Isolation and Structural Assignment of β-Sitosterol Compound from the Whole Plant of *Uncaria macrophylla* Wall.

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ABSTRACT

One of the Myanmar traditional medicinal plants, *Uncaria macrophylla* Wall. (local name-Nabu-saymakhan) was selected for the isolation of bioactive compound and its structure identification. The phytochemical screening of the whole plant of *Uncaria macrophylla* was done and it consists of some phytochemical constituents such as alkaloid, glycoside, flavonoid, polyphenol, sugar, lipophilic, terpene, sterol, saponin and phenolic compounds respectively. The pure organic compound (TLN-1) was isolated from the whole plant of *Uncaria macrophylla* by thin layer and column chromatographic separation techniques. In addition, the antimicrobial activities of the pure isolated compound were tested using Agar-well diffusion method on six microorganisms. The molecular formula determination was done by using FT-IR, ^{1}H NMR, ^{13}C NMR, DEPT and HMQC spectroscopy and Mass spectrometry. The complete structure of sterol derivative compound (TLN-1) was elucidated by applying 1D and 2D NMR spectroscopy as well as EI-Mass spectrometry.

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Keywords : *Uncaria macrophylla* Wall.; FT-IR and NMR Spectroscopy; EI- Mass spectrometry

1 INTRODUCTION

Medicinal plants have been used throughout human history. Plants are the source of variety of chemical compounds to be synthesized for their important biological functions, and to defend against insects, fungi, bacteria and viruses. At least 12,000 such compounds have been isolated so far; a number estimated to be less than 10% of the total. [1,2] Chemical compounds in plants mediate their effect on the human body through processes identical to those already well understood for the chemical compounds in conventional drugs; thus herbal medicines do not differ greatly from conventional drugs in terms of how they work. This enables herbal medicines to be as effective as conventional medicines, but also gives them the same potential to cause harmful side effects. [1, 2]

The use of plants as medicines predates written human history. Ethno botany (the study of traditional human uses of plants) is recognized as an effective way to discover future medicines. In 2001, researchers identified 122 compounds used in modern medicine which were derived from "ethno-medical" plant sources; 80% of these have had an ethno-medical use identical or related to the current use of the active elements of the plant. [3] The use of herbs for the treatment of diseases is almost universal among developing countries, and is often more affordable than purchasing expensive modern pharmaceuticals. The World Health Organization (WHO) estimates that 80 percent of the population of some Asian and African countries presently uses herbal medicine for some aspect of primary health care.

The genus *Uncaria* is widely distributed in tropical regions, including Southern Asia, Africa and South America [4], and most plants of the genus *Uncaria* have been used as important sources of medicinal natural products in the family of Rubiaceae. Many species of the genus *Uncaria* have been used to cure fevers, nervous disorders, apasmolytic, analgesic, hypertension [5], cancer, arthritis, diabetes, inflammation, [6-8], asthma, cirrhosis, stroke and rheumatism. Previous studies show that alkaloids, triterpenes and flavones [6, 9] are the most widespread of the secondary metabolites isolated from the genus *Uncaria*. As a part of the systematic isolation of phytochemical constituents, a new triterpene, 3β, 6β, 19α-trihydroxy-12-oleanen-28-oic acid, was isolated from the chloroform-soluble fraction of the alcohol-water extract of *U. macrophylla*. [10]

In this present research work, one of the Myanmar traditional indigenous medicinal plants, Nabu-saymakhan, (*Uncaria macrophylla*) grown in Kachin State was selected for chemical investigation because of its medicinal properties.

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2 MATERIALS AND METHODS

2.1 Materials

Commercial grade solvents and reagents were applied without purification. The whole plant of *Uncaria macrophylla* Wall. was collected from Myitkyina Township, Kachin State, Myanmar. Analytical preparative thin layer Chromatography was performed by using precoated silica gel (Merk. Co. Inc, Kiesel gel 60 F_{256}). Silica gel (70-230 mesh, Merck Co. Inc Kiesel gel 60 F_{254}) was used for column chromatography. UV-Lamp (Lambda – 40, Perkin – Elmer Co, England) and iodine vapor were used as developing agents in column chromatography. FT-IR spectrometer (Shimadsu, Japan) was used for the identification of the functional groups of the isolated compounds. ^{1}H-and ^{13}C NMR spectroscopy was carried out on JEOL at 500MHz and 125MHz respectively. Chemical shifts values are given in δ-value (ppm) with tetramethylsilane (TMS) as internal standard. EI-mass spectrometer (JEOL, JMS-600 MHz) was employed to assign the molecular formula of the compound.

2.2 Preliminary Phytochemical Tests of the Whole Plant of *Uncaria macrophylla* Wall.

The phytochemical tests were carried out at Department of Chemistry, University of Mandalay, Myanmar to detect the different kinds of chemical constituents in the sample [11] [12] [13] [14].

2.3 Extraction and Isolation of Pure Organic Compound

The air dried sample (540g) was percolated with 95% ethanol (3L) at room temperature for about two months. The ethanol crude extract was again extracted with ethyl acetate (250 mL) under normal condition. The ethyl acetate crude sample (2.34 g) was separated by using column chromatography. Silica gel (70-230 mesh) was used as adsorbent and n-hexane: ethyl acetate mixture was used as eluent with various solvent ratios from non-polar to polar. Totally (142) fractions were obtained. Each and every fraction was checked on TLC using iodine as visualizing agent. The fractions with same R_f values were combined and ten combined fractions were obtained. Among them, fraction (IX) was again purified by using same adsorbent and same eluent as mentioned in the previous column. Pure colorless crystal was obtained and checked on TLC for purity. It gave one spot on TLC in (R_f = 0.45*)* with n-hexane: ethyl acetate (3:2) (v/v). The weight of the isolated compound (TLN-l) was 20.3 mg and its yield percent was found to be (0.87%) based on the ethyl acetate crude extract [15].

2.4 Determination of Antimicrobial Activity of the Pure Organic Compound

The antimicrobial activities of the isolated compound were determined using Agar-well diffusion method on six selected microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomas aeruginosa, Bacillus pumilus, Candida albicans* and *E.coli* in PRD (Pharmaceutical Research Department), Yangon, Myanmar [16].

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3 RESULTS AND DISCUSSION

3.1 Preliminary Phytochemical Tests of the Whole Plant of *Uncaria macrophylla* Wall.

According to the phytochemical tests of the crude extracts from the whole plant of *Uncaria macrophylla*, the sample contains many chemical constituents such as alkaloid, flavonoid, terpenoid, steroid, glycoside, reducing sugar, polyphenol, saponin and lipophilic compounds respectively.

Table (1) Results of Phytochemical Tests of the Whole Plant of *Uncaria macrophylla* Wall.

No.	Tests	Reagents	Observation	Results
1.	Alkaloid	Dragendorff's reagent	Pale Orange ppt	+
		Wagner's reagent	Reddish brown ppt	+
2.	Flavonoid	conc: HCl, Mg tunning	Pink color solution	+
3.	Terpene	Pet ether, Acetic anhydride , conc: H_2SO_4, $CHCl_3$	Reddish brown color solution	+
4.	Steroid	EtOH, Acetic anhydride, conc: H_2SO_4, $CHCl_3$	Greenish blue color	+
5.	Glycoside	10 % lead acetate	Yellow ppt	+
6.	Reducing Sugar	Benedict's solution	Red ppt	+
7.	Polyphenol	10 % $FeCl_3$, $K_3[Fe(CN)_6]$	Greenish blue color solution	+
8.	Saponin	Ethanol, conc: H_2SO_4	Red color Froth	+
9.	Lipophilic	0.5N KOH	Deep color solution	+

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in literature and all these data is shown in the table (4).

Table (3) ^{1}HNMR, ^{13}CNMR, DEPT, HMQC, COSY and HMBC Spectral Data of the Compound (TLN-1) (at 500 and 125 MHz, resp. in $CDCl_3$; 296 K; δ in ppm, J in Hz)

Position C/H	DEPT/ HMQC	δ_H	δ_C	^{1}H- ^{1}H correlations (COSY)	J correlations (HMBC)
1	CH_2	1.08, 1.85 (o)	37.35	H-2a, 2b	
2	CH_2	1.47, 1.82 (o)	31.74	H-1a, 1b, 3	H-4a, 4b
3	CH	3.52 (m)	71.90	H-2a, 4a, 4b	H-1b, 2b, 4a, H4b,
4	CH_2	2.23, 2.28 (m)	42.39	H-3	H-6
5	C		140.85		H-4a, 4b, 19
6	CH	5.34 (bd, J= 4.9 Hz)	121.77	H-7a, 7b	H-4a, 4b, 7a, 7b
7	CH_2	1.52, 1.97 (o)	32.01	H-6, 8	H-6
8	CH	1.45 (o)	31.95	H-7b, 9, 14	H-6
9	CH	0.93 (o)	50.25	H-8, 11a, 11b	H-19
10	C		36.59		H-1b, 2b, 4b, 6, 9, 19
11	CH_2	1.48, 1.53 (o)	21.17	H-9, 12b	H- 19
12	CH_2	1.16, 2.02 (o)	39.88	H-11a, 11b	H- 18
13	C		42.42		H-12a, 17, 18
14	CH	1.02 (o)	56.87	H-8, 15a, 15b	H- 9, 18
15	CH_2	1.56, 1.58 (o)	24.39	H-14, 16a, 16b	H- 16a, 16b
16	CH_2	1.55, 1.57 (o)	24.44	H-15a, 15b, 17	H-15a, 15b,
17	CH	1.10 (o)	56.18	H-16a, 16b	H-18, 21
18	CH_3	0.68 (s)	11.94		H-11a, 11b, 17
19	CH_3	1.0 (s)	19.47		H-1a, 1b, 9
20	CH	1.67 (o)	36.23	H-21, 22a, 22b	H-17, 21
21	CH_3	0.93 (d, J= 6.3 Hz)	19.06	H-20	H-17, 22
22	CH_2	1.24, 1.26 (o)	34.06	H-20, 23a, 23b	H-21
23	CH_2	1.14, 1.17 (o)	26.23	H-22a, 22b, 24	H-
24	CH	0.91 (o)	45.96	H-23a, 23b, 25, 28a, 28b	H-22a, 22b, 26, 27
25	CH	1.28 (o)	29.29	H-24, 26, 27	H-23a, 23b, 26, 27
26	CH_3	0.86 (o)	21.14	H-25	
27	CH_3	0.80(o)	18.86	H-25	
28	CH_2	1.24, 1.25 (o)	23.18	H-24, 29	H-24
29	CH_3	0.85(o)	12.06	H-28a, 28b	

(s= singlet, d= doublet, bd= broad doublet, o= overlap, m= multiplet)

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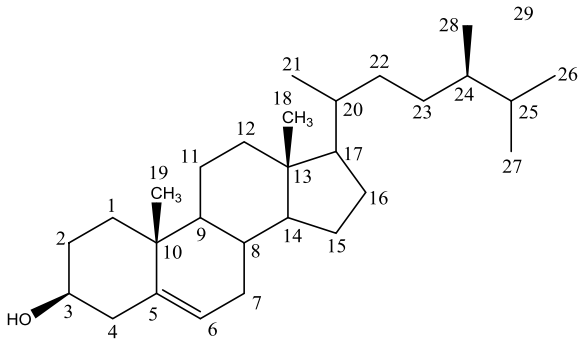

Figure 2. Structure of isolated pure compound (TLN-1)

Table (4) ^{1}HNMR and ^{13}CNMR Spectral Data for Compound (TLN-1) and from the literature

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Position	Experimental		Literature [20]	
	δ_H	δ_C	δ_H	δ_C
C-1		37.35		37.5
C-2		31.74		31.9
C-3	3.52 (m)	71.90	3.53 (tdd, 1H, *J*= 4.5, 4.2, 3.8 Hz)	72.0
C-4		42.39		42.5
C-5		140.85		140.9
C-6	5.34 (bd, 1H, *J*= 4.9 Hz)	121.77	5.36 (t, 1H, *J*= 6.4 Hz)	121.9
C-7		32.01		32.1
C-8		31.95		32.1
C-9		50.25		50.3
C-10		36.59		36.7
C-11		21.17		21.3
C-12		39.88		39.9
C-13		42.42		42.6
C-14		56.87		56.9
C-15		24.39		26.3
C-16		24.44		28.5
C-17		56.18		56.9
C-18	0.68 (s, 3H)	11.94	0.68 (s, 3H)	12.0
C-19	1.0 (s, 3H)	19.47	1.01 (s, 3H)	19.0
C-20		36.23		36.3
C-21	0.93 (d, 3H, *J*= 6.3 Hz)	19.06	0.93 (d, 3H, *J*= 6.5 Hz)	19.2
C-22		34.06		34.2
C-23		26.23		26.3
C-24		45.96		46.1
C-25		29.29		29.4
C-26	0.86 (3H, o)	21.14	0.83 (d, 3H, *J*= 6.4 Hz)	20.1
C-27	0.80 (3H, o)	18.86	0.81 (d, 3H, *J*= 6.4 Hz)	19.6
C-28		23.18		23.3
C-29	0.85 (3H, o)	12.06	0.84 (t, 3H, *J*= 7.2 Hz))	12.2

(s= singlet, d= doublet, bd= broad doublet, t= triplet, o= overlap, m= multiplet)

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4 CONCLUSION

The whole plant of *Uncaria macrophylla* Wall. (local name- Nabu-saymakhan) was used to determine the phytochemical constituents, to isolate the pure organic compound and to investigate its antimicrobial activities. This plant contains alkaloids, glycosides, flavonoids, polyphenols, sugar, lipophilic, terpene, steroid, saponin and phenolic compounds. The pure organic compound (TLN-1) could be isolated by thin layer and column chromatographic separation techniques. Phytochemical test for pure compound (TLN-1) was done and it showed the positive test for sterol (Liebermann Burchard reaction). The melting point of this compound was determined and was found to be 137-139ºC. The yield percent of the isolated compound was found as 1.67% based upon the EtOAc crude extract. Moreover, this pure compound (TLN-1) was to possess high activity on all selected organisms by Ager well diffusion method.

The structural elucidation of this sterol compound (TLN-1) could be done by some modern spectroscopic techniques such as COSY, HMQC, HMBC, and EI-MS spectral data compared with literature. The FT-IR spectrum indicated hydroxyl group at (3425.69 cm^{-1}) and olefinic group at (1645.33 cm^{-1}). The MS displayed (M^+) at m/z 414 (corresponding to $C_{29}H_{50}O$). The ^1H NMR spectrum reflected six methyl groups (δ_H 0.68, 0.80, 0.85, 0.86, 0.93, and 1.0) corresponding to δ_C 11.94, 18.86, 12.06, 21.14, 19.06, and 19.47; a hydroxymethylene (δ_H 3.52, corresponding to δ_C 71.90), one olefinic proton (δ_H 5.34 corresponding to δ_C 121.77). These data point out that the isolated compound is a sterol derivative compound. From the comparison of the experimental data with literature value, the compound (TLN-1) could be assigned as β- sitosterol. According to the references, this isolated β- sitosterol compound was isolated for the first time from the whole plant of *Uncaria macrophylla* Wall. [10, 21]

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