Malaysian Journal of Analytical Sciences (MJAS)
Published by Malaysian Analytical Sciences Society

ISOLATION OF PTEROPODIC ACID FROM MALAYSIAN *Uncaria lanosa* VAR. *ferrea* BY USING LIQUID CHROMATOGRAPHY MASS SPECTROMETRY DEREPLICATION APPROACH

(Pemencilan Asid Pteropodik daripada *Uncaria lanosa* var. *ferrea* Malaysia dengan Pendekatan Direplikasi Kromatografi Cecair Spektrometri Jisim)

Nursyaza Husna Shaharuddin[1], Rohaya Ahmad[1], Fatimah Salim[2,3]*

[1]*Faculty of Applied Sciences,*
Universiti Teknologi MARA, 40450 Shah Alam, Selangor Darul Ehsan
[2]*Atta-ur-Rahman Institute for Natural Product Discovery (AuRIns),*
Universiti Teknologi MARA Selangor Branch, Puncak Alam Campus, 42300 Bandar Puncak Alam, Selangor Darul Ehsan, Malaysia
[3]*Centre of Foundation Studies,*
Universiti Teknologi MARA Selangor Branch, Dengkil Campus, 42800 Dengkil, Selangor, Malaysia

**Corresponding author: fatimah2940@uitm.edu.my*

Received: 15 September 2021; Accepted: 10 November 2021; Published: xx December 2021

Abstract

Previous chemical profiling on Malaysian *Uncaria lanosa* var. *ferrea* using liquid chromatography mass spectrometry (LCMS) had successfully identified three alkaloids namely pteropodine, isopteropodine, and isopteropodic acid along with a flavonoid known as rutin. Continuing our interest on this species, a dereplication strategy was applied to target unknown peak from the plant material. This had successfully led to the isolation of a new alkaloid. The molecular structure of the isolated new alkaloid was elucidated by using various spectroscopic techniques and was recognized as an acidic derivative of the alpha-beta unsaturated carbonyl methyl ester of pteropodine, one of the previously identified alkaloids from the plant. The new alkaloid was named as pteropodic acid. This study demonstrates that LCMS dereplication strategy will not only lead to an increased possibility of discovery of new compounds but also will [illegible]y time, energy, and resources.

Keywords: *Uncaria*, liquid chromatography mass spectrometry, dereplication, alkaloid

Abstrak

Profil kimia sebelumnya oleh *Uncaria lanosa* var. *ferrea* Malaysia melalui kromatografi cecair spektrometer jisim (LCMS) berjaya mengenal pasti tiga alkaloid iaitu pteropodin, isopteropodin, dan asid isopteropodik bersama dengan flavonoid yang dikenali sebagai rutin. Meneruskan minat kami terhadap spesies ini, strategi direplikasi digunakan untuk menyasarkan puncak yang tidak diketahui dari bahan tumbuhan yang sama dan ini berjaya memencilkan alkaloid baru. Struktur molekul alkaloid baru yang dipencilkan telah dipastikan dengan menggunakan pelbagai teknik spektroskopi dan ditentukan sebagai molekul berasid terbitan karbonil metil ester tak tepu alpha-beta dari pteropodin, iaitu salah satu alkaloid yang dikenal pasti sebelumnya dalam tumbuhan yang sama. Oleh itu alkaloid baru ini dinamakan sebagai asid pteropodik. Dapatan kajian ini menunjukkan

bahawa strategi direplikasi LCMS tidak hanya akan meningkatkan kemungkinan dalam menemui sebatian baru tetapi juga mampu menjimatkan waktu, tenaga, dan sumber bahan.

Kata kunci: *Uncaria*, kromatografi cecair spektrometer jisim, direplikasi, alkaloid

Introduction

Genus *Uncaria*, a major part of Rubiaceae family, comprising 34 species has been used to treat various diseases worldwide [1]. To date, over 400 compounds have been identified in *Uncaria* species, primarily alkaloids, terpenoids, and flavonoids [2]. *Uncaria lanosa* Wall, locally known as "gegambir paya" or "gegambir hitam", is one of 14 species of *Uncaria* available in Malaysia [3]. The plant is reported to be used as an infusion for intestine inflammation and a decoction for cleaning wounds and ulcers. According to Turner [4], there are 11 varieties of *Uncaria lanosa* Wall. These are *Uncaria lanosa* var. *appendiculata* (Benth.) Ridsdale, *Uncaria lanosa* var. *ferrea* (Blume) Ridsdale, *Uncaria lanosa* f. *ferrea* (Blume) Ridsdale, *Uncaria lanosa* var. *glabrata* (Blume) Ridsdale, *Uncaria lanosa* f. *glabrescens* (Merr. & Perry) Ridsdale, *Uncaria lanosa* f. *gynogumna* Ridsdale, *Uncaria lanosa* var. *korrensis* (Kaneh.) Ridsdale, *Uncaria lanosa* f. *philippinensis* (Elmer) Ridsdale, *Uncaria lanosa* f. *setiloba* (Benth.) Ridsdale, *Uncaria lanosa* f. *sumatrana* Ridsdale, and *Uncaria lanosa* var. *toppingii* (Merr.) Ridsdale.

In the last two decades, the mass spectrometry technique had undergone a rapid development, allowing analysis of natural products with differentiated physicochemical properties. This is due to the instrument versatility and sensitivity that could be applied to structural characterization of plant metabolite mixtures, particularly for identifying minor components [5]. Liquid chromatography-mass spectrometry (LCMS)-based fingerprinting is one of the methods that would be able to determine the genetic diversity and the authentication of a plant species. In addition, an application of a strategic method such as dereplication would further facilitate and accelerate the metabolite identification, hence search for a novel active compound. The dereplication method requires to parison of molecular chromatographic and spectroscopic data characteristics possibly obtained from an online database or an in-house library [6,7].

Our previous chemical profiling on the dichloromethane (DCM) stem extract of Malaysian *Uncaria lanosa* var. *ferrea* through Orbitrap LCMS successfully identify three alkaloids namely pteropodine, isopteropodine, and isopteropodic acid along with a flavonoid known as rutin [8]. In the present work, an LCMS dereplication strategy was applied to target for unknown peak from the same plant material, and this had led to the isolation of a new alkaloid. The dereplication process which had led to the isolation and the structural identification of the new alkaloid are discussed here.

Materials and Methods

Chemicals and raw materials

Stems of *U. lanosa var farrea* (Blume) Ridsd. were collected from Hutan Simpan Pasir Raja, Dungun Terengganu, Malaysia. This plant was identified by Mr. Ahmad Zainuddin Ibrahim from Universiti Kebangsaan Malaysia and the specimen voucher (HTBP4324) was deposited at the Herbarium Taman Botani Putrajaya, Malaysia. Ultrapure water was obtained from a PURELAB Ultra Laboratory Water Purification Systems while HPLC grade acetonitrile (MeCN) and methanol (MeOH) were purchased from Merck. Solvents used for fractionation and isolation were of industrial grade and were distilled prior to use.

Sample preparation

Dried and ground stems of *Uncaria lanosa* (1.38 kg) was extracted through 3-day maceration with methanol. The MeOH was removed until complete dryness, and crude MeOH extract (88.50 g) was further triturated using two types of solvents; non-polar solvent (hexane) and medium- polar solvent (DCM). Each mixture was filtered and evaporated under reduced pressure which produced 3 extracts; hexane (5.78 g), DCM (15.15 g) and MeOH (49.45 g). The DCM extract was subjected to an on open column chromatography [DCM/MeOH

(100:0 – 20:80)] with gradual increase in solvent polarity to yield seven potential fractions. 1 mg of the crude extract and fractions were weighed accurately and dissolved in 1 mL of MeOH: water containing 0.1% formic acid (30:70, v/v). Samples were filtered through a 0.22 µm PTFE filter into 2 ml screw cap vials prior to LCMS analysis.

High performance liquid chromatography mass spectrometry conditions

The optimized methodology in this study is slightly different compared to previous paper [7]. Samples including extracts and 16 reference compounds (1 mg/mL) were separated using GL Sciences - Inertsil Column C-18 (250 mm x 4.6 mm, i.d., 5µm). The flow rate was set at 0.6 mL/min, the absorbance was set to 254 and 215 nm, and the injection volume was 5 µL. After each injection the needle was washed with 100% MeCN. The mobile phase was a mixture of water (A) and MeCN (B) both containing 0.01% formic acid with gradient elution program of 5-55% B (0.00-30 min), 55-100% B (30-40 min), and 100% B (10-45 min).

HPLC-DAD-TOF MS was conducted on Agilent 1100 Series of high-performance liquid chromatography coupled with diode-array and time-of-flight mass detectors, electrospray ionization (ESI) source system, dual pump, autosampler and column. Nitrogen was used as the nebulizing and dissolving gas with a flow rate of 50 and 400 Lit/h, respectively. The optimum source of ESI conditions were: 3.5kV spray voltage, 25V cone voltage, 3V extractor, 0.2V rf lens, 100°C source temperature, and 350°C probe temperature. Data processing and library searching was performed using Qualitative Analysis on Mass Hunter Acquisition Data.

Isolation of pteropodic acid using recycling HPLC

One of the potential fractions, D4 (639.80 mg) was targeted for further isolation. The fraction was subjected to recycling HPLC using two column Jaigel- ODS- AP, SP- 120-15 (250 mm x 20 mm, i.d., 5µm) with guard column and GL Sciences- Inertsustain Column C-18 (250 mm x 20 mm, i.d., 5µm). The flow rate of the system was set at 4 mL/min, the absorbance at 254 nm, and the injection volume was 1 mL. The fraction was separated using an isocratic elution (20:80) of water and MeCN both containing 0.01% formic acid. The new alkaloid, pteropodic acid (5.7 mg) was eluted in the first cycle at minutes 35.

Results and Discussion

Development of in-house database

The LC-ESI-MS profiles of previously isolated compounds from *Uncaria* species were added into in-house database. This database was used as reference for the identification of compounds from LCMS data analysis of the crude extracts and fractions of *Uncaria* species for the isolation of targeted compounds. The LCMS profiles are shown in Table 1.

LCMS dereplication analysis of *Uncaria lanosa* extract

The HPLC-DAD-TOF-MS system was employed for the metabolite profiling and identification of the chromatographic peak of the DCM stem extract of *U. lanosa var. ferrea*. From the analysis, a positive mode affords higher sensitivity for most of the peaks compared to a negative mode. Therefore, a positive mode was chosen and applied in further analyses. By comparing the retention time and *m/z* value to the reference compounds in the in-house database, only four peaks were identified, including one flavonoid which was rutin (6), and three alkaloids which were pteropodine (8), isopterpodic acid (10), and isopteropodine (13). As shown in Figure 1, four peaks were successfully dereplicated. These were peaks (6), (8), (10), and (13) with $[M+H]^+$ ion of *m/z* 611.1624, 369.2173, 355.1779 and 369.2250, respectively. A major unknown $[M+H]^+$ ion peak at *m/z* 355.2707 was targeted for further isolation using recycling HPLC.

The targeted compound was isolated as white amorphous powder and its mass spectrum gave an $[M+H]^+$ peak at *m/z* 355.2707 which corresponded to the molecular formula $C_{20}H_{22}N_2O_4$. Its UV spectrum displayed absorption bands at 209.97 and 244.00 nm indicating that a hydroxyl substituent may be present. The IR spectrum revealed the presence of non-hydrogen-bonded OH (sharp peak) at 3421.96 cm^{-1}. Typical IR characteristics of heteroyohim B bine-type oxindole alkaloids absorption frequencies at 3114 cm^{-1} (NH), 1622.02 cm^{-1} (C=O acid), 1674 cm^{-1} (C=O

amide), 1636 cm^{-1} (C=C olefinic), 1472.8 cm^{-1} (C=C aromatic) and 1191.42 cm^{-1} (C-O cyclic ether) were also observed suggesting that the compound belonged to pentacyclic oxindole alkaloid. The ^{1}H NMR spectrum of this compound showed that four aromatic proton signals resonating at δ 7.29, δ 7.21, 7.07 and 6.88 confirming that the presence of aromatic ring is unsubstituted in the oxindole moiety. Proton resonances were also observed as a singlet at δ 7.50 belonging to an olefinic proton H-17 and a broad singlet at δ 8.56 attributed to a proton attached with nitrogen. A quartet and three-proton doublet resonances at δ 4.46 (*J* = 6.1 Hz) and 1.41 (*J* = 6Hz) were also present corresponding to H-19 and H-18 respectively. There was no carbomethoxy (OCH_3) resonance which was typically observed in other POAs H^1 NMR spectrum [9, 10].

In the ^{13}C NMR spectrum, the presence of 20 carbons including a methyl group, an acid carbonyl and oxindole carbonyl resonances at δ 17.59, δ 170.40 and δ 181.92 respectively. The slightly higher ^{13}C NMR resonance value for the acid carbonyl (δ 170.40) compared to the ester carbonyl in the pteropodine system (δ 167.77) further confirmed the existence of a carboxylic acid moiety in the structure [11]. Therefore, based on these spectral data as well as comparisons with literature data, the compound was deduced to be pteropodic acid which is the acidic derivative of the alpha-beta unsaturated carbonyl methyl ester of pteropodine, one of the previously identified alkaloids from the plant. The structure of the dereplicated and the isolated alkaloid is shown in Figure 2.

Physical and spectral data of isolated compound

Pteropodic acid: White amorphous powder, wt: 5.7 mg (MeOH), mp 268 - 271 °C. MS *m/z*: 355.2707 $[M+H]^+$, $C_{20}H_{22}N_2O_4$; UV (MeOH) λ_{max} nm: 209.97, 244.00; IR (KBr) υ_{max} cm^{-1}: 3421.96 (OH), 3114 (NH), 1622.02 (C=O acid), 1674 (C=O amide), 1636 (C=C olefinic), 1472.8 (C=C aromatic) and 1191.42 (C-O cyclic ether); ^{1}H NMR (MeOD, 600 MHz) δ ppm: 8.56 (1H, *br s*, N-H), 7.50 (1H, *s*, H-17), 7.29 (1H, *d*, *J* = 7.2 Hz, H-9), 7.21 (1H, *ddd*, *J* =7.7, 1.0 Hz, H-11), 7.07 (1H, *ddd*, *J*= 7.6, 0.8 Hz, H-10), 6.88 (1H, *d*, *J* = 7.7 Hz, H-12), 4.46 (1H, *q*, *J* = 6.1 Hz, H-19), 3.39 - 3.34 (2H, *m*, H-21β, H-5β), 2.50 – 2.27 (5H, *m*, H-15, H-21α, H-6β, H-5α, H-3), 2.07-1.99 (1H, *m*, H-6α), 1.70 – 1.64 (2H, *br m*, H-14α, H-20), 1.63-1.57 (1H, *m*, H-14β), 1.41 (3H, *d*, *J* = 6 Hz, CH_3); ^{13}C NMR (MeOD, 150 MHz) δ ppm: 181.92 (N-C=O), 170.40 (O-C=O), 154.60 (C-17), 141.50 (C-13), 133.30 (C-8), 127.70 (C-11), 122.79 (C-9), 122.27 (C-10), 110.39 (C-16), 109.09 (C-12), 74.11 (C-19), 71.85 (C-3), 56.42 (C-7), 54.59 (C-5), 53.03 (C-21), 38.11 (C-20), 33.56 (C-6), 31.16 (C-15), 29.55 (C-14), 17.59 (CH_3).

Table 1. Characterization of reference compounds by developed HPLC-DAD-TOF MS method

No.	Compound (RT (min))	Proposed Molecular Formula	Observed Mass $[M+H]^+$	Calculated Mass $[M+H]^+$	Observed Mass $[M-H]^-$	Calculated Mass $[M-H]^-$
1	Formosaninol (10.23)	$C_{21}H_{26}N_2O_5$	387.2092	387.1920	-	385.1764
2	Catechin (10.63)	$C_{15}H_{14}O_6$	291.1058	291.0868	289.0612	289.0712
3	Isoformosaninol (11.65)	$C_{21}H_{26}N_2O_5$	387.2108	387.1920	-	385.1764
4	(-)-catechin (12.77)	$C_{15}H_{14}O_6$	291.1058	291.0868	289.0600	289.0712
5	(-)*Epi*-afzelechin (15.15)	$C_{15}H_{14}O_5$	275.1117	275.0919	273.0661	273.0763
6	Rutin (16.88)	$C_{27}H_{30}O_{16}$	611.1745	611.1612	609.1128	609.1456
7	Scopoletin (17.49)	$C_{10}H_8O_4$	193.0726	193.0501	-	191.0345
8	Pteropodine (17.64)	$C_{21}H_{24}N_2O_4$	369.1971	369.1814	-	367.1658
9	Longiflorine (17.69)	$C_{11}H_{15}NO_4$	226.1280	226.1079	-	224.0923
10	Isopteropodic acid (18.32)	$C_{20}H_{22}N_2O_4$	355.1841	355.1658	-	353.1502

Table 1 (cont'd). Characterization of reference compounds by developed HPLC-DAD-TOF MS method

No.	Compound (RT (min))	Proposed Molecular Formula	Observed Mass $[M+H]^+$	Calculated Mass $[M+H]^+$	Observed Mass $[M-H]^-$	Calculated Mass $[M-H]^-$
11	Methyl paraben (21.72)	$C_8H_8O_3$	153.0777	153.0552	151.0387	151.0396
12	Uncariechin (22.64)	$C_{18}H_{14}O_6$	327.1062	327.0868	325.0564	325.0712
13	Isopterodine (23.67)	$C_{21}H_{24}N_2O_4$	369.1996	369.1814	-	367.1658
14	Uncarine F (23.75)	$C_{21}H_{24}N_2O_4$	369.2063	369.1814	367.1625	367.1658
15	Speciophylline (23.79)	$C_{21}H_{24}N_2O_4$	369.2126	369.1814	367.1634	367.1658
16	Rauniticine-*allo*-oxindole B (23.83)	$C_{21}H_{24}N_2O_4$	369.2182	369.1814	367.1566	367.1658
17	Quercetin (24.72)	$C_{15}H_{10}O_7$	303.0699	303.0505	301.0248	301.0349

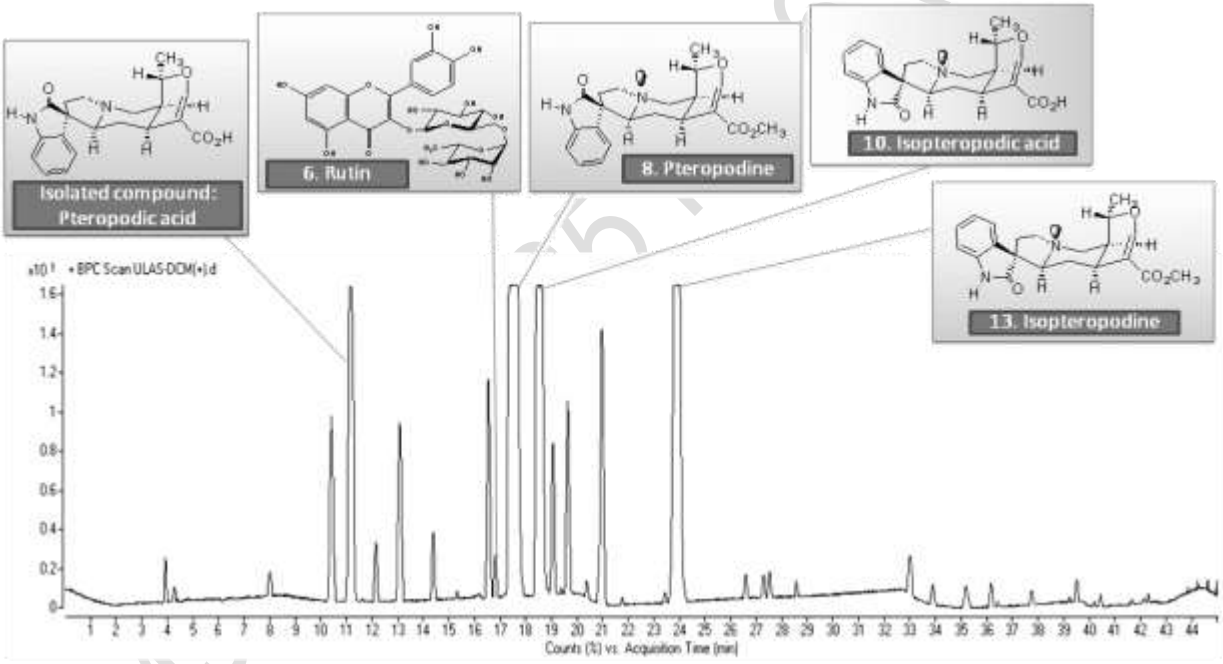

Figure 1. MS profile of *U. lanosa* DCM extract; the total ion current (TIC) in positive mode with structure of identified peaks; numbers of identified peak are according to the no. of reference compound in Table 1.

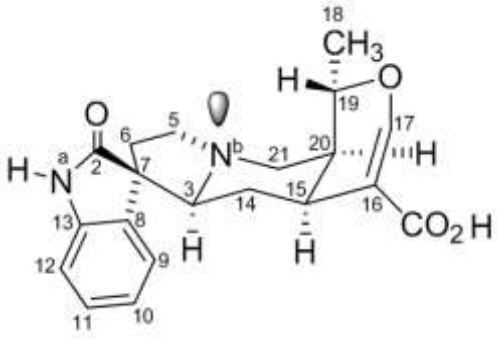

Figure 2. Structure of isolated alkaloid from *Uncaria lanosa* var. *ferrea* identified as pteropodic acid

Conclusion

The proposed HPLC-DAD-TOF-MS dereplication strategy using in-house developed database was applied to identify known peaks and target unknown peak from *U. lanosa* var. *ferrea*. This has successfully led to the identification of one flavonoid and three alkaloids, and the isolation of a new alkaloid which was deduced as pteropodic acid. This study demonstrates that the LCMS dereplication strategy will not only increase the possibility discovery of new compounds but will save time, energy and resources by minimizing repetitive isolation of known compounds.

Acknowledgement

Authors would like to thank the Ministry of Science, Technology and Innovation Malaysia (MOSTI) for funding this research through E-Sciences grant 100-RMI/SF 16/6/2 (23/2012), and the Faculty of Pharmacy, Universiti Teknologi MARA for the HPLC-DAD-TOF-MS facility.

References

1. Ahmad, R., and Salim, F. (2015). Oxindole alkaloids of *Uncaria* (Rubiaceae, subfamily cinchonoideae): A review on its structure, properties, and bioactivities. In *Book Studies in Natural Products Chemistry*, 45: 486-525.
2. Qin, N., Lu, X., Liu, Y., Qiao, Y., Qu, W., Feng, F. and Sun, H. (2021). Recent research progress of *Uncaria* spp. based on alkaloids: phytochemistry, pharmacology and structural chemistry. *European Journal of Medicinal Chemistry*, 210: 112960.
3. Burkill, I. H. (1966). A dictionary of the economic products of Malaysia Peninsula. 2nd edition. Kuala Lumpur: pp. 2236-2245.
4. Turner, I. M. (2018). A revised conspectus of *Uncaria* (Rubiaceae). *Journal of Plant Taxonomy and Geography*, 73(1): 9-21.
5. Alvarez-rivera, G., Ballesteros-vivas, D., Parada-alfonso, F., Ibanez, E., and Cifuentes, A. (2019). Recent applications of high resolution mass spectrometry for the characterization of plant natural products. *Trends in Analytical Chemistry*, 112: 87-101.
6. Lone, S. A., Kushwaha, M., Wani, A., Kumar, A., Gupta, A. P., Hassan, Q. P., Chandra, S. and Gupta, S. (2017). Genetic diversity, LCMS based chemical fingerprinting and antioxidant activity of *Epimedium elatum* Morr & Decne. *Journal of Applied Research on Medical and Aromatic Plants*, 5: 72-81.
7. Zhang, X., Li, Q., Xu, Z. and Dou, J. (2020). Mass spectrometry-based metabolomics in health and medical science: A systematic review. *RSC Advances*, 10: 3092-3104.
8. Shaharuddin, N. H., Ismail, N. H., Manshoor, N., Salim, F. and Ahmad, R. (2016). Chemical profiling and identification of alkaloids and flavonoids in *Uncaria lanosa* var. *ferrea* via UHPLC-Orbitrap MS. *Malaysian Journal of Analytical Sciences*, 20(2): 318-323.

9. Salim, F., Ismail, N. H., Awang, K. and Ahmad, R. (2016). Rauniticine-*allo*-oxindole B and rauniticinic-*allo* acid B, new heteroyohimbine-type oxindole alkaloids from the stems of Malaysian *Uncaria longiflora* var. *pteropoda*. *Molecules*, **16**(8): 6541-6548.
10. Salim, F., Yunus, Y. M., Anouar, E. H., Awang, K., Langat, M. K., Cordell, G. A., and Ahmad, R. (2019). Absolute configuration of alkaloids from *Uncaria longiflora* var. *pteropoda* through experimental and theoretical approaches. *Journal of Natural Products*, 82: 2933-2940.
11. Salim, F. and Ahmad, R. (2010). Isopteropodic acid from Malaysian *Uncaria longiflora* var. *pteropoda*. *World Applied Science Journal*, 10(11): 1334-1337.