African Journal of Biotechnology Vol. 10(72), pp. 16337-16341, 16 November, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.2239
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Cytotoxic constituents of *Clausena excavata*

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Accepted 7 October, 2011

Phytochemical investigation on leaves, stem bark and roots of Malaysian *Clausena excavata* has led to the isolation and identification of limonoid compounds, clausenolide-1-methyl ether (1) and clausenarin (2), carbazole alkaloids, 3-formyl-2,7-dimethoxycarbazole (3) and clausine-K (4) together with coumarins, xanthyletin (5), dentatin (6) and nordentatin (7). Extracts of roots and isolated compounds (1), (2), (5) and (6) were subjected to cytotoxic screening against various cancer cell lines (HL-60, MCF-7, HeLa and HT-29). All roots extracts except methanol showed strong activity against HL-60 and MCF-7 cancer cell lines with IC_{50} values ranging from 4 to 6 µg/ml. Dentatin (6) was found to be the most cytotoxic constituent against all cancer cell lines with IC_{50} values ranging from 5 to 10 µg/ml.

Key words: *Clausena excavata*, carbazole alkaloids, limonoids, coumarins, cytotoxic.

INTRODUCTION

The Rutaceae family is one of the largest plant family with approximately 150 genera and 1,500 species (Jones, 1995), distributed largely in tropical and subtropical parts of the world. The Rutaceae family is known throughout the world for its citrus fruits such as oranges, lemons and grape fruit (Sharma, 1993). Essential oils obtained from the leaves and fruit peel of various species of Rutaceae family especially from the genus *Clausena, Citrus* and *Murraya* are popularly used in medicine and perfumery. *Clausena excavata* Burm.F. locally known as “Pokok Kemantu” (ghostly tree) or “Pokok Cemamar” (diarrhea tree) is one of Malaysian species of “ulam” with high anti-oxidant properties. The plant has been claimed to be a useful folk medicine in the treatment of various diseases such as cough, rhinitis, fever and stomach disorder.

This plant has been reported to possess various biological activities such as anti-inflammatory, anti-platelet, antiplasmodic, antimicrobial, antinociceptive and anti-immunomodulatory (Wu et al., 1994). Previous phytochemical investigations have reported isolation of some carbazole alkaloids, coumarins and limonoids (Su et al., 2009; Taufiq et al., 2007; Ito et al., 1997; Wu et al., 1997). In more recent study, several natural and synthesized analogues of pyranocoumarins obtained from this plant were found to be potent against hepatitis B virus and showed significant cytotoxicity against a panel of cancer cell lines (Su et al., 2009). In this paper, we reported the isolation and characterization of alkaloids, coumarins and limonoids from the plant, and the cytotoxic activity of the plant extracts and isolated compounds against different cancer cell lines (HL-60, MCF-7, HT-29 and HeLa). The work reported here is the first on cytotoxic screening of roots extracts and isolated compounds, clausenolide-1-methyl ether **(1)** and clausenarin **(2)** from Malaysian *C. excavata* against various cancer cell lines.

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MATERIALS AND METHODS

C. excavata Burm.F. was collected from Pendang, Kedah in December 2006. The plant was identified by Mr. Shamsul Khamis from Institute of Bioscience, Universiti Putra Malaysia. A voucher specimen of this plant was deposited in the herbarium of the institute. The plant materials were separated into leaves, stem bark and roots, air-dried and ground prior to use.

Extraction and isolation

Different parts of *C. excavata* were extracted successively with hexane, chloroform and methanol at room temperature. The extracts were evaporated to dryness under reduced pressure using

rotary evaporator to give crude extracts. Air-dried and ground leaves (778 g) yielded hexane (8.7 g), chloroform (11.9 g) and methanol (15.8 g) extracts, respectively while stem bark (780 g) yielded hexane (3.5 g), chloroform (35.1 g) and methanol (25.2 g) extracts, respectively. Similar procedures on roots of the plant (686 g) yielded hexane (12.6 g), chloroform (35.1 g), acetone (11.0 g) and methanol (50.0 g) extracts, respectively. Each of these extracts was subjected to column chromatography over silica gel using a stepwise gradient elution system (hexane/ethyl acetate and ethyl acetate/methanol). Column chromatography separation of hexane extract of the leaves (6.7 g) yielded stigmasterol (35 mg), while its chloroform extract (9.9 g) yielded 3-formyl-2,7-dimethoxycarbazole (**3,** 20 mg). Similar column separation of hexane extract of stem bark (2 g) yielded stigmasterol (10 mg) and β-sitosterol (15 mg), while the chloroform extract (33.1 g) yielded also β-sitosterol (15 mg), together with clausenarin (**2,** 30 mg), clausenolide-1-methyl ether (**1,** 15 mg) and clausine-K (**4,** 20 mg). In addition, clausenarin (**2,** 35 mg) was also obtained from the methanol extract (23.2 g). Meanwhile, column chromatography fractionation of hexane extract of the roots of the plant (10.6 g) yielded coumarins xanthyletin (**5,** 36 mg) and dentatin (**6,**100 mg), while the chloroform extract yielded also dentatin (**6,** 50 mg) together with nordentatin (**7,** 10 mg).

Clausenolide-1-methyl ether (1) was isolated as colourless powder, $C_{26}H_{34}O_8$, HR-FAB-MS: $[M+H]^+$ *m/z* 475.2322. m.p. 235 to 237°C (Wu et al., 1993, m.p. 190-191°C). IR (KBr disc, v_{max}, cm^{-1}): 3502, 1724, 1680, 1160, 922. EIMS: 474 ($[M]^+$, 2), 443 (15), 459 (3), 351 (64), 319 (66), 277 (70), 217 (44), 95 (100), 69 (84), 55 (51). ^1H and ^{13}C NMR spectral data are in a good agreement with the published data (Wu et al., 1993).

Clausenarin (2) was isolated as colourless needle-shaped crystal, $C_{26}H_{32}O_9$, m.p. 292 to 294°C (Ngadjui et al., 1989, m.p. 293-294°C). IR (KBr disc, v_{max}, cm^{-1}): 3483, 1719, 1638, 1162, 875. EIMS: 488 ($[M]^+$, 10), 474 (5), 445 (3), 365 (100), 289 (8), 277 (27), 133 (35), 107 (34), 95 (64). ^1H and ^{13}C NMR spectral data are in good agreement with the published data (Ngadjui et al., 1989).

3-Formyl-2,7-dimethoxycarbazole (3) was isolated as greenish needle, $C_{15}H_{13}NO_3$, m.p 217-219°C (Peh, 2001, m.p. 217 to 219°C). IR (KBr disc, v_{max}, cm^{-1}): 3438, 2924, 1664, 1158. EIMS: 255 ($[M]^+$, 100), 240 (43), 226 (6), 209 (20), 197 (11), 191 (3), 184 (17), 179 (4), 169 (30), 161 (7), 153 (15), 147 (3), 141 (22). ^1H and ^{13}C NMR spectral data are in good agreement with the published data (Peh, 2001).

Clausine-K (4) was isolated as yellow needle-shaped crystal, $C_{15}H_{13}NO_4$, m.p. 254 to 256°C (Wu et al., 1996, m.p. 250-256 ºC). IR (KBr disc, v_{max}, cm^{-1}): 3412, 3317, 1665, 1163. EIMS: 271 ($[M]^+$, 100), 256 (28), 240 (16), 212 (15), 196 (15). ^1H and ^{13}C NMR spectral data are in a good agreement with the published data (Wu et al., 1996).

Xanthyletin (5) was isolated as colourless needle-shaped crystal, $C_{14}H_{12}O_3$, m.p. 119 to 121°C (Wu et al., 1997, m.p. 120 to 121°C). IR (KBr disc, v_{max}, cm^{-1}): 1722, 1622, 1562, 1160. EIMS: 228 ($[M]^+$, 20), 213 (100), 185 (21), 128 (10), 115 (8), 91 (24), 51 (18). ^1H and ^{13}C NMR spectral data are in good agreement with the published data (Wu et al., 1997).

Dentatin (6) was isolated as colourless needle-shaped crystal, $C_{20}H_{22}O_4$, m.p. 90 to 92°C (Xin et al., 2008, m.p. 91 to 92°C). IR (KBr disc, v_{max}, cm^{-1}): 1681, 1592, 1460, 1168. EIMS: 326 ($[M]^+$, 20), 311 (100), 281 (17), 269 (3), 253 (8), 241 (3), 227 (5), 213 (3). ^1H and ^{13}C NMR spectral data are in good agreement with the published data (Xin et al., 2008).

Nordentatin (7) was isolated as colourless needle-shaped crystal, $C_{19}H_{20}O_4$, m.p. 183 to 186°C (Wu and Furukawa, 1982, m.p. 178 to 180°C). IR (KBr disc, v_{max}, cm^{-1}): 3311, 1681, 1593, 1184. EIMS: 312 ($[M]^+$, 45), 297 (100), 283 (3), 269 (15), 255 (10), 241 (30). ^1H and ^{13}C NMR spectral data are in good agreement with the published data (Wu and Furukawa, 1982).

Cytotoxic assay

The crude extracts and selected pure compounds including clausenolide-1-methyl ether **(1)**, clausenarin **(2)**, xanthyletin **(5)** and dentatin **(6)** were screened for cytotoxic activity against HL-60 (human promyelocytic leukemia), MCF-7 (human breast cancer), HT-29 (human colon cancer) and HeLa (human cervical cancer) cancer cell lines. The assay was carried out according to the methods previously described (Sukari et al., 2010). The cytotoxic index used was IC_{50}, which is the concentration that gave 50% inhibition of the cell as compared to the untreated control. Extracts and pure compounds which exhibits cytotoxic index IC_{50} less than 10 µg/ml were considered to have significant cytotoxic activity (Mackeen et al., 1997).

RESULTS AND DISCUSSION

Extraction and isolation work on different parts of Malaysian *C. excavata* have led to the identification and characterization of limonoid compounds, carbazole alkaloids and coumarins. Clausenolide-1-methyl ether **(1)**, clausenarin **(2)** and carbazole alkaloid, clausine-K **(4)** were obtained from chloroform extract of stem bark of *C. excavata.* Clausenarin **(2)** was also gotten from fractionation of methanol extract of stem bark. Another carbazole alkaloid, 3-formyl-2,7-dimethoxycarbazole **(3)** was isolated from chloroform extract of the leaves. Besides, three coumarins identified as xanthyletin **(5)**, dentatin **(6)** and nordentatin **(7)** were isolated from hexane and chloroform extracts of the plant. Figure 1 shows the chemical structures of isolated compounds from different parts and various extracts of Malaysian *C. excavata*. The structures of the compounds were elucidated using spectroscopic methods and comparison of their spectral and physical data with the literature values.

Clausenolide 1-methyl ether **(1)** has been reported only once and this is the first isolation of the compound from Malaysian species. The compound was obtained as colourless powder and the molecular formula was determined to be $C_{26}H_{34}O_8$ by HR-FAB-MS at *m/z* 475.2322 $[M+H]^+$ (calculated for $C_{26}H_{35}O_8$ 475.2332). The infrared spectrum showed a lactone carbonyl peak at 1724 cm^{-1} and a ketone carbonyl at 1680 cm^{-1}, whereas low intensity peak at 922 cm^{-1} was due to β-substituted furan. Hydroxyl group displayed a strong absorption band at 3502 cm^{-1}. Its ^1H NMR spectrum was similar to clausenolide (Ngadjui et al., 1989), except the present of singlet at δ 3.23 due to methoxyl group attached to β-substituted tetrahydrofuran ring. All the compounds **(1)** to **(7)** have been previously isolated from *C. excavata* collected from different Asian regions. However, out of

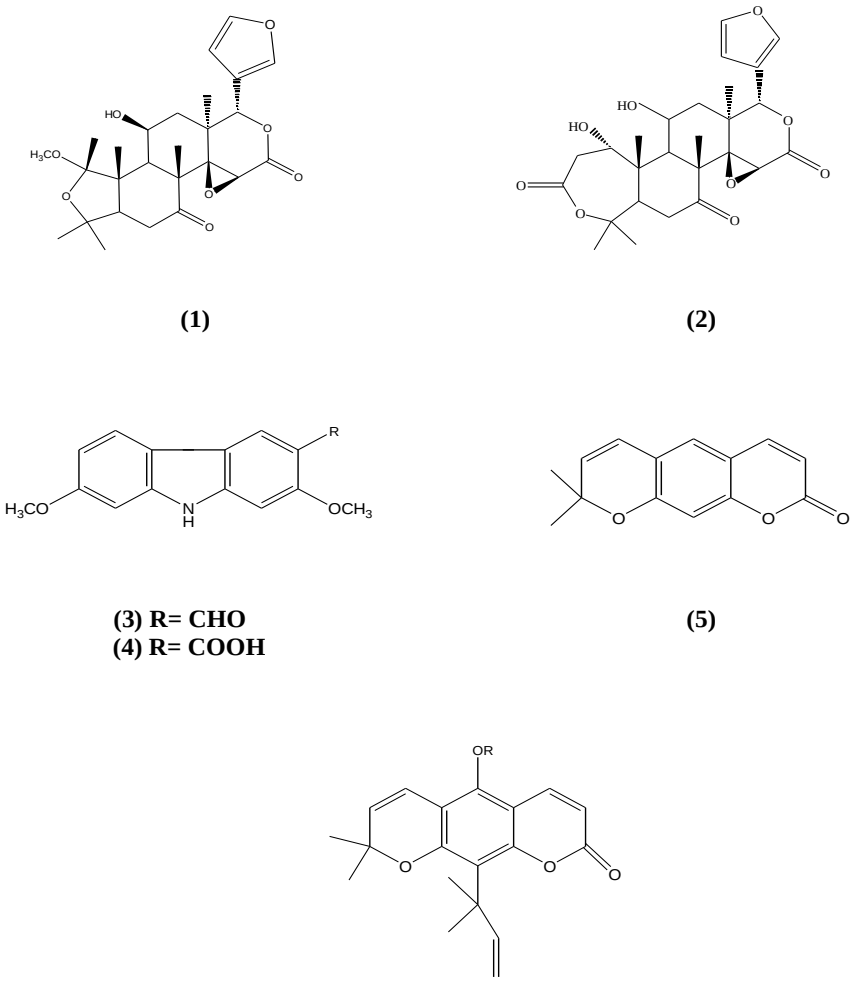

Figure 1. Structures of isolated compounds (1-7).

seven constituents mentioned here, only clausenarin **(2)**, 3-formyl-2,7-dimethoxycarbazole **(3)** and clausine-K **(4)** has been isolated from another collection of Malaysian species reported by Peh (2001). In both cases, the plant materials were collected from different locations of Kedah in north Malaysia peninsula. Apparently, there are variations of chemical constituents obtained which might be due to different soil conditions.

Extracts of roots together with isolated compounds, clausenolide-1-methyl ether **(1)**, clausenarin **(2)**, xanthyletin **(5)** and dentatin **(6)** were subjected to cytotoxic screening against various cancer cell lines (HL-60, MCF-7, HeLa and HT-29). The results are summarized in Table 1. Hexane, chloroform and acetone extracts exhibited strong activity against HL-60 and MCF-7 cancer cell lines with IC_{50} values ranging from 4 to 6 µg/ml. The extracts also showed moderate to strong effects against HT-29 and HeLa cancer cell lines, except non-active action of chloroform extract against HT-29 cancer cell line.

Dentatin **(6)** was the most cytotoxic compound against all cancer cell lines tested with IC_{50} values ranging from 5 to 10 µg/ml as compared to coumarin and xanthyletin **(5)** which showed low to moderate activity against all cancer cell lines tested. However, limonoid compounds **(1)** and **(2)** showed insignificant cytotoxicity against all cancer cell lines tested with IC_{50} values more than 30 µg/ml, except for compound **(1)** which showed moderate activity against HL-60 and MCF-7 cancer cell lines. The cytotoxic activity of compounds **(3)**, **(4)** and **(7)** were not carried out due to insufficient amount of the samples. Most of the crude extracts from roots part were more cytotoxic than the isolated compounds. These results suggest the synergistic effects shown by the isolated compounds

Table 1. Cytotoxicity of roots extracts and compounds against various cancer cell lines.

Plant part	Extracts/pure compounds	*IC_{50} (µg/ml) value			
		HL-60	MCF-7	HT-29	HeLa
Roots	Hexane	4.8±0.21	4.8±0.32	12.5±0.27	6.8±0.32
	Chloroform	5.8±0.12	5.5±0.21	>30	5.0±0.28
	Acetone	5.0±0.23	6.0±0.29	11.5±0.23	11.9±0.24
	Methanol	23.8±0.27	>30	>30	10.9±0.32
Roots	Xanthyletin **(5)**	19.5±0.23	19.5±0.25	26.8±0.29	25.5±0.30
	Dentatin **(6)**	5.2±0.24	8.0±0.26	9.5±0.22	9.6±0.27
Stem	Clausenolide 1-methyl ether **(1)**	18.5±0.23	21.5±0.28	>30	>30
Bark	Clausenarin **(2)**	>30	>30	>30	>30
Standards	Goniothalamin Tamoxifen 5-Fluorouracil	1.5±0.20	3.0±0.20	1.5±0.30	1.2±0.21

< 10 µg/ml = Strong activity, 10 to 20 µg/ml = moderate activity, 20 to 30 µg/ml= low activity.
*Values are means ± standard deviation of triplicate analyses

towards the cytotoxic properties of the crude extracts. Previous study on cytotoxic activity of Malaysian *C. excavata* have shown that the stem bark extract and isolated compound, 3-carbomethoxy-2-hydroxy-7-methoxycarbazole (Clausine-TY) exhibit significant cytotoxicity against CEMss (human T4 lymphoblastoid) cancer cell line (Taufiq et al., 2007). On the other hand, the leaves extract of the plant was found to be not active.

Xanthyletin **(5)** has been reported to show broad activity against a panel of cancer cell lines (Kawaii et al., 2001; Yong et al., 2001; Lie et al., 2003; Pettit et al., 2004; Anaya et al., 2005). Our results reveal that dentatin **(6)** showed strong activity against MCF-7 cancer cell line, while its analogue nordentatin (7) was reported to exhibit moderate activity against the same cancer cell (Su et al., 2009). The replacement of hydroxyl group with methoxyl group at C-5 position has increased the cytotoxicity of these analogues against MCF-7 cell lines. Another investigation by Kawaii et al. (2001) reported that dentatin **(6)** has been implicated as a promising chemopreventive agent against several cancer cell lines. However, the compound demonstrated insignificant cytotoxic activity against other cancer cell lines tested (Sunthitikawinsakul et al., 2003; Songsiang et al., 2011). The work reported here is the first on cytotoxic screening of roots extracts and isolated compounds, clausenolide-1-methyl ether **(1)** and clausenarin **(2)** from Malaysian *C. excavata* against different cancer cell lines mentioned earlier.

ACKNOWLEDGEMENT

We thank The Ministry of Science, Technology and Innovation (MOSTI) for the Fundamental Research Grant Scheme and Graduate Research Fellowship (UPM) given to one of the authors (N. W. Muhd Sharif).

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