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Biological Characterization of Crude Extract & Pure Compound Isolated from *Swertia chirata* Ham

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Abstract The fresh stem of the plant *Swertia chirata* Ham was extracted by rectified spirit. The crude rectified spirit extract was fractionated by using standard chromatographic techniques, on alumina gave several fractions (A, B, C, D, E & F). Fraction D, when subjected to column chromatographic analysis on neutral alumina, yielded a pure compound X-1 m.p. 180°C. X-1 was screened for its antibacterial activities against 12 pathogenic bacteria, 6 Gram positive and 6 Gram negative, by disc diffusion method at a concentration of 200 µg/disc. The results obtained were compared with those for a standard antibiotic Kanamycin. X-1 showed significant activity against *Bacillus megaterium* (13 mm), *Bacillus subtilis* (11 mm), *Salmonella typhi-A* (12 mm), *Shigella flexeneriae* (12 mm) and *Klebsiella* sp (13 mm) but a little activity against *Staphylococcus aureus*. The Minimum Inhibitory Concentrations (MIC) of X-1 determined against *Bacillus megaterium* and *Salmonella typhi-A* were 128 µg/ml and 132 µg/ml, respectively when tested in a nutrient broth medium. X-1 also showed significant activity against the brine shrimp (*Artemia salina*) nauplii (LC_{50} value of 10 µg/ml), in which the mortality rate increased with the increasing concentration of the compound, suggesting a positive correlation between brine shrimp toxicity and cytotoxicity.

Keywords: *biological activity, Swertia chirata, gentianaceae, pathogenic bacteria*

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1. Introduction

The plant S*wertia chirata* Ham. (F. Gentianaceae) is a tropical family of small trees and herb. It consists of 180 species of which 8-10 species exist in Indian subcontinent [4]. This plant is indigenous to Himalayas at altitudes above 4000 ft from Kashmir, Nepal to Bhutan [1]. All members of this family are used as medicinal plants. The main chemical constituents of this plant are two bitter principles, *viz*, ophelic acid, an amorphous bitter principle and chiratin, a yellow bitter glucoside. The plant also contains resins, tannin, gum, carbonates, phosphates and 4-6% ash, lime and magnesia [2,11]. A number of workers have shown that the plant contains bitter glucosidal components, chiratin and amarogentin, swerchirin, gentiopicrin, phytosterd and also a number of acid, yellow crystalline phenols and saccharine [3,5,6]. The present work describes the biological activity of the various fractions of the crude as well as pure extracts of the whole plant.

2. Materials and Methods

Collection of the plant: Dried stems of *S. chirata* (locally known as Chirata) were collected from Bhangura Kabiraji Shop at Rajshahi Shaheb Bazar.

Preparation of plant materials*:* The stems along with the leaves of the plant were cut into small pieces by a sharp knife and dried in the sun for 72 hrs. This was further dried in the oven for 24 hrs at a temperature below 40°C. About 650 g of the dried plant materials were weighed by an electric balance and grinded with a grinding machine.

Extraction of plant materials: The powdered materials were taken in a clean flat bottomed glass container (2.5 L) and macerated with sufficient amount of rectified spirit and with occasional shaking. After 15 days the solvent was decanted and filtered by Tincture filter press (Karl Kolb, Scientific-Technical Supplies, Frankfurt, Germany); and then filtered through fresh cotton. The filtrate thus obtained was taken in a beaker.

Evaporation of the solvent*:* The solvent of the extract was evaporated under temperature and pressure to obtain a gummy mass, which was preserved in a refrigerator at 4°C for chemical investigation.

Isolation of the compounds*:* Rectified spirit extract of the plant was a crude product that contained a mixture of compounds. Thin layer chromatographic (TLC) examination of the extract under petroleum ether: ethyl acetate (1:2) system showed 3 spots having Rf values of 0.1, 0.54 and 0.73, respectively. After isolating, different fractions were obtained. The fractions were combined on the basis of their preliminary TLC examination. Each examination gave combined fractions designated as A, B,

C, D, E and F, each one was evaporated to dryness under reduced pressure. Fraction D showed two spots (R_f = 0.53 and 0.73) on TLC plates using solvent system Petroleum ether: Ethyl acetate (1:2). The fraction D was further subjected on mini column chromatography using Petroleum ether: Ethyl acetate (1:1). The two eluants were collected and evaporated to get two components designated as X-1 (45gm) and X-2 (8mg). The component X-1 was crystalline but the component X-2 was not crystalline and was insufficient quantities was not considered for further investigation.

Purification of pure compound: The compound X-1 showed single spot on TLC analysis with some impurities. The compound X-2 was recrystallized dissolving in Petroleum ether: Ethyl acetate (1:1) and the crystals were washed with different solvents of varying polarity. The isolated compound X-1 was tested in different solvent systems (Table -1) for its purity. The compound showed a single spot on TLC examination. So, this compound was pure. Finally its R_f values (Table-1) were determined using the various solvent systems.

Table 1. TLC analysis of the pure compound X-1 on silica gel.

Solvent system and ratio	R_f value
Petroleum ether: Ethyl acetate (1:2)	0.73
Chloroform: Ethyl acetate (49:1)	0.92
Ethyl acetate: Chloroform (19:1)	0.79
Toluene: Ethyl acetate (3:1)	0.88
Ethyl acetate: Pyridine: Water (5:1:4)	0.98
Ethyl acetate: Chloroform: Methanol (2:2:1)	0.95
Benzene : Ethyl acetate (19:1)	0.86
Chloroform: Ethyl acetate (4:1)	0.91
Ethyl acetate: Acetone (9:1)	0.94

Bioassays of the crude and pure extracts: Brine shrimp lethality bioassay is a recent development in the bioassay for bioactive compounds, which indicates cytotoxicity as well as a wide range of pharmacological activities (*e.g*. anticancer, antiviral, pesticidal, AIDS etc.) of the compounds. Bioactive compounds are almost always toxic in high doses. Pharmacology is simply toxicology at a lower dose or toxicology is simple pharmacology at a higher dose. Brine shrimp lethality bioassay is a bench top bioassay method for evaluating anticancer, antimicrobial and pharmacological activities natural products. Natural product extracts, fractions or pure compounds can be tested for their bioactivity by this method. Here in vivo lethality of a simple zoological organism (brine shrimp nauplii) is used as a convenient monitor for screening a fractionation in the discovery of new bioactive natural products. Generally the median effective dose (ED_{50}) values for cytotoxicities are one tenth (1/10) of median lethal dose (LC_{50}) values in the brine shrimp test.

3. Results and Discussion

Test organisms used for the study: Fourteen pathogenic bacteria (five gram positive and nine gram negative) were selected for the test (Table 2). These organisms were available in the microbiological research laboratory of Biochemistry and Molecular Biology department, University of Rajshahi, Bangladesh. The pure cultures were collected from the Microbiological Laboratory of the Institute of Nutrition and food Science (INFS) and Department of Microbiology, University of Dhaka, Bangladesh.

Table 2. List of the test pathogenic bacteria

Serial No	Name of test organism	Strain number
Gram positive		
1.	Staphylococcus aureus	ATCC-259233
2.	*Bacillus megaterium*	QL-38
3.	*Bacillus subtilis*	QL-40
4.	*Streptococcus-β-haemolyticus*	CRL
5.	*Sarcina lutea*	QL-166
Gram negative		
6.	*Salmonella typhi*	-
7.	*Shigella dysenteriae*	AL-35587
8.	*Shigella flexneri*	AL-30372
9.	*Shigella shiga*	ATCC-26107
10.	*Shigella sonnei*	AJ-8992
11.	*Shigella boydil*	AL-17313
12.	*Beudomonas aeruginosa*	CRL
13.	*Escherichia coli*	FPFC-1407
14.	*Kelesiella species*	-

Antibacterial activity of test sample: The antibacterial activities of the crude extract and the pure compound X-1 were tested. The results obtained are shown in Table 3 and Table 4. The inhibitory activities are shown in Figure 1, 2 and 3.

Antibacterial activity of rectified spirit extract: Antibacterial activity of rectified spirit extract was tested against eight bacteria at concentrations of 30 µg/disc and 90 µg/disc. Standard antibiotic disc of chloramphenicol (30 µg/disc) was used for comparison. The results obtained were shown in Table 3 and Figure 1, 2 and 3. The produced zone of inhibition for rectified spirit extract against *Staphylococcus aureus*, *Bacillus megaterium* and *Escherichia coli* were 10 mm, 9 mm and 9 mm at 30 µg/disc dose respectively. At 90 µg/disc dose, the produced zone of inhibition against the same bacteria was 17 mm, 14 mm and 13 mm respectively. It was evident that the antibacterial activity of rectified spirit extract against the above bacteria showed decrease dose dependency.

Table 3. *In vitro* antibacterial activity test of the crude extract of *S. chirata*

Zone of Inhibition (Diameter in mm)			
Test organisms	Rectified spirit extract		Standard Chloramphenicol (30 µg/disc)
	30 µg	90 µg	
Staphylococcus aureus	10	17	33
Bacillus subtilis	-	-	35
Bacillus megaterium	9	14	29
Sarcina lutea	-	-	27
Salmonella typhi	-	-	23
Shigella sonnei	-	-	35
Shigella shiga	-	-	26
Escherichia coli	9	13	34

Table 4. Antibacterial activity of pure compound X-1

Bacterial strain	**Pure Compound X-1 200 µg/disc**
Gram positive	
Bacillus megaterium	13
Bacillus subtilis	11
Staphylococcus aureus	-
Sarcina lutea	-
Gram negative	
Salmonella typhi	12
Shigella flexeneriae	12
Klebsiella species	13
Shigella dysenteriae	-

Values indicate zone of inhibition (diameter in mm)

Antibacterial activity of pure compound X-1

The pure compound X-1 was screened for their antibacterial activities against 12 pathogenic bacteria, 6 Gram-positive and 6 Gram-negative, by disc diffusion method at a concentration of 200µg/disc. The results obtained were shown in Table 4 compared with those for a standard antibiotic Kanamycin. Pure compound X-1 showed significant activity against *Bacillus megaterium* (13 mm), *Bacillus subtilis* (11 mm), *Salmonella typhi-A* (12 mm), *Shigella flexeneriae* (12 mm) and *Klebsiella* sp. (13 mm). A little activity of pure compound X-1 against *Staphylo coccus aureus*, *Sarcina lutea* and *Shigella dysenteriae* was observed.

Minimum inhibitory concentration (MIC) of the test sample

Minimum Inhibitory Concentration (MIC) of rectified spirit extract was determined by serial dilution technique [13] against two Gram-positive bacteria *Staphylococcus aureus* and *Sarcina lutea* and two Gramnegative bacteria *Escherichia coli* and *Salmonella typhi-A*. The MIC value of pure compound X-1 was determined against a Gram-positive bacterium (*Bacillus megaterium)* and a Gram-negative bacterium (*Salmonella typhi-A*) These values are shown in (Table 5 and Table 6).

MIC of the crude extract

For the Rectified spirit extract the growth was observed in the test tube containing 128 µg/ml of extract against *Escherichia coli, Sarcina lutea, Bacillus megaterium* and for *Staphylococcus aureus* in the test tube containing 64 µg/ml and *Salmonella typhi-A* containing 132 µg/ml of rectified spirit extract. So the MIC values of Rectified spirit extract for *Sarcina lutea, Escherichia coli, Bacillus megaterium* were 128 µg/ml and for *Staphylococcus aureus* was 64 µg/ml and *Salmonela typhi-A* was 132 µg/ml (Table 5).

MIC of pure compound X-1

The Minimum Inhibitory Concentrations of the pure compound X-1 was determined against *Bacillus megaterium* and *Salmonella typhi-A*. The MIC of the pure compound X-1 against *Bacillus megaterium* and *Salmonella typhi-A* were 128 µg/ml and 132 µg/ml respectively when tested in nutrient broth medium. The results are shown in (Table 6).

Table 5. Minimum Inhibitory Concentration (µg/ml) values of rectified spirit extract of *S. chirata*

Test Bacteria	Rectified spirit extract (µg/ml)
Gram positive	
1. Staphylococcus aureus	64
2. Sarcina lutea	128
Gram negative	
1. Escherichia coli	128
2. Salmonela typhi	132

Table 6. Minimum inhibitory concentration (MIC) of pure compound X-1

Minimum inhibitory concentration (µg/ml)		
Sample	Gram positive	Gram negative
	Bacillus megaterium	Salmonella typi-A
Pure compound X-1	128	132

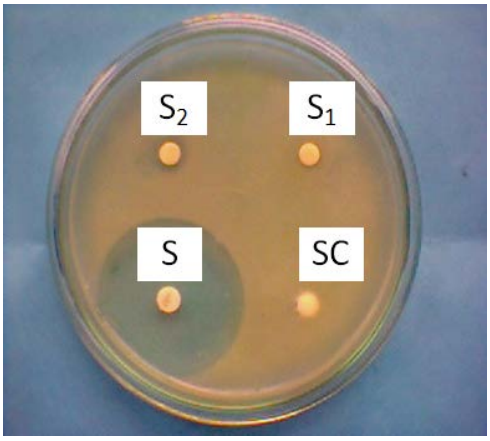

Figure 1. Antibacterial activity of **Rectified spirit** extract against ***Bacillus megaterium***

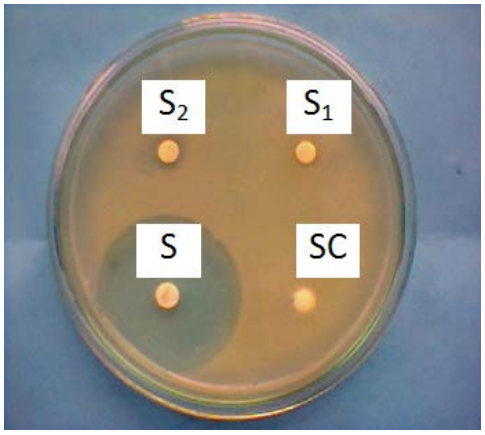

Figure 2. Antibacterial activity of **Rectified spirit** extract against ***Escherichia coli***

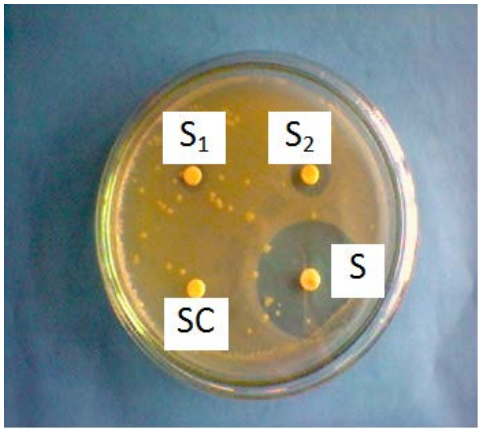

Figure 3. Antibacterial activity of **Rectified spirit** extract against ***Staphylococcus aureus***

S_1 = 30 µg/disc
S_2 = 90 µg/disc
SC = Solvent control
S = Standard (Chloramphenicol 30 µg/disc)

Brine shrimp mortality of test sample

Brine shrimp mortality test is a recent development in the bioassay for the bioactive compounds [9]. Bioactive compounds are almost always toxic in high dose. There is a positive correlation between brine shrimp toxicity and cytotoxicity [7,10,12]. The crude rectified spirit extract and pure compound X-1 showed positive result in brine shrimp lethality bioassay. The results were shown in (Table 7 and Table 8).

Brine shrimp mortality of rectified spirit extract

To determine the cytotoxic effect of rectified spirit extract, the lethal concentration, LC_{50} (concentration at which 50% mortality of the nauplii occurred) was measured and found to be 80.50 µg/ml. This was obtained from a plot of percentage of mortality versus log of concentration on the graph, which produced approximate linear correlation between them (Figure 4). The result of brine shrimp lethality of rectified spirit extract was given in the (Table 7).

Table 7. Results of the Rectified spirit extract on brine shrimp lethality bioassay

Sample	Concentration µg/ml	No of nauplii taken	No of nauplii alive	No of nauplii died	% of Mortality	LC50 µg/ml
Rectified spirit extract	10	10	9	1	10	
	20	10	7	3	30	
	40	10	6	4	40	**80.50**
	80	10	5	5	50	
	120	10	3	7	70	

Brine shrimp mortality by pure compound of X-1

To determine the cytotoxic effect of pure compound X-1 medium lethal concentration (LC_{50}) of brine shrimp lethality was measured and it was found to be 10 µg/ml, which was obtained from a plot of percentage of mortality versus log of concentration (µg/ml) on the graph. This afforded an approximate linear correlation between them (Figure 5). The results of brine shrimp mortality of pure compound X-1 are shown in the (Table 8).

Table 8. Results of the pure compound of X-1 on brine shrimp lethality bioassay

Concentration µg/ml	No of nauplii taken	No of nauplii alive	No of nauplii died	% of mortality	LC_{50} µg/ml
5	10	8	2	20	
10	10	6	4	40	
20	10	4	6	60	10
40	10	2	8	80	
80	10	0	10	100	

*10 nauplii used per concentration.

The pure compound X-1 was found to show significant activity against the brine shrimp nauplii. In this bioassay, the mortality rate of brine shrimp is found to increase with the increasing concentration of the compound. So it was observed that there existed a positive correlation between brine shrimp toxicity and cytotoxicity. The very low value of LC_{50} (10.10 µg/ml) indicated the high cytotoxic effect of the pure compound X-1.

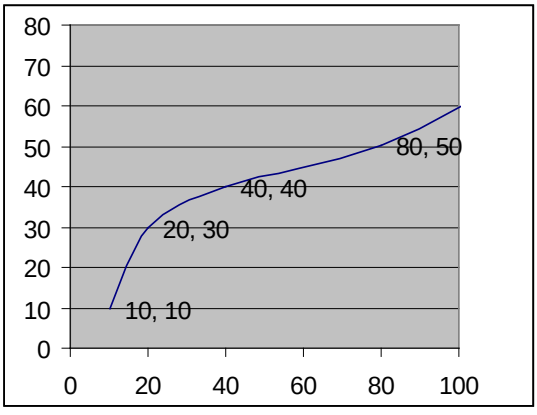

Figure 4. Determination of LC_{50} of Rectified spirit extract against brine shrimp nauplii.

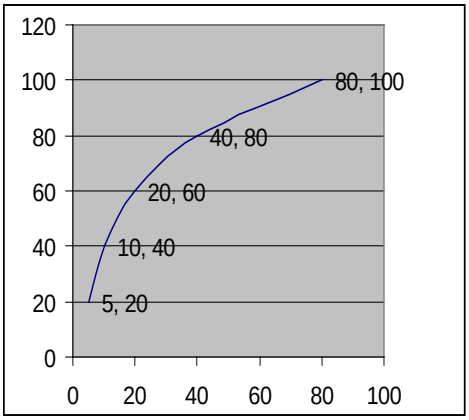

Figure 5. Determination of LC_{50} of the pure compound X-1 against brine shrimp nauplii.

4. Conclusion

The antibacterial as well as biological activities of the rectified spirit extract and the pure compound of the medicinal plant *S. chirata* showed desirable characterization for various applications.

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