SCREENING ON CYTOTOXICITY, ANTI-INFLAMMATORY, ANTIPROLIFERATIVE, AND ANTIARTHRITIC ACTIVITIES OF THE *STEPHANIA VENOSA* (BLUME) SPRENG. (TAUNG-KYA) TUBER

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Abstract

Stephania venosa (Blume) Spreng. (Taung-kya) belongs to the family Menispermaceae and is a rich source of alkaloids. The research focused on the screening of the cytotoxicity, anti-inflammatory, antiproliferative, and antiarthritic activities of the tuber of *S. venosa*. The cytotoxicity activity of the watery and ethanol extracts determined by a brine shrimp lethality bioassay showed LD_{50} values > 1000 µg/mL, a non-toxic effect. The anti-inflammatory activity of the methanol and watery extracts of *S. venosa* was determined by inhibition of nitric oxide (NO) production against LPS-induced RAW 264.7 cells. The IC_{50} value of nitric oxide inhibition was found to be less than the IC_{50} value of cell viability. The watery extract showed mild antiproliferative activity against the A549 cell line with an IC_{50} value of 144.86 µg/mL. The watery extract was found to possess significant antiproliferative activity against the HeLa (Cervix cancer) cell line, with the IC_{50} value less than 20 µg/mL. Additionally, the methanol extract had significant antiproliferative activity against the A549 (Lung cancer) cell line, with an IC_{50} value of less than 20 µg/mL. But the methanol extract possessed weaker antiproliferative activity for HeLa (Cervix cancer) cell line, with IC_{50} values >200 µg/mL than the standard 5 FU (IC_{50} =19.06 µg/mL against Lung cancer cell line and IC_{50} =15.84 µg/mL Cervix cancer cell line). The antiarthritic activity of ethanol and watery extracts was determined by using fresh hen's egg albumin denaturation method in different concentrations ranging from 50 to 1600 µg/mL. According to the results, the IC_{50} values of ethanol and watery extracts were found to be 743 µg/mL and > 1600 µg/mL, respectively. Therefore, the two extracts showed lower antiarthritic activity than the standard drug diclofenac sodium (IC_{50} = 266 µg/mL).

Keywords: *Stephania venosa* (Blume) Spreng., cytotoxicity, anti-inflammatory, antiproliferative and antiarthritic activities

Introduction

Stephania venosa (Blume) Spreng. is an herbaceous perennial vine growing to around four metres tall with a large tuber on the ground. It belongs to the Menispermaceae family and is known as Taung-kya in Myanmar (Figure 1). It is widely distributed in East and South Asia and Australia. Its leaves are spirally arranged on the stem, with the leaf petiole attached near the centre of the leaf. It is a plant rich in alkaloids. Its tubers have been used in traditional medicine as nerve tonics, aphrodisiacs, and appetizers. Moreover, it is also used for the treatment of asthma, hyperglycemia, antimalarial activities, microbial infections, and cancer. *S. venosa* leaves have been used to treat ringworm, tinea versicolor, chronic cancer, and acne. It has been reported to have a range of biological effects (Moongkarndi *et al*., 2004). The phytochemical screening from the tuber of the plant showed the presence of a wide variety of isoquinoline and aporphine alkaloids with different structural types including tetrahydropalmatine, crebanine, *O*-methylbulbocapnine, and *N*-methyltetrahydropalmatine. *S. venosa* possesses various pharmacological activities reported by Ingkaninan *et al*. (2006). The aim of the present work is to

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screen the cytotoxicity, anti-inflammatory, antiproliferative, and antiarthritic activities of the tuber of *S. venosa.*

Figure 1. The photograph of tuber of *Stephania venosa* (Blume) Spreng.

Materials and Methods

Collection and Identification of Plant Samples

The tuber of *S. venosa* (Blume) Spreng. was collected from Thanlyin Township, Yangon Region, Myanmar, in June, 2019. The plant was identified at the Department of Botany, Hpa-an University. The sample was washed, dried in the shade for a week, cut into very small pieces, and then ground into a fine powder using an electric grinder. The powdered sample was stored in an air-tight container.

Chemicals

Phosphate buffer saline (PBS) powder, fetal bovine serum (FBS, Sigma 172012), trypsin, alcohol (MeOH and EtOH), Minimum Essential Medium α (MEM, Wako 135-15735), 0.1 mM Non-Essential Amino Acid (NEAA, Gibco 11140-050), Lipopoly saccharide (LPS), (naphthyl)ethylenediamine dihydrochloride, sulphanilamide, 1mM sodium pyruvate (SM, Gibco-11360-170) and MTT reagent, dimethyl sulphoxide (DMSO), distilled water, bovine serum albumin (BSA), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), monosodium dihydrogen phosphate (NaH_2PO_4), diclofenac sodium, potassium dichromate, caffeine, artificial seawater (37 %)

Instruments

Quartz cuvette (4 mL), UV-visible spectrophotometer (UV-7504), a stirrer, an autoclave (Tomy Seiko Co., Ltd, Tokyo, Japan), a constant temperature bath (Yamato Scientific Co., Ltd, Japan), sterile Petri-dish, spirit burner, polyethylene plastic bag, a refrigerator and an incubator, multipipette, 96 well plate, aluminum foil, centrifuge tube, Haemacytometer, microscope and vibrator, syringe (3 mL, 5 mL), beakers, chambers, Pasteur pipette, lamp and water bottles (1.5 mL)

Preparation of Extracts from the *S. venosa* Tuber

Each 50 g of air-dried powder sample was percolated with ethanol (100 mL), methanol (100 mL), and water (100 mL) for one week at room temperature, and then the filtrate was individually evaporated under reduced pressure by a rotatory evaporator to yield different solvent extracts.

Determination of Cytotoxicity of *S. venosa* by Brine Shrimp Lethality Bioassay

The cytotoxicity of ethanol and watery extracts of *S. venosa* was investigated by a brine shrimp lethality bioassay, according to the procedure described by Olowa *et al*. (2013). The brine shrimp (*Artemia salina*) was used in this bioassay (Ali *et al.,* 2013). The sample solution was prepared by dissolving 5 mg of the respective crude extract in 5 mL of distilled water. The stock solution was tenfold diluted serially with distilled water to get the sample solutions with concentrations of 1000, 100, 10, and 1 µg/mL. Artificial seawater (9 mL) was mixed with the sample solution (1 mL) and placed in the chamber of the ice cup. Alive brine shrimp (10 nauplii) were taken with a Pasteur pipette and then placed into each chamber, which was kept at room temperature for about 24 h. After 24 h incubation, the number of surviving brine shrimp was counted, and a 50 % lethal dose (LD_{50}) was calculated. Cytotoxic effect of control solutions ($K_2Cr_2O_7$ and caffeine) was also determined according to the above procedure.

Determination of *in vitro* Anti-inflammatory Activity of Crude Extracts from Tuber of *S. venosa*

In vitro anti-inflammatory activity of methanol and watery extracts of the tuber of *S. venosa* was evaluated by a nitric oxide (NO) inhibition assay against LPS-induced RAW cells according to the method (Jin *et al.,*2012) modified by Win *et al*. (2015). These tests were done at the Department of Natural Products Chemistry, the Institute of Natural Medicine, and the University of Toyama, Japan. When the cell proliferation reached about 70 % confluence, the cells were harvested using a cell scraper and diluted to a suspension in fresh medium. The 100 µL of cells (1×10^4/well) were seeded in the 96 well plates, and then, they were incubated for 24 h at 37 °C. The cells were treated with 50 µL each of LPS (100 ng/mL) and 100 µg/mL and 10 µg/mL of different doses of samples for 24 h. After incubation at 37 °C for 24 h, 100 µL each of the supernatants from 96 well was mixed with an equal volume of Griess reagent (0.5 % sulphanilamide and 0.05 % naphthylenediamide dihydrochloride in 2.5 % H_3PO_4) in the new 96 well plates and allowed to stand for 5 min at room temperature. The resulting colour was assayed for absorbance at 540 nm using a microplate reader. L-NMMA monoacetate was used as a positive control. On the other hand, the effect of the samples on cell proliferation was evaluated by the MTT reagent. The 100 µL of MTT (5 mg/mL) in the medium was added to the remaining 96 wells. After 2 h incubation, the medium was discarded and 100 µL each of DMSO was added to dissolve the formazan crystals, and the absorbance at 570 nm was recorded by a microplate spectrophotometer. The percentage of NO inhibition and that of cell viability were calculated as follows:

$$\text{NO inhibition (\%)} = [(Abs_{(control)} - Abs_{(sample)})/Abs_{(control)}] \times 100$$

where, $Abs_{(control)}$ = the absorbance of LPS treated control group,

$Abs_{(sample)}$ = the absorbance of the sample

$$\text{Cell viability (\%)} = [(Abs_{(test\ sample)} - Abs_{(blank)})/(Abs_{(control)} - Abs_{(blank)})] \times 100$$

where, $Abs_{(test\ sample)}$ = absorbance of test sample solution,

$Abs_{(control)}$ = absorbance of DMSO solution,

$Abs_{(blank)}$ = absorbance of MTT reagent.

IC_{50} (50 % inhibitory concentration) in % NO inhibition should be less than IC_{50} in % cell viability if the extract exhibits only an anti-inflammatory effect.

Determination of *in vitro* Antiproliferative Activity of Watery and Methanol Extracts Against Human Cancer Cell Lines

The antiproliferative activity of watery and methanol extracts from the tuber of *S. venosa* was tested against two human cancer cell lines, A549 (lung cancer) and HeLa (cervical cancer). The antiproliferative activity was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Win *et al*., 2015). These tests were done at the Department of Natural Products Chemistry, the Institute of Natural Medicine, and the University of Toyama, Japan. This assay detects the reduction of MTT by mitochondrial dehydrogenase to a blue formazan product, indicating the normal function of mitochondria and cell viability. A minimum essential medium with L-glutamine and phenol red (α-MEM, Wako) was used for cell cultures. All media were supplemented with 10 % fetal bovine serum (FBS, Sigma) and 1 % antibiotic antimycotic solution (Sigma). From the above medium solution, 100 mL of this supplemented medium was mixed with 1 mL of non-essential amino acid (NAA) for A549. The antiproliferative activity of the crude extracts was determined by the procedure described by Win *et al.* (2015). The assay was carried out using MTT dye and measuring the absorbance at 570 nm with a SH-1200 microplate reader (Corona, Hitachinaka, Japan). Briefly, each cell line was seeded in 96-well plates (2×10^3 per well) and incubated in the respective medium at 37 °C under 5 % CO_2 and 95 % air for 24 h. After that, the cells were treated with serial dilutions of the tested samples. After 72 h of incubation, the cells were washed twice with PBS, and 100 µL of MTT reagent solution was added to the wells. After 3 h incubation, the medium will be aspirated, followed by the addition of 100 µL of DMSO to the 96 well plates. The concentrations of the crude extracts were 200, 20 µg/mL, and 20, 10, and 2 mm for the positive control prepared by serial dilution. Cell viability was calculated from the mean values of the data from three wells using the equation below. The antiproliferative activity was expressed as the IC_{50} (50 % inhibitory concentration) value compared to the 5-fluorouracil (5 FU) as a positive control.

$$\text{Cell viability (\%)} = [(\text{Abs}_{(\text{test sample})} - \text{Abs}_{(\text{blank})}) / (\text{Abs}_{(\text{control})} - \text{Abs}_{(\text{blank})})] \times 100$$

where, $\text{Abs}_{(\text{test sample})}$ = absorbance of test sample solution,

$\text{Abs}_{(\text{control})}$ = absorbance of DMSO solution,

$\text{Abs}_{(\text{blank})}$ = absorbance of MTT reagent.

Determination of *in vitro* Antiarthritic Activity of Watery and Ethanol Extracts by Protein Denaturation Method

The *in vitro* antiarthritic activity was studied by the protein denaturation method using fresh hen's egg albumin and bovine serum albumin (Rahman *et al*., 2012). The reaction mixture consisted of test solutions with different concentrations (1600, 800, 400, 200, 100, and 50 µg/mL), 0.2 mL of fresh hen's egg albumin, and 2.8 mL of phosphate buffered saline (pH 6.3) were mixed to form a total volume of 5 mL. An equal volume of distilled water served as the control solution. The test samples and standard were incubated at 37 °C in an incubator for 15 min, and then, followed by heating at 70 °C for 5 min. The absorbance of these solutions was

measured at 660 nm using a UV-visible spectrophotometer. The percentage inhibition of protein denaturation was calculated by the following formula:

$$\% \text{ Inhibition} = \frac{A_{control} - (A_{sample} - A_{blank})}{A_{control}} \times 100$$

where, A_{sample} = absorbance of the sample (with egg albumin),

A_{blank} = absorbance of blank (only sample? without egg albumin),

$A_{control}$ = absorbance of control (only egg albumin).

Standard deviation (SD) and 50 % inhibition concentration (IC_{50}) value were calculated by linear regressive excel program.

Results and Discussion

Cytotoxicity of Ethanol and Watery Extracts from the Tuber of *Stephania venosa*

The cytotoxic activity of ethanol and watery extracts of tuber of *Stephania venosa* (Blume) Spreng. was evaluated by a brine shrimp lethality bioassay. The nauplii were exposed to different concentrations of plant extracts for 24 h. The number of motile nauplii was calculated to determine the effectiveness of the extract. The cytotoxic effect was expressed as LD_{50} values (50 % lethal dose). The potassium dichromate ($K_2Cr_2O_7$) and caffeine were chosen as a positive control and a negative control, respectively, because $K_2Cr_2O_7$ is a toxic agent in this assay and caffeine is a natural product. From the results, the LD_{50} values of ethanol and watery extracts were found to be > 1000 µg/mL, and the standard potassium dichromate and caffeine were 509 µg/mL and > 1000 µg/mL, respectively. In the presence study, the two extracts did not exhibit cytotoxic effects comparable to those of the standard potassium dichromate. Therefore, ethanol and watery extracts of *S. venosa*, were relatively safe to be consumed as antioxidants for cancer chemoprevention. After 24 h, it could be deduced that the two extracts were not cytotoxic to the brine shrimp up to the maximum dose of 1000 µg/mL compared with the Deciga-Campos criteria. For the standard potassium dichromate, the LD_{50} was 509 µg/mL, and for caffeine, it was > 1000 µg/mL. The experimental data are shown in Table 1 and Figure 2.

Table 1. Cytotoxicity of Ethanol and Watery Crude Extracts from the Tuber of *S. venosa*

Sample	No. of dead of brine shrimp (Mean ± SD) at various concentrations (µg/mL)				
	1	**10**	**100**	**1000**	**LD_{50}**
EtOH extract	6 ± 0.1	6 ± 0.1	6 ± 0.1	18 ± 0.1	>1000
watery extract	0± 0	0± 0	18 ± 0.1	34± 0.1	>1000
*$K_2Cr_2O_7$	0±0	0±0	17±0.5	90±0.3	509
**caffeine	3 ±0.5	10±0.9	20±0.3	27±0.9	>1000

* positive control, ** negative control

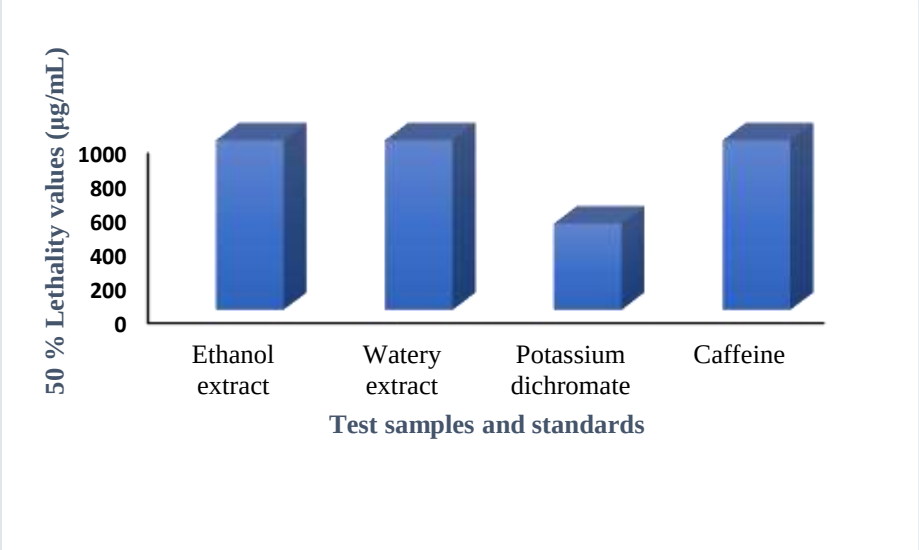

Figure 2. Brine shrimp lethality of crude extracts from the tuber of *S. venosa*

Anti-inflammatory Activity of Crude Extracts from the Tuber of *S. venosa*

In vitro anti-inflammatory activity of crude extracts (methanol, and watery extracts) of the tuber of *S. venosa* was determined by the inhibition of NO production against LPS-induced RAW 264.7 cells. NO production is a typical phenomenon that occurs in LPS-stimulated macrophages and is used as an indicator of a typical inflammatory response. The anti-inflammatory effect of tested extracts can be estimated by comparing the IC_{50} values of the % NO inhibition and cell viability. If the percentage of cell viability is greater than that of NO inhibition, both extracts will have an anti-inflammatory effect; the ethanol and watery extracts of *S. venosa* showed significant inhibition of cellular NO production at cytotoxic concentrations. According to the results, they exhibited anti-inflammatory activity because the IC_{50} value of % NO inhibition was less than the IC_{50} value of cell viability. The watery and methanol extracts showed lower IC_{50} values (79.82 and 76.86 µg/mL) than the standard (98.25 µg/mL) in % NO inhibition. So, *S. venosa* can potentially be used as a natural anti-inflammatory ingredient for the treatment of arthritis. The experimental data are shown in Table 2 and Figure 3.

Table 2. Anti-inflammatory Activity of Various Crude Extracts of Tuber of *S. venosa*

Sample	% NO inhibition (µg/mL)		IC_{50} (µg/mL)	% NO inhibition (µg/mL)		IC_{50} (µg/mL)
	10	100		10	100	
methanol extract	20.99	60.03	76.86	106.51	68.70	>100.00
watery extract	20.99	58.37	79.82	106.10	93.16	>100.00
[a]L-NMMA	18.49	50.35	98.25	100.32	92.01	>100.00

[a]L-NMMA positive control

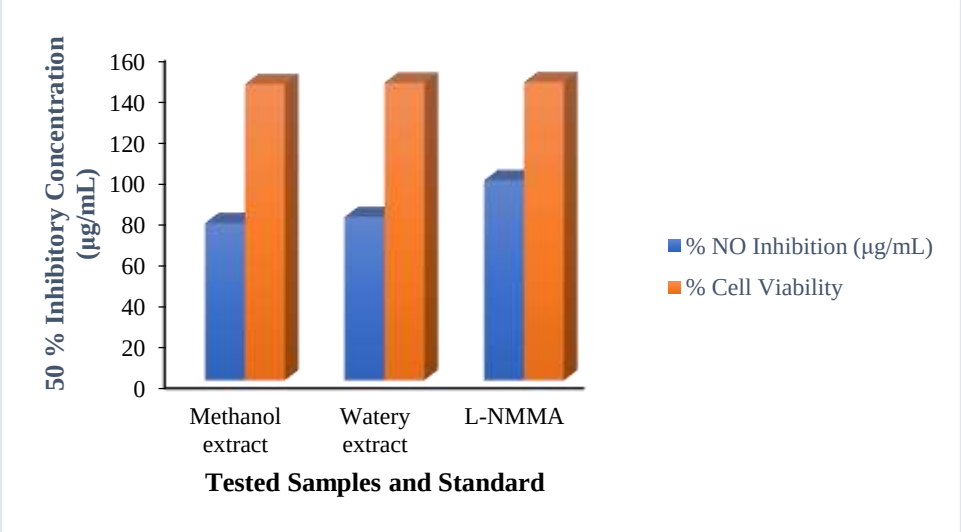

Figure 3. A bar graph diagram for anti-inflammatory activity of crude extracts of tuber of *S.venosa*

Antiproliferative Activity of Crude Extracts from the Tuber of *S. venosa*

In vitro antiproliferative activities of methanol, and watery extracts of the tuber of *S. venosa* were evaluated by MTT assay on two human cancer cell lines, such as A549 (lung cancer) and HeLa (Cervix cancer) cell lines. The antiproliferative effect was expressed as IC_{50} values (50 % inhibitory concentration). The lower the IC_{50} values, the higher the antiproliferative activity. According to these results, the H_2O extract showed mild antiproliferative activity against the A549 cell line with IC_{50} value of 144.86 µg/mL. The H_2O extract had significant antiproliferative activity against the HeLa (Cervix cancer) cell line, with an IC_{50} value of less than 20 µg/mL. But the methanol extract possessed weaker antiproliferative activity for HeLa (Cervix cancer), which was compared with watery extracts because of their IC_{50} values of > 200 µg/mL. However, the methanol extract had significant antiproliferative activity against the A549 (lung cancer) cell line, with an IC_{50} value of less than 20 µg/mL. The test samples had weaker antiproliferative activity compared with the standard 5 FU. The experimental data is shown in Table 3 and Figure 4.

Table 3. Antiproliferative Activity of Various Crude Extracts of tuber of *S. venosa* Against Two Human Cancer Cell Lines

Test Sample	Antiproliferative activity					
	Cervix (HeLa)			Lung (A549)		
	20 µg/mL	200 µg/mL	IC_{50} µg/mL	20 µg/mL	200 µg/mL	IC_{50} µg/mL
watery extract	47.66± 9.40	47.32± 0.07	<20	100.65± 6.65	27.63± 0.14	144.86
methanol extract	65.86± 1.56	53.36± 0.00	>200	78.87± 6.43	98.57± 4.88	<20

Positive control	2 µg/mL	10 µg/mL	20 µg/mL	IC_{50} µg/mL
5- Fluorouracil (lung cancer cell line)	136.24±12.94	70.45± 5.59	47.89± 8.21	19.06
5- Fluorouracil (cervix cancer cell line)	91.44±24.93	85.22± 4.95	24.93± 0.28	15.84

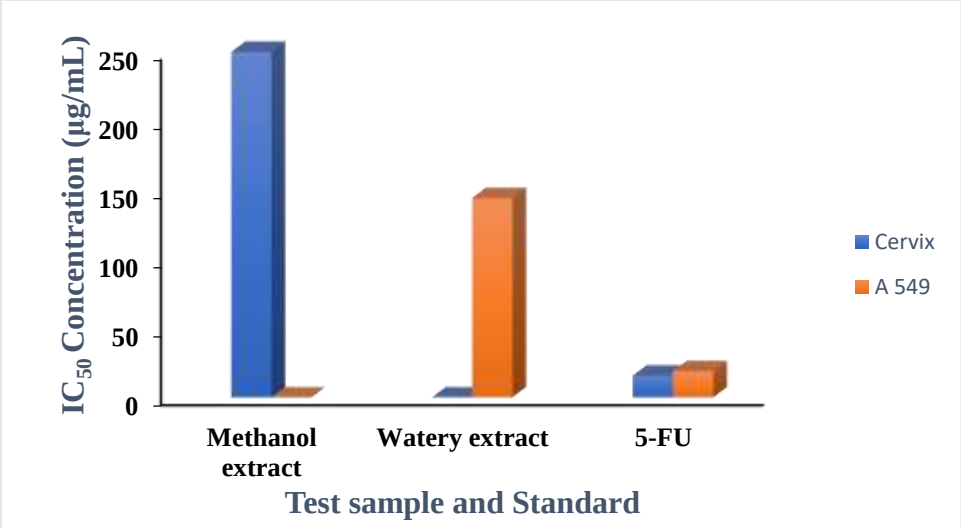

Figure 4. IC_{50} values of crude extracts of the tuber of *Stephania venosa* against two human cancer cell lines

In vitro* Antiarthritic Activity of Ethanol and Watery Extracts from the Tuber of *S. venosa

Protein denaturation is one of the primary causes of arthritis. *In vitro* antiarthritic activity of EtOH and watery extracts from the tuber of *Stephania venosa* was investigated by using the protein denaturation method (using egg albumin). According to the results of the egg's albumin denaturation, the IC_{50} values of ethanol and watery extracts were found to be 743 µg/mL and >1600 µg/mL. Therefore, the two extracts were found to be mild activity comparable with the

standard drug diclofenac sodium (IC_{50} value: 266µg/mL). The results of the antiarthritic activity of EtOH and watery extracts are shown in Table 4 and Figure 5.

Table 4. Antiarthritic Activity of EtOH and Watery Extracts from the Tuber of *S. venosa*

Sample	% Inhibition (mean± SD) at different concentrations (µg/mL)						IC_{50} (µg/mL)
	50	100	200	400	800	1600	
ethanol extract	33.42 ± 0.03	41.16 ± 0.03	41.55 ± 0.03	48.59 ± 0.05	53.14 ± 0.03	61.51 ± 0.07	743
watery extract	10.47 ± 0.05	12.20 ± 0.03	16.17 ± 0.03	21.55 ± 0.05	27.62± 0.03	29.86 ± 0.03	>1600
diclofenac sodium	42.73 ± 0.03	43.09 ± 0.03	48.61 ± 0.03	53.48 ± 0.03	61.79± 0.05	63.63 ± 0.08	266

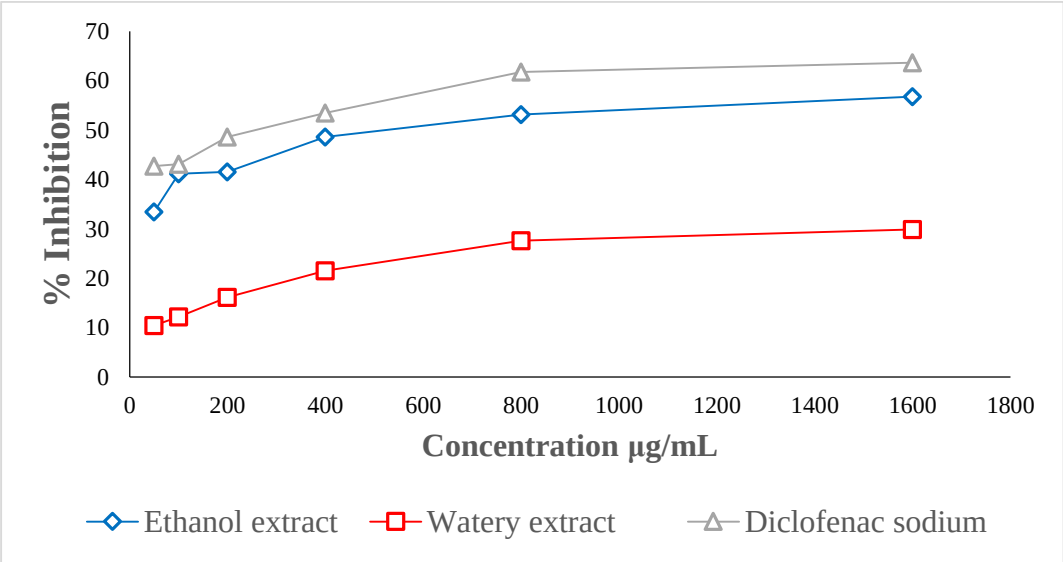

Figure 5. Antiarthritic activities of crude extracts from the tuber of *S. venosa*

Conclusion

In this research work, the screening of cytotoxicity, anti-inflammatory, antiproliferative, and antiarthritic activities based on ethanol, methanol, and watery extracts from the tuber of *S. venosa* is carried out. The cytotoxicity activity of the watery and ethanol extracts was (LD_{50} values >1000), indicating a non-toxic effect, by a brine shrimp lethality bioassay. Methanol and watery extracts exhibited anti-inflammatory activity because their IC_{50} values (data) of % NO inhibition against LPS-induced RAW 264.7 cells are less than the IC_{50} values (data) of cell viability. The watery extract showed mild antiproliferative activity against the A549 cell line with an IC_{50} value of 144.86 µg/mL, and significant antiproliferative activity against the HeLa (Cervix) cell line with an IC_{50} value less than 20 µg/mL. However, the methanol extract had significant antiproliferative activity against the A549 (lung cancer cell line), with an IC_{50} value of

less than 20 µg/mL, but weaker antiproliferative activity for the HeLa (Cervix) cell line, with an IC_{50} value greater than 200 µg/mL, comparable to the standard 5 FU (IC_{50} =19.06 µg/mL for lung cancer cell line and IC_{50} =15.84 µg/mL for cervix cancer cell line). According to the results of ethanol and watery extracts tested by the fresh egg's albumin protein denaturation, the IC_{50} values of ethanol and watery extracts were found to be 743 µg/mL and >1600 µg/mL, respectively. Therefore, the two extracts showed lower antiarthritic activity than the standard drug diclofenac sodium (IC_{50} value = 266 µg/mL). From the above results, it could be concluded that the tuber of *Stephania venosa* (Blume) Spreng. possessed potential pharmacological activities such as cytotoxicity, anti-inflammatory, antiproliferative, and antiarthritic activities. This finding provided potential for the tuber of *S. venosa* as a therapeutic drug for traditional medicinal areas.

Acknowledgements

The authors would like to thank the Department of Higher Education, Ministry of Education, Myanmar, for permission to do this research and the Myanmar Academy of Arts and Science for allowing to submit this paper.

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