *n*-butanol. The extraction was repeated with a new portion of

methanol extract in aqueous form and fresh organic solvents in order to gain as much extract as possible. Each organic extract was combined and evaporated to dryness under reduced pressure. Chloroform extract (CE) yielded a yellow solid (6.3570 g), ethyl acetate extract (EE) gave reddish brown syrup (10.5951 g), while *n*-butanol extract (BE) gave a brown gum (4.2385 g). The entire extraction process is summarized schematically in Figure 1.

Wood impregnation was adopted from the method described by Nakayama et al.,[21] with slight modification. A cylindrical, glass made chamber (5.0 cm diameter $\times$ 35.0 cm tall) was used instead of a metal pressure chamber. First, dried and defect-free pine sap wood (*Pinus* sp.) was cut into small blocks of dimensions (2.0 $\times$ 2.0 $\times$ 1.0) cm^3 and smoothed with sandpaper. Then, six wood blocks were placed into the chamber and evacuated at about 10^{-3} mmHg (Dynavac) for five hours to remove air from the blocks. After evacuation, 100 ml of 500 ppm PE (weight/volume, w/v) was introduced by releasing the vacuum to atmospheric pressure to fill the chamber above the wood blocks. After 6 hours soaking in the extract, the wood blocks were removed; excess solution was wiped off and allowed to dry in an oven at 60°C before weighing. The impregnation process was repeated for 500 ppm of CE, EE and BE, each with six replications. Weight percent gain (WPG %) was computed from the weight of untreated and treated wood.

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Fractionation and Impregnation of Ethyl Acetate Extract

Of the bark extracts tested, EE appeared to have superior anti-termite activity. It was believed that this extract might contain certain natural chemicals that were potent inhibitor against termite attack. Thus, interest had been taken in an attempt to separate and characterize these bioactive substances.

Column chromatography was used for separation on a larger scale to achieve adequate amounts of purified materials used for further biological tests and chemical characterizations. Fractionation of EE was carried out using this method. A clean glass column (4.0 cm diameter $\times$ 60.0 cm length), containing glass wool at the bottom, was packed with a slurry of silica gel 60 (250 g, 230–400 mesh ASTM, Fluka) mixed with solvent system of hexane/ethyl acetate (9:1, v/v). After leaving it for settlement, 9.8971 g of the EE was then dissolved in a minimum amount of the solvent mixture stated earlier and applied onto the silica gel column. Elution was performed sequentially with step-wise solvent systems (450 ml each) of hexane/ethyl acetate mixture (9:1; 5:5, v/v), and ethyl acetate, respectively. The eluate was collected in 15 ml fractions and the average rate of collection was 3 ml per minute. There were totally 86 fractions collected.

The collected 86 fractions were then examined by TLC, and those with the same profile (fraction 1–28, 29–65, and 66–86) were combined to yield the corresponding 3 main fractions F1–F3, respectively. Solvent in each fraction

was removed under reduced pressure. F1 appeared as yellowish oil (0.8085 g), F2 as yellow solid (5.6425 g), while F3 as brown syrup (3.2340 g).

Wood impregnation process was carried out using 500 ppm of each fraction (F1, F2, and F3; w/v; ethyl acetate used as solvent) as described previously. Assessment was based on weight percent gains of the treated wood blocks.

DETERMINATION OF TOTAL PHENOLIC CONTENT (TPC) BY FOLIN CIOCALTEAU ASSAY

Total phenolic content of the bark extracts was determined by a colorimetric assay described by Loo et al.[22] with slight modifications. A 1 ml methanolic PE extract was pipetted into a test tube containing 5 ml of 10% Folin Ciocalteu reagent and 4 ml of 7.5% (w/v) sodium carbonate solution. The contents were vortexed for 15 s and then left to stand at room temperature for 30 min. The absorbance of the mixture solution was measured using a Jasco V-530 UV/VIS Spectrophotometer at 765 nm. This procedure was repeated for CE, EE, and BE extracts. Gallic acid solutions (1 ml) with a concentration range of 20–200 μg/ml were used to make the calibration curve. Estimation of phenolic compounds in the extracts was carried out in triplicate. The results reported were mean values expressed as gallic acid equivalents (GAE), which indicates the phenolic content as the amount of gallic acid in milligrams per gram of dry weight of a sample.

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EVALUATION OF ANTIOXIDANT ACTIVITY

DPPH Free Radical-Scavenging Activity

The radical scavenging activity of the bark extracts was assayed by the DPPH (1, 1-diphenyl-2-picrylhydrazyl) method as described by Loo et al.[22] and Gao et al.[13] The DPPH radical standard solution (9.25 mg in 250 ml of methanol) was prepared fresh daily, covered with aluminium foil and stored at 4°C between measurements. A 100 μl of methanolic PE extract at different concentration (20–160 μg/ml) was added to 3 ml of this DPPH solution. The reaction mixture was shaken by hand and then kept in darkness for 30 min at ambient conditions. The absorbance was measured at 517 nm. The decrease in absorbance is due to the reduction of the DPPH radical (i.e., lower absorbance indicates higher free radical scavenging activity). The procedure was repeated for CE, EE, and BE extracts. Two synthetic antioxidants, butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), were used as reference compounds. The

antioxidant capacity was expressed as percent inhibition, which was calculated by the Eq. (3):

$$\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} \times 100 \quad (3)$$

where A_0 equals absorbance without extracts/reference compounds and A_1 equals absorbance with extracts/reference compounds at 517 nm. The IC_{50} is the antioxidant concentration that inhibits the DPPH reaction by 50% under the experimental conditions. This was calculated by plotting the inhibition percentage against the extract concentration. Low IC_{50} values indicate high free-radical-scavenging activities. All tests were performed in triplicate and the values were averaged.

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RESULTS AND DISCUSSION

Termite Bioassay on Different Bark Species (1st Round)

A comparison of the resistance levels of five bark species against *C. gestroi* in the no-choice feeding test is summarized in Table 1. The ranking of the bark preference, which was determined from the mean mass loss, was as follows: rubber > pine > acacia > mangrove > mahogany.

Termite Bioassay on Different Extracts Impregnated Wood Blocks (2nd Round)

The comparison of the efficacy levels of each bark extract impregnated wood blocks on termite feeding is tabulated in Table 2.

Generally, as the wood consumption rate increased, wood mass loss also increased in the study. It is naturally believed that high wood mass loss is due to high wood consumption by termites.[20] Therefore, at the same concentration level, the ranking of the termiticidal reactivity of each bark extract on the basis of wood mass loss, in descending order, was as follows: EE > CE > PE > BE.

Counting termites' mortality may be more accurate than weighing, especially where different size castes are present.[14] The sterile sand was used as matrix to attain humidity of the test containers and therefore the tendency of the termites tunneling into sand and the survival rates could not be calculated by this adopted standard method.

It is perceptible that the *R. apiculata* bark extracts overall showed improvement of termite inhibition as compared to the controls. It was presumed that the extract contained mostly non-polar or neutral compounds, such as cutins, lipids,

Table 2. Mean weight percent gains (WPG), weight losses, and visual ratings of the tested wood blocks, and approximate termite mortality after six weeks of no-choice laboratory test (2nd round).

Treatment	WPG (%)	Weight loss[a] g	Weight loss[a] %	Visual rating	Approximate mortality
PE	10.65	0.8339 ± 0.1358	35.79 ± 4.31	5.5	Slight
CE	10.70	0.6030 ± 0.0954	27.37 ± 3.91	6.3	Slight
EE	11.26	0.2413 ± 0.0244	10.54 ± 0.78	9.0	Moderate
BE	13.02	0.8983 ± 0.1834	38.24 ± 7.03	5.8	Slight
Control					
U1	—	1.0135 ± 0.2349	50.33 ± 10.26	2.0	Slight
P	0.02	1.2639 ± 0.2124	61.82 ± 9.10	1.3	Slight
C	0.01	1.1952 ± 0.1974	61.60 ± 9.37	1.3	Slight
E1	0.00	1.2362 ± 0.1806	58.92 ± 6.66	2.0	Slight
B	0.05	1.0535 ± 0.0919	52.73 ± 7.83	2.7	Slight

[a]Values are means ± standard deviations from six replications. PE, petroleum ether; CE, chloroform; EE, ethyl acetate; BE, *n*-butanol extracts impregnated wood blocks (500 ppm each) and U1, untreated; P, petroleum ether; C, chloroform; E1, ethyl acetate; B, *n*-butanol solvents treated wood blocks served as control and added controls, respectively.

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fats, and waxes, as it is usually used as defatting agent.[15,23,24] By increasing the solvent polarity, average weight losses of the treated wood blocks were reduced apparently. Chloroform, a less polar solvent, did not remove all the toxic substances. Successive extraction with ethyl acetate produced extra toxic substance to the termites. The lethal substance in both extracts might have differed only quantitatively since they elicited similar termite resistant activities. In spite of the fact that *n*-butanol is the most polar solvent among the others, BE impregnated woods did not appear to show the lowest mean mass loss and the mortality was also not that great compared to both the former solvents. It is suggested that ethyl acetate was enough polar to remove the detrimental ingredients from the aqueous methanol extract.

It is noted that one complete separation of the extract constituents is rarely achieved and the same compounds may be recovered (in varying proportion) in several fractions. Extraction methods and sample preparations can significantly affect the yield and the profile of natural occurring compounds.[15,25] By using these solvents in succession, the research attempted to obtain reasonable complete extractions of the termite-detrimental ingredients, but the data clearly illustrate that no single solvent is applicable to remove all the lethal materials.

Table 3. Mean weight percent gains (WPG), weight losses, and visual ratings of the tested wood blocks, and approximate termite mortality after six weeks of no-choice laboratory test (3rd round)

Treatment	WPG(%)	Weight loss[a]		Visual rating	Approximate Mortality
		g	%		
F1	10.68	0.9828 ± 0.0715	42.59 ± 2.42	4.0	Slight
F2	10.16	0.1227 ± 0.0299	5.53 ± 1.21	9.2	Moderate
F3	11.20	0.6325 ± 0.1851	27.76 ± 8.15	8.0	Slight
Control					
U2	—	1.1496 ± 0.2062	54.36 ± 7.74	2.7	Slight
E2	0.01	1.2290 ± 0.1566	61.20 ± 5.51	2.7	Slight

[a]Values are means ± standard deviations from six replications. F1, fraction 1; F2, fraction 2; F3, fraction 3 impregnated wood blocks (500 ppm each) and U2, untreated; E2, ethyl acetate solvent treated wood blocks served as control and added control, respectively.

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Termite Bioassay on Different Fractions Impregnated Wood Blocks (3rd Round)

Fractions F1–F3 was subsequently subjected to termite test in order to monitor sudden activity changes due to the chromatographic fractionation step. The comparison of the efficacy levels of each fraction impregnated wood blocks on termite feeding is given in Table 3.

The average weight percent gains (WPG) of the impregnated wood blocks at 500 ppm were around 10% of the initial weights. All timbers are not equally suitable for impregnation by wood preservatives. Recognizing that the sapwood is actively involved in transporting inorganic nutrients from the roots to the rest of the tree, it seems logical that this tissue would have the highest metal-chelating capacity.[15] Therefore, sapwood is much easier to impregnate than heartwood.

All the containers assembled with sand, water, and termites, but without test blocks showed virtually complete survival after the first week of bioassay. Besides, the great tunneling made by the termites was thereby indicative that test procedures have been followed and vigorous termites were being used. Thus, the mortality reported here could be probably due to the exposure of the termites to the chemicals impregnated into the test blocks. Considering mean weight loss was the termite resistant measurement, the ranking of the reactivity of these fractions at the same concentration level, in descending order, was as follows: F2 > F3 > F1. The added control exhibited no accountable effects toward termite inhibition as the average wood loss was greater than half of its

initial weight. The volatile solvent, ethyl acetate, was likely to be removed after being oven-dried at 50°C and contributed to merely nil of weight percent gain.

All the three fractions had performed in different degrees of wood protection against termite attack, particularly F2, suggesting that the fractionation step by means of TLC profiles analysis did not destroy the anti-termite activity, or rather, it showed a decreasing in the mean wood loss compared to before fractionation. Therefore, according to Millar and Sims.[26] we had probably separated antagonistic constituents from the EE.

Therefore, we believed that the termiticidal activities shown by these fractions (F1, F2, and F3) were most probably triggered by some bioactive compounds.

Chemical and Spectral Analyses on EE

Chemical and spectral analyses conducted suggested that EE might be associated with the class of compound of aromatic carboxylic acids or phenolics.

Determination of pH and Ferric Chloride ($FeCl_3$) Test

The pH of the aqueous EE was about 3, suggesting the existence of acidic compounds. Meanwhile, dark color formation was observed after putting two to three drops of $FeCl_3$ solution into approximately 1 ml of this extract in a test tube. According to Seikel[16] and Harborne,[15] most phenols and a number of other compounds with moderately acidic hydroxyl groups will react with $FeCl_3$to give colored complexes (black colored). Hence, it elicited the presence of phenolic compounds.

UV and Visible Spectroscopy

The value of UV and visible spectra in identifying unknown constituents is obviously related to the relative complexity of the spectrum and to the general position of the wavelength maxima. The utility of spectral measurements for identification purposes can be greatly enhanced by repeating measurement made in neutral solution, either at a range of different pH values or in the presence of particular inorganic salts. UV spectra of the EE in 95% ethanol and after the addition of the shift reagent, 2M NaOH were obtained by the method described earlier. In 95% ethanol, this extract displayed a UV spectrum with wavelength maxima at 218, 274, and 288 nm (Figure 2). These bands were shifted toward shorter wavelengths with increases in absorbance on addition of 2 drops of 2M NaOH. The wavelength maxima were at 204, 236, and 274 nm. This characteristic phenomenon infers the presence of aromatic carboxylic acids as they undergo hypsochromic shifts when alkali is added to their neutral solutions.[15]

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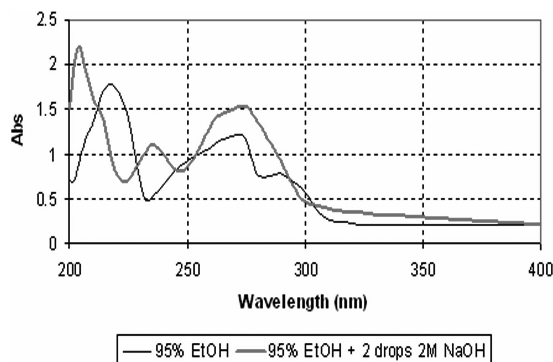

Figure 2. Ultraviolet absorption spectra of the EE before and after the addition of shift reagent.

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On the other hand, alkaline solutions of phenolic compounds in which the hydroxyl group is conjugated through the ring with a carbonyl group will have maxima greater than 300 nm. However, no manifest peaks could be observed in this region, indicating the absence of the conjugated (esterified) side chains and the maxima at approximately 250–300 nm of these spectra are characteristic for the absorption of the phenolate ion of simple non-conjugated aromatic hydroxyl groups.[27] Consequently, we ascribed the bioactive compounds in the EE to a mixture comprised mainly of aromatic carboxylic acids or phenolic acids and most probably they existed in non-bound form.

Determination of Total Phenolic Content (TPC)

Phenols are very important plant constituents because of their radical scavenging ability due to their hydroxyl groups. The phenolic content may contribute directly to the antioxidative action.[13,22] The content of phenolics in the bark

Table 4. Total phenolic content (TPC) of different bark extracts of *Rhizophora apiculata*

Extract	TPC in dry extract (mgGAE/g)*
PE	216.3 ± 5.2
CE	596.8 ± 8.9
EE	804.2 ± 6.0
BE	347.7 ± 11.3

PE, petroleum ether; CE, chloroform; EE, ethyl acetate; BE, *n*-butanol.
*Values are means ± standard deviations from three replications; GAE, gallic acid equivalent.

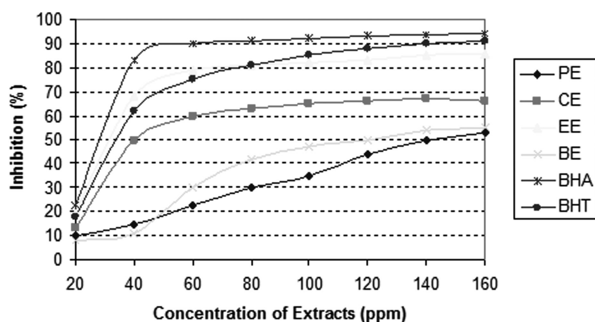

Figure 3. Correlation of total phenolic content (expressed as gallic acid equivalent, GAE) and termite bioactivity (weight loss %).

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extracts was determined from a regression equation of the calibration curve ($y = 0.0052x + 0.0279$, $R^2 = 0.9894$) and was expressed in gallic acid equivalents (GAE). As can be seen from Table 4, the distribution of phenolic compounds in the bark extracts of *R. apiculata* demonstrated that EE contained the highest amount, 804.2 mgGAE/g extract, followed by CE (596.8 mgGAE/g extract), BE (347.7 mgGAE/g extract), and PE (216.3 mgGAE/g extract). The result elicited that different organic solvent has different ability in extracting phenolic compounds from aqueous methanolic extract. It has been reported that ethyl acetate is a recommended organic solvent for extraction of low molecular weight phenols from oak wood.[28] The relation between total phenolic content (GAE) and termite bioactivity (weight loss %) has been elucidated in Figure 3.

Effect of DPPH Radical-Scavenging Activity

DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. A freshly prepared DPPH solution exhibits a deep purple color with an absorption maximum at 517 nm. This purple color generally fades when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH (by providing a hydrogen atom or by electron donation) and convert it to a colorless product, resulting in a decrease in absorbance at 517 nm. In brief, the reduction capacity of DPPH was determined by the decrease in its absorbance at 517 nm, which is reduced by antioxidants.[13,22] The radical-scavenging properties of the various bark extracts of *R. apiculata* are given in Figure 4 and Table 5. All the extracts exhibited concentration-dependent DPPH radical scavenging activity. Among these extracts, EE showed the most activity (85.73%), which was comparable to BHT control with the $IC_{50} = 31.47$ ppm higher than BHT (33.49 ppm). Both the synthetic antioxidants revealed a different degree of scavenging activity. BHA (94.24%) performed better than BHT (91.61%) and this result correlated

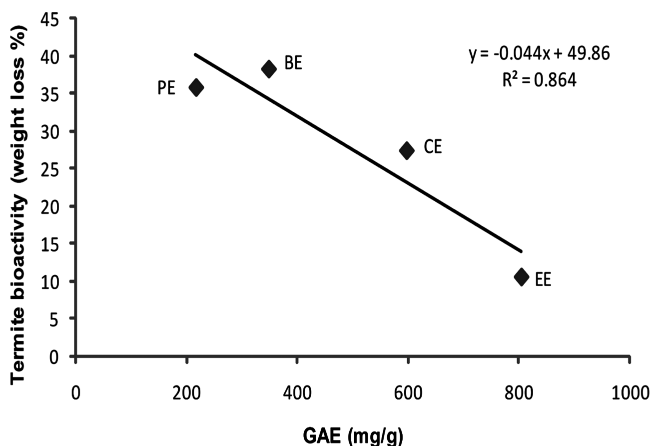

Figure 4. Radical scavenging ability of the bark extracts of *Rhizophora apiculata* and the reference compounds, that is, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) against 1,1-diphenyl-2-picrylhydrazyl hydrate radical (DPPH).

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well to that reported by Loo et al.[22] The CE exhibited moderate antioxidant activity with inhibition percentages of 66.37%, followed by BE (55.80%) and PE (53.42%). On the basis of IC_{50} value (Table 5), the ranking of the free radical scavenging activity of these bark extracts, in descending order, was as follow: EE > CE > BE > PE.

The trend of antioxidant activity is similar to that of second round termite bioassay (EE > CE > PE > BE), although discrepancies in the ranking of the latter two extracts (PE and BE) were present. PE with the lowest TPC, 216.3 mgGAE/g exhibited the least free radical scavenging activity (Tables 4 and 5). In

Table 5. Percentage inhibition (at 160 μg/ml) and 50% inhibition concentration, IC_{50}values of the bark extracts and reference compounds

Extract	Inhibition (%)*	IC_{50} (μg/ml)
PE	53.42 ± 4.76	140.01
CE	66.37 ± 7.13	39.96
EE	85.73 ± 6.53	31.47
BE	55.80 ± 5.08	120.00
Control		
BHA	94.24 ± 7.12	28.23
BHT	91.61 ± 5.85	33.49

PE, petroleum ether; CE, chloroform; EE, ethyl acetate; BE, *n*-butanol; BHA, butylated hydroxyanisole; BHT, butylated hydroxytoluene.

*Values are means ± standard deviations from three replications.

the second round termite bioassay, however, it showed greater activity than BE (TPC = 347.7 mgGAE/g). It is suggested that PE contained other non-phenolic compounds, most probably of non-polar compounds, which was able to deter termite infestation although its activity was not significant compared to EE and CE with relatively higher TPC (804.2 and 596.8 mgGAE/g, respectively). In addition to the inherent objectionable flavors of phenolic compounds, the results suggested that antioxidants might also play a role in preventing termite attack. In fact, recent reports have suggested that free radical species may help to disrupt the cell walls of wood and thus facilitate the penetration of white- and brown-rot fungal enzymes.[29–31] Accordingly, the free-radical-scavenging properties of methanol extracts from some decay-resistant tree species may inhibit this early step in the decay process. Recent studies have also shown that the combination of antioxidants and an organic biocide gives wood enhanced protection against termite attack and fungal decay.[32] Recent work has found that a non-biocidal antioxidant repelled and caused some mortality with two species of termites, and the authors suggested that both the toxicity and inherent antioxidant properties of phenolic extractives were important in natural termite resistance of certain heartwoods.[10]

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CONCLUSION

Wood blocks treated with mangrove bark extracts exhibited different degrees of termite inhibitions as compared to the controls, with the ranking of EE > CE > PE > BE. As a result, EE showed potent anti-termite activity on the basis of the least wood loss test, and was further fractionated into fractions F1, F2 and F3. The ranking of the reactivity of these fractions in terms of termite resistance was F2 > F3 > F1, at 500 ppm. The slight improvement in the percentage of wood loss suggested antagonistic compounds have been separated and the fractionation of the EE using silica gel column chromatography did not destroy its anti-termite property. The distribution of phenolic compounds in the bark extracts of *R. apiculata* demonstrated that EE contained the highest amount, 804.2 mgGAE/g extract, followed by CE (596.8 mgGAE/g extract), BE (347.7 mgGAE/g extract), and PE (216.3 mgGAE/g extract). On the basis of IC_{50} value, the ranking of the free radical scavenging activity of these bark extracts, in descending order, was found to be as follows: EE > CE > BE > PE. Thus, in addition to the inherent objectionable flavors of phenolic compounds, the results suggested that antioxidants also play a role in preventing termite attack.

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