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Phytochemicals, Antimicrobials and Antioxidants Studies of the Stem Bark Extract from *Calophyllum ferrugineum*

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ABSTRACT

Traditionally, Calophyllum genus was used for swollen gums, arthritis, diarrhea, chronic abscess, skin infections, and lesions treatment. From the reported literature, C. ferrugineum from Sarawak has least studied about their phytochemical constituents and biological activities. The objectives of this study are to isolate and characterise the chemical components from C. ferrugineum and to determine their antimicrobial and antioxidant activity. The plant stem barks were collected from National Park in Sarawak and underwent extraction process. The extracts underwent the isolation and purification processes by using several chromatographic methods. Structural elucidation was achieved by using infrared, MS and NMR spectra. The isolation process from the species has afforded five known compounds namely isocalanone (1) and 1-hydroxy-7-methoxy-9H-xanthen-9-one (2), lupeol (3), friedelin (4) and diethylene glycol dibenzoate (5). The chloroform and methanol extracts showed strong inhibitions against S. aureus, B. subtilis, P. aeruginosa and E. coli with the MIC and MBC values ranging from 225 and 112.5 µg/mL. Isocalanone (1) and the methanol extract of C. ferrugineum showed potent antioxidant activity with the IC50 values of 28 ± 5.23 and 35 ± 3.69 µg/mL compared to ascorbic acid, respectively. The results obtained from this study emphasized the potential of the species as antimicrobial and antioxidant agent.

Keywords: *Calophyllum ferrugineum, coumarin, Isocalanone*

INTRODUCTION

Numerous plant species were found belongs to the family of Clusiaceae. Plant from the Clusiaceae family is crucial and known throughout tropical Asia and Africa [4]. One of them was *Calophyllum* genus known as Bintangor among the local [3, 7]. This genus exhibited the presence of various secondary metabolites for example triterpenoids, xanthones, coumarins, benzophenones, bioactive chromanones and flavonoids [1,6]. The biological test on the xanthones and coumarins afforded from this genus revealed their abilities as anti-agents against tumour, cancer, microorganism and human immunodeficiency virus (HIV) [5]. In addition, traditionally the other species have been used for swollen gums, arthritis, diarrhoea, chronic abscess, skin infections, and lesions treatment [8]. Therefore, a detailed phytochemical study was conducted on C. *ferrugineum* stem barks. This paper contains the overview of the isolated compounds and the plant extracts on their antimicrobial and antioxidant activity.

PLANT MATERIAL

The stem barks of C. *ferrugineum* were gathered from Sarawak National Park under management of Sarawak Forestry Corporation Sdn. Bhd.

CHEMICALS

Analytical grade solvents by MERCK has been used; n-hexane, chloroform and methanol, also silica gel 60 (230-400 mesh). Ascorbic acid and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) were obtained from Sigma-Aldrich Chemical.

REFERENCE DRUG

Streptomycin sulfate were purchased from Duchefa Biochemie (Haarlem, Netherland).

CULTURE MEDIA

The medium (Nutrient Broth (NB)) to culture the bacteria in the preparation of inoculum and also used for Minimum Inhibitory Concentration (MIC). Mueller Hinton Agar (MHA) was used for Minimum Bactericidal Concentration (MBC).

PREPARATION OF INOCULUM

The inoculum was prepared by adding the bacterial strains into the NB, and nurtured overnight at 37°C. The turbidity of the NB solution showed the equivalent concentration of 0.5 McFarland solutions.

MICROBIAL STRAIN

The types of microorganisms that were used in this study such as Staphylococcus aureus ATCC 29737, *Bacillus subtilis* ATCC 6633, *Pseudomonas aeruginosa* ATCC 9027 and *Escherichia coli* ATCC 10536.

GENERAL

The determination of infrared spectra was analysed via Universal Attenuated Total Reflection (UATR) on a Perkin-Elmer 2000 Series. Perkin Elmer Claruz 680 Mass Spectrometer was used to record the EIMS. A BRUKER 400 MHz FT NMR spectrometer with tetramethylsilane (TMS) was used to obtain the 1D and 2D NMR spectra.

EXTRACTION AND ISOLATION

C. ferrugineum (1.5 kg) plant materials were dried and pulverised. Three types of solvents namely as n-hexane followed by chloroform and methanol were used consecutively to extract the sample. The extraction yielded 15.10 g of *n*-hexane, 11.71 g of chloroform and 27.43 g of methanol extracts, respectively. The n-hexane extract was fractionated using VLC which afforded 27 fractions. Then, after the TLC profiling, the targeted fraction

was obtained from column method using binary solvent system of n-hexane: EtOAc followed with EtOAc: MeOH. A fine needle-like triterpene namely friedelin (102.9 mg) (4) was isolated. Besides, another type of triterpene known as lupeol (3) (10 mg) was afforded from the neighbouring fraction. Meanwhile, an ester was afforded and identified as diethylene glycol dibenzoate (5) by using the solvent system of a mixture of n-hexane: EtOAc. The chloroform extract was fractionated and afforded 27 fractions. Then, the targeted fraction was chromatographed and afforded 1-hydroxy-7-methoxyxanthone (4.98 mg) (2). A coumarin known as isocalanone (206.52 mg) (1) was afforded from the methanol extract after the purification process.

Isocalanone **(1)**. Yellow crystal (206.52 mg); m.p. 171-173 °C. IR (uATR, ν_{max}, cm^{-1}): 2925, 1719, 1598, 1271, 1110. EI-MS m/z (ret. int.) 424, 409, 331, 281. ^{1}H NMR (400 MHz, $CDCl_3$): δ_H: 5.92 (1H, *s*, H-3), 7.24 (1H, *m*, H-2′, H-6′), 7.42 (1H, *m*, H-3′, H-4′, H-5′), 7.66 (1H, *dd*, *J* = 7.3, 1.8 Hz, H-3″, H-7″), 7.50 (1H, t, J = 7.3 Hz, H-4″, H-6″), 7.60 (1H, *t*, *J* = 7.3 Hz, H-5″), 6.69 (1H, *d*, *J* = 10.1 Hz, H-1‴), 5.47 (1H, *d*, *J* = 10.1 Hz, H-2‴), 1.52 (3H, *s*, H-4‴, H-5‴), 12.50 (1H, *s*, 5-OH). ^{13}C NMR (100 MHz, $CDCl_3$): δ_C: 156.4 (C-2), 112.6 (C-3), 155.3 (C-4), 102.4 (C-4a), 161.5 (C-5), 103.8 (C-6), 155.9 (C-7), 105.8 (C-8), 156.4 (C-8a), 139.7 (C-1′), 127.2 (C-2′), 128.2 (C-3′), 127.8 (C-4′), 128.5 (C-5′), 127.2 (C-6′), 198.9 (C-1″), 140.4 (C-2″), 128.2 (C-3″), 127.6 (C-4″), 132.4 (C-5″), 127.6 (C-6″), 128.2 (C-7″), 115.3 (C-1‴), 127.0 (C-2‴), 79.1 (C-3‴), 27.5 (C-4‴, C-5‴).

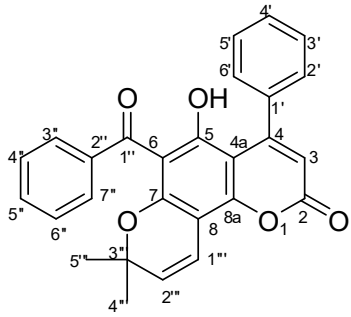
1-hydroxy-7-methoxy-9H-xanthen-9-one **(2)**. Yellow crystal (4.98 mg); m.p. 262-264 °C. IR (uATR, ν_{max}, cm^{-1}): 2923, 2853, 1735, 1643, 1606, 1472, 1365, 1277, 1208, 1027, 807. EI-MS m/z (ret. int.) 242, 227, 199, 171, 139, 127, 69, 63, 50. ^{1}H NMR (400 MHz, $(CD_3)_2CO$): δ_H: 12.71 (1H, *s*, 1-OH), 6.81 (1H, d, J = 8.2 Hz, H-2), 7.73 (1H, t, J = 8.3 Hz, H-3), 7.04 (1H, d, J = 8.4 Hz, H-4), 7.61 (1H, d, J = 9.2 Hz, H-5), 7.52 (1H, dd, J = 12.2, 3.08 Hz, H-6), 3.94 (3H, s, 7-OCH3), 7.64 (1H, d, J = 3.1 Hz, H-8). 13C NMR (100 MHz, (CD3)2CO): δC: 161.8 (C-1), 109.8 (C-2), 137.0 (C-3), 107.0 (C-4), 156.4 (C-4a), 119.6 (C-5), 125.6 (C-6), 156.4 (C-7), 105.1 (C-8), 121.1 (C-8a), 182.0 (C-9), 108.5 (C-9a), 151.1 (C-10a), 55.5 (7-OCH^3).

Lupeol **(3)**. Amorphous solid (10.0 mg); m.p. 212-213°C. IR (uATR, ν_{max}, cm^{-1}): 3263, 2924, 1615, 1468, 1133, 1058, 830. EI-MS m/z (ret.

int.) 426, 411, 315, 229, 218, 207, 189, 109, 95, 81, 68, 55, 43. ^{1}H NMR (400 MHz, CDCl3): δH: 3.21 (1H, dd, J = 11.2, 5.0 Hz, H-3), 2.39 (1H, m, H-19), 1.93 (1H, m, H-21), 0.99 (3H, s, H-23), 0.78 (3H, s, H-24), 1.04 (3H, s, H-25), 0.96 (3H, s, H-26), 0.81 (3H, s, H-27), 4.71 (3H, s, H-28), 4.71-4.59 (1H, s, H-29), 1.70 (3H, s, H-30). 13C NMR (100 MHz, CDCl3): δC: 37.17 (C-1), 27.41 (C-2), 79.10 (C-3), 38.71 (C-4), 55.29 (C-5), 18.02 (C-6), 34.27 (C-7), 40.01 (C-8), 50.43 (C-9), 42.83 (C-10), 20.93 (C-11), 25.12 (C-12), 38.87 (C-13), 43.01 (C-14), 27.45 (C-15), 35.58 (C-16), 48.29 (C-17), 40.83 (C-18), 48.0 (C-19), 151.0 (C-20), 28.0 (C-21), 38.04 (C-22), 29.85 (C-23), 14.46 (C-24), 16.14 (C-25), 15.98 (C-26), 15.40 (C-27), 18.33 (C-28), 109.35 (C-29), 19.32 (C-30).

Friedelin **(4)**. Amorphous solid (102.9 mg); m.p. 262-265 °C. IR (uATR, νmax, cm-1): 2926, 1714, 1457. EI-MS m/z (ret. int.) 426, 411, 341, 302, 273, 125, 109, 95, 69. 1H NMR (400 MHz, CDCl3): δH: 1.98 (1H, m, H-1a,), 1.71 (1H, m, H-1b), 2.40 (1H, dd, J = 11.3, 5.4 Hz, H-2a), 2.28 (1H, m, H-2b), 2.26 (1H, m, H-4), 0.90 (3H, d, J = 6.8 Hz, H-23), 0.74 (3H, s, H-24), 0.89 (3H, s, H-25), 0.97 (3H, s, H-26), 1.07 (3H, s, H-27), 1.20 (3H, s, H-28), 1.02 (3H, s, H-29, H-30). 13C NMR (100 MHz, CDCl3): δC: 22.3 (C-1), 41.3 (C-2), 213.4 (C-3), 58.2 (C-4), 42.8 (C-5), 41.5 (C-6), 18.2 (C-7), 53.1 (C-8), 37.4 (C-9), 59.5 (C-10), 35.3 (C-11), 30.5 (C-12), 39.7 (C-13), 38.3 (C-14), 32.8 (C-15), 36.0 (C-16), 30.0 (C-17), 42.8 (C-18), 35.6 (C-19), 28.2 (C-20), 32.8 (C-21), 39.3 (C-22), 6.8 (C-23), 14.7 (C-24), 18.0 (C-25), 20.3 (C-26), 18.7 (C-27), 32.1 (C-28), 31.8 (C-29), 35.0 (C-30).

Diethylene glycol dibenzoate **(5)**. Colourless oil (6.42 mg); m.p. 24.0 °C. IR (uATR, νmax, cm-1): 2953, 1714, 1451, 1268, 1097, 1070, 707. EI-MS m/z (ret. int.) 315, 149, 105, 77. 1H NMR (400 MHz, CDCl3): δH: 8.05 (1H, d, J = 7.6 Hz, H-2, H-6, H-2′, H-6′), 7.57 (1H, t, J = 7.2 Hz, H-4, H-4′), 7.42 (1H, t, J = 7.4 Hz, H-3, H-5, H-3′,H-5′), 4.52 (1H, s, H-8, H-8′), 3.91 (1H, s, H-9, H-9′). 13C NMR (100 MHz, CDCl3): δC: 130.0 (C-1, C-1′), 129.9 (C-2, C-6, C-2′, C-6′), 128.4 (C-3, C-5, C-3′, C-5′), 133.0 (C-4, C-4′), 166.6 (C-7, C-7′), 64.0 (C-8, C-8′), 69.2 (C-9, C-9′).



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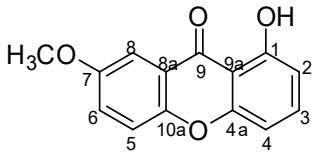

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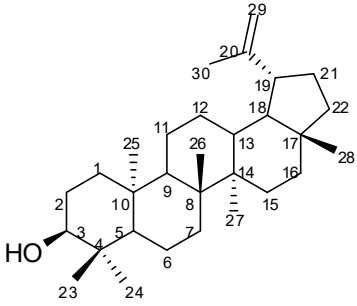

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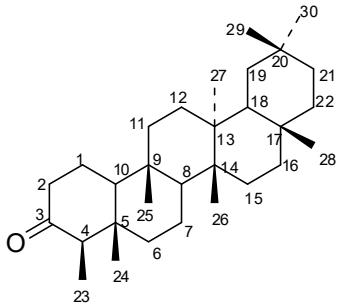

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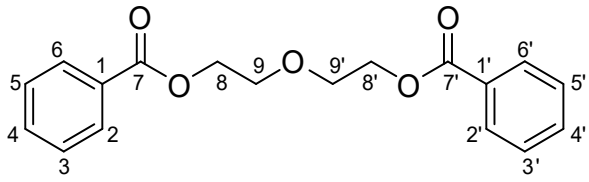

(5)

Figure 1: Compounds Isolated from C ferrugineum

DPPH RADICAL SCAVENGING ASSAY

The antioxidant properties were measured via DPPH free radical method with the ascorbic acid as standards. The equation was used to resolve the half maximal inhibitory concentration (IC50):
% Inhibition = ((A-B)/A) x 100 %
A = negative control absorbance (DPPH + Ethanol)
B = sample test absorbance

ANTIMICROBIAL ASSAY

The antimicrobial activity was analysed via Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) method. The tested bacteria were inoculated into the medium with varied concentrations of the plant extracts in the 96-well microtiter plates. The MIC and MBC value was measured after a defined incubation period (24 hours). The lower concentration of the dilution with no visible bacteria growth means good antimicrobial agents and reported as MBC results.

RESULTS AND DISCUSSION

Isocalanone **(1)** (206.52 mg) was isolated as a yellow crystal with the melting point of 171-173 °C. The mass spectrum exhibited at *m/z* 424 corresponding to the formula of $C_{27}H_{20}O_5$.

The ^{1}H NMR spectrums presented a single proton at δ_H 12.50 belongs to the hydroxyl group (O-H). The resonance at δ_H 7.42 exhibited multiplet peaks belong to the protons H-3', H-4' and H-5', meanwhile at signal δ_H 7.24 showed the protons H-2' and H-6'. The triplets showed at δ_H 7.50 (H-4″, H-6″) and δH 7.60 (H-5″) with similar coupling constant 7.3 Hz, δ_H 7.66 (1H, dd, J = 7.3 Hz, 1.8 Hz, H-3″ and H-7″) indicated the benzoyl moiety in the structure. Another singlet peak (H-3) appeared at δH 5.90. The protons H-1‴ (δH 6.69) and H-2‴ (δH 5.47) as cis-olefinic protons appeared as doublets with 10.1 Hz coupling constant. In addition, a singlet at δ_H 1.02 (6H, H-4‴ and H-5‴) belongs to two tertiary methyl groups. The ^{13}C NMR spectrum exhibited 2 methyl, 13 methine, 12 quartenary which sum up to 27 carbons in the structure.

The HMBC spectrum exhibited the H-3 (δ_H 5.90) was correlated with the C-2 (δ_C 158.0), the carbonyl carbon and C-4a (δ_C 102.4). Besides, the proton H-3 also correlated with C-1' (δ_C 139.7) and attached to the C-4. A hydroxyl group (5-OH) correlated with C-5 (δ_C 161.5) and attached to C-6 (δC 103.8), then correlated to the H-3'' and H-7''. The protons H-1''' (δ_H 6.69) and H-2''' (δ_H 5.47) (each J = 10.1 Hz) ascribed a pyrano ring, along with the germinal- dimethyl group at δH 1.52 indicated as H-4''' and H-5'''. Hence, the ring was bonded to the carbon C-7 (δ_C 155.9) and C-8 (δ_C 105.8). This is the first isolation of isocalanone from *C. ferrugineum*.

Compound **2-5** were identified as 1-hydroxy-7-methoxy-9H-xanthen-9-one **(2)**, lupeol **(3)**, friedelin **(4)**, diethylene glycol dibenzoate **(5).** Spectral data are in agreement with previous reports.

The methanolic extracts showed slightly higher IC_{50} (IC_{50} = 35 ± 3.69 µg/mL) compared to the n-hexane and chloroform extracts (Table 2). The scavenging activity of isocalanone **(1)** (IC_{50} = 28 ± 5.23 µg/mL) which was isolated from the methanolic extract displayed the same potent antioxidant activity compared to the ascorbic acid (Table 1). The determination of IC50 value was to measure the radical scavenging activity in the plant samples; the lower values of the IC50 showed the stronger antioxidant potential.

Table 3 displayed the MIC and MBC results for C. *ferrugenium* extracts. The chloroform and methanol extracts showed MIC values ranged from 112.5 and 225 µg/mL. The MIC ≤ 500 µg/mL exhibited strong MIC values [2]. Meanwhile the *n*-hexane extract showed the MIC values ranged from 900 and 1800 µg/mL. The MBCs for all the samples were higher compared to the MICs. All the extracts showed ranged from 112.5 – 1800 µg/mL for the MBCs. The chloroform extract have the potential as a bactericidal agent against *Pseudomonas aeruginosa* and *Escherichia coli*. Generally, the plants have the inhibitory activity against the tested strains.

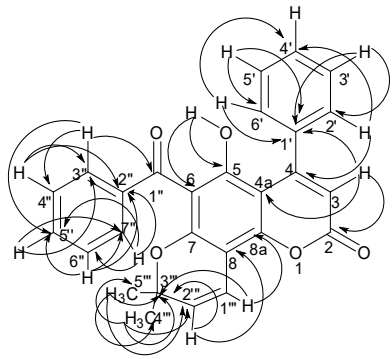

Figure 2: HMBC Correlation of Compound (1)

Table 1: ^{1}H NMR (400 MHz, $CDCl_3$), ^{13}C NMR (100 MHz, $CDCl_3$) and HMBC Spectral Data of Isocalanone (1)

Position	δ_H	δ_C	HMBC
2	-	158.1	-
3	5.92 (1H, *s*)	112.6	C-2, C-4a, C-1′
4	-	155.3	-
4a	-	102.4	-
5	-	161.5	-
6	-	103.9	-
7	-	156.0	-
8	-	105.8	-
8a	-	156.4	-
1′	-	139.7	-
2′	7.24 (1H, *m*)	127.2	C-4′
3′	7.42 (1H, *m*)	128.2	C-1′,C-2′
4′	7.42 (1H, *m*)	127.8	-
5′	7.42 (1H, *m*)	128.2	C-1′, C-6′
6′	7.24 (1H, *m*)	127.2	C-4′
1″	-	198.9	-
2″	-	140.4	-
3″	7.66 (1H, *dd, J* = 7.3, 1.8 Hz)	128.2	C-1″, C-4″, C-5″

4″	7.50 (1H, *t*, *J* = 7.3 Hz)	127.6	C-2″, C-3″
5″	7.60 (1H, *t*, *J* = 7.3 Hz)	132.4	C-3″, C-7″
6″	7.50 (1H, *t*, *J* = 7.3 Hz)	127.6	C-2″, C-7″
7″	7.66 (1H, *dd*, *J* = 7.3 Hz, 1.8 Hz)	128.2	C-1″, C-5″, C-6″
1‴	6.69 (1H, *d*, *J* = 10.1 Hz)	115.3	C-8a, C-3‴
2‴	5.47 (1H, *d*, *J* = 10.1 Hz)	127.1	C-8, C-3‴
3‴	-	79.1	-
4‴	1.02 (3H, *s*)	27.5	C-2‴, C-3‴, C-5‴
5‴	1.02 (3H, *s*)	27.5	C-2‴, C-3‴, C-4‴
5-OH	12.50 (1H, *s*)	-	C-5, C-6

Table 2: IC50 Results of Isolated Compounds and Extracts of C. *ferrugineum*

Samples	IC_{50} (µg/mL)
Ascorbic acid (standard)	15 ± 1.38
Isocalanone **(1)**	28 ± 5.23
Lupeol **(3)**	108 ± 7.46
Friedelin **(4)**	176 ± 11.46
n-Hexane	96 ± 11.03
Chloroform extract	90 ± 9.37
Methanol extract	35 ± 3.69

Table 3: The MIC and MBC Results of C. ferrugineum Extracts

	***n*-Hexane**		**Chloroform**		**Methanol**		**Streptomycin sulphate**
Bacteria	MIC	MBC	MIC	MBC	MIC	MBC	MIC
	value	value	value	value	value	value	value
	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)	(µg/mL)
S. aureus	450	900	225	225	225	225	28.13
B. subtilis	450	900	225	225	225	225	14.07
P. aeruginosa	900	1800	112.5	112.5	225	225	28.13
E. coli	900	1800	112.5	225	225	225	14.13

CONCLUSION

Isocalanone **(1)** was afforded from C. *ferrugineum* stem bark extract for the first time together with 1-hydroxy-7-methoxy-9H-xanthen-9-one **(2)**, lupeol **(3)** and friedelin **(4)** and an ester, diethylene glycol dibenzoate **(5)**. Isocalanone (1) and the methanol extract of C. *ferrugineum* were active as antioxidant with mechanism by reduction of DPPH radical.

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