ISOLATION, STRUCTURE ELUCIDATION AND SCREENING OF THE ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF SECONDARY METABOLITES FROM THE STEM BARK OF *Aegle marmelos* (L.) Corrêa. (Ohshit)

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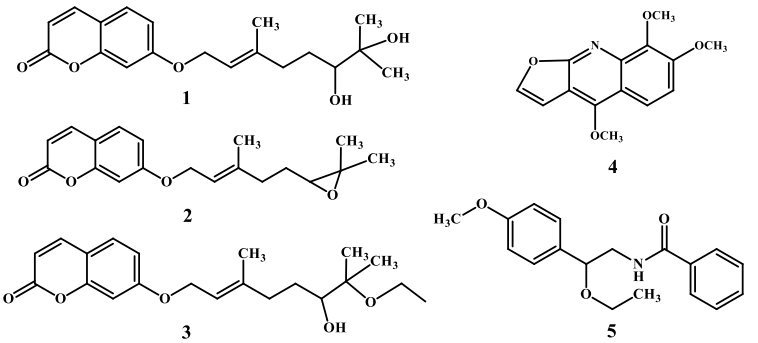
Abstract

Three coumarin compounds (**1**), **(2)**, **(3)** and two alkaloid compounds **(4)** and **(5)** were isolated from the stem bark of *Aegle marmelos*, collected from Sagaing Region, Myanmar. The structures of these compounds were elucidated mainly by extensive spectroscopic analysis (1D and 2D NMR). The isolated compounds were screened for free radical scavenging activity using DPPH radical-scavenging assay. In addition, compounds (**1-5)** were evaluated in vitro for their cytotoxic activity against Hela cells. It was observed that pure compounds (**1**), **(2)**, **(3)**(EC_{50}>100 μM) exhibited lowerantioxidant activity than ascrobic acid(EC_{50} 27.5 μM). The compound **(4)** (IC_{50} 55 μM) exhibited low cytotoxic activity against Hela cell lines while compounds **(1)** and **(5)** displayed very weak cytotoxic activity with IC_{50} value > 100 μM.



1 **2** **3** **4** **5**

Keywords: *Aegle marmelos*, coumarin, alkaloid, Hela cells

Introduction

Aegle marmelos (L.)Corrêa. is locally known as Ohshit belonging to the family Rutaceae. It is medium sized tree growing throughout the forest of

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India of altitude 1200 meter. It is found all over India, from sub-Himalayan forest, Bengal, central and south India. It is also found in Bangladesh, Egypt, Malaysia, Myanmar, Pakistan, Sri Lanka and Thailand (Lambole *et al.*, 2010).In all natural habitats, plants are surrounded by an enormous number of potential enemies (biotic) and various kinds of abiotic environmental stress. Nearly all ecosystems contain a wide variety of bacteria, viruses, fungi, nematodes, mites, insects, mammals and other herbivorous animals, greatly responsible for heavy reduction in crop productivity. By their nature, plants protect themselves by producing some compounds called as secondary metabolites. Secondary metabolites, including terpenes, phenolics and nitrogen (N) and sulphur (S) containing compounds, defend plants against a variety of herbivores and pathogenic microorganisms as well as various kinds of abiotic stresses (Mazid *et al.*, 2011).The different parts of *Aeglemarmelos* plant contain number of coumarins, alkaloids, sterols and essential oils(Lambole *et al.*, 2010).According to Khare (2007) a number of coumarins (including xanthotoxol and alloimperatorin methyl ether), flavonoids (including rutin and marmesin), alkaloids (including alpha-fagarine), sterols and essential oils have been isolated from plant parts. According toKumar *et al.*, *Aeglemarmelos* is used to treat fevers, abdomen pain, palpitation of the heart, urinary troubles, melancholia, anorexia, dyspepsia, diabetes and diarrhea in Indian traditional systems of medicine (Kumar *et al.*, 2013).Plant parts like root, stem bark and fruit have been reported for various medicinal properties such as antidiabetic, anticancer, antibacterial, antifungal, antipyretic, analgesic, antioxidant, cardio protective, radioprotective, antidiarrheal, antidysentery, antiulcer, wound healing and many more (Verma *et al.*, 2013). The main aim of the present research work is to perform the isolation and structural elucidation of secondary metabolites from the stem bark of one Myanmar indigenous medicinal plant, *Aeglemarmelos* (L.) Corrêa., Ohshit and screening of the antioxidant and cytotoxic activities of the isolated compounds.

Materials and Methods

General Procedure

FTIR spectra were recorded on FT IR-410 spectrophotometer. NMR spectra were recorded in methanol-d_4 using a Bruker Avance III HD600 spectrophotometer equipped with Prodigy liq. Nitrogen cryoprobe. Chemical shifts for ^{1}H and ^{13}C NMR are given in parts per million (δ) relative to solvent signal (MeOH-d_4: δ_H 3.30 and δ_C49.0; $CDCl_3$: δ_H7.26 and δ_C77.0) as internal standard. Positive mode FAB mass was obtained by using a JEOL JMS HX-110 mass spectrophotometer with m-nitrobenzyl alcohol as matrix. Optical rotations were recorded on a JASCO P-1020 polarimeter (cell length 100 mm). HPLC reversed phase column (C-8-UG-5) was used for purification of compounds. The specific optical rotations were measured on a JASCO P-2200 Polarimeter.The melting point of the pure compounds in crystal form was measured by Stuart SMP 30 melting point apparatus. For cytotoxic assay, cell densities were measured at 450 nm by using Bio-RAD Model 550 Microplate Reader.

Plant Material

Thestembark of *Aeglemarmelos*(L.) Corrêa., Ohshitwas collected from Ywa-thit-kyi village, Sagaing Township, Sagaing Region in October 2014. The plant materials were authenticated by the authorized botanist from Botany Department, University of Mandalay.

Extraction and Isolation

The air dried sample of the stem bark of *A.marmelos* (1 kg) was extracted with95% ethanol (3.0 L) at room temperature for one month to obtain the ethanolic extract. The ethanolic extract was concentrated by evaporator, and dried extract (96.6 g) was obtained. The ethanolic extract was then extracted with ethyl acetate to give 26.44 g of ethyl acetate extract. Then, 2 g of ethyl acetate extract was separated on silica gel eluting with the solvent systems with various ratios:such as n-hexane only, n-hexane : EtOAc (19:1 to 1:4) and EtOAc only. Totally 500 fractions were obtained. Each fraction was checked by TLC, iodine vapour and UV lamp. Then, the fractions of the same R_f value were combined and 26 combined fractions were obtained. The

combined fraction 7 (178 mg) was purified by HPLC reversed phase column (C-8-UG-5) using a gradient system of (A) methanol-water (2:98) and (B) MeOH with A: B (50:50 to 0:100) as a mobile phase to yieldcompounds **(1)** (5.1 mg), **(2)** (4.1 mg), **(3)** (5.2 mg) and **(5)** (11.7 mg) as colourless oil forms and compound **(4)** (0.8 mg) as a white crystal (m.p. 177-179°C).The yield percentages of compounds (**1-5)** were found to be 0.26%, 0.21%, 0.26%, 0.04% and 0.59 % based on ethyl acetate extract respectively.

Antioxidant Activity Screening of the Isolated Compounds (1-5)

The antioxidant activities of the isolated pure compounds were determined by using 2, 2-diphenyl-1- picrylhydrazyl radical scavenging activity assay (Yamaguchi *et al*., 1998).Its reaction principle was based on mechanism of free radicals inhibition by hydrogen transfer.The antioxidant activity of sample was expressed in EC_{50} (50% effective concentration). 500 μL of test solutions in various concentrations (100 μg/mL, 50μg/mL and 10 μg/mL) and 500 μLof 0.2 M acetate buffer(pH 5.5)solution were mixed in a test tube. 250 μL of $5x10^{-4}$ M DPPH solution was added to the mixture in dark. The mixture was homogenized by using a vortex mixer in a dark room (resistant to UV light) and stands for 30 min at room temperature. After that, absorbance of the mixture was measured at λ_{max} 517 nm by a UV-Vis spectrophotometer. Vitamin C was used as a reference compound in the same concentration range as the test compounds. A control solution was prepared by mixing 500 μL of buffer (pH 5.5) solution, 500 μL of ethanol and 250 μL of 5×10^{-4} M DPPH solution in the test tube. Blank solution was prepared by mixing 500 μL of buffer (pH 5.5) solution with 750 μL of ethanol in the test tube. The mean values were obtained from triplicate experiments. The capability of scavenging DPPH radicals as a percentage of DPPH remaining in the resulting solution was determined using the following equation:

$$\text{DPPH}\ (\%) = \frac{\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}}{\text{Abs}_{\text{control}}} \times 100\ \%$$

where $Abs_{control}$ is absorbance of control and Abs_{sample} is absorbance of sample. The antioxidant power (EC_{50}) is expressed as the concentration of test substance (μg/mL) that result in a 50% reduction of initial absorbance of DPPH solution and that allows to determine the concentration. EC_{50} (50%

effective concentration) values were calculated by linear regressive excel program (Yamaguchi *et al*., 1998).

Cytotoxicity Test of the Isolated Compounds (1-5)

In vitro cytotoxic effects of the isolated compounds (1-5) were measured on a Hela(cervix adenocarcinoma) cell line by the colorimetric method using a Cell Counting kit-8 that was based on the tetrazolium salt/formazan system (Ishiyama *et al*., 1993). HeLa cell (JCRB9004) was obtained from Japanese Collection of Research Bioresources (JCRB) cell bank. Cells were cultured in minimum essential media (MEM) supplemented with 10 % fetal bovine serum. For the cytotoxic assay, cells were seeded at a density of 5×10^3 cells/well in 0.2 mL of medium in 96-multiwell plates and adhered. Samples were dissolved in saline containing 10% DMSO and sterilized by filtration. Series of the diluted samples (0.2 mL) were then added to the cells. The plate was incubated at 37°C under 5% CO_2 atmosphere for 48 h. Twenty microliters of cell counting kit-8 (based on the tetrazolium salt/formazan system) was added to each well, and the microplate was incubated for 1 h, after which cell densities were measured at 450 nm using Bio-RAD Model 550 Microplate Reader.

Results and Discussion

The crude ethyl acetate extract of the stem bark of *Aegle marmelos*was separated by silica gel column chromatography and purified by HPLC reversed phase column to furnish three coumarin compounds (**1**), **(2)**, **(3)** and two alkaloid compounds **(4)** and **(5)**. The structures were elucidated by FT IR and detailed NMR investigations including ^{1}H and ^{13}CNMR, COSY, HSQC and HMBC spectra (Silverstein *et al*., 2005).

Compound (1): colourless oil; $[\alpha]_D$+ 25.45 (c 0.022, $CHCl_3$),FTIR (KBr) ν_{max} cm^{-1}: 3423, 3080, 2972, 2862, 1730,1613, 1507, 1464, 1402, 1350, 1278, 1231, 1199, 1128,1078, 999, 893. FAB-MS *m/z*: 333 $[M+H]^+$ (calc. 332 for $C_{19}H_{24}O_5$), ^{13}Cand ^{1}HNMR: (Table 1).

Compound (2): colourless oil; $[\alpha]_D$: + 16 (c 0.022, $CHCl_3$). FT IR (KBr) ν_{max} cm^{-1}: 3078, 2927, 2852, 1732,1612, 1507, 1456, 1402, 1349,1277, 1230,

1197, 1124, 891. FAB-MS *m/z*: 315 $[M+H]^+$ calc. 314 for $C_{19}H_{22}O_4$), ^{13}Cand ^{1}HNMR: (Table 1).

Compound (3): colourless oil; $[\alpha]_D$+ 32 (c 0.03, $CHCl_3$).FTIR (KBr) ν_{max} cm^{-1}: 3471, 3082, 2974, 2876, 1733, 1613, 1507, 1463, 1402, 1350, 1278, 1231, 1197, 1125, 1071, 1000, 891. FAB-MS *m/z*: 361 $[M+H]^+$ (calc. 360 for $C_{21}H_{28}O_5$),^{13}Cand ^{1}HNMR: (Table 1).

Compound (4): white crystals; m.p. 177 – 179°C**.**FT IR(KBr) ν_{max} cm^{-1}: 3042, 2940, 2842, 1614, 1577, 1507, 1456, 1394, 1270, 1239. Mass spectrum *m/z* (% intensity): 259 (M+, 59), 244 (29), 216 (10), 162 (100), 153 (57), 134 (41). ^{13}Cand ^{1}HNMR: (Table 2).

Compound (5): colourless oil; $[\alpha]_D$+ 10.2 (c 0.02, $CHCl_3$). FT IR (KBr) $\nu_{max}$$cm^{-1}$:3316,3063, 2968, 2937, 1652, 1611, 1509, 1462, 1294, 1246, 1095, 831, 711.FAB-MS *m/z*: 322 $[M+Na]^+$ (calc. 299 for $C_{18}H_{21}NO_3$), ^{13}Cand ^{1}HNMR: (Table 3).

Structural Elucidation of Coumarin Compound (1)

Compound **(1)** was isolated as a colourless oil with a molecular formula of $C_{19}H_{24}O_5$ determined by FAB-MS with *m/z*333 $[M + H]^+$which indicated the molecular mass (*m/z*332).The unsaturated degree of 8 was calculated from the molecular formula. FT IR spectrum showed the presence of broad peak at 3423cm^{-1} exhibited OH group.Peak at 3080 cm^{-1} revealed the aromatic C-H, whereas peaks at 2972 and 2862 cm^{-1} were due to aliphatic C-H stretching vibration. The band at 1730 cm^{-1} indicated the presence of δ- lactone group. Intense bands at 1613, 1507 and 1464 cm^{-1} were the characteristics of conjugated C=Cbonds. The absorption bands at 1350 and1278-1078 cm^{-1} confirmed the presence of gem dimethyl group and ether groups respectively. The DEPT spectrum indicated the presence of three methyl carbons, three methylene carbons, seven methine carbons, five quaternary C-atoms and one carbonyl carbon. The ^{1}HNMR spectrum (Table 1) exhibited three aliphatic methyl signals [δ_H 1.78 (3H, s); 1.17 (3H, s); 1.21 (3H, s)], an olefinic signal [δ_H 5.53, (1H, t, 6.5 Hz)] and three methylene signals [δ_H 4.62 (2H, d, 6.5 Hz); 2.37, 2.17, (2H, m); 1.65, 1.46 (2H, m)]. In addition, there were five sp^2 signals, including double doublet signal of δ_H 6.85 with 7.36 [an *o*-coupling (J = 8.6 Hz)] and that of δ_H 6.85 with 6.82

[a*m*-coupling (*J* = 2. 3 Hz)], as well as two doublet signals at δ_H 6.24 and 7.63, with an *o*-coupling (*J* = 9.4 Hz), each with one integrated proton. A pair of doublets, each with coupling constant 9.4 Hz at δ_H6.24 and 7.63 in the ^{1}HNMR spectrum was typical of H-3 and H-4 of the coumarinnucleus and another pair of doublets, each with coupling constant 8.6 Hz at δ_H7.36 and 6.85 was assigned to H-5 and H-6, respectively. The ^{1}H, ^{1}H-COSY analysis of **(1)** led to the four substructures indicated by boldfaced lines in (Figure1) which were supported by HMBCs (Figure1). The COSY correlations (Figure 1) between H-3 (δ_H6.24)/ H-4 (δ_H 7.63), H-6 (δ_H6.85)/H-5 (δ_H7.36) and H-6 (δ_H6.85)/H-8 (δ_H 6.82) and HMBC correlations (Figure1)observed from H-5 to C-7 (δ_C162.07) and C-8a (δ_C155.87), from H-6 to C-8 (δ_C101.59) and C-4a (δ_C 112.48), from H-8 to C-2, C-6, C-7, C-8a and C-4a, from H-4 to C-3 (δ_C113.02), C-2 (δ_C161.24) and C-8a (δ_C155.87)proved the presence of coumarin nucleus (chromen-2-one moiety). The COSY correlations (Figure1)between H-5′ ($\delta_H\delta$ 1.46, δ_H1.65)/H-4′ (δ_H2.17, δ_H2.37) and H-1′ (δ_H4.62)/H-2′ (δ_H5.53) and HMBC correlations (Figure 1) of H-7' -Me (δ_H 1.21 and δ_H1.17) with C-6′ (δ_C77.98) and C-7′ (δ_C73.05), H-3' -Me (δ_H 1.78) with C-2′ , C-3′ and C-4′ , H-2′ with C-3' -Me (δ_C16.75) and C-3′ (δ_C142.14), H-1′ (δ_H4.62) with C-2′ , C-3′ and H-5′ with C-3′ and C-4′ suggested the presence of dimethyl oct-2-ene moiety. The two moieties could be logically connected by an oxygen atom because of the presence of HMBC correlation (Figure1)between the H-1′ (δ_H4.62) and C-7 (δ_C162.07) that occurred in the lower magnetic field. From this it was presumed that this carbon was attached to oxygen and the proposed molecular formula ($C_{19}H_{22}O_3$) required two hydroxyl groups. Therefore, these two hydroxyl groups could be connected to the downfield chemical shift carbons at C-6′ (δ_C77.98) and C-7′ (δ_C 73.05). Hence, compound **(1)** was identified as (*E*)-7-((6, 7-dihydroxy-3, 7-dimethyl oct-2-en-1-yl) oxy)-*2H*- chromen-2-one (Figure2).

Structural Elucidation of Coumarin Compound (2)

Compound **(2)** was also isolated as colourless oil, and assigned as the molecular formula of $C_{19}H_{22}O_4$ on the basis of FAB-MS spectral data

$[M+H]^+$, *m/z* at 315 which indicated the molecular mass (*m/z* 314).The FT IR absorption bands at 3078, 1732, 1456, 1349,1277 and 1124 cm^{-1} confirmed the presence of the aromatic C-H, an ester carbonyl group, a conjugated double bond (C=C), gem dimethyl group and ether group, respectively. The unsaturated degree of 9 was calculated from the molecular formula, $C_{19}H_{22}O_4$. The ^{1}HNMR and ^{13}CNMR spectral data of compound **(2)**(Table 1) were very similar to those of compound **(1)**, except for the appearance of an epoxide ring instead of two hydroxyl groups of compound **(1).** The ^{1}HNMR and ^{13}CNMR data for the coumarin nucleus of compound **(2)**were almost identical with those of compound **(1).**In the side chain, two hydroxyl groups in compound **(1)** were replaced by an oxymethine at δ_H2.75 (1H, t, 6.2, 12.5 Hz), typical of an epoxide. The C-6' methine signal was also shifted to higher field at δ_H2.75 indicating the characteristics of an epoxide ring. Selective C-H long range correlations provided the carbon framework of **(2)** as shown in (Figure 1).The^{1}HNMR spectrum (Table 1) exhibited three aliphatic methyl signals [δ_H 1.77 (3H, s); 1.26 (3H, s); 1.26 (3H, s)], an olefinic signal [δ_H 5.53 (1H, t, 6.5 Hz)], three methylene signals [δ_H 4.68 (2H, d, 6.5 Hz); 2.23, 2.24 (2H, m), 1.68, 1.69 (2H, m)]. In addition, there were five sp^2 signals, including double doublet signal of δ_H 6.92 with 7.52 [an *o*-coupling (*J* = 8.7 Hz)] and that ofδ_H 6.92 with 6.89 [a*m*-coupling(*J* = 2.3 Hz)], as well as two doublet signals at δ_H 6.23 and 7.87 with an *o*-coupling (*J* = 9.5 Hz), each with one integrated proton. ^{13}CNMR spectrum (Table 1) showed 19 carbon signals, including one ester carbonyl (δ_C163.40), five quaternary carbons, seven methine carbons, three methylene carbons and three methyl carbons. The ^{1}HNMR and ^{13}CNMR assignments of **(2)** were confirmed by analysis of the 2D NMR spectra (COSY, HSQC and HMBC). The ^{1}H, ^{1}H-COSY analysis of **(2)** led to the four substructures indicated by boldfaced lines in (Figure1).The COSY spectrum of **(2)** showed the connection of H-3↔H-4, H-5↔H-6↔H-8, H-4'↔H-5'↔H-6' and H-1'↔H-2' (Figure1)**.** The HMBC spectrum revealed that H-3 correlated with C-2; H-4 with C-2, C-3and C-8a; H-5 with C-7 and C-8a, H-6 with C-4a and C-8; H-8 with C-2, C-6 and C-4a; H-1' with C-7; H-5' with C-3' and C-7'; H-7' -Me with C-5', C-6' and C-7'; H-3' -Me with C-2' and C-3' ; H-2' with C-3' , C-4' which supported the structure of **(2)** (Figure1) and identified as (*E*)-7-((5-(3, 3-dimethyloxiran -2-yl) - 3-methylpent-2-en-1-yl) oxy)-*2H*-chromen- 2-one (Figure 2).

Structural Elucidation of Coumarin Compound (3)

Compound **(3),** colourless oil,$C_{21}H_{28}O_5$was identified as (*E*)-7-((7-ethoxy-6-hydroxy-3,7-dimethyloct-2-en-1-yl)oxy)-*2H*-chromen-2-one (Figure2) by the following spectroscopic data. The result of FAB-MS spectrum gave a molecular formula of $C_{21}H_{28}O_5[M+H]^+$, *m/z* at 361 which indicated the molecular mass (*m/z* 360).The unsaturated degree of 8 was calculated from the molecular formula. The FT IR absorption bands at 3471, 3082, 1733, 1613, 1507, 1463, 1350, 1278 and 1071 cm^{-1} confirmed the presence of a hydroxyl group, aromatic C-H, an ester carbonyl group, conjugated double bonds (C=C), gem dimethyl group and ether groups respectively. The IR spectrum was informative with the presence of δ lactone carbonyl functionality at 1733 cm^{-1}.The ^{1}HNMR spectrum of compound **(3)**(Table 1) was very similar to those of compound **(1)**, except that a hydroxyl group at C-7 was replaced by an ethoxy group. The ^{1}HNMR spectrum (Table 1) exhibited four aliphatic methyl signals [$\square_H$1.78 (3H, s); 1.09 (3H, s); 1.12 (3H, s); 1.15 (3H, t)], an olefinic signal [$\square_H$ 5.52 (1H, t, 6.5 Hz)], three methylene signals[$\square_H$ 4.62 (2H, d, 6.5 Hz);2.41, 2.16 (2H, m),1.55, 1.47 (2H, m)]. In addition, there were five sp^2 signals, including double doublet signal of $\square_H$ 6.85 with 7.36 [an *o*-coupling (*J* = 8.5 Hz)] and that of $\square_H$ 6.85 with 6.82 [a*m*-coupling (*J* = 2.4 Hz)], as well as two doublet signals at$\square_H$ 6.24 and 7.63, with an *o*-coupling (*J* = 9.4 Hz), each with one integrated proton.^{13}CNMR spectrum of compound **(3)**(Table 1) shows 21 carbon signals, including one ester carbonyl ($\square_C$161.24), five quaternary carbons, seven methine carbons, four methylene carbons and four methyl carbons.The ^{1}H, ^{1}H-COSY analysis of **(3)** led to the five substructures indicated by boldfaced lines in (Figure 1).The COSY correlations (Figure 1)between H-3 ($\square_H$6.24)/H-4 ($\square_H$7.63), H-6 ($\square_H$ 6.85)/H-5 ($\square_H$ 7.36) and H-6 ($\square_H$ 6.85)/H-8 ($\square_H$ 6.82) and HMBC correlations (Figure 1)observed from H-5 to C-7 ($\square_C$ 162.16) and C-8a ($\square_C$155.89), from H-6 to C-8 ($\square_C$101.61) and C-4a ($\square_C$112.44), from H-8 to C-2, C-6, C-7, C-8a and C-4a, from H-4 to C-3 ($\square_C$112.99), C-2 ($\square_C$161.24) and C-8a proved the presence of coumarinnucleus (chromen-2-one moiety). The COSY correlations (Figure 1)between H-5′ ($\square_H$1.55, δ 1.47)/H-4′ ($\square_H$2.41,$\square_H$2.16) and H-1′ ($\square_H$4.62)/H-2′ ($\square_H$ 5.52) and HMBC

correlations between H-7' -Me ($\square_H$1.09 and $\square_H$1.12)/C-6′ ($\square_C$76.40)/C-7′ ($\square_C$78.0), H-3' -Me ($\square_C$1.78)/C-2′ /C-3′ and C-4′ , H-2′ /C-3' -Me ($\square_C$16.86)/C-3′ , H-1′ ($\square_H$ 4.62)/C-2′ /C-3′ and H-5′ /C-3′ /C-4′ suggested the presence of dimethyl oct-2-ene moiety. The two moieties could be logically connected by an oxygen atom because of the presence of HMBC correlation between the H-1′ ($\square_H$ 4.62)/C-7 ($\square_C$162.16) that occurred in the lower magnetic field. Based on HMBC data (Figure 1),there were two methyl proton signals that were substituted on the quaternary carbon (C-7') and showed HMBC correlations to C-6' and C-7' . Moreover, the COSY correlation between CH_2 (7' -OEt) ($\square_H$3.42)/CH_3(7' -OEt) ($\square_H$1.15) gave an ethyl group which could be connected to C 7' by an oxygen atom due to the presence of down field chemical shift carbon atoms CH_2 (7' -OEt) (δ_C 56.43) and C 7' (δ_C 78). It was proven by HMBC correlation (Figure 1) of sp^3 methylene protons at [δ_H3.42, CH_2 (7' -OEt)] with C-7' (δ_C 78).

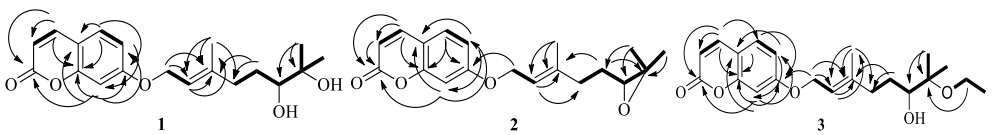

Figure 1 Key (—) COSY and ( ) HMBC correlation of (1-3)

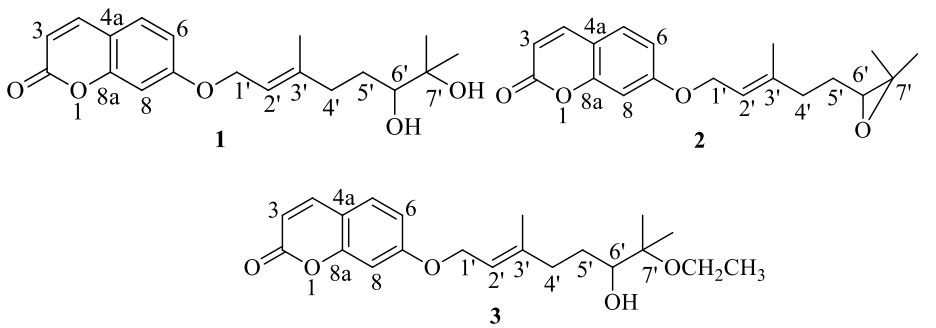

Figure 2: Structures of compounds (1-3)

Table 1: ^{1}H NMR (600 Hz) and ^{13}C-NMR (150 MHz) Data for Compounds (1), (2) and (3)

No.	(1) (chloroform-d)		(2) (chloroform-d)		(3) (chloroform-d)	
	^{13}CNMR δ_C, (ppm)	^{1}HNMR δ_H, (ppm)	^{13}CNMR δ_C, (ppm)	^{1}HNMR δ_H, (ppm)	^{13}CNMR δ_C, (ppm)	^{1}HNMR δ_H, (ppm)
2	161.24	-	163.40	-	161.24	-
3	113.02	6.24, d, 9.4 Hz	113.27	6.23, d, 9.5 Hz	112.99	6.24, d, 9.4 Hz
4	143.4	7.63, d, 9.4 Hz	145.78	7.87, d, 9.5 Hz	143.39	7.63, d, 9.4 Hz
4a	112.48	-	113.97	-	112.44	-
5	128.68	7.36, d, 8.6 Hz	130.39	7.52, d, 8.7 Hz	128.66	7.36, d, 8.5 Hz
6	113.26	6.85, d, 2.3, 8.6 Hz	114.48	6.92, d, 2.3,8.7 Hz	113.22	6.85, d, 2.4, 8.5 Hz
7	162.07	-	163.81	-	162.16	-
8	101.59	6.82, d, 2.3 Hz	102.51	6.89, d, 2.3 Hz	101.61	6.82, d, 2.4 Hz
8a	155.87	-	157.11	-	155.89	-
1'	65.40	4.62, d, 6.5 Hz	66.46	4.68, d, 6.5 Hz	65.49	4.62, d, 6.5 Hz
2'	118.94	5.53, t, 6.5 Hz	120.99	5.53, t, 6.5Hz	118.66	5.52,t, 6.5Hz
3'	142.14	-	141.52	-	142.48	-
4'	36.53	2.37, m 2.17, m	37.13	2.23, 2.24, m	36.64	2.41, m 2.16, m
5'	29.43	1.65, m 1.46, m	28.05	1.68, 1.69, m	29.20	1.55, m 1.47, m
6'	77.98	3.35, dd, 2.0,10.4 Hz	65.55	2.75, t, 6.2, 12.5Hz	76.40	3.40, dd, 2.0, 10.4 Hz
7'	73.05	-	60.25	-	78.0	-
3' -Me	16.75	1.78, s	16.66	1.77, s	16.86	1.78, s
7' -Me	23.28	1.17, s	18.86	1.26, s	19.34	1.09, s
7' -Me	26.53	1.21, s	24.95	1.26, s	21.47	1.12, s
CH_2 (7' -OEt)	-	-	-	-	56.43	3.42, t
CH_3 (7' -OEt)	-	-	-	-	16.10	1.15, t, 7.0, 14 Hz

C=C

Structural Elucidation of Alkaloid Compound (4)

Compound (4): Skimmianine appeared as white crystals withm.p. 177-179°C**.**The EI MS spectrum shows molecular ion at odd number *m/z* 259, corresponds to molecular formula $C_{14}H_{13}NO_4$.FT IR spectrum exhibited the presence of the C-H aromatic and C-H aliphatic at 3042 and 2940 cm^{-1}. Peaks at 1614, 1577, 1507, 1489 and 1456 cm^{-1} appeared due to C=C and C=N ring skeletal stretching vibrations, while C-O-C bond was represented by peaks at 1270 and 1239 cm^{-1}. The strong singlet ^{1}H NMR signals (Table 2) at δ_H4.49, 4.02 and 3.97 were typical of methoxy groups. The rest of the signals occurred in the aromatic region. A pair of doublet at δ_H8.06 and δ_H7.37 with coupling constant (*J*=9.4 Hz) was assigned to ortho coupling protons at C-5 and C-6. Another pair of doublet at δ_H7.76 and δ_H7.33 (*J*=2.82 Hz) was typical of adjacent of furan protons (H-2' and H-3'). ^{13}C NMR spectrum showed the presence of fourteen carbons in which three of them were methyl carbons resonated at δ_C60.08, 57.25and 61.72 ppm. Other signals included four methine and seven quaternary carbon absorptions. The assignment of ^{1}H and ^{13}CNMR data was further substantiated by HSQC, COSY and HMBC spectral data (Table 2). The ^{1}H, ^{1}H-COSY analysis of **(4)** gave rise to the two substructures indicated by boldfaced lines in (Figure 3) which were supported by HMBCs (Table 2 and Figure 3).The complete assignment and correlations are summarized in (Table 2). Based on these data, the structure of the compound (4) was identified as 4,7,8-trimethoxyfuro [2,3-b] quinoline or skimmianine (Figure 4).

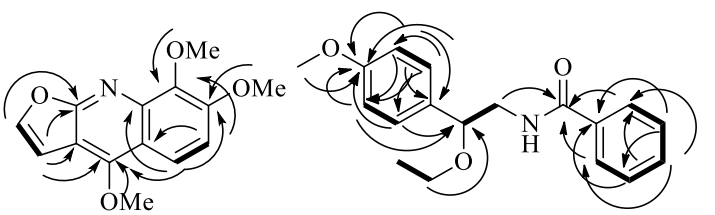

Figure 3. Key (—) COSY and (↷) HMBC correlation of (4) and (5)

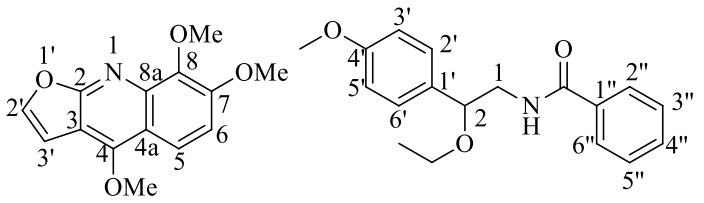

Figure 4: Structures of compounds (4) and (5)

Table 2: ^{1}H NMR (600 Hz) and ^{13}C NMR (150 MHz) Data for the Isolated Compound (4)

C position	^{13}CNMR δ_C, (ppm)	^{1}HNMR δ_H, (ppm)	COSY correlation	HSQC correlation	HMBC correlation
2	166.09	-	-	-	H-2' , H-3'
3	103.40	-	-	-	H-3'
4	159.29	-	-	-	H-3' ,4-Me, H-5
4a	115.93	-	-	-	H-6
5	119.65	8.06,d 9.4 Hz	H-6	H-5	-
6	113.55	7.37,d,9.4 Hz	H-5	H-6	-
7	153.90	-	-	-	H-5,7-OMe
8	142.70	-	-	-	H-6,8-OMe
8a	142.07	-	-	-	H-5
2'	144.47	7.76,d,2.82 Hz	H-3'	H-2'	-
3'	106.30	7.33, d, 2.82 Hz	H-2'	H-3'	-
4-OMe	60.08	4.49, s	-	H-4-OMe	-
7-OMe	57.25	4.02, s	-	H-7-OMe	-
8-OMe	61.72	3.97, s	-	H-8-OMe	-

Structural Elucidation of Alkaloid Compound (5)

Compound (**5)** appeared as colourless oily form, had molecular formula $C_{18}H_{21}NO_3$ by FAB-MS because of the $[M+Na]^+$, *m/z* at 322 which indicated the molecular mass (*m/z* 299). FT IR spectrum showed the presence of N-H as sharp peak around 3316 cm^{-1}. Peak at 3063cm^{-1} revealed the aromatic C-H, whereas peaks at 2968 and 2937 cm^{-1} were due to aliphatic C-H stretching vibrations. The existence of carbonyl group conjugated to a double bond was represented by the strong peak at 1652 cm^{-1}. In addition, peaks at 1611 and 1509cm^{-1} were attributed of C=C and aromatic ring system.

Weaker band at 1294 cm^{-1}resulted from interaction between N-H bending and C-N stretching. The presence of ether group was shown by peaks at 1246 and 1095 cm^{-1}. The band appeared at 711 cm^{-1} was due to the absorption of N-H wagging. ^{1}HNMR spectrum exhibited that a singlet atδ_H 3.78, integrated for three protons was attributed a methoxy group (H-4' -OMe). Mono-substituted aromatic protons were represented by H-2'' and H-6'' which appear as doublet of doublet at δ_H7.75 (*J*=7.4,*J*=1.3 Hz), while H-3'' and H-5'' resonate as triplet at δ_H7.44 (*J*=7.4, *J*=1.44 Hz) and H-4'' exhibits a doublet of doublet at δ_H7.52(*J* = 7.4,*J* = 1.44 Hz). Another pair of doublet (*J*=8.64Hz) were given by H-3' and H-5' (δ_H 6.92) which ortho-coupled to H-2' and H-6' (δ_H 7.29). The inequivalent methylene protons at C-1 appeared as two doublet of doublet at δ_H3.52 (*dd, J*=13.59, *J*=7.95 Hz) and δ_H 3.54(*dd, J*=13.59,*J*=7.95 Hz). The neighbouring methine proton at C-2 which is coupled to protons (H-1) resonates to give another doublet of doublet atδ_H 4.51 (*dd, J*= 8.28, *J*=3.0 Hz). Another inequivalent methylene protons at CH_2 (2-OEt) appeared as multiplets at δ_H 3.42 and δ_H 3.36. The neighbouring methyl protons at CH_3 (2-OEt) are coupled to inequivalent methylene protons CH_2 (2-OEt) to give another doublet of doublet at δ_H1.16 (*dd, J*= 7.02, *J*=14.04 Hz). All of the coupling interactions between protons were confirmed by COSY spectrum such as the cross peaks which were due to the interaction between H-1 and H-2, between inequivalent methylene protons at CH_2 (2-OEt) and neighbouring methyl protons at CH_3 (2-OEt) and between appropriate aromatic protons (Table 3 and Figure 3). The ^{1}H, ^{1}H-COSY analysis of **(5)** produced the five substructures indicated by boldfaced lines in (Figure 3) which were supported by HMBCs (Table 3 and Figure 3).^{13}C NMR spectrum gave absorption peaks representing mostly aromatic and sp^3 carbons. The presence of carbonyl group was shown by peak at δ_C 170.33. The assignment of ^{1}H and ^{13}C NMR data was further substantiated by HSQC and HMBC spectrum, which the complete assignment and correlations are summarized in (Table 3). Based on these spectral data the compound **(5)** was identified as *N*-(2-ethoxy-2-(4-methoxyphenyl) ethyl) benzamide(Figure 4).

Table 3:^{1}H NMR (600 Hz) and ^{13}C-NMR (150 MHz) Data of the Isolated compound (5)

C position	^{13}CNMR δ_C, (ppm)	^{1}HNMR δ_H, (ppm)	COSY correlation	HSQC correlation	HMBC correlation
1	47.46	3.54, 3.52, dd, 13.59, 7.95 Hz	H 2	H 1	-
2	81.05	4.51,dd, 8.28, 3.0 Hz	H 1	H 2	H 5' , H 6' ,CH_2 (2-OEt)
1'	133.50	-	-	-	H 3'
2' , 6'	129.07	7.29, d, 8.64 Hz	H 3' , H 5'	H 2' , H 6'	H 2' , H 6'
3' , 5'	114.94	6.92, d, 8.64 Hz	H 2' , H 6'	H 3' , H 5'	H 2' ,H 3' , H 5'
4'	161.03	-	-	-	H 2' , H 6' , H 3' , H 5' , H 4' -OMe
1' '	135.80	-	-	-	H 3' ' , H 5' '
2 ' ' , 6' '	128.23	7.75, dd, 7.4, 1.3 Hz	H 3' ' , H 5' ' H 4' '	H 2 ' ' , H 6' '	H 4' ' H 2 ' ' , H 6' '
3' ' , 5' '	129.53	7.44, t, 7.4, 1.3 Hz	H 2 ' ' , H 6' ' H 4' '	H 3' ' , H 5' '	H 3' ' , H 5' ' H 4' '
4' '	132.61	7.52, dd, 7.4, 1.44 Hz	H 3' ' , H 5' ' H 2' ' , H 6' '	H 4' '	-
4' -OMe	55.71	3.78, s	-	H 4' -OMe	-
-CONH	170.33	-	-	-	H 1, H 2 ' ' , H 6' '
CH_2(2-OEt)	65.09	3.42, 3.36, m	CH_3(2-OEt)	CH_2(2-OEt)	-
CH_3(2-OEt)	15.59	1.16, dd, 7.02,14.04Hz	CH_2(2-OEt)	CH_3(2-OEt)	-

The Antioxidant Activity and Cytotoxic Activity of the Isolated Compounds (1-5)

The antioxidant activity and cytotoxic activity of the isolated compounds are shown in Tables 4 and 5. Compounds (**1) – (5)** were tested for antioxidant activity by using DPPH assay. According to these data, DPPH radical scavenging activities of isolated pure compounds**(1), (2), (3)** (EC_{50}>100 μM) exhibited lower activity than ascrobic acid(EC_{50} 27.5 μM). However, the pure compounds **(4)** and **(5)** did not show any DPPH radical scavenging activity. In addition, compounds (**1) – (5)**were further evaluated for their *in vitro* cytotoxic activities against Hela cell lines by using cell counting kit 8. In accordance with these data, compound (**4)**displayed low cytotoxicactivity against Hela cell lines with IC_{50} values of (55 μM) while compounds (**1)** and (**5)** displayed very weak cytotoxic activity with IC_{50} value > 100 μM.

Table 4: Antioxidant Activity (EC_{50}) of Ascorbic acid and Compounds (1-5) Isolated from *Aeglemarmelos*

Compounds	EC_{50}(μM)
Ascorbic acid	27.5
1	>100
2	>100
3	>100
4	ND
5	ND

ND = Not detected

Table 5:The Cytotoxic Activity of Isolated Compounds (1-5) from *Aeglemarmelos*

Compounds	Cytotoxic Activity IC_{50} (μM)
1	> 100
2	-
3	-
4	55
5	> 100

Conclusion

In this research work, one of Myanmar medicinal plants, stem bark of *Aegle marmelos* was used for isolation and structural elucidation of secondary metabolites and screening of the antioxidant and cytotoxic activities. The structures of three coumarin compounds (**1**), **(2)**, **(3)** and two alkaloid compounds **(4)** and **(5)** could be assigned by advanced spectroscopic techniques.Moreover, evaluation of antioxidant activities and cytotoxic activities of the pure compounds were also performed. According to these data, it was found that DPPH radical scavenging activities of isolated pure compounds(**1**), **(2)**, **(3)**(EC_{50}>100 μM) exhibited lower activity than ascrobic acid(EC_{50} 27.5 μM). The compound **(4)**(IC_{50} 55 μM) showed low cytotoxic activity against Hela cell lines while compounds **(1)** and **(5)** indicated very weak cytotoxic activity with IC_{50} value > 100 μM.

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