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PHYTOCHEMICAL ANALYSIS AND ISOLATION OF SECONDARY METABOLITES FROM *Persea declinata*

(Analisis Fitokimia dan Pengasingan Metabolit Sekunder Daripada *Persea declinata*)

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Abstract

Phytochemical analysis of *Persea declinata* leaves and stembark extracts exhibited the presence of cardiac glycosides, coumarins, reducing sugars, steroids, flavonoids, tannins, and phenolic compounds. Sesamin **(1)**, γ-sitosterol **(2),** and palmitic acid **(3)** were isolated for the first time from ethyl acetate extract of the stembark of *P. declinata* by chromatographic methods. Their structures were elucidated based on spectroscopic analysis such as infrared (IR), gas chromatography-mass spectroscopy (GC-MS), nuclear magnetic resonance (NMR), and also involved comparison with data from literatures.

Keywords: *Persea declinata*, phytochemicals, sesamin, gamma sitosterol, palmitic acid

Abstrak

Analisis fitokimia ke atas daun dan kulit batang *Persea declinata* menunjukkan kehadiran sebatian glikosida kardiak, kumarin, gula penurun, steroid, flavonoid, tanin dan sebatian fenolik. Sesamin **(1)**, γ-sitosterol **(2)**, dan asid palmitik **(3)** telah diasingkan buat pertama kali daripada ekstrak etil asetat kulit batang *P. declinata* menggunakan kaedah kromatografi. Struktur sebatian telah dicirikan berdasarkan analisis spektroskopi seperti inframerah (IR), kromatografi gas-spektroskopi jisim (GC-MS), resonans magnet nuklear (NMR) dan juga melibatkan perbandingan data dengan kajian lepas.

Kata kunci: *Persea declinata*, fitokimia, sesamin, gamma sitosterol, asid palmitik

Introduction

Diseases such as diabetes, cancer, and hypertension continue to pose a significant threat to humanity globally. The use of conventional drugs in the treatment of diseases is usually associated with side effects and is typically costly. It is, therefore, necessary to continue to search for effective, safe, and affordable medicines from natural sources. Phytochemicals contained in plant parts, including fruits and vegetables, effectively reduce the risk of cardiovascular disease, cancer, Alzheimer's disease, stroke, cataracts, and help delay ageing [1].

Persea declinata is a plant that belongs to the genus *Persea* from the Lauraceae family. Species of the genus *Persea* have been used traditionally in treating diarrhoea, hypertension, infectious diseases [2], as well as gastric analgesic and anti-inflammatory agents [3]. *P. declinata* belongs to the same genus (*Persea*) with the popularly consumed healthy plant, *Persea americana* (avocado), which is extensively studied and reported for many pharmacological activities including antidiabetic, anti-inflammatory, anti-convulsion, antibacterial, antifungal, anthelmintic [4], initiating the hypolipidemic effect [5], and as trypanocidal agent [6]. Previous studies indicate that compounds isolated from the *Persea* species (avocado) had cytotoxic, insecticidal [7, 8], and antimycobacterial activity [9]. Various phytochemicals, including flavonoids, coumarins, phenolic compounds, aliphatic acetogenins (alkanols), had been isolated from the *Persea* species [3, 7, 9, 10, 11]. A recent review showed that the Lauraceae family is generally a good source of lignans and neolignans, the most chemotaxonomic characteristics of many species of the family [12]. Previous bioactivities studies suggest that certain plant compounds, such as sesamin, were potent antioxidant agents [13] and play a role in the amelioration of oxidative stress induced by CCl_4 [14]. γ-Sitosterol had been reported for antidiabetic property [15, 16]. Palmitic acid previously isolated from *Terminalia glaucescens* root bark [17] and *Canthium parviflorum* leaves [18] and the active crude extract from which it was identified in *Kigelia pinnata* leaves exhibited a good cytotoxic effect on human colon cancer cell line (HCT-116) [19]. *P. declinata* was recently reported as having apoptotic property [20], indicating that it could be an appropriate candidate as the principal drug for many other diseases.

However, despite promising medicinal values within the *Persea* species, no single report on *P. declinata* phytochemicals is currently available in the literature. The biological and chemical characterisation of plant species has led to promising sources of biologically potent compounds [21]. Therefore, this study was carried out to evaluate the classes of phytochemicals present in the leaves and stembark of *P. declinata*, and the isolation of compounds from its stembark. To our knowledge, this is the first report on the phytochemical screening and isolation of compounds from *P. declinata*.

Materials and Methods

Chemicals and reagents

All chemicals and reagents used in this work were of analytical grade.

Plant materials

The plant samples (leaves and stembark) were collected from Gunung Jerai, Kedah, Malaysia and verified by Dr. Shamsul Khamis, Universiti Kebangsaan Malaysia. The samples were deposited at the UKMB Herbarium with a voucher No. UKMB 4350.

General experimental procedure

^1H and ^{13}C NMR spectra were recorded on Bruker Avance (400 MHz) spectrometer, using deuterated chloroform ($CDCl_3$) as the solvent. The IR was recorded on PerkinElmer FT-IR spectrometer, while GC-MS data were acquired using Agilent 7820A (G4350). Thin-layer chromatography (TLC) was done on 0.20 mm precoated silica gel aluminium sheets (Merk Kieselgel 60 F_{254}). TLC spots were observed under the UV light at 254 nm and 365 nm. Vanillin sulphuric acid was used as the spraying reagent.

Extraction and phytochemical screening

The leaves and stembark of *P. declinata* were air-dried and triturated to a coarse powder using a grinding machine. Each sample (50 g) was separately macerated with methanol, and another set (50 g each) were extracted using Soxhlet for comparison of yield and phytochemicals present. The extracts were concentrated under vacuum using a rotary evaporator to obtain methanol leaves and stembark extracts from maceration and Soxhlet methods.

Phytochemical screening of the leaf and stembark extract of *P. declinata*

Preliminary phytochemical screening of leaves and stembark of *P. declinata* were done using the standard procedures of Harborne [22] and Trease and Evans [23], with slight modification as follows;

Tests for simple sugar (Fehling's test)

Approximately 1 mL of Fehling's solutions **A** and **B** were mixed and warmed for 1 minute. The mixture was added to an equal volume of the test solution. The solution was heated in a boiling water bath for 5-10 minutes. A yellow precipitate was formed, which then turned to brick red.

Test for anthraquinone glycosides (Borntrager's test)

Each test sample was mixed with 3 mL of dilute H_2SO_4, warmed, and filtered. The filtrate was cooled, and an equal volume of benzene was added to it. The solution was shaken vigorously to separate the organic layer. An equal volume of dilute ammonia solution was added to the organic layer. The inability of the ammonia layer to turn pink indicates the absence of anthraquinone glycosides.

Test for cardiac glycosides (Keller-Killiani test)

A volume of 1 mL concentrated H_2SO_4, 2 mL of glacial acetic acid, and 1 drop of $FeCl_3$ solutions were added to 5 mL of the extract. The appearance of a brown ring indicated the presence of cardiac glycosides.

Test for coumarins

The concentration of 10% NaOH was added to 2 mL of the extract. The mixture was shaken vigorously for 5 min. The appearance of yellow colouration indicated the presence of coumarins.

Test for saponins (foam test)

About 20 mL of distilled water was added to 1 mL of the extract in a measuring cylinder. The mixture was shaken well for 15 min, and no layer of foam was observed.

Tests for quinone

Concentrated H_2SO_4 was mixed with 2 mL of the extract in a test-tube and shaken vigorously for 5 min. No visible red colour was observed.

Test for steroids (Salkowski test)

About 2 mL of concentrated H_2SO_4 and 2 mL of chloroform were added to 2 mL of the extract. The solution was shaken vigorously. The chloroform layer changed to red, while the acid layer showed greenish-yellow fluorescence.

Test for alkaloids (Mayer's test)

Approximately 1 mL of dilute HCl and 1 mL of Mayer's reagent were added to 2-3 mL of filtrate, and well shaken. The formation of yellow precipitate indicated the presence of alkaloids.

Test for Flavonoids (Shinoda test)

Concentrated hydrochloric acid (HCl) was added in drops to the test solution. Magnesium turnings were put into the solution. The pink-red colour observed indicated the presence of flavonoids.

Test for tannins and phenolic compounds $FeCI_3$ solution test

A solution of 5% $FeCl_3$ was added to 2 mL of the extract. A deep blue-black colouration indicated tannins and phenolic compounds in the test sample.

Extraction and isolation procedure

Powdered stembark sample of *P. declinata* (1.5 kg) was macerated successively in increasing solvent polarity with *n*-hexane, ethyl acetate, and methanol each for 72 hours. The extracts were separately concentrated under vacuum yielding *n*-hexane, ethyl acetate, and methanol crude extracts – PDHSE, PDESE, and PDMSE. Silica gel 60 Å (Mesh size 230–400) was used for Vacum Liquid Chromatography (VLC) of the EtOAc extract, PDESE (36.44 g; extract: silica gel = 1:30) yielding 35 fractions. Fractions were pooled based on TLC profiles resulting in five sub-fractions and were labelled as A–E. Sub-fraction B (3.48 g) was further fractionated using VLC and 44 sub-fractions were obtained, which were then pooled based on the Thin-Layer Chromatography (TLC) profile and labelled as PDSE1-PDSE8. Further purification of PDSE2 by column chromatography (CC) resulted in the isolation of sesamin (1), γ-sitosterol (2), and palmitic acid (3).

Results and Discussion

The yield of extraction of leaves and stembark of *P. declinata* using maceration and Soxhlet are shown in Table 1. Leaves extract yielded higher (13.33%) using Soxhlet compared to the maceration method (6.11%). The results of phytochemical screening of leaves and stembark maceration and Soxhlet extracts of *P. declinata* revealed the presence of reducing sugars, cardiac glycosides, coumarins, steroids, flavonoids, tannins, and alkaloids in all extracts (Table 2). Therefore, the plant under study may possess bioactive compounds with specific medicinal values.

Isolation and purification of compounds from *P. declinata* stembark

Sesamin (1)

Gravity column chromatographic separation on PDSE2 yielded compound **(1)** (5.9 mg) as white needle solid R_f = 0.52 in PE:EtOAc 4:1. The IR spectrum of **(1)** showed a band at 2881 cm^{-1} for (C-H) and an aromatic (C=C) ring stretch band at 1653 cm^{-1}. The ^1H NMR spectrum of **(1)** showed signals at δ 6.87 (2H, *d*, *J* =1.2 Hz, H-2′,2″), 6.80-6.84 (4H, m, H-5′,5″,6′,6″) representing aromatic protons. The singlet peak at δ 5.97 (4H, *s*, H-7′,7″) was assignable to methylenedioxy protons, while the signal at δ 3.05 (2H, *m*, H-1,5) was assigned to the methine protons. Other prominent chemical shifts at δ 3.89 (2H, *dd*, *J* = 3.0 and 9.0 Hz, H-2,6) and δ at 4.26 (2H, d, *J*=4.0 Hz, H-4,8β), 4.74 (2H, dt, *J*=7.0 and 9.0 Hz, H-4,8α) were assigned to methine and methylene protons, respectively. The ^{13}C spectrum showed 10 peaks representing 20 carbons in the compound being symmetrical. The peaks at chemical shifts δ 106.5, 108.1, and 119.3 were assigned to aromatic carbons at positions 2′/2″, 5′/5″, and 6′/6″ respectively. C-7′/7″ were represented by the peak at δ 101.0, while δ 135.1 was assigned to C-1′/1″. The GC-MS data shows molecular ion peak [M^+] of *m/z* 354.2 (cal. 354) corresponding to the molecular formula of compound **(1)** $C_{20}H_{18}O_6$, which has been reported previously [24]. These spectroscopic data and the data from the literature [24] confirmed compound **(1)** as sesamin.

γ- Sitosterol (2)

Continuous gravity elution of PDSE2 resulted in the isolation of compound **(2)** as a white solid (13 mg) R_f = 0.55 in PE:EtOAc 4:1. Treatment of the TLC spot with vanillin sulphuric acid reagent gave a purple colouration, indicating a terpene type of compound. The IR spectrum of **(2)** shows a broad absorption band at 3390 cm^{-1} characteristics of O-H group, while the band at 2933 cm^{-3} corresponds to sp^3 C-H stretch. The ^1H NMR of compound **(2)** shows chemical shifts at δ 0.69 (3H, *s*, H- 18), δ 1.02 (3H, *s*, H-19), (3H, *d*, *J*=6.4 Hz, H-21), δ 0.82 (3H, *d*, *J*=2.0 Hz, H-26), δ 0.84 (3H, *d*, *J*=2.0 Hz, H-27), δ 0.86 (3H, H-29) representing methyl groups in the compound. Evidence for oxymethine proton at position C-3 was indicated by the peak at δ 3.54. The signal at δ 5.37 was for the proton attached to C=C at position C-6. ^{13}C chemical shifts at δ 121 and δ 140 confirm the presence of olefinic carbons C-6 and C-5, respectively. Oxymethine carbon C-3 was represented by the signal at δ71.82. The various peaks at δ 11.85–19.80 represent the methyl groups. The GC-MS spectrum showed a molecular ion peak [M^+] at *m/z* 414.5 (calc. 414) corresponding to the molecular formula of $C_{29}H_{50}O$ [16].

Palmitic acid (3)

Compound **(3)** was also isolated from PDSE2 as a white solid (10.8 mg), R_f = 0.78 in PE:EtOAc 4:1. The IR sharp band at 3325 cm^{-1} and band at 2929 cm^{-1} correspond to O-H and C-H stretch. Evidence for the presence of a carbonyl group was seen by the appearance of C=O stretch band at 1713 cm^{-1}. The ^1H NMR of compound **(3)** indicates the presence of long-chain of multiple methylene protons at δ 1.277, while methyl protons were represented by a triplet peak at δ 0.90. Protons at C-2 were represented by the triplet signal at 2.35 (2H, *t*, *J*=7.2 Hz, H-2) shifted further being near to -COOH group. The ^{13}C spectrum shows peak at δ 179, which represents the carbonyl carbon (C=O) at position C-1 and the chemical shift position of methylene carbon at position C-2 was further shifted to δ 34.03 due to de-shielding effect of the neighbouring carbonyl group. Other methylene carbons (C-4 to C-12) were represented by the intense peak and other peaks at δ 24.69-30.86. Its molecular formula $C_{16}H_{32}O_2$, was confirmed by the presence of molecular ion peak [M^+] at *m/z* 256.3 (cal. 256) in the GC-MS [17].

Spectroscopic data for isolated compounds

Sesamin (1)

IR (Neat) v_{max} cm^{-1} ($CDCl_3$): 2881.30 (C-H), 1653.15 (C=C aromatic). **^{1}H-NMR** ($CDCl_3$, 400 MHz): **δ_H** 3.05 (2H, *m*, H-1,5), 3.89 (2H, *dd*, *J* = 3.0 and 9.0 Hz, H-2,6), 4.26 (2H, d, *J*=4.0 Hz, H-4,8β), 4.74 (2H, dt, *J*=7.0 and 9.0 Hz, H-4,8α), 5.97 (4H, *s*, H-7′,7″), 6.80-6.84 (4H, m, H-5′,5″,6′,6″), 6.87 (2H, *d*, *J* =1.2 Hz, H-2′,2″). **^{13}C-NMR** ($CDCl_3$, 100 MHz): **δ_C** 135.1 (C-1'/1''), 54.3 (C-1/5), 85.7 (C-2/8), 71.7 (C-4/6), 106.5 (C-2'/2''), 147.9 (C-3'/3''), 147.1 (C-4'/4''), 108.1 (C-5'/5''), 119.3 (C-6'/6''). **GC-MS**: 65,103,149,203,239, 281, 317, 354.

γ- Sitosterol (2)

IR (Neat) v_{max} cm^{-1} ($CDCl_3$): 3390.10 (OH), 2933.58 (C-H). **^{1}H-NMR** ($CDCl_3$, 400 MHz): **δ_H** 3.50 (1H, *m*, H-3), 5.36 (1H, *t*, *J* = 2.16 Hz, H-6), 0.69 (3H, *s*, H- 18), 0.93 (3H, d, *J* = 6.4 Hz, H-21), 0.82 (3H, *d*, *J* = 2.0 Hz, H-26), 0.84 (3H, *d*, *J* = 2.0 Hz, H-27), 0.86 (3H, H-29). **^{13}C-NMR** ($CDCl_3$, 100 MHz): **δ_C** 37.26 (C-1), 32.41 (C-2), 71.82 (C-3), 42.26 (C-4), 140 (C-5), 121.71 (C-6), 31.92 (C-7), 31.63 (C-8), 50.23 (C-9), 33.72 (C-10), 21.09 (C-11), 39.79 (C-12), 42.33 (C-13), 56.08 (C-14), 26.13 (C-15), 28.24 (C-16), 56.78 (C-17), 11.85 (C-18), 19.39 (C-19), 36.51 (C-20), 19.04 (C-21), 33.97 (C-22), 26.13 (C-23), 45.86 (C-24), 29.19 (C-25), 18.25 (C-26), 19.80 (C-27), 23.09 (C-28), 12.22 (C-29). **GC-MS:** 57, 69, 81, 95, 105, 119, 133, 145, 159, 173, 187, 199, 213, 222, 231, 241, 255, 273, 283, 303, 329, 341, 354, 371, 381, 396, 414.

Palmitic acid (3)

IR (Neat) v_{max} cm^{-1} ($CDCl_3$): 3325.78 (OH), 1713.95 (C=O), 2929.34 (C-H). **^{1}H-NMR** ($CDCl_3$, 400 MHz): **δ_H** 2.35 (2H, *t*, *J* = 7.2 Hz, H-2), 1.61 (2H, *m*, 7.2, 7.6 Hz, H-3), 1.27 (22H, *s*, H-4 to H-13), 1.33, 0.91 (3H, *t*, *J* = 6.4 Hz). δ ^{13}C: 179.87, 34.03, 24.19, 29.06-30.86, 30.86, 22.68, 14.08. **^{13}C-NMR** ($CDCl_3$, 100 MHz): **δ_C** 179.87 (C-1), 34.03 (C-2), 24.19 (C-3), 29.06-30.86 (C-4-13), 30.86 (C-14), 22.68 (C-15), 14.08 (C-16). **GC-MS:** 51, 60, 73, 83, 97, 107, 115, 129, 143, 157, 171, 185, 199, 213, 227, 239, 143, 157, 171, 185, 199, 213, 227, 239, 248, 256.

Phytochemical investigation of the leaves and stembark of *P. declinata* indicated the presence of various classes of secondary metabolites, including reducing sugars, cardiac glycosides, coumarins, steroids, flavonoids, tannins, and alkaloids. The classes of compounds were known to possess medicinal values. The compounds **(1)**, **(2)**, and **(3)** belonging to lignans, sterols, and fatty acids respectively were isolated and reported for the first time from *P. declinata* and the genus *Persea* (Figure 1). Therefore, *P. declinata* containing these bioactive principles is a good candidate for further investigations of its medicinal potentials. As such, isolation and bioactivity studies of its various parts are currently on-going.

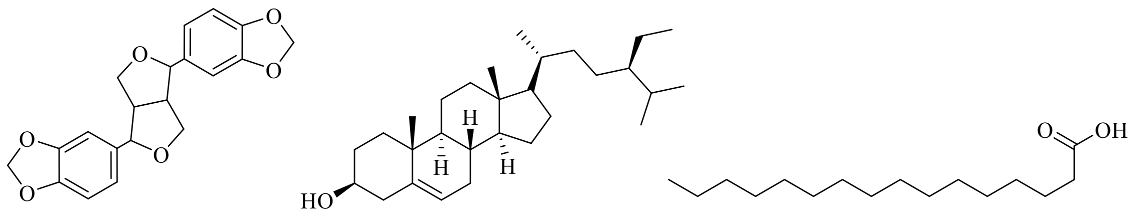
Table 1. Comparison of the yield of maceration and Soxhlet extraction methods

Sample	**Extraction method**	**Maceration**	**Soxhlet extraction**
	Sample weight (g)	50	50
Stembark	Yield (g)	3.96	3.78
	Percentage (%)	7.92	7.55
Leaf	Yield (g)	3.06	6.67
	Percentage (%)	6.11	13.33

Table 2. Summary of phytochemical screening of the leaf and stembark methanol extracts of *P. declinata*

Sample test	**Stembark**		**Leaf**	
	Maceration	**Soxhlet**	**Maceration**	**Soxhlet**
Reducing sugars	+	+	+	+
Cardiac glycosides	+	+	+	+
Coumarins	++	++	++	++
Saponins	-	-	-	-
Quinones	-	-	-	-
Steroids	+	+	+	+
Flavonoids	+	+	+	+
Phenolic compounds	++	++	++	++
Alkaloids	+	+	+	+
Anthraquinones glycosides	-	-	-	-

+ = presence, ++ = intense, - = not present



(1) **(2)** **(3)**

Figure 1. Structures of Compounds **(1)**, **(2)** and **(3)**

Conclusion

Phytochemicals found present in both the leaves and stembark of *P. declinata* include reducing sugars, cardiac glycosides, coumarins, steroids, flavonoids, tannins, and phenolic compounds. Three compounds identified as sesamin **(1)**, γ- sitosterol **(2)**, and palmitic acid **(3)** were successfully isolated and elucidated using spectroscopic techniques. It is hoped that more isolation and pharmacological tests would be undertaken in the on-going work.

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