ISOLATION AND CHARACTERIZATION OF A BIOACTIVE COMPOUND AND SCREENING OF ANTIMICROBIAL AND ANTIOXIDANT ACTIVITIES OF *STEPHANIA VENOSA* (BL.) SPRENG (TAUNG-KYA) TUBER

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Abstract

Stephania venosa (Bl.) Spreng (Taung-kya), belonging to the family Menispermaceae and is a rich source of alkaloids commonly found as herbaceous perennial vine with a large tuber. The aim of the research includes preliminary phytochemical investigations, isolation and identification of bioactive chemical constituent and *in vitro* antimicrobial and antioxidant activities of different crude extracts from the tuber of *S. venosa*. The preliminary phytochemical screening revealed the presence of alkaloids, α-amino acid, carbohydrates, glycosides, flavonoids, organic acids, phenolic compounds, reducing sugars, steroids, saponins and tannins in the samples by using the standard methods. The air-dried powder plant sample was subjected to cold percolation with 80 % methanol and fractionation with petroleum ether only, petroleum ether and ethyl acetate, ethyl acetate only, and ethyl acetate and methanol. The isolated compound (0.03 g, 0.06%) was separated from ethyl acetate fraction by using silica gel column chromatographic separation method. It was identified by using modern spectroscopic methods such as UV-visible, FT IR, ^{1}H NMR, ^{13}C NMR and mass spectrometry and also by comparing with the reported data. The antimicrobial activity of different crude extracts (PE, EtOAc, EtOH, MeOH and Watery) from tuber of *S. venosa* was tested by the agar well diffusion method. Ethyl acetate extract has the most potent antimicrobial activity (the inhibition zone diameter range between 17 to 22 mm) against tested microorganisms except as *Bacillus pumilus.* The antioxidant activity was investigated by using DPPH free radical scavenging assay method. The IC_{50} values of watery and ethanol extracts were found to be 173.61 and 134.93 μg/mL, respectively. The IC_{50} value of isolated compound was also observed as 24.43 μg/mL. Therefore, the isolated compound possesses higher potent antioxidant activity than the extracts.

Keywords: *Stephania venosa* (Bl.) Spreng, bioactive compound, antimicrobial activity, antioxidant activity

Introduction

Nowadays, the use of traditional medicine has greatly increased in Myanmar and all over the world. Depression and anxiety disorders are the most common mental illnesses in humans (Wong and Licinio, 2002). According to the World Health Organization (WHO), the majority of medicinal plants in disease management have established their use in the primary health care delivery system. *Stephania venosa* (Bl.) Spreng is a herbaceous perennial vine growing to around four metres tall with a large tuber on the ground. It is widely distributed in eastern, southern 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 such as nerve tonics, aphrodisiacs and appetizers. Moreover, it is also used for the treatment of asthma, hyperglycemia, 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 biological compounds that belong to secondary metabolites include phenolic compounds, steroids, flavonoids, and alkaloids.

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

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tetrahydropalmatine, crebanine, *O*-methylbulbocapnine and *N*-methyltetrahydropalmatine. *S. venosa* contains pharmacological activities such as cytoxic, antimicrobial, anti-inflammatory, antihyperglycemic, anti-acetylcholinesterase, antitubercular, antioxidant and antiprotozoal activities (Ingkaninan *et al*., 2006; soares *et al*., 1997).

The aim of the present work is to an isolate alkaloid and to screen the antimicrobial and antioxidant activities of the tuber of *S. venosa.*

Scientific classification of *Stephania venosa* (Bl.) Spreng

Family : Menispermaceae

Botanical name : *Stephania venosa* (Bl.) Spreng

Myanmar name : Taung-kya

Part used : Tuber

Tuber

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

Materials and Methods

Collection and Identification of Plant Samples

The tuber of *S. venosa* was collected from Than-Hlwin Township, Yangon Region, Myanmar, in June, 2019. The plant was identified at the Department of Botany, Hpa-an University.

Preliminary Phytochemical Tests on the Tuber of *S. venosa*

The tuber of *S. venosa* (Taung-kya) was screened for the presence or absence of bioactive constituents such as alkaloids, α-amino acid, carbohydrates, glycosides, flavonoids, organic acids, phenolic compounds, reducing sugars, steroids, saponins and tannins (Harborne, 1984).

Extraction and Isolation from the Tuber of *S. venosa*

The air-dried powder of *S. venosa* tuber (1000 g) was macerated with 80 % methanol (5L) for one week at room temperature. Then, the filtrate was filtered through Whatman no.1 filter paper. The extraction was performed three times as the same procedure mentioned above. Total filtrate was concentrated under reduced pressure by using rotary evaporator to provide the methanol crude extract (50 g). The 50 g of methanol extract was subjected to column chromatographic separation over silica gel (200 g, 70-230 mesh) using petroleum ether: ethyl acetate solvent system with increasing polarity to give four fractions. The ethyl acetate fraction (Fraction-III) was rechromatographed over silica gel with solvents increasing order of polarity using pure petroleum ether, petroleum ether: ethyl acetate, pure ethyl acetate and ethyl acetate: methanol. A white crystalline compound was obtained from (PE: EtOAc, 5:1) fraction and the yield percent is 30 mg (0.06 %) based on the methanol extract (50 g).

Identification of The Isolated Compound

The isolated compound from *S. venosa* was identified by TLC, UV-visible, FT IR, ^{1}H NMR, ^{13}C NMR and MS from the Institute of Chemistry, Vietnam Academy of Science and Technology, Hanoi.

The ^{1}H and ^{13}C NMR of compound was recorded on an Advance NEO NMR spectrometer, and the chemical shifts were expressed in the δ (ppm) scale with TMS as an internal standard. The mass spectrum was recorded on an LC-MSD-Trap-SL Print mass spectrometer.

Screening of Antimicrobial Activity of *S. venosa*

In vitro, antimicrobial assay of the petroleum ether, ethyl acetate, ethanol, methanol and watery extracts from tuber of *S. venosa* was performed at Department of Chemistry, Pa-thein University. The tested eight microorganisms such as *Escheerichia coli, Candida albicans, Bacillus subtilis, Staphylococcus aureus, Pseudomonas fluorescens, Agrobacteriumb tumefaciens, Bacillus pumilus and Micrococcus luteus.* The antimicrobial activity was determined by the agar well diffusion method (Selvamohan *et al*., 2012).

In vtro Antioxidant Activity by DPPH Free Radical Scavenging Assay

The effect on DPPH free radical scavenging activity of Taung-kya was determined by a UV-visible spectrophotometric method according to the procedure described by (Marinova and Batchvarov, 2011). The control solution was prepared by mixing 1.5 mL of 180 µM DPPH solution with 1.5 mL of ethanol using a shaker. The sample solution was also prepared by mixing thoroughly 1.5 mL of 180 µM DPPH solution and 1.5 mL of test sample solution with concentration (1000 - 62.5 µg/mL). The mixed solutions were allowed to stand at room temperature for 30 min. After 30 min, the absorbance of each sample solution was measured at 517 nm by using UV-visible spectrophotometer. The percent radical scavenging activity was calculated by the following equation.

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

Where % RSA = % radical scavenging activity

$A_{control}$ = absorbance of DPPH in EtOH solution

A_{Sample} = absorbance of sample and DPPH solution

A_{blank} = absorbance of sample and EtOH solution

The experimental results were performed in triplicate. The data were recorded as mean ± standard deviation.

Results and Discussion

Preliminary Phytochemical Investigation for *S. venosa*

The phytochemical tests revealed the presence of secondary metabolites including alkaloids, α-amino acids, carbohydrates, glycosides, flavonoids, phenolic compounds, reducing sugars, steroids, saponins, and tannins in the tuber of *S. venosa*. The preliminary phytochemical results are shown in Table 1.

Table 1. Results of Preliminary Phytochemical Tests on the Tuber of *S. venosa*

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

No.	Test	Extracts	Test Reagent	Observation	Remark
1	Alkaloids	1% HCl	(i)Wagner's reagent	bown ppt	+
			(ii)Dragendorff's reagent	orange ppt	+
			(iii) Mayer's reagent	white ppt	+
			(iv) sodium picrate	yellow ppt	+
2	α-Amino acids	H_2O	ninhydrin	purple colour	+
3	Carbohydrates	H_2O	10 % α-naphthol and conc:H_2SO_4	red ring	+
4	Cyanogenic glycosides	H_2O	sodium picrate solution	no colour	-
5	Flavonoids	EtOH	Mg ribbon & conc: HCl	pink colour	+
6	Glycosides	H_2O	10 % lead acetate	white ppt	+
7	Phenolic compounds	EtOH	1 % $FeCl_3$	reddish-brown ppt	+
8	Reducing sugars	H_2O	Benedict's solution	brick red ppt	+
9	Saponins	H_2O	distilled water	frothing	+
10	Starch	H_2O	iodine solution	no blue colour	-
11	Steroids	PE	acetic anhydride and conc:H_2SO_4	greenish brown colour	+
12	Tannins	EtOH	ferrous sulphate	reddish-brown ppt	+
13	Terpenoids	$CHCl_3$	acetic anhydride and conc:H_2SO_4	no colour	-

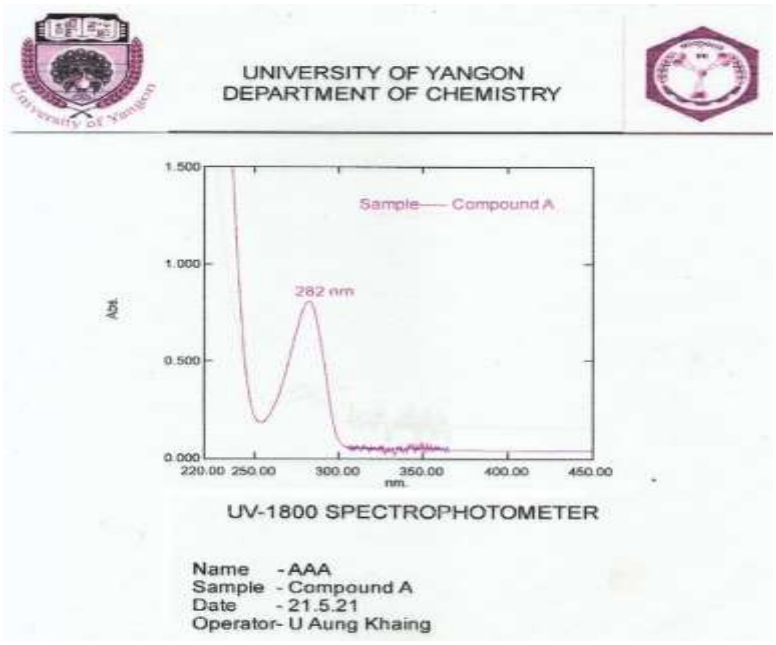
(+) = presence (-) = absence (ppt) = precipitation

Characterization of an Isolated Compound from the Tuber of *S. venosa*

A compound was isolated from ethyl acetate fraction of *S. venosa* tuber by the column chromatographic separation technique. The isolated compound was obtained as a white crystal after recrystallization in methanol. The R_f value of compound was found to be 0.57 (PE:EtOAc, 1:1 v/v) and it is soluble in ethyl acetate and methanol solvent. It is provided in orange colour with Dragendorff 's reagent by checking on TLC. The UV-visible and FT IR spectra of the isolated compound in methanol are shown in Figure 2. The wavelength of maximum absorption was observed at 282 nm and it may be due to the ($\pi \rightarrow \pi^*$) transition of C=C. The FT IR spectral data and band assignments are described in Table 2. The FT IR spectral data revealed the presence of =C-H stretching vibration (3050 and 3020 cm^{-1}), an aromatic ring (1635, 1611, and 1513 cm^{-1}), an ether linkage (1137, 1078, and 1050 cm^{-1}), and *o*-disubstituted benzene (785 cm^{1}). In addition, the structure of compound was identified by ^{1}H NMR, ^{13}C NMR, and ESI MS (Figures 3, 4 and 5). In the 600 MHz ^{1}H NMR spectrum of the isolated compound, the signals were observed at δ 6.89 ppm for H-1, δ 6.73 ppm for H-4, δ 6.92 ppm for H-11 and δ 6.96 ppm for H-12 exhibited on the aromatic ring and the methylene proton H-8 showed a pair doublet at δ 4.22 and δ 3.53 ppm. The four methoxy groups were observed at δ 3.85 ppm H-2, δ 3.86 ppm H-3, δ 3.84 ppm H-9 and δ 3.83 ppm H-10 displayed on the aromatic ring. Additionally, the aliphatic signals were assigned at δ 3.11 and δ 2.66 ppm (5a, b) and δ 3.23 and δ 2.76 ppm (6a, b) due to CH_2-CH_2 spin system.

In 150 MHz ^{13}C NMR spectrum of a compound, the spectrum indicated the presence of twenty-one carbon signals. Including, the four methoxy carbons were found to be at δ 56.72, δ 56.42, δ 60.80 and δ 56.37 ppm for carbon position (2, 3, 9 and 10). Furthermore, the downfield shift of the ^{13}C NMR signal of CH (δ 60.56 ppm C-13a) and CH_2 (δ 52.68 ppm C-6) and (δ 54.85 ppm C-8) groups indicated these carbon atoms were adjacent to the nitrogen atom. Moreover, the eight quaternary carbon were found at δ 149.30 ppm for C-2, δ 149.16 ppm for C-3, δ 127.83 ppm for C-4a, δ 128.70 ppm for C-8a, δ 146.28 ppm for C-9, δ 151.76 ppm for C-10, δ 128.89

ppm for C-12a and δ 130.70 ppm for C-13b (Table 3). According to the ^{1}H NMR and ^{13}C NMR results, the isolated compound was identified as tetrahydropalmatine (Joanne *et al*., 2003). The MS spectrum, the molecular weight of compound was confirmed by the MS spectrum, which shows an *m/z* value of $[M + H]^{+}$ at 356.0. Finally, the structure of the isolated compound was assigned as tetrahydropalmatine.



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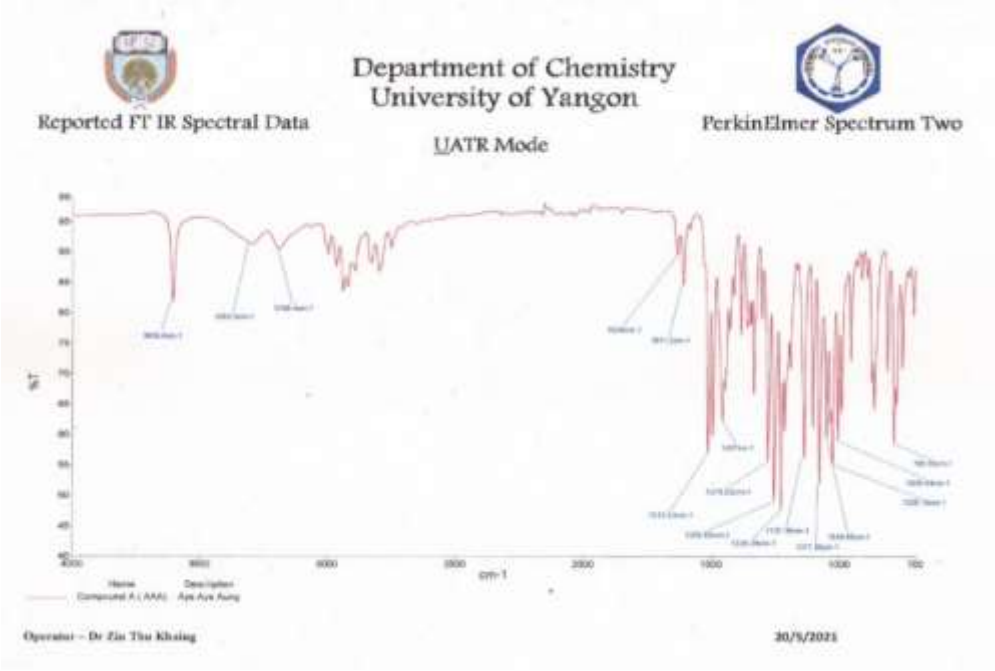

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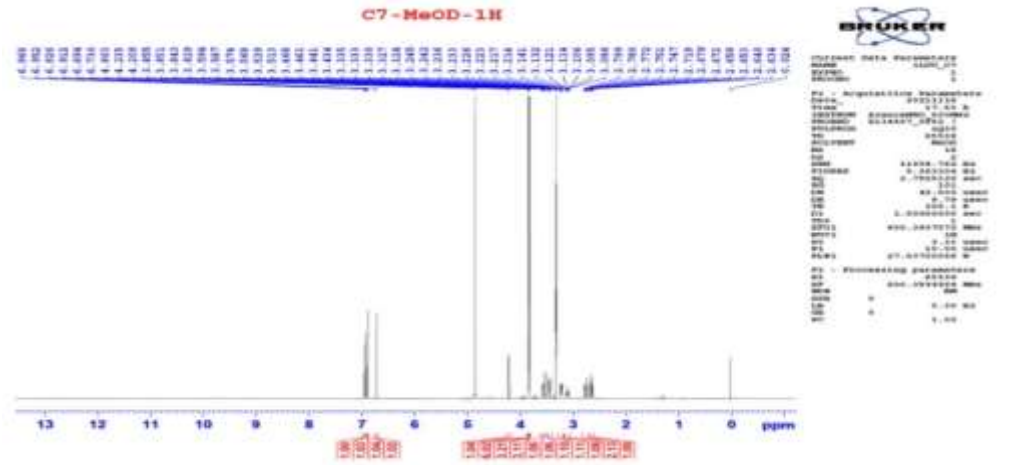
Figure 2. (a) UV and (b) FT IR spectra of the isolated compound from *S. venosa*

Table 2. FT IR Spectral Data and Band Assignment of the Isolated Compound

Wavenumber (cm^{-1})	Assignments
3050 and 3020	=C-H stretching vibration
2970 and 2890	C-H asymmetric stretching vibration
2850	C-H symmetric stretching vibration
1635, 1611 and 1513	C=C stretching vibration in aromatic ring
1457	C-H bending vibration
1279, 1255 and 1228	C-N aliphatic stretching vibration
1137 ,1078 and 1050	C-O-C stretching vibration
1026 and 1005	C-C ring stretching vibration
785	C-H out of plane bending vibration



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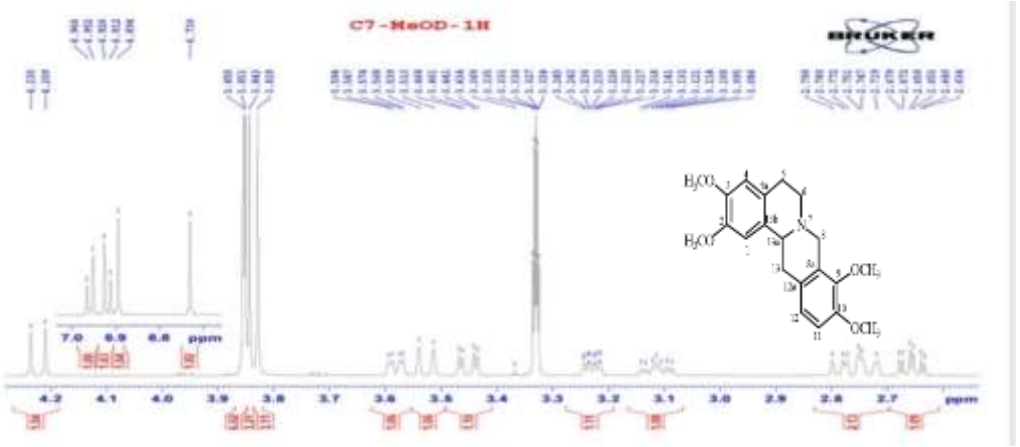

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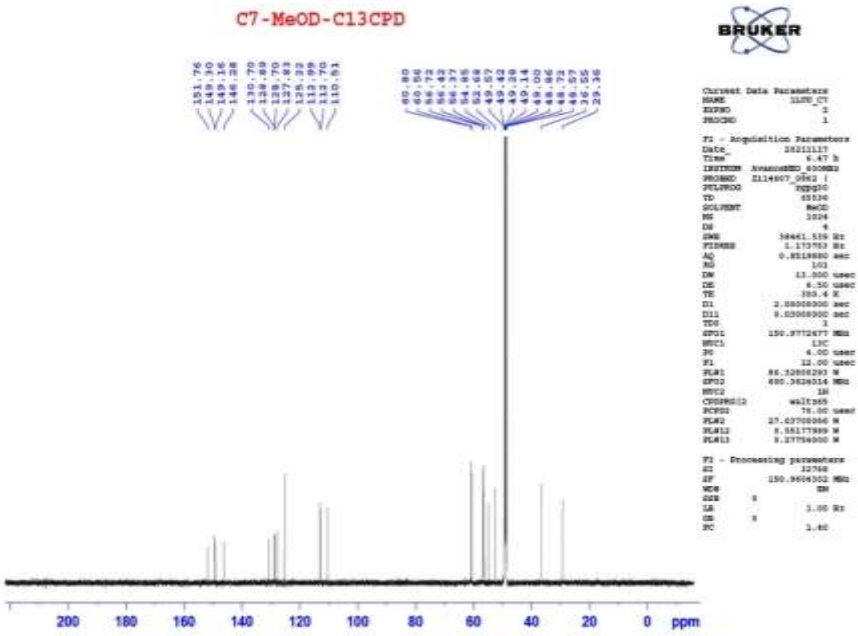
Figure 3. (a) and (b) ^{1}H NMR spectra (600 MHz, MeOD) of the isolated compound



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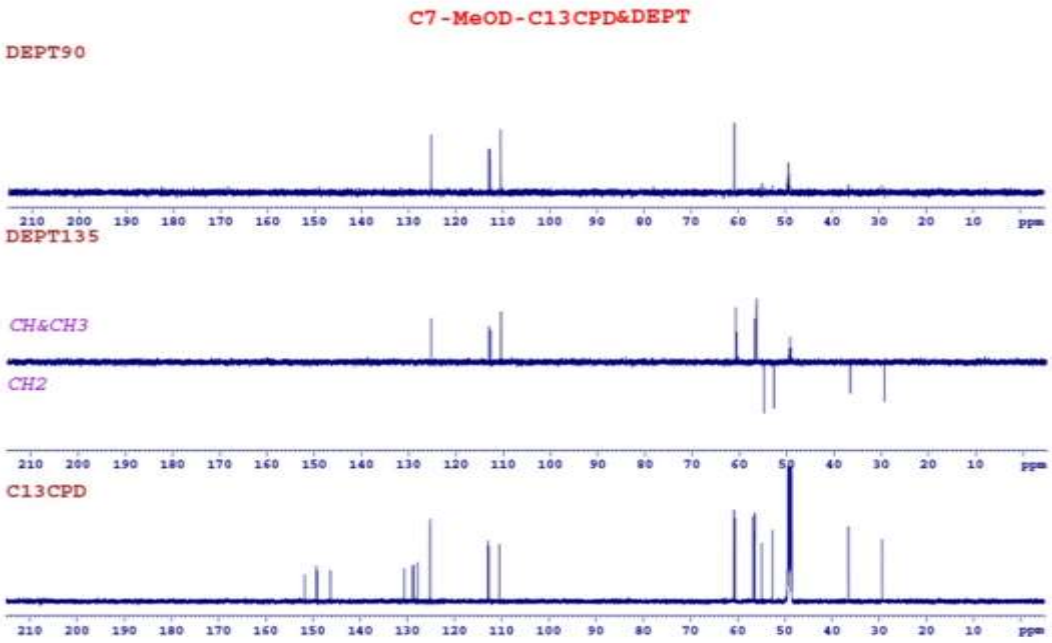


(b)

Figure 4. (a) ^{13}C NMR and (b) ^{13}C NMR (DEPT) spectra (150 MHz, MeOD) of the isolated compound

Table 3. ^{1}H NMR and ^{13}C NMR Spectra Data of the Isolated Compound

Position	Isolated compound		Literature * $CDCl_3$	
	δ_H (J Hz)	**δ_C (DEPT)**	**δ_H (J Hz)**	**δ_C (DEPT)**
1	6.89 (s)	110.51 (CH)	6.73 (s)	108.59 (CH)
2	-	149.30 (C)	-	147.48 (C)
3	-	149.16 (C)	-	147.42 (C)
4	6.73 (s)	112.99 (CH)	6.62 (s)	111.33 (CH)
4a	-	127.83 (C)	-	126.76 (C)
5	(a) 3.11 (obsc) (b) 2.66 (obsc)	29.36 (CH_2)	(a) 3.13 (obsc) (b) 2.66 (obsc)	29.06 (CH_2)
6	(a) 3.23 (obsc) (b) 2.66 (obsc)	52.68 (CH_2)	(a) 3.21 (obsc) (b) 2.66 (obsc)	51.48 (CH_2)
8	(a) 4.22 (d,15.60) (b) 3.53 (d,15.60)	54.85 CH_2)	(a) 4.24 (d, 15.63) (b) 3.54 (d, 15.63)	53.96 (CH_2)
8a	-	128.70 (C)	-	127.07 (C)
9	-	146.28 (C)	-	145.06 (C)
10	-	151.76 (C)	-	150.24 (C)
11	6.92d, 8.40)	112.70 (CH)	6.79 (d,8.38)	110.94 (CH)
12	6.96 (d, 8.40)	125.22 (CH)	6.88 (d,8.38)	123.82 (CH)
12a	-	128.89 (C)	-	128.61 (C)
13	(a)3.45(dd, 16.20, 4.20) (b)2.76 (obsc)	36.55 (CH_2)	(a) 3.27 (dd,15.76, 3.65) (b) 2.83(dd,15.76, 11.49)	36.28 (CH2)
13a	3.58 (obsc)	60.56 (CH)	3.54 (obsc)	59.29 (CH)
13b	-	130.70 (C)	-	129.66 (C)
2-OCH_3	3.85 (s)	56.72 (CH_3)	3.87 (s)	56.05 (CH_3)
3-OCH_3	3.86 (s)	56.42 (CH_3)	3.89 (s)	55.84 (CH_3)
9-OCH_3	3.84 (s)	60.80 (CH_3)	3.85 (s)	60.14 (CH_3)
10-OCH_3	3.83 (s)	56.37 (CH_3)	3.84 (s)	55.81 (CH_3)

*(Joanne *et al*., 2003). obsc = obscure

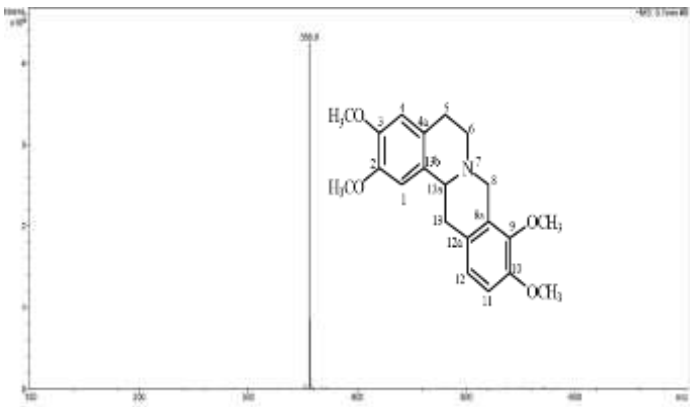
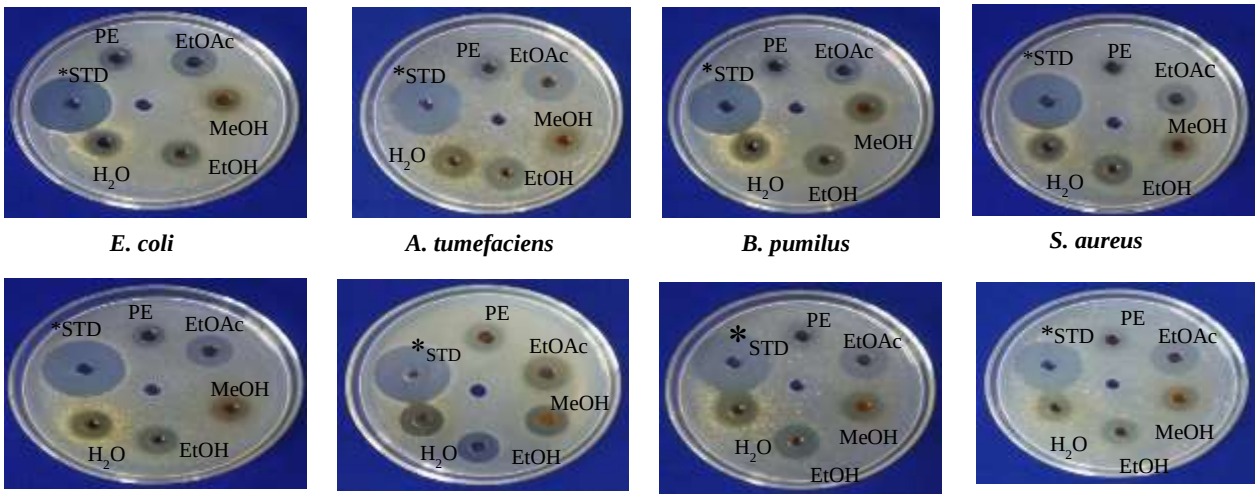


Figure 5. ESI Mass Spectrum of the isolated compound (*m/z* of +MS=356)

Screening of Antimicrobial Activity of Crude Extracts and the Isolated Compound

The present study was aimed to evaluate the potency of petroleum ether, ethyl acetate, ethanol, methanol, and watery extracts from the tuber of *S. venosa* against eight different strain of microorganisms such as *Escheerichia coli, Candida albicans, Bacillus subtilis, Staphylococcus aureus, Pseudomonas fluorescens, Agrobacteriumb tumefaciens, Bacillus pumilus, and Micrococcus luteus* by using agar well diffusion method. The measurable zone diameter, including the well diameter, shows the degree of antimicrobial activity. The agar well diameter is 10 mm. The greater zone diameter indicates the higher potency of the antimicrobial activity. According to the results shown in Table 4 and Figure 6, the petroleum ether, ethyl acetate, ethanol, methanol, and watery extracts showed antimicrobial activity against all tested microorganisms (12 mm to 22 mm). Among these extracts, the ethyl acetate extract (17 mm to 22 mm) showed higher inhibition zone diameter than the other extracts, except for *Bacillus pumilus*. Therefore, the ethyl acetate extract may be traditionally used for treating antimicrobial infections.



*STD = Chloramphenicol for bacteria, *STD = Nystatin for fungus

Figure 6. Antimicrobial activity of various extracts from tuber of *S. venosa*

Table 4. Antimicrobial Activity Results of *S. venosa*

Microorganisms	Inhibition Zone Diameter (mm)					
	PE extract	EtOAc extract	EtOH extract	MeOH extract	H_2O extract	*STD
E. coli	14	19	16	15	13	32
C. albicans	16	20	20	20	20	33
B. subtilis	14	19	17	16	15	33
S. aureus	12	19	18	14	14	32
P. fluorescens	13	21	17	16	15	30
A. tumefaciens	12	22	19	18	18	30
B. pumilus	13	17	20	20	13	33
M. luteus	12	21	20	18	17	33

Agar well diameter = 10 mm (1µg/mL)
10 mm – 14 mm = (+) low activity
15 mm – 19 mm = (++) medium activity
20 mm and above = (+++) very high activity

Antioxidant Activity of Crude Extracts of *S. venosa*

The antioxidant activity was determined in terms of hydrogen donating or radical scavenging ability in the present of the stable free radical (DPPH) using spectrophotometric method. *In vitro* antioxidant activity of the 95% ethanol, watery extracts, and a compound from the tuber of *S. venosa* and the standard ascorbic acid were determined by using free radical scavenging DPPH assay. In this study, the various concentration (15.625, 31.25, 62.5, 125, 250, and 500 µg/mL) of ethanol and watery extracts and six different concentration (3.125, 6.25, 12.5, 25, 50 and 100 µg/mL) of an isolated compound were used. An increase in radical scavenging activity evaluates a decrease in absorbance. The radical scavenging activities of the different crude extracts and a compound were expressed in % RSA and IC_{50} (50% inhibitory concentration). The absorbance of these solutions was measured at 517 nm by using a UV spectrophotometer. These results are shown in Tables (5 and 6) and Figures (7 and 8). According to the results, the IC_{50} values of ethanol, watery extracts and a compound were found to be 134.93, 173.61, and 24.43 µg/mL. Therefore, the lower the IC_{50} value exhibits the higher the antioxidant activity in the sample. The antioxidant activity was found to be lower than that of standard ascorbic acid (2.83 µg/mL).

Table 5. Antioxidant Activity of Crute Extracts *S. venosa tuber*

Samples	% RSA (mean ± SD) in different concentrations						IC_{50} (µg/mL)
	15.625	31.25	62.5	125	250	500	
Ethanol	16.62 ± 0.008	29.31 ± 0.026	32.48 ± 0.017	46.44 ± 0.025	91.24 ± 0.005	98.09 ± 0.016	134.93
Watery	20.55 ± 0.005	25.85 ± 0.029	30.96 ± 0.030	42.54 ± 0.001	61.13 ± 0.000	82.58 ± 0.009	173.61

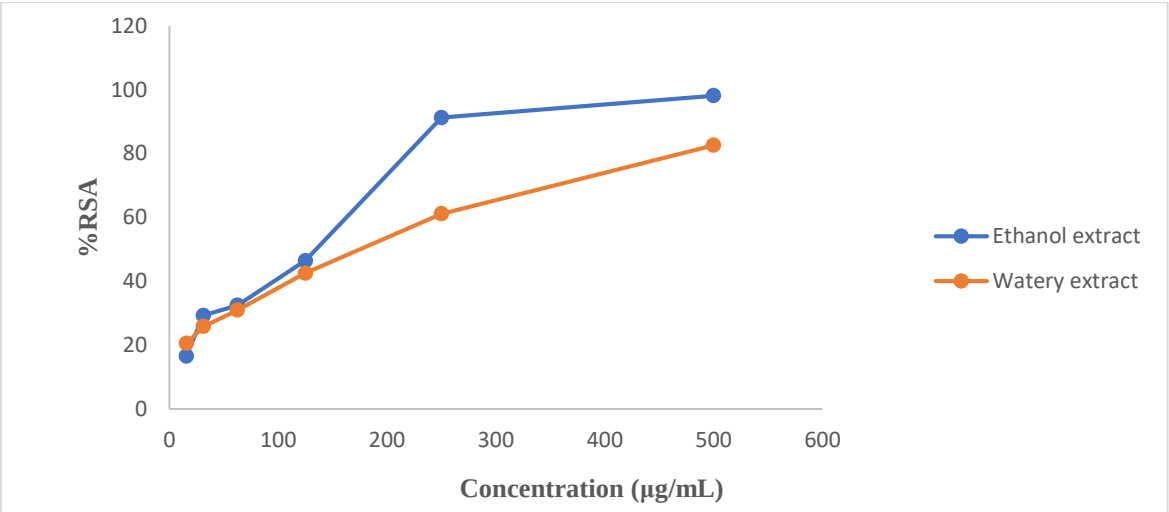


Figure 7. % RSA of ethanol and watery extracts of *S. venosa versus concentration*

Table 6. Antioxidant Activities of Isolated Compound and Standard Ascorbic Acid

Samples	% RSA (mean ± SD) in different concentrations (µg/mL)						IC_{50} (µg/mL)
	1.5625	3.125	6.25	12.5	25	50	
Compound	31.57 ± 0.063	45.48 ± 0.009	47.39 ± 0.041	48.95 ± 0.004	50.57 ± 0.003	52.02 ± 0.001	24.43
Ascorbic acid	33.03 ± 0.040	53.94 ± 0.095	90.48 ± 0.017	94.16 ± 0.000	96.94 ± 0.000	96.76 ± 0.000	2.83

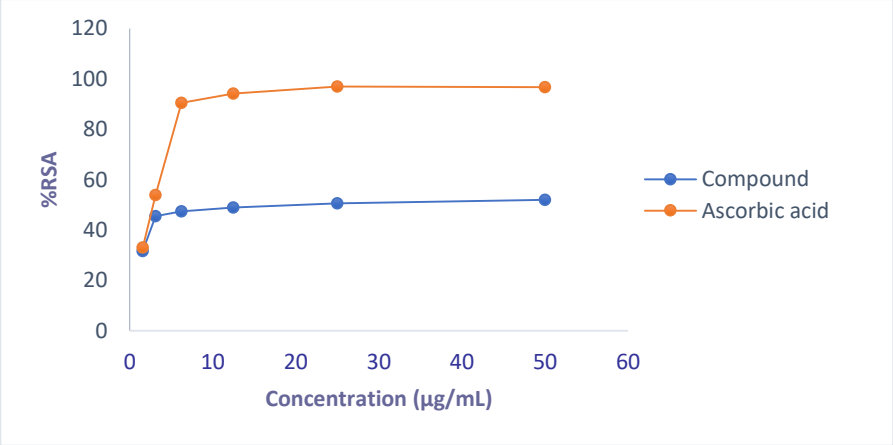


Figure 8. % RSA of isolated compound and standard ascorbic acid versus concentration

Conclusion

The overall assessments of the research work include preliminary phytochemical investigations, isolation, and identification of a bioactive compound, *in vitro* antimicrobial and antioxidant activities from the tuber of *S. venosa*. The important bioactive molecules of the sample were explored with preliminary phytochemical screening and reported to consist of alkaloids, α-amino acid, carbohydrates, glycosides, flavonoids, organic acids, phenolic compounds, reducing sugars, steroids, saponins, and tannins. The isolated compound (0.06 % yield and white crystal) was separated from the ethyl acetate extract by using a thin layer and column chromatographic separation techniques. The isolated compound was identified by the modern spectroscopic methods such as TLC, UV-visible, FT IR, ^{1}H NMR, ^{13}C NMR, and MS. According to the results, the isolated compound would be deduced as an alkaloids compound: tetrahydropalmatine ($C_{21}H_{25}NO_4$, R_f = 0.57). The different crude extracts such as ethyl acetate, ethanol, methanol, and watery extracts exhibited the significant antimicrobial activity by the agar well diffusion method. The ethyl acetate extract has the most potent antimicrobial activity than the other extracts, except for *B. pumilus* against test microorganisms. The antioxidant activity of the crude extracts and the isolated compound was determined by DPPH assay method. According to the results, the ethanol extract (IC_{50} value =134.93 µg/mL) indicated antimicrobial activity than the watery extract (IC_{50} value =173.61 µg/mL). Furthermore, the isolated compound also showed higher antioxidant activity (IC_{50} value =24.43 µg/mL) than the extracts. Alkaloids are bioactive compounds and it can be used for therapeutic agents in several ailments. Therefore, *S. venosa* is a very popular medicinal plant in traditional medicine.

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