Malaysian Journal of Chemistry, 2022, Vol. 24(4), 26-35
(Special Issue: *8th International Conference for Young Chemists (ICYC) 2022*)

Cytotoxic Activity of Eburnane-type Indole Alkaloids Isolated from *Kopsia terengganensis* Against HT-29 Human Colon Cancer Cell

Wan Nur Huda Wan Hanafi[1], Ibrahim Bello[1], Nurhisyam Zakaria[2], Norhafiza Mohd Arshad[3], Pandian Bothi Raja[1], Mohd Hazwan Hussin[1], Khalijah Awang[4], Marc Litaudon[5] and Mohamad Nurul Azmi Mohamad Taib[1]*

[1]School of Chemical Sciences, Universiti Sains Malaysia, 11800 Penang, Malaysia
[2]Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[3]Centre for Research in Biotechnology for Agriculture (CEBAR), University of Malaya, 50603 Kuala Lumpur, Malaysia
[4]Department of Chemistry, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia
[5]Institut de Chimie des Substances Naturelles, CNRS-ICSN UPR2301, Univ. Paris-Sud 11, av. de la Terrasse, 91198 Gif-sur-Yvette, France
*Corresponding author (e-mail: mnazmi@usm.my)

The genus *Kopsia* from the Apocynaceae family bears a large group of indole alkaloids which possessed active biological activities. The focus of this study was to elucidate the isolated alkaloids and investigate their biological activity. Six indole alkaloids from the eburnane-type; eburnamine (**1**), isoeburnamine (**2**), eburnaminol (**3**), larutensine (**4**), eburnamenine (**5**), eburnamonine (**6**), and one from aspidospermane-type; quebrachamine (**7**) were isolated from the bark of *Kopsia terengganensis*. These alkaloids were characterized using various chromatographic techniques and spectroscopic methods and were compared with the literature. Later, alkaloids **1–4** were subjected to cytotoxic activity using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay against HT-29 colorectal adenocarcinoma. The observed result showed that only compound **3** was able to suppress the growth of cancer cells in a dose-dependent manner with the half-maximal inhibitory concentration (IC_{50}) of 75.8± 3.06 µM. It also shows a good cytotoxic activity compared to cisplatin with IC_{50} value of >100 µM as a control drug. This illustrated that further exploration on the eburnane-type alkaloid should be taken on other cancer cell lines since it has shown a profound result on human colon cancer.

Key words: Apocynaceae; *Kopsia*; alkaloid; cytotoxic activity; HT-29 colon cancer

Received: October 2022; Accepted: November 2022

Colon cancer is a global health issue that affects both men and women. According to the statistics by GLOBACAN 2020, colon cancer is ranked second in terms of mortality. Statistics showed that one in 10 of new cases and new deaths are represented by colon cancer. It is estimated that in 2020, more than 1.9 million (6.0%) out of 19.3 million new cases and 0.935 million (5.8%) out of 10 million new deaths were reported due to colon cancer [1]. This reflects the effect of modern lifestyle and changes in diet, i.e., high intake of animal-source products and a more sedentary life [2]. Therefore, there is a demand to find novel and environmentally benign drug therapies for colon cancer. Recently, the uses of natural product-based cancer treatments such as alkaloids have been emphasized and are presently under development [3,4]. Developing cancer drugs from natural resources has shown its potential in reducing the risk of colon cancer and decelerating its progression [5].

Kopsia terengganensis L.Allorge & Wiart (*K. terengganensis*) is a grey bark tree with 3m height found in the lowland of the evergreen forest at 230 to 330 m altitude in Peninsular Malaysia [6]. The plant belongs to the genus *Kopsia*, which belongs to the large family Apocynaceae. Plants from this family have been reported for bearing a large group of indole alkaloids [7,8,9]. By tradition, *Kopsia* species have been used medicinally in poulticing ulcerated noses in tertiary syphilis, relieving headaches as well as treatment for rheumatoid arthritis and gout [10]. Some researchers have reported that the *Kopsia* species possess several properties, such as anti-hypertensive [11], anti-proliferative [12], anti-plasmodial [13], anti-cancer [14], anti-allergic [15], anti-leishmanial [16], and cytotoxic [17,18].

Hence, the present work aims to report the isolation and structure determination by 1D- and 2D-

†Paper presented at the 8th International Conference for Young Chemists (ICYC) 2022

NMR analyses of eburnane-type and aspidospermane-type of alkaloids from *Kopsia terengganensis* and to investigate the cytotoxic activity of the selected eburnane-type indole alkaloids isolated from the bark of *K. terengganensis*.

MATERIALS AND METHOD

General Method

All chemicals and solvents were obtained from Qrec (Asia) and Merck (Germany). Thin-layer chromatography (TLC) was carried out using a pre-coated 4×10 cm aluminum plate, 0.25 mm thickness of silica gel 60 F_{254} (Merck, Germany) which will be visualized under UV-Vis's light (254 and 365 nm). Dragendorff's reagent was used to identify alkaloids on the TLC plate. Silica gel in column chromatography (CC) was prepared using silica gel 60 of 230-400 mesh (Merck, Germany) depending on the weight of the fractions. All spectral data were obtained on the following instruments: the 1D- and 2D- nuclear magnetic resonance (NMR) spectra were recorded with Bruker AVN 700 or 600 spectrometer (Bruker Bioscience, Billerica, Massachusetts, United States). Data were analysed using TopSpin 3.6.2. Chemical shifts are reported in parts per million (ppm), referenced to the peak of tetramethylsilane, defined at δ = 0.00 (^{1}H- and ^{13}C-NMR), or the residual solvent peak, defined at δ =4.78, 3.31 (^{1}H-NMR, MeOH-d_4), 7.26 (^{13}C-NMR, MeOH-d_4) or 77.0 (^{13}C-NMR, $CDCl_3$). Multiplicities are abbreviated as follow: s (singlet), d (doublet), t (triplet), q (quartet), quint (quintet), m (multiplet), br (broad). Coupling constants *J* are given in Hz and are rounded to the closest multiple of 0.5. The mass spectrometry was performed with high-resolution mass spectrometry (HRMS) using Waters 2795 Liquid Chromatography Time-of-Flight Mass Spectrometer (LC-TOF-MS) (Waters®, Milford, Massachusetts, United States). The infrared spectra (IR) were recorded using a Perkin Elmer Universal ATR FT-IR spectrometer (PerkinElmer, Waltham, Massachusetts, United States) with dichloromethane (DCM) as solvent. The ultraviolet-visible (UV-Vis) spectrum was obtained by using Shimadzu UV-2600 UV-Vis Spectrophotometer (Shimadzu, Kyoto, Japan) with DCM as solvent. Preparative radial chromatography (PRC) was used on Deluxe Cyclograph™ Centrifugal Chromatography System A87-40d (Miles Scientific, Newark, Denmark) by preparing a round glass plate coated with 1 mm thickness of silica gel 60 PF_{254} containing gypsum (Merck, Germany).

Plant Material

K. terengganensis with a number code of KL 4432 was collected in 1995 at Dungun, Terengganu. It was identified by Teo L.E., a botanist from the Faculty of Science, University of Malaya. The voucher specimen was deposited in the Herbarium Department of Chemistry, Faculty of Science, University of Malaya.

Extraction and Isolation

The air-dried ground bark of the plant (1 kg) was first defatted with hexane (3 L) for 3 days at room temperature. The crude was combined and evaporated to give 3.74 g of hexane extract. The dried plant material was made alkaline and moistened with 28% of ammonia solution (NH_4OH) for 3 h to aggregate the nitrogen-containing components. The extraction process was continued by soaking the plant material in DCM for another 3 days at room temperature. The supernatant was finally concentrated and dried to give 6.5 g of crude DCM. This crude was further subjected to acid-base extraction using 5% hydrochloric acid (HCl) and basified with NH_4OH solution to give a crude alkaloid extract of 2.00 g. The alkaloid content was determined by staining Dragendorff's reagent on the TLC plate giving an orange spot as a positive result [19].

The crude alkaloid of bark extract was subjected to CC over silica gel using DCM: methanol (MeOH) solvent system starting from 100:0 followed by increasing the MeOH gradient and finally flushed with ethanol. The elution resulted in 15 major fractions (Fr.1 – Fr.15). Further purification by PRC on the Fr-2 afford compound **7** (19.6 mg, 0.98% yield), Fr-3 afford compound **4** (3.6 mg, 0.18% yield), Fr-7 afford compound **1** (90.0 mg, 4.50% yield) and **2** (13.1 mg, 0.66% yield), Fr-10 afford compound **5** (3.4 mg, 0.17% yield) and **6** (1.4 mg, 0.07% yield), and Fr-12 afford compound **3** (40.4 mg, 2.00% yield).

Eburnamine (**1**): brownish oil; IR ν_{max} 3299 (O-H), 2924 (C-H), 2855, 1457 (C-H), 1123 (C-O) cm^{-1}; UV (CH_2Cl_2) λ_{max}, nm: 233, 256,282; ^{1}H-NMR and ^{13}C-NMR data see Tables 1 and 2; HRMS (TOF-ES$^-$) *m/z* 295.2165 $[MH]^-$(calc. for $C_{19}H_{23}N_2O$ *m/z* 295.1810).

Isoeburnamine (**2**): light brownish oil; IR ν_{max} 3360 (O-H), 2924 (C-H), 2854, 1143 (C-O), 857 cm^{-1}; UV (CH_2Cl_2) λ_{max}, nm: 236, 283; ^{1}H- and ^{13}C-NMR data see Tables 1 and 2; HRMS (TOF-ES$^-$) *m/z* 295.2144 $[MH]^-$ (calcd for $C_{19}H_{23}N_2O$: *m/z* 296.1889)

Eburnaminol (**3**): white solid; IR ν_{max} 3400 (O-H), 2924 (C-H), 2854, 1487, 1142 (C-O), 855 cm^{-1}; UV (CH_2Cl_2) λ_{max}, nm: 229, 249, 257; ^{1}H-NMR and ^{13}C-NMR data see Tables 1 and 2; HRMS (TOF-ES$^+$) *m/z* 313.2257 $[MH]^+$ (calcd for $C_{19}H_{25}N_2O_2$: *m/z* 313.1916)

Larutensine (**4**): yellowish oil; IR ν_{max} 2924 (C-H), 2854, 1349, 1142 (C-O-C), 856 cm^{-1}; UV (CH_2Cl_2) λ_{max}, nm: 229, 249, and 257; ^{1}H-NMR and ^{13}C-NMR data see Tables 1 and 2; HRMS (TOF-ES$^-$) *m/z* 293.1925 $[MH]^-$ (calcd for $C_{19}H_{21}N_2O$: *m/z* 293.1654)

Eburnamenine (**5**): yellowish oil; IR ν_{max} 2928 (C-H), 2856, 1698, 1640 (C=C), 1455 (C-H) cm^{-1}; UV (CH_2Cl_2) λ_{max}, nm: 207, 232, and 253; ^{1}H-NMR and ^{13}C-NMR data see Tables 1 and 2; HRMS (TOF-ES$^-$)

m/z 277.2092 $[MH]^-$ (calcd for $C_{19}H_{21}N_2$: *m/z* 277.1705)

Eburnamonine (**6**): yellowish oil; IR ν_{max} 2926 (C-H), 1698 (C=O), 1455 (C-H), 1372, 1143 cm^{-1}; UV (CH_2Cl_2) λ_{max}, nm: 239, 264 and 272; ^{1}H-NMR and ^{13}C-NMR data see Tables 1 and 2; HRMS (TOF-ES$^+$) *m/z* 295.2145 $[MH]^+$ (calcd for $C_{19}H_{23}N_2O$: *m/z* 295.1810)

Quebrachamine (**7**): colourless crystal; IR ν_{max} 3450 (N-H), 2924 (C-H), 775 cm^{-1}; UV (CH_2Cl_2) λ_{max} , nm: 225, 286, and 289; ^{1}H-NMR and ^{13}C-NMR data see Tables 1 and 2; HRMS (TOF-ES$^+$) *m/z* 283.2174 $[MH]^+$ (calcd for $C_{19}H_{27}N_2$: *m/z* 283.2174).

Cell Viability Assay (MTT Assay)

Cytotoxicity test was carried out using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) assay to measure the metabolic activity of the cell. HT-29 colon cancer cell was used as a targeted human cancer cell and cisplatin (Merck, Germany) as the positive standard. The cell line was cultured in Minimum Essential Medium Eagle (EMEM) (Sigma-Aldrich, USA) with 10% foetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA) as a supplement. Then, the cell was allowed to grow as a monolayer in a humidified 5% CO_2 incubator at 37°C. The cell has seeded a total of 10000 cells/well in a 96-well plate and treated with different concentrations of extract: 0–100 µg/mL for DCM crude and 0–100 µM for alkaloid and cisplatin. After 24 h of incubation, a volume of 20.0 µL of MTT reagent (5.0 mg/mL) was added into each well and further incubated for 1.5 h. Spent media were discarded and 200.0 µL of dimethyl sulfoxide (DMSO) was added to dissolve the purple formazan precipitates. The result was obtained by measuring the absorbance at the test wavelength, 570 nm, and reference wavelength, 650 nm using a microtiter plate reader (Tecan Sunrise™, Männedorf, Switzerland). The half maximal inhibitory concentration (IC_{50}) was calculated from the graph of percentage cell viability versus the concentration of alkaloids. The percentage of cell viability was calculated as follows [20]:

$$\text{Viable cell (\%)} = \frac{(\text{Absorbance sample} - \text{absorbance blank})}{(\text{Absorbance control} - \text{absorbance blank})} \times 100$$

Statistical Analysis

Result was expressed as mean values ± standard deviation (SD). All data collected from the experiments were performed in three replicates and analysed using the one-way analysis of variance (ANOVA) at a significance level of $p<0.05$ indicated by * and $p<0.0005$ indicated by **.

RESULTS AND DISCUSSION

Isolation Structural Elucidation

The alkaloid crude of the *K. terengganensis* was subjected to silica gel chromatography to afford fifteen fractions Fr.1–Fr.15. the fractions were further purified using PRC leading to the isolation of eburnamine (**1**), isoeburnamine (**2**), eburnaminol (**3**), larutensine (**4**), eburnamenine (**5**), eburnamonine (**6**), and quebrachamine (**7**). All assignments of ^{1}H- and ^{13}C-NMR data were established through in depth analysis of 2D-NMR; COSY; HSQC and NOESY. The spectroscopic data were compared with literatures [21-22, 24-27].

Table 1. ^{1}H-NMR data of compounds **1–2** and **4–6** (in $CDCl_3$), and **3** (in MeOH-d_4)[a]

No.	1	2	3	4	5	6	7
3	2.55 m	2.60 m 2.52 m	2.40 dd (13.9, 5.1) 2.66 m	2.86 m	3.05 m	2.74 m 2.49 m	2.23 td (13.4, 3.0 2.41 m
5	3.29 dd (9.5, 3.4)	3.27 m 3.18 m	3.27 m 3.23 m	3.13 m	3.26 d (10.9) 3.02 d (11.5)	3.38 m 3.29 m	2.29-2.42 m
6	2.54 m 2.93 m	2.92 m 2.50 br d	2.57 dd 2.98 m	2.73 dd 2.92 m	3.70 dd (13.4, 5.8) 3.58 m	2.88 m 2.61 m	2.81-2.94 m
9	7.47 d (7.6)	7.42 d (7.6)	7.42 d (7.6)	7.30 d (7.7)	7.44 d (7.6)	7.39 d (7.5)	7.21-7.25 m
10	7.14 t (7.6)	7.07 t (7.6)	7.08 t (7.6)	7.02 t (7.7)	7.14 t (7.6)	7.26 t (7.5)	7.03-7.07 m
11	7.18 t (7.6)	7.11 t (7.6)	7.13 t (7.6)	7.09 t (7.7)	7.25 t (7.6)	7.31 t (7.5)	7.04-7.08 m
12	7.74 d (7.6)	7.35 d (7.6)	7.71 d (7.6)	7.36 d (7.7)	7.32 d (7.6)	8.32 d (7.5)	7.45-7.48 m

14	1.30 d (13.4) 1.73 m	1.70 m 1.31 m	1.82 m 1.40 m	1.64 m 2.14 m	1.23 m	1.47 br d (13.2) 1.97 m	1.57 m 1.28 m
15	0.87 td (13.5, 3.7) 1.38 d (13.5)	1.59 m 1.46 d (13.7)	0.97 td (13.6, 4.0) 1.47 m	1.31 m 1.60 m	1.59 m 2.20 m	1.50 d (14.3)	1.12 m
16	5.59 dd (9.5,5.1)	5.97 d (4.4)	5.59 dd (9.5, 5.2)	5.73 dd (2.5, 2.0)	6.96 d (7.8)	-	2.62 m 2.73 m
17	1.57 dd (14.5, 4.1) 2.35 m	1.90 dd (14.9,4.6) 2.10 m	2.39 m 1.90 m	1.63 m	5.12 d (7.8)	2.93 d (16.9) 2.48 d (16.8)	1.88 m 1.56 m
18	0.91 t (7.6)	0.86 t (7.5)	3.77 m (7.1)	3.69 dd (12.3, 5.7) 3.84 m	1.03 t (7.4)	0.81 t (7.0)	0.84 t (7.5)
19	2.08 m 1.53 m	2.12 d (6.4) 1.38 m	2.22 m 1.80 m	1.43 d (13.4) 1.72 m	2.25 m 1.98 m	1.20 d (4.5)	1.22 m 1.10 m
21	3.85 s	3.79 s	3.97 s	3.04 s	4.73 s	4.53 s	3.22 dt (11.8, 1.5) 1.49 d (11.9)
NH	-	-	-	-	-	-	7.75 br s

[a]δH (ppm), (*J* in Hz). Compounds **1**–**4** were recorded at 700 MHz, and compounds **5**–**7** at 600 MHz

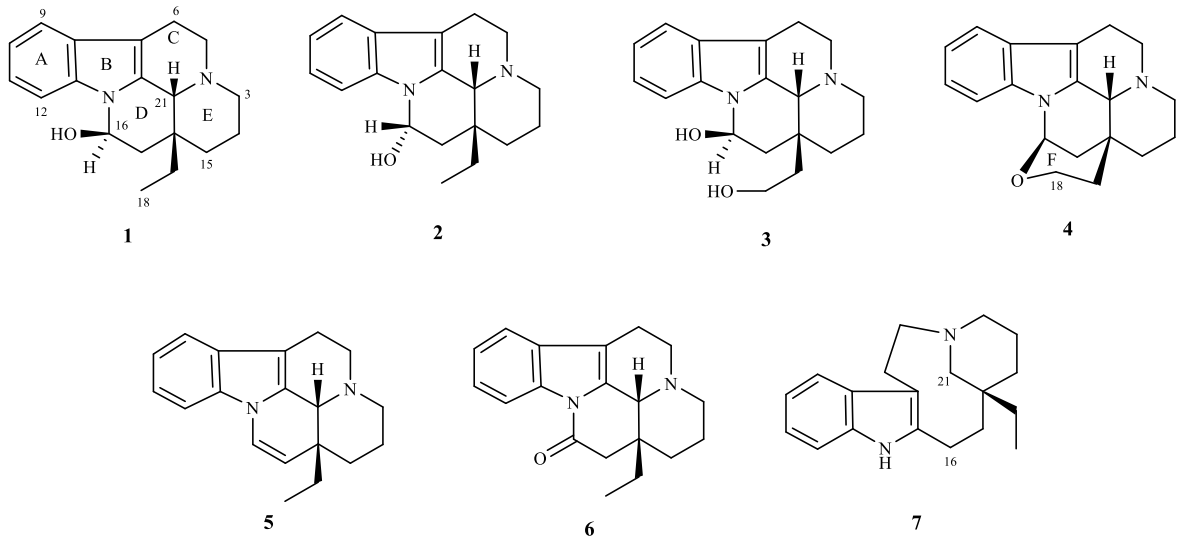

Figure 1. Structures of compounds **1** – **7**.

Compound **1** (Eburnamine) was isolated as brownish oil. The UV-Vis spectrum showed absorbance at 233, 256, and 282 nm, highlighting the indole alkaloid chromophore [21]. The HRMS spectrum of compound **1** resulted in a molecular ion peak at *m/z* 295.2165 $[MH]^-$, suggesting a molecular formula of $C_{19}H_{24}N_2O$ (calc. for $C_{19}H_{23}N_2O$ *m/z* 295.1810). There are 9 degrees of unsaturation, where six observed the indole structure and the remaining three degrees were consistent for three cyclohexane. The IR spectrum of **1** showed the absorption bands at 3299, 2924, 1457, and 1123 cm^{-1} corresponding to the O-H stretching, C-H stretching of an alkane, C-H bending of a methyl group, and C-O stretching of a secondary alcohol, respectively. In the ^{1}H-NMR spectral data (Table 1), four aromatic protons were indicated with δ_H 7.47 (*J* = 7.6 Hz, H-9), 7.14 (*J* = 7.6 Hz, H-10), 7.18 (*J* = 7.6 Hz, H-11), and 7.74 (*J* = 7.6 Hz, H-12), which suggested the presence of aromatic ring [21]. Three protons with a splitting pattern of triplet at the most upfield region, δ_H 0.91

indicated a methyl group was observed. A singlet peak at δ_H 3.85 which most probably belonged to C-21 hydrogen has also appeared. A doublet of doublet (*dd*) peak appeared at δ_H 5.59 and was assigned to H-16 with coupling constant of J = 9.5 and 5.1, which indicated the proton was situated in the axial position [22]. The ^{13}C spectral data (Table 2) showed the presence of 19 carbons: one methyl, seven methylene, eight methine, and five quaternary carbons. The bicyclic structure of indole represented by ring-A, an aromatic ring fused to the ring-B, a pyrrole ring could be accurately depicted with the HMBC correlation (Figure 2) between H-16 (δ_H 5.59) to C-13 (δ_C 136.7), H-12 (δ_H 7.74) to C-8 (δ_C 128.5), H-9 (δ_H 7.47) to C-7 (δ_C 105.6), and H-6 (δ_H 2.54, 2.93) to C-2 (δ_C 132.3). The position of C-21 which connected the ring-C, D, and E was verified via HMBC correlation of H-5 (δ_H 3.20, 3.29) and H-15 (δ_H 0.85, 1.38) to C-21 (δ_C 58.8). The ethyl side chain which connected to C-20 was shown by HMBC correlation between H-18 (δ_H 0.91) and H-19 (δ_H 2.08, 1.53) to C-20 (δ_C 36.9). The structure of compound **1** was further confirmed by the ^{1}H-^{1}H COSY spectrum via correlation of H-16/H-17, H-18/H-19, H-5/H-6, and H-14/H-3 and H-15. The spectral data of **1** were compared to literature [22].

Compound **2** (Isoeburnamine) was obtained to be a light brown oil and its molecular formula was $C_{19}H_{24}N_2O$ determined by the HRMS ion signal at *m/z* 295.2144 $[MH]^-$ (calc. for $C_{19}H_{23}N_2O$ *m/z* 295.1810) from which 9 degree of unsaturation was deduced. The UV-Vis spectrum showed absorption at 236 and 283 nm which indicated another indole chromophore alkaloid. The presence of the OH group was supported by the IR spectrum which had an absorption band at 3360 cm^{-1}. Clearly, the ^{1}H- and ^{13}C-NMR data of compound **2** showed a similarity with compound **1** which brought to the conclusion that compound **2** has an eburnane skeleton. In the ^{1}H-NMR spectral data (Table 1), the only difference that can be distinguished from compound **1** was the position of H-16 (δ_H 5.97). The chemical shift moved downfield, and the coupling constant showed a value of J = 4.4 Hz which indicated the hydrogen was in an equatorial position [22]. From the ^{13}C-NMR spectrum, aromatic carbon C-12 (δ_C 110.0), methine carbon C-16 (δ_C 74.5), and methylene carbon C-17 (δ_C 40.0) shifted to the upfield region compared to compound **1**. Since compound **2** possessed the same carbon skeleton, the same correlation of ^{13}C-^{1}H in HMBC and ^{1}H-^{1}H in COSY was expected when elucidating the compound. The HMBC correlations between H-16 (δ_H 2.50, 2.92) to C-2 (δ_C 130.8) and C-20 (δ_C 34.6), H-12 (δ_H 7.35) to C-8 (δ_C 128.5), H-9 (δ_H 7.42) to C-7 (δ_C 105.6), H-3 (δ_H 2.52, 2.60) to C-21 (δ_C 59.3), and H-15 (δ_H 1.46, 1.59) to C-19 (δ_C 29.0) confirmed the eburnane skeleton of compound **2.** The ^{1}H- and ^{13}C-NMR spectral data of **2** are consistent with the literature data [22].

Compound **3** (Eburnaminol) was isolated as a white solid. The HRMS spectrum of compound **3** showed a molecular ion peak at *m/z* 313.2257 $[MH]^+$ (calcd for $C_{19}H_{25}N_2O_2$: *m/z* 313.1916) which agreed with the molecular formula of $C_{19}H_{24}N_2O_2$. Other fragmentations observed were *m/z* 295.2515 $[M+H-H_2O]^+$ and 251.1882 $[M+H-C_2H_5O-OH]^+$. These peaks led to the hypothesis that there are two hydroxyl groups presented in compound **3**. From the IR value, a broad peak at 3400 cm^{-1} confirmed the presence of the OH group. This was further verified by ^{1}H- and ^{13}C-NMR spectral data. In the ^{1}H-NMR data (Table 1), a multiplet was observed at δ_H 3.77 for C-18 hydrogen and it shifted further downfield compared to compound **1** which is a triplet. This can be deduced that the presence of hydrogen bonding from electronegative atoms in neighbouring protons leads to a higher chemical shift value. A *dd* peak at δ_H 5.59 with coupling constant J = 9.5 and 5.2 was assigned to H-16 which is vicinal to the OH group. This chemical shift and J value of H-16 are the same as compound 1 indicated that the ring-D in the eburnane structure preferred a more stable chair conformation in which the hydrogen is always in an axial position instead of equatorial as in the case of compound **2** [22]. In the 13C-NMR spectrum, the data are similar to compounds 1 and 2. However, in

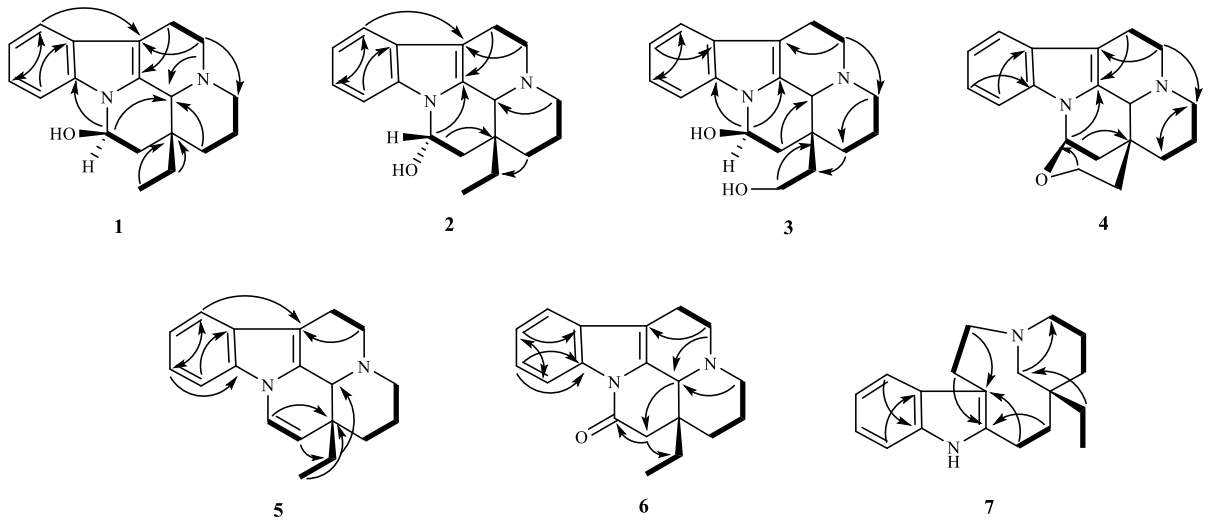

Figure 2. Key ^{1}H-^{1}H COSY (bold) and HMBC (^{1}H→^{13}C) correlations of **1–7**.

Table 2. ^{13}C NMR data of of compounds **1–2** and **4–6** (in $CDCl_3$), and **3** (in MeOH-d_4)[a]

Position	**1**	**2**	**3**	**4**	**5**	**6**	**7**
2	132.3	130.8	131.2	136.6	128.0	134.4	140.0
3	44.3	44.9	44.0	51.8	16.3	43.2	55.2
5	50.9	51.3	50.4	54.3	45.3	50.2	53.3
6	16.8	16.7	16.2	21.1	52.3	16.5	22.5
7	105.6	105.6	104.7	107.8	106.1	112.1	108.7
8	128.5	128.9	128.3	128.6	126.9	129.5	129.0
9	118.1	118.5	117.4	109.7	119.1	118.4	110.2
10	120.2	120.2	119.5	120.2	121.4	124.2	118.7
11	121.4	121.3	120.9	121.7	123.8	125.1	120.3
12	112.2	110.0	111.7	118.4	109.3	116.5	117.4
13	136.7	135.0	136.8	137.9	134.6	134.4	134.9
14	20.3	20.8	20.2	20.1	29.6	20.8	22.8
15	25.0	26.4	25.5	35.6	18.1	29.0	32.2
16	76.6	74.5	75.3	77.6	120.7	167.0	22.0
17	43.5	40.0	43.7	38.1	115.2	46.2	33.6
18	7.5	7.49	57.6	58.6	8.6	14.5	7.8
19	28.6	29.0	38.7	40.7	27.4	29.7	34.9
20	36.9	34.6	36.1	28.9	38.3	38.4	37.2
21	58.8	59.3	58.9	63.4	58.0	57.2	56.8

[a]δ_C (ppm). Compounds **1–4** were recorded at 175 MHz, and compound **5–7** at 150 MHz.

C-18 and C-19, the signal shifted further downfield and appeared at δ_C 57.6 and 38.7, respectively. This corresponded to CH_2CH_2OH in compound **3** [21]. The structure of compound **3** was further confirmed by HMBC correlation of H-5 (δ_H 3.23, 3.27) to C-7 (δ_C 104.7) and C-3 (δ_C 44.0), H-16 (δ_H 5.59) to C-2 (δ_C 131.2) and C-13 (δ_C 136.8), H-18 (δ_H 3.77) to C-20 (δ_C 36.1), and H-15 (δ_H 0.97, 1.47) to C-19 (δ_C 38.7). The structure of compound **3** was accurately depicted by ^{1}H - ^{1}H COSY correlation as in compound **1**. The signals from ^{1}H- and ^{13}C-NMR spectral data were identical to the literature data by Awang et al.[21].

Compound **4** (Larutensine) was obtained as a yellowish oil. The UV-Vis spectrum exhibited a typical indole chromophore at 238, 249, and 262 nm. The molecular formula, $C_{19}H_{22}N_2O$, was established by HRMS spectrum with molecular ion peak at *m/z* 293.1925 $[MH]^-$ (calcd for $C_{19}H_{21}N_2O$: *m/z* 293.1654); from which 10 degrees of unsaturation was deduced, where nine observed the eburnane skeleton and the remaining observed the tetrahydropyran ring. The difference of 18 amu in the molecular formula of compound **4** compared to compound **3** suggested that compound **4** is formed through the loss of water molecules [21]. This hypothesis is supported by the IR value of an ether group (1142 cm^{-1}) and no absorption for the OH group was observed. The ^{1}H- and ^{13}C-NMR spectral data suggested an eburnane derivative oxygenated at C-16 with a missing ethyl substituent at C-20. In the ^{13}C-NMR data, 19 carbon signals were observed, including eight methylene, six methine, and five quaternary carbons. The signal of two ether carbons at δ_C 77.6 for C-16 and δ_C 58.6 for C-18 indicated that ring formation had occurred in which ether oxygen linked C-18 to C-16. The incorporation of oxygen in the ring structure also causes the chemical shift of C-20 to be more upfield compared to compounds **1** and **3**. In the ^{1}H-NMR data, a small coupling constant was observed in H-16 (δ_H 5.73, dd *J* =2.5, 2.0) due to the resonance with H-17 that revealed at δ_H 1.63. This small coupling constant indicated a typical fused six-membered ring system and the presence of an electronegative atom in the ring. The same reason is used for the chemical shift of H-18 (δ_H 3.84, 3.69) to move further downfield [21,22]. Further analysis using HMBC showed a correlation of H-16 (δ_H 5.73) to C-18 (δ_C 58.6)/C-2 (δ_C 136.6)/C-20 (δ_C 28.9), H-15 (δ_H 1.31, 1.60) and H-5 (δ_H 3.13) to C-3 (δ_C 51.8), and H-6 (δ_H 2.73, 2.92) to C-2 (δ_C 131.6). The structure of compound **4** was confirmed by the ^{1}H-^{1}H COSY correlation between H-5/H-6, H-14/H-3/H-15, H-16/H-17 and H-18/H-19. The signals from ^{1}H- and ^{13}C-NMR spectral data were identical to the literature data by Awang et al. [21].

Compound **5** (Eburnamenine) was isolated as light yellowish oil and gave a positive reaction with Dragendorff's reagent. The UV-Vis spectrum showed an indole chromophore when absorption occurred at 233, 247, and 261 nm. Its molecular formula was determined as $C_{19}H_{22}N_2$ by HRMS at *m/z* 277.2092 $[MH]^-$ (calcd for $C_{19}H_{21}N_2$: *m/z* 277.1705) corresponding to 10 degrees of unsaturation. The IR spectrum showed absorption at 1455 and 1640 cm^{-1} implying the presence of a C-H stretch of methyl group and a disubstituted alkene with *cis* form. No absorption for OH or NH was observed. The ^{1}H- and ^{13}C-NMR spectral data showed that compound **5** has an eburnane

skeleton. 19 carbon signals were observed in the ^{13}C-NMR spectrum, including one methyl, six methylene, seven methines, and five quaternary carbons. Two signals at δ_C 120.7 and 115.2 were assigned to C-16 and C-17, respectively, representing a typical peak for the alkene group. Analysis of its NMR data manifested that compound **5** was highly similar to compound **1**, except in C-16 and C-17 hydrogens. Two doublet peaks were observed at δ_H 6.96 (J = 7.8 Hz) and 5.12 (J = 7.8 Hz) which were assigned to H-16 and H-17, respectively. This suggested that the hydroxyl group in compound **1** was replaced by the olefin group with a coupling constant showing a vicinal coupling of *cis* protons [23]. The structure of compound **5** was further depicted by HMBC and COSY correlations as shown in Figure 5. In the HMBC spectrum, the structure of **5** was depicted accurately by the correlation of H-16 (δ_H 6.96) to C-20 (δ_C 38.3), H-17 (δ_H 5.12) to C-19 (δ_C 27.4), H-5 (δ_H 3.02, 3.26) and H-9 (δ_H 7.44) to C-7 (δ_C 106.1), and H-19 (δ_H 1.98, 2.25) to C-21 (δ_C 58.0). Meanwhile, the COSY spectrum highlighted the correlation between H-18/H-19, H-14/H-3/H-15, H-16/H-17, and H-5/H-6. The NMR data of compound **5** were compared and resemble those in the literature [24].

Compound **6** (Eburnamonine) was obtained as a yellowish oil. The HRMS spectrum displayed molecular ion peak at *m/z* 295.2145 $[MH]^+$ (calcd for $C_{19}H_{23}N_2O$: *m/z* 295.1810), consistent with the molecular formula of $C_{19}H_{22}N_2O$, corresponding to 10 degrees of unsaturation. A strong band at 1698 cm^{-1} and no broad peak at 3500 cm^{-1} and above in the IR spectrum indicated the presence of a carbonyl group (C=O) and the absence of an OH group. In the ^{1}H NMR spectral data (Table 1), there was no signal recorded for C-16 hydrogen, and the chemical shift of H-17 was shifted further downfield as compared to compound **1**. This gave the idea that the position of the carbonyl group was connected to C-16. Other signals such as aromatic protons at δ_H of 7.39 (J = 7.6 Hz, H-9), 7.26 (J = 7.2 Hz, H-10), 7.31 (J = 7.2 Hz, H-11), and 8.32 (J = 8.0 Hz, H-12) were observed. In the ^{13}C NMR spectrum, 19 carbon signals were displayed which were composed of one methyl, seven methylene, five methines, and six quaternary carbons. The presence of a peak at δ_C 167.0 confirmed the carbonyl group in compound **6**. The structure of **6** was further confirmed by the HMBC correlation between H-17 (δ_H 2.48, 2.93) to C-16 (δ_C 167.0, C=O) and C19 (δ_C 29.7), H-21 (δ_H 4.53) to C-17 (δ_C 46.2), H-3 (δ_H 2.49, 2.74) and H-5 to C-21 (δ_C 57.2), and H-5 (δ_H 3.29, 3.38) to C-7 (δ_C 112.1). The ^{1}H-^{1}H COSY correlation for compound **6** was no longer observed for H-17 and H-16 as compound **1,** indicating that hydrogen has been replaced by the carbonyl group (C=O). The ^{1}H and ^{13}C NMR spectral data were identical to the previously reported data by Kam et al.[25].

Compound **7** (Quebrachamine) was obtained as colourless crystal with molecular formular of $C_{19}H_{26}N_2$ deduced from the HRMS molecular ion peak at *m/z* 283.2174 $[MH]^+$(calcd for $C_{19}H_{27}N_2$: *m/z* 283.2174). The IR spectrum showed the characteristic absorption (3450 cm^{-1}) assignable to secondary amine group (N-H). Analysis of the ^{1}H- and ^{13}C-NMR spectra of compound **7** showed signals attributed to a total of 19 carbons: one methyl, four aromatic methines, nine methylene, and five quaternary carbons. With the aid of 1D- and 2D-NMR experiments, all the ^{1}H- and ^{13}C-NMR signals of **7** were assigned. In the ^{1}H-NMR spectral data (Table 1), a triplet peak was observed at δ_H 0.84 indicated to a methyl proton assigned as H-18. Compared to other eburnane-type alkaloids, compound **7** exhibited two signals for C-21 hydrogens at δ_H 3.22 (dt, J = 11.8, 1.5 Hz) and 1.48 (J =11.9 Hz). The coupling constant suggested that both protons were geminal coupling. An additional broad singlet peak at the most deshielded region, δ_H 7.75 was recorded and assigned to N-H. Through the analysis of the ^{13}C-NMR spectrum, NMR data of compound **7** revealed similarity with compound **1** except the signal for C-16 (δ_C 22.0) appeared at the upfield region indicating no OH group was attached to the carbon. The structure of **7** can be deduced from the HMBC correlation between H-16 (δ_H 2.62, 2.73) and H-5 (δ_H 2.32, 2.39) to C-7 (δ_C 108.7), H-17 (δ_H 1.56, 1.88) and H-6 (δ_H 2.81, 2.94) to C-2 (δ_C 140.0), H-9 (δ_H 7.23) to C-13 (δ_C 134.9), H-12 (δ_H 7.45) to C-8 (δ_C 129.0), and H-19 (δ_H 1.10, 1.22) to C-21 (δ_C 56.8). From the ^{1}H-^{1}H COSY spectrum, the structure of **7** was further confirmed by the correlation of H-5/H-6, H-16/H-17, H-18/H-19, and H-14/H-3 and H-15. The spectral data of ^{1}H- and ^{13}C-NMR were compared to the literature [26,27].

Cytotoxic Activity

Secondary metabolites from natural products have attracted many researchers to study their biological properties and part of them has already been used to treat various chronic and terminal diseases in humans [28]. Compounds such as indole alkaloids have significant biological activities in which some of the chemical constituents have been commercialized as an anti-cancer drug [29,30]. In this study, the cytotoxic activity of the DCM crude, compounds **1**–**4**, and cisplatin as reference were evaluated on HT–29 colon cancer cell using the MTT assay by measuring the metabolic activity in viable cells. Figure 3 depicted that the percentage of viable cells decreased as the concentration increased. The same pattern can be observed for the cell treated with cisplatin. The IC_{50} values were deduced from the percentage of cell viability graph and summarized in Table 3. The IC_{50} value for the DCM crude was recorded as not active (>100 µg/mL) with 56.16 ± 1.49% of cell viable at 100 µg/mL. However, the test for the pure compounds demonstrated that only compound **3** was able to suppress the growth of HT-29 colon cancer cell lines in a dose-dependent manner. The IC_{50} value of treated cell lines after 24 h was 75.81 ± 3.06 µM. In contrast, there was no IC_{50} value was recorded for compound **1**, **2**, and **4** indicating the significant effect of compound **3** on colon cancer cell. Since the cytotoxicity of a chemical compound on cell functions depend on its functional group and the chemical structure [31], it was predicted that the hydroxyl group in **3** contributed as the toxicophores [32].

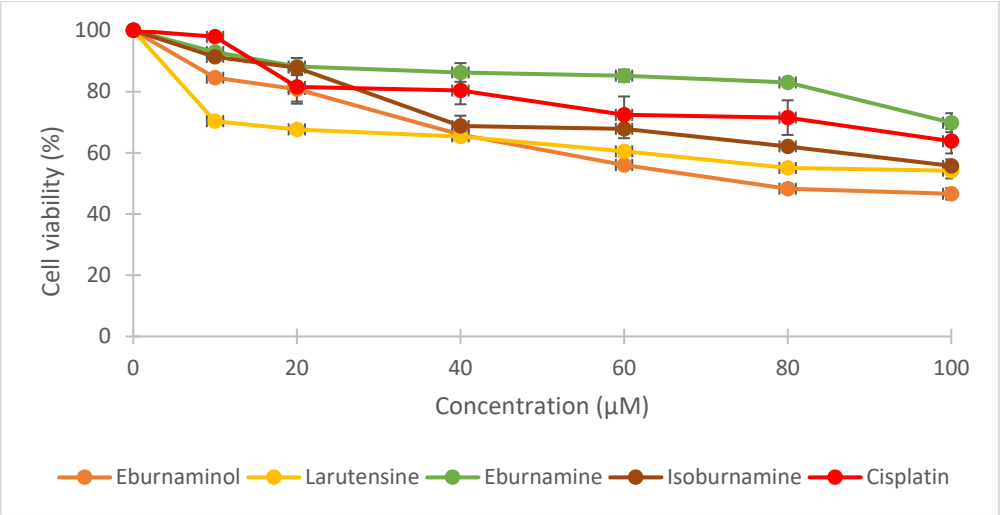

Figure 3. The graph of the concentration of isolated alkaloids against the percentage of cell viability

Table 3. Half-maximal inhibitory concentration (IC_{50}) of selected alkaloids of *K. terengganensis*. The IC_{50} was determined from the cell viability graph from the MTT assay.

Compound	IC_{50} (µM)	Viable cells (%) at 100 µM
Eburnaminol	75.81 ± 3.06	46.62 ± 1.87**
Larutensine	>100	54.18 ± 2.54*
Eburnamine	>100	69.87 ± 3.10
Isoburnamine	>100	55.74 ± 2.12
Cisplatin	>100	63.81 ± 4.01

P-value ANOVA between the compound and cisplatin, *P<0.05, **P<0.005

CONCLUSION

Seven indole alkaloids were extracted from the bark of *K. terengganensis* DCM extract named eburnamine (**1**), isoeburnamine (**2**), eburnaminol (**3**), larutensine (**4**), eburnamenine (**5**), eburnamonine (**6**), and quebrachamine (**7**). These alkaloids were characterized using various spectroscopic methods and selected compounds were subjected to cytotoxic activity. The findings suggest that only eburnaminol showed a cytotoxic effect towards HT-29 colorectal adenocarcinomas with an IC_{50} value of 75.81 ± 3.06 µM superior to cisplatin at > 100 µM. Results from this study showed that the bioactive compounds in *K. terengganensis* have the potential to be explored further with cytotoxic activities.

ACKNOWLEDGEMENTS

The authors would like to thank Ministry of Higher Education for FRGS Grant (FRGS/1/2018/STG01/USM/01/5), Universiti Sains Malaysia (USM) for the research facilities and the University of Malaya for conducting the cytotoxic activity. We are grateful to Graduate Excellence Programme (GrEP) MARA for the scholarship. This research was conducted within the frame of the collaboration between USM-CNRS (LoI) and auspices of CNRS-UM; Associated International Laboratory (LIA) under FM-NatProLab. We thank Din M.N., Rafly S. and Teo L.E. (UM) for the collection and identification of plant material.

REFERENCES

1. Sung, H., Ferlay, J., Siegel, R. L., Laversanne, M., Soerjomataram, I., Jemal, A. & Bray, F. (2021) Global cancer statistics 2020: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA: A Cancer Journal for Clinicians*, **71(3)**, 209–249.

2. Kuppusamy, P., Yusoff, M. M., Maniam, G. P., Ichwan, S. J. A., Soundharrajan, I. & Govindan, N. (2014) Nutraceuticals as potential therapeutic agents for colon cancer: a review. *Acta Pharmaceutica Sinica B*, **4(3)**, 173–181.

3. Cai, Y., Xia, Q., Luo, R., Huang, P., Sun, Y., Shi, Y. & Jiang, W. (2014) Berberine inhibits the growth of human colorectal adenocarcinoma in vitro and in vivo. *Journal of Natural Medicines*, **68(1)**, 53–62.

4. Han, M. S., Lee, H. J., Park, S. J., Hong, S. P., Cheon, J. H., Kim, W. H. & Kim, T. I. (2017) The effect of metformin on the recurrence of colorectal adenoma in diabetic patients with previous colorectal adenoma. *International Journal of Colorectal Disease*, **32(8)**, 1223–1226.

5. Pandey, N., Meena, R. P., Rai, S. K. & Pandey-Rai, S. (2011) Medicinal plants derived nutraceuticals:

a re-emerging health aid. *International Journal of Pharma and Bio Sciences*, **2(4)**, 420–441.

6. Middleton, D. J. (2004) A revision of Kopsia (Apocynaceae: Rauvolfioideae). *Harvard Papers in Botany*, 89–142.

7. Bitombo, A. N., Zintchem, A. A. A., Atchadé, A. D. T., Nyemeck Ii, N. M., Bikobo, D. S. N., Pegnyemb, D. E. & Bochet, C. G. (2021) Antiplasmodial activities of indole alkaloids from *Tabernaemontana penduliflora* K. Schum (Apocynaceae). *Fitoterapia*, **153**, 104941.

8. Vrabec, R., Maříková, J., Ločárek, M., Korábečný, J., Hulcová, D., Hošťálková, A., Kuneš, J., Chlebeka, J., Kučera, T., Hrabinová, M., Jun D., Soukup, O., Andrisano, V., Jenčo, J., Šafratová, M., Nováková, L., Opletala, L. & Cahlíková, L. (2022) Monoterpene indole alkaloids from *Vinca minor* L. (Apocynaceae): Identification of new structural scaffold for treatment of Alzheimer's disease. *Phytochemistry*, **194**, 113017.

9. Xie, J., Zou, X., Sang, C., Song, M., Chen, Q. & Zhang, J. (2021) Three new monoterpenoid indole alkaloids from Alstonia rostrata. *Tetrahedron Letters*, **75**, 153180.

10. Sevenet, T., Allorge, L., David, B., Awang, K., Hamid, A., Hadi, A., Kan-Fan, C., Quirion, J.-C., Remy, F., Schaller, H. & Teo, L. E. (1994) A preliminary chemotaxonomic review of *Kopsia* (Apocynaceae). *Journal of Ethnopharmacology*, **41(3)**, 147–183.

11. Mok, S. L., Yoganathan, K., Lim, T. M. & Kam, T. S. (1998) Cardiovascular effects of aspidofractinine-type alkaloids from *Kopsia*. *Journal of Natural Products*, **61(3)**, 328–332.

12. Kam, T. S., Lim, K. H., Yoganathan, K., Hayashi, M. & Komiyama, K. (2004) Lundurines A–D, cytotoxic indole alkaloids incorporating a cyclopropyl moiety from *Kopsia tenuis* and revision of the structures of tenuisines A–C. *Tetrahedron*, **60(47)**, 10739–10745.

13. Khozirah, S., Noor Rain, A., Siti Najila, M. J., Imiyabir, Z., Madani, L. & Rodaya, C. (2011) *In vitro* antiplasmodial properties of selected plants of Sabah. *Pertanika Journal of Science and Technology*, **19**, 11–17.

14. Kam, T. S., Subramaniam, G., Sim, K. M., Yoganathan, K., Koyano, T., Toyoshima, M., Rho, M.-C., Hayashi, M. & Komiyama, K. (1998) Reversal of multidrug resistance (MDR) by aspidofractinine-type indole alkaloids. *Bioorganic & Medicinal Chemistry Letters*, **8(19)**, 2769–2772.

15. Shahari, M. S., Ismail, A. F., Kumolosasi, E., Rajab, N. F. & Husain, K. (2017) Isolation of indole alkaloids from *Kopsia larutensis* King & Gamble and their effects on histamine and β-hexosaminidase inhibitory in RBL-2H3 cell line. *Journal of Innovations in Pharmaceutical and Biological Sciences*, **4**, 80–86.

16. Kam, T. S., Sim, K. M., Koyano, T. & Komiyama, K. (1999) Leishmanicidal alkaloids from *Kopsia griffithii*. *Phytochemistry*, **50(1)**, 75–79.

17. Subramaniam, G., Hiraku, O., Hayashi, M., Koyano, T., Komiyama, K. & Kam, T. S. (2007) Biologically active aspidofractinine, rhazinilam, akuammiline, and vincorine alkaloids from *Kopsia*. *Journal of Natural Products*, **70(11)**, 1783–1789.

18. Wong, S. K., Yeap, J. S. Y., Tan, C. H., Sim, K. S., Lim, S. H., Low, Y. Y. & Kam, T. S. (2021) Arbolodinines A−C, biologically active aspidofractinine-aspidofractinine, aspidofractinine-strychnan, and kopsine-strychnan bisindole alkaloids from *Kopsia arborea*. *Tetrahedron*, **78**, 131802.

19. Raal, A., Meos, A., Hinrikus, T., Heinämäki, J., Romāne, E., Gudienė, V., Jak.tas, V., Koshovyi, O., Kovaleva, A., Fursenco, C., Chiru, T. & Nguyen, H. T. (2020) Dragendorff's reagent: Historical perspectives and status of a versatile reagent introduced over 150 years ago at the University of Dorpat, Tartu, Estonia. *Die Pharmazie-An International Journal of Pharmaceutical Sciences*, **75(7)**, 299–306.

20. Zakaria, N., Mahdzir, M. A., Yusoff, M., Mohd Arshad, N., Awang, K. & Nagoor, N. H. (2018) Cytotoxic effects of pinnatane A extracted from *Walsura pinnata* (Meliaceae) on human liver cancer cells. *Molecules*, **23(11)**, 2733.

21. Awang, K., Pais, M., Sévenet, T., Schaller, H., Nasir, A. M. & Hadi, A. H. A. (1991) Eburnaminol and larutensine, alkaloids from *Kopsia larutensis*. *Phytochemistry*, **30(9)**, 3164–3167.

22. Toh-Seok, K., Poh-Suan, T., & Chen, W. (1993). Absolute configuration of C-16 of eburnane alkaloids from *Kopsia larutensis*. *Phytochemistry*, **33(4)**, 921–924.

23. Breitmaier, E. & Sinnema, A. (1993) Structure Elucidation by NMR in Organic Chemistry, John Wiley & Sons Ltd, England.

24. Yap, W. S., Gan, C. Y., Sim, K. S., Lim, S. H., Low, Y. Y. & Kam, T. S. (2016) Aspidofractinine and eburnane alkaloids from a north Borneo *Kopsia*. Ring-contracted, additional ring-fused, and paucidactine-type aspidofractinine alkaloids from *K. pauciflora*. *Journal of Natural Products*, **79(1),** 230–239.

25. Kam, T. S., Tan, P. S. & Chuah, C. H. (1992) Alkaloids from leaves of *Kopsia larutensis*. *Phytochemistry*, **31(8)**, 2936–2938.

26. Santos, T. A. D., Ferreira, D. T., Pinto, J. P., Faccione, M. & Braz-Filho, R. (2008) New alkaloid from Aspidosperma polyneuron roots. *Natural Product Communications*, **3(2)**, 171–174.

27. Torrenegra, R., Pedrozo, J. A., Achenbach, H. & Bauereiß, P. (1988) Alkaloids of *Stemmadenia grandiflora*. *Phytochemistry*, **27(6)**, 1843–1848.

28. Mushtaq, S., Abbasi, B. H., Uzair, B. & Abbasi, R. (2018) Natural products as reservoirs of novel therapeutic agents. *EXCLI Journal*, **17**, 420.

29. Rosales, P. F., Bordin, G. S., Gower, A. E. & Moura, S. (2020) Indole alkaloids: 2012 until now, highlighting the new chemical structures and biological activities. *Fitoterapia*, **143**, 104558.

30. riya, M. L., Priya, K. B., Kotakadi, V. S. & Josthna, P. (2015) Herbal and medicinal plants molecules towards treatment of cancer: A mini review. *American Journal of Ethnomedicine*, **2(2)**, 136–142.

31. Kubiak-Tomaszewska, G., Roszkowski, P., Grosicka-Maciąg, E., Strzyga-Łach, P. & Struga, M. (2022) Effect of hydroxyl groups esterification with fatty acids on the cytotoxicity and antioxidant activity of flavones. *Molecules*, **27(2)**, 420.

32. Lim, K. W., Sharma, B., Das, P., Chenthamarakshan, V. & Dordick, J. S. (2020) Explaining chemical toxicity using missing features, in *Proc. Int. Conference on Machine Learning '37, Vienna, Austria*, **108**.