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Cytotoxic Carbazole Alkaloid from the Root of *Clausena excavata* on HeLa Cell Line

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Abstract: In a search for bioactive constituents from Myanmar medicinal plants, a carbazole alkaloid, named 7-hydroxy heptaphylline (**1**) was isolated from the root of *Clausena excavata*. The structure of isolated compound was elucidated based on spectrophotometric data such as UV-vis, FT-IR, NMR and HRMS data. The cytotoxicity of the isolated compound (**1**) was evaluated by MTT assay against on HeLa cancer cells. The compound (**1**) exhibited moderate inhibition activity with IC₅₀ 41.4 µg/ml.

1 INTRODUCTION

Clausena excavata Burm. f. is a wild shrub, a member of Rutaceae family predominantly distributed in India, China and Southeast Asia. The leaves, twigs, and root barks of *C. excavata* have long been used in Asian folk medicine for the treatment of colic, cough, rhinitis, sore, wounds, malaria, abdominal pain, snake-bite, preliminary stage of AIDS and dermatopathy, dysentery, enteritis, and urethra infection (Waziri et al., 2016a) (Kumar et al., 2012) (Peh et al., 2013).

The constituents of *C. excavata* have been frequently studied. Phytochemical analyses in the past have revealed that *C. excavata* is a rich source of coumarins, carbazole alkaloid along with a small group of flavonoids, limonoids and triterpenoids (Cheng et al., 2009) (Mohan, 2012) (Sunthitikawinsakul et al., 2003) (Kumar et al., 2012) (Peh et al., 2013) (Peng et al., 2013) (Thant et al., 2019). Many compounds reported from *C. excavata* showed diverse therapeutic activities which are antibacterial, antifungal, antiplatelet, antiplasmodial,

antitumor, antinociceptive, antimycobacterial, and anti-HIV-1 activities (Kongkathip and Kongkathip, 2009).

The coumarins isolated from this plant have attracted attention due to its bioactive properties such as the furanone-coumarins named clauslactones A–J isolated from leaves exhibited tumor promotion inhibitory effects, nordentatin showed antibacterial properties and a pyranocoumarin clausenidin isolated from roots displayed anti-HIV-1 activity (Kumar et al., 2012). Nevertheless, the other potent bioactivities of the constituents from *C. excavata* are still unknown and worthy of exploration (Cheng et al., 2009). Moreover, four isolated pyranocoumarins from *C. excavata* and screened their cytotoxic potentials in cancer cells. The study revealed that the pyranocoumarins are good modulators of tumor cell death (Waziri et al., 2016b) (N. W. Muhd Sharif, 2011).

Cancer is the second leading cause of death worldwide. Cervical cancer is one of the most dead list diseases among women and it is occurred when the abnormal cells are undergoing to the rapid and uncontrolled growth on the cervix. Current treatments

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for cervical cancer may include surgery, drugs (hormonal therapy and chemotherapy), radiation and/or immunotherapy. Conventional cancer treatments such as chemotherapy and radiotherapy have shown some effectiveness for reducing or eradicating cancers; however, they can produce unpleasant side effects, e.g. nausea, vomiting, changes in bowel habits, fatigue and hair loss. Complementary and alternative medicine (CAM), herbals and multivitamin supplements, or herbal medicine is increasingly used as an adjunctive treatment for cancer patients to reduce or manage side effects of conventional cancer treatments. Several studies have confirmed the anti-proliferative and cell cycle regulatory effects of some plants which behave as cancer prevention (Amin et al., 2009) (Azarifar et al., 2015). More than 25% of drugs used during the last 20 years are directly derived from plants, while the other 25% are chemically altered natural products. Still, only 5-15% of the approximately 250,000 higher plants have ever been investigated for bioactive compounds. The advantage of using such compounds for cancer treatment is their relatively non-toxic nature and availability in an ingestive form (Amin et al., 2009).

In continuing our research, isolation of bioactive constituents from natural product, isolated compound (1) was tested on his cytotoxicity activity on HeLa cancer cell by MTT assay.

13 2 MATERIALS AND METHODS

2.1 Plant Material

The plant sample of *C. excavata* was collected from Pyin Ma Nar Township, Mandalay Division, Myanmar in October 2016. It was identified by Prof. Soe Myint Aye, botanist from Department of Botany, Mandalay University, Myanmar.

2.2 MTT Assay

Anticancer activity tests on isolated compound was carried out using the MTT assay method (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) following the protocol of Suwito et al (Suwito et al., 2018). The cancer cells were seeded in a 96-well plate at a density of 1×10^4 cells/well with a phenol red-free RPMI (Roswell Park Memorial Institute medium) 1640 medium (containing 10% FBS (fetal bovine serum)) and maintained for 24 h.

Subsequently, the tested compound (various concentrations) was applied for 24 h. After addition of 0.5% MTT solution, the incubation was continued for a further 4 h at 37 °C/5% CO₂. The stop solution (0.04 N HCl in isopropanol) was added to the culture medium to each well. Then, the spectroscopic measurement was carried out at 570 nm (peak) and 630 nm (bottom) using an ELISA (Enzyme-Linked Immunosorbent Assay) reader. It was conducted in triplicate. Doxorubicin was used as a positive control. The value of IC₅₀ was determined using a probit analysis (SPSS 17, IBM Analytics, New York, NY, USA).

12 2.3 Extraction and Isolation

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The dried roots (3.6 kg) were extracted successively with 95% EtOH (12.0 L) over a period of two weeks at ambient temperature. After removing the solvent 156 g of ethanolic extract was obtained. Among them the extract (100g) was partitioned by using solvents; *n*-hexane: methanol (1:1, v/v). Then methanol portion (80.4 g) was subjected to vacuum liquid chromatography over silica gel (150g) eluted with different mixtures of *n*-hexane: ethylacetate by stepwise increasing gradient polarity gave a total of 7 combined fractions (MF-1 to -7) were obtained. MF-6.2.1.1 was purified by using *n*-hexane : ethylacetate, ethylacetate (0-30%) with gradient polarity and silica gel column, gave 44 fractions. Among them 7-21 showed one spot on tlc with three different solvent elution system (*n*-hex: EtOAc ; *n*-hexane: CHCl₃; *n*-hexane : Acetone) afforded as pure compound (1).

2.4 Statistical Analysis

Results were presented as mean ± SD in triplicate experiment. Differences were determined using SPSS 17, IBM Analytics, New York, NY, USA at significant difference of 0.05.

3 RESULTS AND DISCUSSIONS

Spectroscopic data of Compound-1 (7-hydroxyheptaphylline)

Compound-1: greenish yellow solid. UV(MeOH), λ_{max} (log_e) 342 (0.629), 303 (2.519), 237 (1.408) nm. IR (KBr) ν_{max} cm⁻¹: 3250, 2958, 2922, 2852, 1743, 1614, 1454, 1327, 1186, 9966 ¹H NMR (600 MHz, DMSO-d₆) δ 11.53, 11.27, 9.88, 9.49, 8.14, 7.80, 7.78, 6.86, 6.86, 6.66, 6.65, 6.64,

6.64, 5.29, 5.28, 5.28, 5.27, 5.27, 5.27, 5.26, 5.26, 5.26, 3.51, 3.50, 1.78, 1.63. ^{13}C NMR (151 MHz, DMSO- d_6) δ 196.45, 156.98, 156.59, 145.16, 143.00, 132.15, 124.25, 122.11, 120.96, 117.83, 115.91, 115.01, 109.84, 109.32, 97.80, 25.92, 23.08, 18.39.

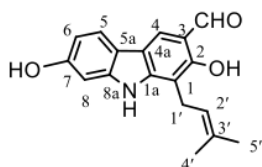


Figure 1: Structure of compound-1 isolated from the root of *C. excavata*

Table 1: Cytotoxic activity of isolated compound (1) against on *Hela* cell lines.

Compounds	IC ₅₀ (ug/mL)
7-hydroxy heptaphylline (1)	41.4
Doxorubicin	1.19

Table 2: ^1H (600 MHz), ^{13}C (151 MHz) NMR and HMBC spectral data of compound-1 (7-hydroxyheptaphylline)

Position	δ_{H} (mult, J Hz)	δ_{C} (ppm)	HMBC
1a	-	145.2	
1	-	109.1	
2	-	157.9	
3	-	123.7	
4	8.03 (s, 1H)	125.3	C-1a, C-2, C-3, C-4a, CHO
4a	-	115.5	
5a	-	140.2	
5	7.97 (dd, J = 7.7 Hz, 1H)	119.8	C-5a, C-8, C-8a
6	7.27 (m, 1H)	120.5	C-5a, C-7, C-8
7	7.40 (dt, J = 7.7, 3.8 Hz, 1H)	125.9	C-6
8	7.41 (dd, J = 7.7, 3.8 Hz, 1H)	110.9	C-5a
8a	-	117.4	
1'	3.64 (d, J = 6.9 Hz, 2H, H-1')	22.9	C-1, C-1a, C-2, C-10, C-11
2'	5.33 (d, J = 6.9 Hz, 1H, H-2')	121.3	C-1, C-9, C-12, C-13
3'	-	134.2	
4'	1.91 (s, 3H)	18.1	C-10, C-11, C-13
5'	1.78 (s, 3H)	25.7	C-10, C-11, C-12
-CHO	9.91	195.4	C-1, C-2, C-4
-NH	8.21		

-OH	11.66
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Compound-1 (7-hydroxyheptaphylline) was afforded as yellow green crystal UV (MeOH), λ_{max} (log ϵ) 342 (0.629), 303(2.519), 237(1.408), 249(1.64) revealed as basic carbazole alkaloid. The IR spectrum of compound-1 showed the presence of -OH and -NH functional groups at (3250 cm^{-1}) and also carbonyl and aromatic benzene groups at (1743, 1612 and 1588 cm^{-1}). In the ^1H NMR spectrum the compound-1 revealed a total of 11 signals representing 13 protons as characteristic of carbazole alkaloid. In the down field region three singlets; intramolecular bonding OH-(δ 11.66) with -CHO (δ 9.91, C-3) and one broad signal due to -NH (δ 8.21) and another singlet proton H-4 at δ 8.03 (s, 1H). In the aromatic region four signals with δ values 7.26 (td, J=3.8, 7.7Hz, 1H), 7.40 (td, J=3.8, 7.7Hz, 1H) showed the absence of substituent in ring A. The presence of prenyl group was revealed by one methylene protons at δ 3.64 (d, J=6.9Hz, 2H), δ 5.33 (t, J= 6.9Hz, 1H) and one dimethyl group (δ 1.89(s, 3H), δ 1.72(s, 3H) (Table 2, Figure 1).

According to ^{13}C NMR and DEPT (90, 135) spectra it was revealed that the presence of 18 signals and 18 carbons. Furthermore, the DEPT spectrum showed the presence of one aldehyde carbon, 8 quaternary carbons, 6 methine carbons, one methylene carbon and two methyl carbon as shown in table 2. The 2D NMR spectra such as DQF-COSY revealed the correlation of two adjacent protons. The HSQC spectrum gave the direct connection between protons and carbon. The HMBC data showed the position of H-4 singlet proton to C-1a, C-2, C-3, C-4a, -CHO. The presence and attachment of prenyl group to core carbazole alkaloid was revealed by the proton H-1' to C-1, C-1a, C-2, C-10, C-11 (Table 2). Finally, the combination of fragments and attachments of substituents were confirmed by HMBC spectrum and compound (1) was elucidated as 7-hydroxyheptaphylline. It was also agreement with literature values.

The isolated compound was tested for their cytotoxicity on *Hela* cell lines (Table 1). Both of them showed toxicity against to cervix cancer cell (*Hela*) with IC₅₀ values 41.4 and $\mu\text{g}/\text{ml}$, where doxorubicin was used as positive control.

4 CONCLUSIONS

Nowadays the demand for anticancer drugs which are effective and less side effects are increasing. To fulfill this aim we conducted the isolation of bioactive compounds from root of *C. excavata*. The isolated compound was evaluated for their cytotoxicity. The compound (1), 7-hydroxyheptaphylline with IC₅₀ 41.4 µg/ml. So the result revealed that the compound-1 should be further study for a potential natural anticancer candidate for HeLa cell in future.

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