Structural Elucidation of an Organic Compound and Some Biological screening of the Root of *Streptocaulon tomentosum* Wight & Arn (Myinsa-gonni)

Aye Aye Thant[1], Yi Yi Win[2], Myint Myint Khine[3] and Ni Ni Than[4]

Abstract

In this research work, the root of *Streptocaulon tomentosum*, a traditional medicinal plant in Myanmar named Myinsa-gonni, was collected for this study in Launglon Township, Tanintharyi Region. The sample was chopped into small pieces and dried in the air. The air-dried tuber sample was extracted with 95 % ethanol for about one month. Furthermore, using a partitional column with pet-ether and ethyl acetate before being subjected to thin layer and column chromatography using the pet-ether and ethyl acetate solvent ratio (PE: EtOAc, 15:1, 90 mg, and R_f value 0.66), a biologically active pure compound (AAT-1) was isolated. The structure of the isolated compound was confirmed by spectroscopic data measurements such as FT IR, ^{1}H NMR, and ^{13}C NMR, and the literature comparisons infer that the isolated compound is a pentacyclic triterpenoid, namely lupeol acetate**.** The study was to evaluate some biological potentials, including cytotoxicity and anti-inflammatory activities. The brine shrimp lethality test was examined for cytotoxic potential. The 50 % lethal concentrations of ethanol extract (LC_{50} = 400 µg/mL), watery extract (LC_{50} = 200 µg/mL), and Lupeol acetate (LC_{50} = 239.88 µg/mL) were also determined. *In vitro*, the anti-inflammatory activity of ethanol and watery extracts was evaluated by a nitric oxide (NO) inhibition assay; these results did not significantly affect cellular nitric oxide (NO) production or cell viability because the two extracts had medium toxic potential.

Keywords*:* Cytotoxicity, anti-inflammatory, *S. tomentosum*, Lupeol acetate, and structural elucidation

Introduction

Streptocaulon tomentosum is a plant in the Asclepiadaceae family and includes five species. Two species, *S. tomentosum* and *S. griffithii* J. D. Hooker, grow in Myanmar. The roots of *S. tomentosum* are used in Myanmar traditional medicine for the treatment of cancer, dysentery, and stomachaches, and the leaves are used externally for the treatment of snake poisoning and abscesses. (Khine *et al.,* 2004; 2007), and its roots are used as tonics for anemia, rheumatism, menstrual disorders, neurasthenia, and dyspepsia. (Jun-ya *et al*., 2003). However, the isolation of cardenolides from the root of *S. Juventus* (Lour.) Merr. and the antiproliferative activity of cardenolides isolated from *S. Juventus* have been reported (Ueda *et al.*, 2003a; 2003b). In Vietnam, the roots of *S. juventus* are used to treat many diseases, including anemia, chronic malaria, rheumatism, menstrual disorders, neurasthenia, and dyspepsia (Can *et al*., 2017). The roots of *S. tomentosum* have been reported to be abundant in cardenolides. Hemiterpenoids, phenylpropanoids, and phenylethanoids have also been isolated from the roots. Pharmacological studies of cardenolides have demonstrated that these compounds selectively and potently inhibit proliferation of the growth of the HT-1080 and A549 cell lines through the induction of apoptosis (Win *et al*., 2021). The current study aims to investigate cytotoxicity, anti-

[1] Lecturer, Daw, Department of Chemistry, University of Yangon
[2] Professor, Dr. Department of Chemistry, Hpa-an University
[3] Pro-rector, Dr. Myingyan University
[4] Professor and Head, Dr. Department of Chemistry, University of Yangon

inflammatory activities, and the isolation and identification of an organic compound (AAT-1) from S. *tomentosum*.

Figure 1 Photograph of the root and the plant of *S. tomentosum*

Material and Methods

Plant Material

The sample was collected from Launglon Township, Tanintharyi Region, Myanmar, in the month of May 2019 and identified as *S. tomentosum* Wight & Arn., by the authorized botanist at the Department of Botany, University of Yangon. The sample was dried under the shade for a week, cut into very small pieces, and then ground into a purely fine powder using an electric grinder. The powdered sample was stored in airtight containers.

Preliminary Phytochemical Tests

Preliminary phytochemical tests on the powdered sample were carried out according to the reported methods in order to classify the types of organic constituents present in the samples such as alkaloids, α-amino acids, carbohydrates, cyanogenic glycosides, flavonoids, glycosides, organic acids, phenolic compounds, reducing sugars, saponins, starch, steroids, tannins and terpenoids by appropriate reported methods (Bandiola, T. M. B, 2018).

Identification of Isolated Pure Compounds AAT-1

The dried powdered root of *S. tomentum* (500 g) was extracted with 95 % ethanol by sonication for 5 L (1.5 h × 3) at 70 ℃, and the sonication was evaporated under reduced pressure to yield an ethanol extract (40.51 g). The ethanol extract was again extracted with petroleum ether, followed by ethyl acetate. The pet-ether extract (20 g) was separated by column chromatography on silica gel GF_{254} adsorbent by increasing the polarity of the eluent solvent system (PE: EA), and five main fractions (f 1 to f V) were obtained. From fraction I., Compound AAT-1 (90 mg, 1.45%, and mpt 216 ℃) was isolated. The isolated pure compound AAT-1 from pet-ether extract was structurally identified by modern spectroscopic methods, including FT IR, ^{1}H NMR, and ^{13}C NMR. The cytotoxic activity was assessed by using a pure compound (AAT-1) and crude extracts (ethanol and watery), and the anti-inflammatory activity was also assessed by using crude extracts (ethanol and watery).

Cytotoxicity Effects by Brine Shrimp Lethality Bioassay

The cytotoxic potency of ethanol and watery extracts of the root of *S. tomentosum* was estimated by a brine shrimp lethality bioassay, according to the procedure described by Olowa *et al*. (2013). The cytotoxicity of test extracts was expressed using Clarkson's criteria, according to which the toxicity of plant extracts was classified: those with an LC_{50} above 1000 μg/mL were non-toxic, those with an LC_{50} of 500-1000 μg/mL were low toxic, those with an LC_{50} of 100-500 μg/mL were medium toxic, and those with an LC_{50} of 0-100 μg/mL were highly toxic (Mentor *et al.*, 2014).

Investigation of Anti-inflammatory and Cell Viability Activity of Ethanol and Watery Extracts

The anti-inflammatory activity of crude ethanol and watery extracts was evaluated using the NO inhibition assay, which was carried out at Toyama University, Toyama, Japan. The percentages of NO inhibition and cell viability were calculated as follows:

Results and Discussion

Phytoconstituents Present in Root of *S. tomentosum*

Primary phytochemical screening of *S. tomentosum* was performed using the appropriate methods. The phytochemical tests revealed the presence of metabolites such as alkaloids, carbohydrates, reducing sugars, glycosides, saponins, organic acids, phenolic compounds, flavonoids, tannins, steroids, and terpenoids, but cyanogenic glycosides were absent in the sample. These results are summarized in Table 1.

Table 1 Preliminary Phytochemical Results on the Root of *S. tomentosum*

No.	Tests	Test reagents	Observation	Results
1.	Alkaloid	Dragendorff's reagent	Orange ppt	+
2.	Flavonoids	EtOH, Mg turning and Conc: HCl	Pink colour solution	+
3.	Glycosides	10 % lead acetate	White ppt	+
4.	Carbohydrates	10 % α-naphthol and Conc: H_2SO_4	Red ring	+
5.	Cyanogenic glycosides	Sodium picrate	No brick red colour	-
6.	Organic acids	Bromocresol green	Green colour	+
7.	Phenolic compounds	10 % $FeCl_3$	Dark green colour	+
8.	Reducing sugars	Benedict's solution	Brick red ppt	+
9.	Saponins	Distilled water	Frothing	+
10.	Tannins	1% Gelatin	White ppt	+
11.	Steroids	$(CH_3CO)_2O$ and Conc:H_2SO_4	Green colour	+
12.	Terpenoids	$(CH_3CO)_2O$and Conc: H_2SO_4	Pink colour	+

(+) = presence (-) = absence ppt = precipitation

Characterization and Identification of an Isolated Compound AAT-1

Compound AAT-1, isolated from the pet-ether extract of the root of *S. tomentosum*, was obtained as a white amorphous compound with a melting point of 216-218 ℃. It is soluble in chloroform, ethyl acetate, and methanol. It was UV inactive, and the R_f value was found to be 0.66 (PE: EtOAc,15:1 v/v). According to chemical tests, the phenolic -OH group was absent in compound AAT-1 because it did not change colour after spraying with a 10 % $FeCl_3$ solution. It did not contain a carbonyl group because the reaction did not give yellow precipitates when tested with a 2,4-DNP solution. Since compound AAT-1 changed pink colour ($CHCl_3$ used as solvent) when treated with Libermann Burchard solution. So, compound AAT-1 must be a terpenoid compound. In the confirmation test, it also gave a purple colour spot on TLC by spraying with vanillin-sulphuric acid reagent after heating. Therefore, it was suggesting that it was a terpenoid compound.

AAT-1 White amorphous

Under UV 254 nm
Solvent = (PE: tOAc,15:1)
R_f = 0.66 (90 mg, 1.45 %)

Sprayed with Vanillin-sulphuric acid reagent

Figure 2 Thin layer chromatograms of isolated compoundAAT-1 from roots of *S. tomentosum*

Structure Elucidation of Isolated Compound AAT-1

The structure of the isolated compound AAT-1 from a pet-ether extract of *S. tomentosum's* root was measured by various spectroscopic data, such as ^{1}H NMR and ^{13}C NMR, at Toyama University, Toyama, Japan. The FT IR spectrum of compound AAT-1 is illustrated in Figure 3, and the corresponding spectral data assignment is tabulated in Table 2. The =CH stretching band appeared at 3070 cm^{-1} indicating the presence of a vinylidene group, the absorption at 2924 and 2851 cm^{-1} represents aliphatic C-H stretching due to symmetric and asymmetric stretching of CH_2 and CH_3 groups. The C=O stretching vibration for the RCOOR group was found at 1735 $cm^{-1,}$ and the C=C stretching vibration was at 1645 cm^{-1}. The deformation vibrations of aliphatic C-H of CH_2 and CH_3 were found at 1453 and 1368 cm^{-1}, respectively. The band that appeared at 1024 $cm^{-1}$was assigned to the C-O stretching vibration. The vinylidene group's out of plane C-H bending vibration was observed at 827 cm^{-1}.

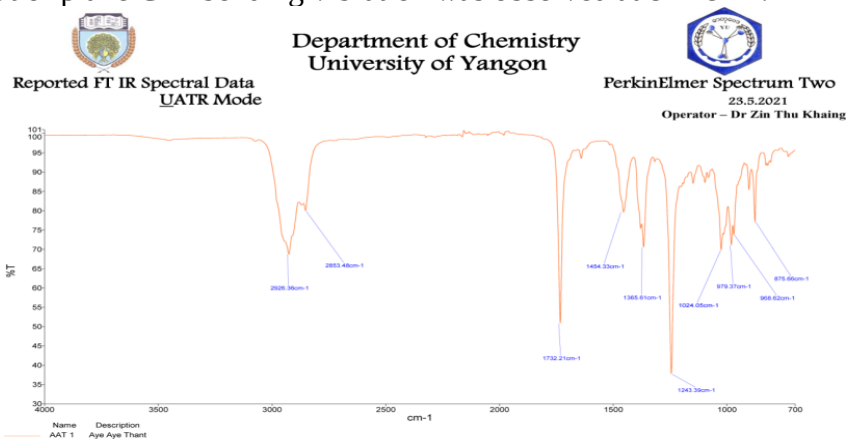

Figure 3 FT IR spectrum of isolated compound AAT – 1

Table 2 FT IR Spectral Data of Isolated compound AAT-1 Compared with Reported Data of Lupeol acetate

Wave number (cm^{-1})		Band Assignment
AAT-1	Lupeol acetate	
3070	-	$\nu_{=CH}$ of vinylidene group
2926, 2853	2920, 2855	ν_{C-H} of asym and sym CH_3 and CH_2 group
1732	1732	Carbonyl group of esters
1645	1645	$\nu_{C=C}$ of olefinic group
1454	1452	δ_{C-H} of CH_3 and CH_2 group
1365	1368	CH_3 deformation of isopropyl group
1243,1024	1244,1026	ν_{C-O} of ester group
827	827	Olefinic C-H out of plane bending

*Muktar, *et al*.,2018

According to proton NMR spectral data (Figure 4 and Table 3), There were 52 protons. The olefinic protons appeared as a doublet at 4.68 and 4.57 in the stretching ^{1}H NMR spectrum of compound AAT-1, and showed eight methyl group signals at δ_H 2.04, 1.68, 1.01, 0.88, 0.85, 0.83, 0.79, and 0.84. The signal of 1.68 ppm was due to the methyl group on the olefinic carbon of lupeol acetate. The methyl group of acetate was observed at δ_H 2.04. The remaining 24 protons indicated signals ranging from δ_H 0.8 to 2.5.

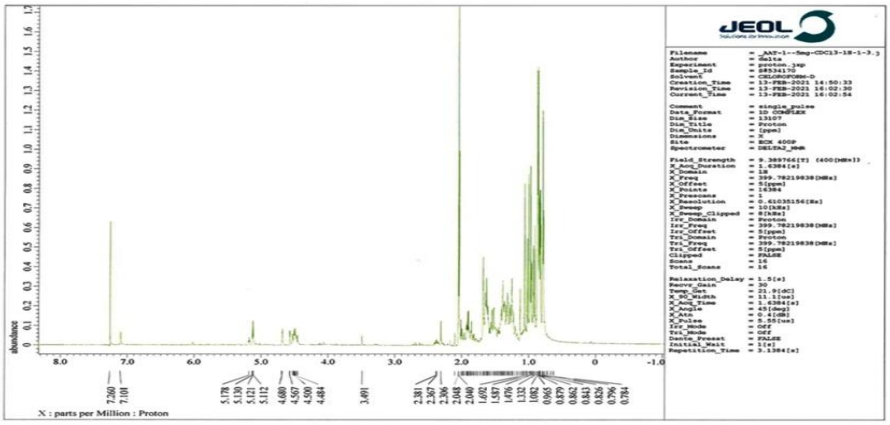

Figure 4 ^{1}H NMR Spectrum of isolated compound AAT-1 ($CDCl_3$, 400 MHz)

In ^{13}C NMR ($CDCl_3$, 100 MHz) spectra, it could be clearly seen that there were 32 carbons, including eight methyl carbons, eleven methylene carbons, six methine carbons, six quaternary carbons, two exo-methylene carbons, and one carbonyl carbon. The peaks were at δ 171.02 for the carbonyl group for C-1' , the terminal double bond carbon at δ 150.9 and 109.34 for C-20 and C-29 and oxygenated carbon at δ81.13 for C-3. (Figure 5 and Table 4).The above spectral features were in closed agreement with those observed for lupeol acetate.

Table 3 ^{1}H NMR Spectral Data of Isolated Compound AAT-1 Compared with Reported Data of Lupeol acetate

H-postion	Types of protons	AAT-1	*Lupeol acetate	H-position	Types of protons	AAT-1	*Lupeol acetate
		δ_H (CDCl3, 400 MHz)				δ_H (CDCl3, 400 MHz)	
1	CH_2	1.6 (m)	1.6 (m)	17	C	-	-
		1.72 (m)	1.72 (m)				
2	CH_2	1.6 (m)	1.6 (m)	18	CH_2	1.6 (m)	1.56 (m)
		1.70 (m)	1.71 (m)			2.37 (m)	2.37 (m)
3	OCH	4.48 (H,m)	4.48 (H,m)	19	CH	2.37 (H,m)	2.37 (H,m)
4	C	-	-	20	C	-	-
5	CH	0.83 (m)	0.83 (m)	21	CH	0.86 (m)	0.86 (m)
						1.08 (m)	1.08 (m)
6	CH_2	1.5 (m)	1.44 (m)	22	CH_2	1.33 (m)	1.33 (m)
		1.6 (m)	1.59 (m)			1.50 (m)	1.45 (m)
7	CH_2	1.27 (m)	1.26 (m)	23	CH_3	0.83 (3H,s)	0.83(3H,s)
		1.5 (m)	1.49 (m)				
8	C	-	-	24	CH_3	0.85 (3H,s)	0.85(3H,s)
9	CH	1.3(m)	1.5(m)	25	CH_3	0.88 (3H,s)	0.88 3H,s)
10	C	-	-	26	CH_3	0.83 (3H,m)	-
11	CH_2	1.27 (m)	1.25 (m)	27	CH_3	0.83 (3H,m)	1.27 (m)
		1.5 (m)	1.47 (m)				1.5 (m)
12	CH_2	1.58 (m)	1.51 (m)	28	CH_3	1.58 (m)	1.58 (m)
		1.6 (m)	1.6 (m)			1.6 (m)	1.6 (m)
13	CH	1.6 (m)	1.6 (m)	29	$=CH_2$	4.68 (H,s)	4.68 (H,s)
						4.57 (H,s)	4.57 (H,s)
14	C	-	-	30	CH_3	1.68 (3H, s)	1.68 (3H, s)
15	CH_2	1.06 (m)	1.06 (m)	1'	(C=O) O	-	-
		1.33 (m)	1.33 (m)				
16	CH_2	1.5 (m)	1.5 (m)	2'	CH_3	2.05 (3H,s)	2.05 (3H,s)
		1,6 (m)	1,6 (m)				

***Nasution *et al.*,2020, Myint Myint Khine,2006**

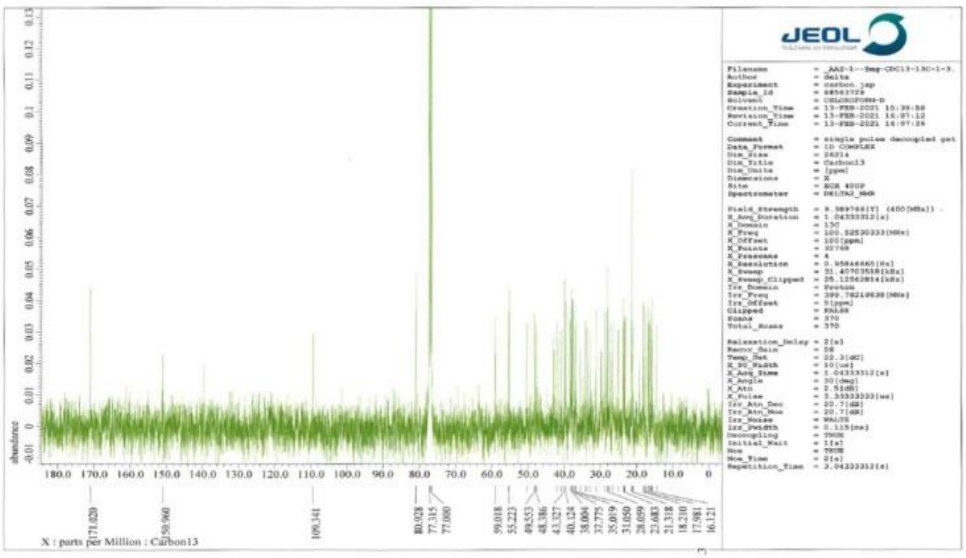

Figure 5 ^{13}C NMR Spectrum of isolated compound AAT-1 ($CDCl_3$, 100 MHz)

Table 4 ^{13}C NMR Spectral Data Assignment of Isolated Compound AAT- 1 ($CDCl_3$,100 Hz)

H-postion	Types of carbon	AAT-1	*Lupeol acetate	H-position	Types of carbon	AAT-1	*Lupeol acetate
		δc (CDCl3, 100 MHz)				δc (CDCl3, 100 MHz)	
1	CH_2	38.0	38.6	17	C	43.3	43.2
2	CH_2	21.3	21.7	18	CH_2	48.3	48.5
3	OCH	80.9	81.2	19	CH	48.2	48.2
4	C	38.0	38.0	20	C	150.9	151.2
5	CH	55.2	55.6	21	CH	31.0	30.0
6	CH_2	18.2	18.4	22	CH_2	39.9	40.2
7	CH_2	35.01	34.4	23	CH_3	28.0	27.6
8	C	39.9	41.0	24	CH_3	17.9	16.7
9	CH	48.3	50.5	25	CH_3	16.3	16.4
10	C	37.1	37.3	26	CH_3	16.3	16.2
11	CH_2	21.3	21.1	27	CH_3	16.3	15.7
12	CH_2	23.7	24.0	28	CH_3	18.2	18.2
13	CH	37.0	36.2	29	$=CH_2$	109.3	109.6
14	C	43.3	43.0	30	CH_3	21.3	19.5
15	CH_2	23.7	25.3	1'	C=O	171.0	171.3
16	CH_2	37.0	35.8	2'	CH_3	28.0	28.2

***Kipkemei, 2017, Myint Myint Khine,2006**

Cytotoxicity Effect of Crude Extracts of the Root of *S. tomentosum*

Most toxicity studies that use the Brine Shrimp Assay to determine the toxicity count the number of survived nauplii after 24 hours of test sample exposure. The cytotoxic activity of ethanol and watery extracts of the root of *S. tomentosum* was evaluated by a brine shrimp lethality bioassay. The cytotoxic effect was expressed as LC_{50} values (50 % Lethality Concentration). Standard Potassium dichromate ($K_2Cr_2O_7$) and Caffeine were chosen because $K_2Cr_2O_7$ is a well-known toxic agent in this assay and caffeine is a natural product. Table 3 and Figure 5 show the results of ethanol and watery extracts of the root of *S. tomentosum*. The LC_{50} values showed 400 µg/mL in ethanol, 200 µg/mL in water, and 293.88 µg/mL in isolated lupeol acetate respectively. For standard, $K_2Cr_2O_7$ was 56.44 µg/mL and caffeine was > 1000 µg/mL. The resulting LC_{50} values were compared with Clarkson's criteria, the ethanol and watery extracts and isolated lupeol acetate of the root of *S. tomentosum* showed significant toxicity due to LC_{50} values below 500 µg/mL.

Table 5 Cytotoxicity of Ethanol and Watery Crude Extracts from the Root of *S.tomentosum*

Test Sample	% Mortality of Brine Shrimp Concentration ± SD in Different Concentration of Sample (µg/mL)					IC 50 (µg/mL)
	50	100	200	400	800	
S. tomentosum (water extract)	33.34 ± 0.58	43.34 ± 0.55	50.00 ± 0.00	60.00 ± 0.00	66.75 ± 0.00	200
S. tomentosum (ethanol extract)	26.67 ± 1.53	36.67 ± 0.58	43.34 ± 0.58	50.00 ± 0.00	66.7 ± 1.15	400
AAT-1 (Lupeol acetate)	20.00 ± 0.53	30.00 ± 3.35	46.67 ± 1.02	63.37 ± 1.82	80.00 ± 3.24	239.88
* patassium dichromate	36.67 ± 0.53	53.34 ± 0.58	70.00 ± 1.00	86.67 ± 1.15	100.00 ± 0.00	56..44
** Caffeine	0.00 ± 0.00	0.00 ± 0.00	6.67 ± 1.53	16.67 ± 0.58	23.34 ± 1.53	>1000

***= Negative control **= Positive control**

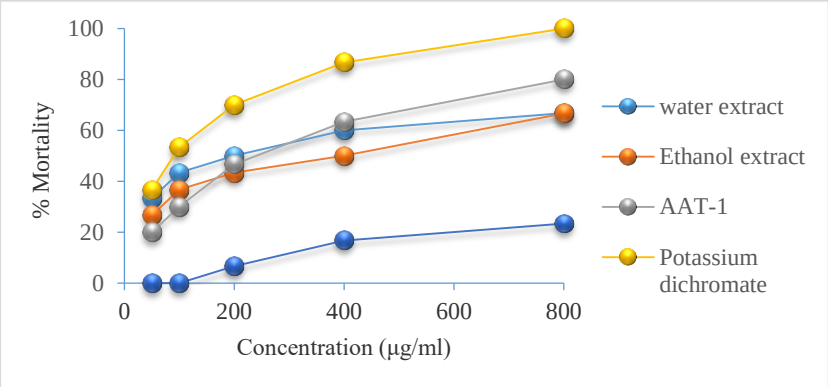

Figure 6 Brine shrimp lethality of crude extract and isolated compound AAT-1 of root of *S. tomentosum*

Anti-inflammatory and cell viability activity of crude extracts

The results of anti-inflammatory and cell viability activity are shown in Table 6. The anti-inflammatory and cell viability effects were expressed as IC_{50} values (50 % inhibitory concentration). If the percentage of cell viability is greater than that of NO inhibition, these extracts will only have any anti-inflammatory effect; the ethanol and watery extracts of *S. tomentosum* roots show no significant inhibition of cellular NO production because they have a moderate cytotoxicity effect.

Table 6 Anti-inflammatory and Cell Viability Activity of Crude Extracts of Root of *S. tomentosum* by Inhibition of NO Production against LPS Induced RAW 264.7 cell

Sample	Anti-inflammatory Activity					
	% NO inhibition			% Cell Viability		
	10 µg/mL	100 µg/mL	IC_{50} µg/mL	10 µg/mL	100 µg/mL	IC_{50} µg/mL
Ethanol-extract	16.07 ± 0.603	32.49 ± 0.656	> 100	95.74 ± 0.848	66.81 ± 0,848	> 100
Watery-extract	4.4 ± 0.173	40.3 ± 0.346	> 100	97.6 ± 5.927	100.3 ± 2.757	> 100
LNMMA	18.49 ± 0.1	50.35 ± 0.1	98.25	100,32 ± 12.41	92.01 ± 1.02	> 100

L-NMMA = Positive Control

The greater percentage of IC_{50} of NO inhibition, the lesser that of Cell Viability (Because of cytotoxicity)

Conclusion

In this research work, the following screenings could be deduced: The preliminary phytochemical analysis revealed the presence of alkaloids, carbohydrates, flavonoids, glycosides, phenolic compounds, organic acids, reducing sugars, saponins, steroids, tannins, and terpenoids in the sample. Moreover, cyanogenic glycosides were absent in this plant sample. In this study, the first isolation of lupeol acetate ($C_{32}H_{52}O_2$) from the root of a pet-ether extract of *S. tomentosum* was accomplished by modern spectroscopic techniques: FT IR, ^{1}H NMR (400 MHz), and ^{13}C NMR (100 MHz). According to the reference data, the compound was recognized as a known compound. As a result, the cytotoxic and anti-inflammatory potentials of these substances were investigated. Isolated compound, ethanol, and watery extracts of the plant's root all demonstrated a considerable cytotoxic impact (LC_{50} < 500 µg/mL), indicating a moderate toxic effect, in a brine shrimp lethality test. The ethanol and watery extracts of *S. tomentosum* root revealed no apparent reduction in cellular NO generation because the measured IC_{50} of NO inhibition and cell viability were both greater than 100, which might be employed in traditional medicine formulations to treat a number of ailments.

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