European Journal of Medicinal Plants
18(3): 1-11, 2017; Article no.EJMP.31958
ISSN: 2231-0894, NLM ID: 101583475

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Phytochemical and Biological Studies on Crude Extract of *Swertia chirata* and Its Fractions

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/EJMP/2017/31958
Editor(s):
(1) Patrizia Diana, Department of Molecular and Biomolecular Sciences and Technologies, University of Palermo, Palermo, Italy.
Reviewers:
(1) Mostafa Abbas Shalaby, Cairo University, Egypt.
(2) Manu Pant, Graphic Era University, India.
(3) Mindaugas Liaudanskas, Lithuanian University of Health Sciences, Lithuania.
(4) Rashidur Rahman, Jessore University of Science & Technology, Bangladesh.
Complete Peer review History: http://www.sciencedomain.org/review-history/18293

Original Research Article

Received 31st January 2017
Accepted 9th March 2017
Published 21st March 2017

ABSTRACT

Aim: The aim of present study is to evaluate the pharmacognostic, phytochemical and some biological studies on *Swertia chirata*.
Methodology: Microscopic evaluation, fluorescence analysis, TLC, FTIR and HPLC techniques used for standardization of Powder and crude extract of *S. chirata*. Biological studies were performed *in vitro* through haemagglutination on all positive and negative blood groups at the dose of 5 mg/ml, 2.5 mg/ml, 1.25 mg/ml, 0.625 mg/ml and 0.3125 mg/ml and brine shrimp bioassay at the doses of 1000, 100 and 10 µg/ml.

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Results: Microscopic examination of powder of *S. chirata* showed important diagnostic features of the plant. The reactions with chemical reagents showed positive results for the presence of triterpenes, tannin, alkaloids, carbohydrate and sterols while the tests for saponins and protein were negative indicated the absence of these components. The fluorescence analysis of powder of *S. chirata* was observed under ordinary light and UV light at 254 nm and 366 nm. TLC, FTIR and HPLC analysis indicated the presence of active constituents in crude extract. The results of haemagglutination activity showed that the drug has highest response (agglutination activity) against B^+ and O^+ blood groups that is 80% at the concentration of 5 mg/ml. The cytotoxicity test showed no lethality.

Conclusion: Phytochemical evaluation provides standardization knowledge of *S. chirata.* Due to presence of triterpenes and alkaloids. *S. chirata* may be used for therapeutic purposes. Haemagglutination and cytotoxic results indicated the safety profile of *S. chirata*.

Keywords: Swertia chirata; brine shrimps; FTIR; microscopic study; TLC; HPLC; haemagglutination.

1. INTRODUCTION

Nature has provided a huge number of medicinal plants which were used in different ailments. This approach of treatment has gained immense popularity especially in subcontinent so it is necessary to investigate the pharmacognostic and phytochemical characteristics of medicinal plants [1].

Swertia chirata Buch-Ham (Family - *Gentianaceae*) is also known as Haima, kirata Tikta, Nidrari, Ramasenka, kairata in Sanskrit, in urdu language it is called Chiravata, Chireta in Bengal and in arabic and Farsi called as Qasabuzzarirah. Its anthelmintic, hypoglycemic, antipyretic and bitterness properties due to presence of amarogentin. Different herbal drugs of chirata are used as antipyretic, hypoglycemic, antibacterial and antifungal drugs [2]. It contains xanthones, which possess anti-inflammatory, CNS stimulant, antiplatelet, anti-cancer, anti-fungal and antimalarial activities [3]. It is a remedy for ulcers, gastrointestinal diseases, skin diseases, cough, hiccup, liver and Kidney diseases, Neurological disorders, and urogenital tract disorders. Also used as purifier of breast milk, and as a laxative and carminative [4-5].

Swertia chirata provides us new lead molecules for the development of drugs against various pharmacological targets [1]. Keep in view a number of phytochemical and biological information [1-2] regarding *S. chirata,* standardization studies along with cytotoxic activity have been performed.

2. MATERIALS AND METHODS

2.1 Collection and Identification of Plant Material

The aerial part of *Swertia chirata* were collected from local market Karachi Pakistan. The plant sample were identified and deposited in Department of Pharmacy, University of Karachi (voucher no. AW2011-3) [6]. The plant samples were air-dried and ground into uniform powder using milling machine. Powder material was macerated with methanol for 15 days at room temperature. The methanol extract was then filtered and evaporated under reduced pressure in rotary evaporator glycerin to obtain a crude extract as thick gummy mass [7]. All the chemicals were obtained from Merck and the glass wares was made of Pyrex.

2.2 Microscopic Studies

Microscopic examination of powder drug was done under the light microscope Olympus (10X) with the help of chemical reagents as chloral hydrate (10%), (50%) and iodine (5%) [8].

2.3 Phytochemical Screening

Phytochemical examinations were carried out for plant extracts as per the standard methods.

2.3.1 Test for triterpenoids

Liebermann Burchard test: Crude extract was mixed with few drops of acetic anhydride, boiled and cooled concentrated sulphuric acid was then added from the sides of the test tube and observed for the formation of a brown ring at the junction of two layers. The formation of deep red color in the lower layer would indicate a positive test for triterpenoids [9-10].

2.3.2 Test for tannins

Ferric chloride Test*:* 2 ml of freshly prepared ferric chloride solution was added to 2 ml of the concentrated extract. Formation of dark blue or green or black colour indicates the presence of tannins [11-12].

2.3.3 Test for saponins

Froth test: 2 ml of the extract was mixed with 20 ml distilled water in a graduated test tube and shaken well for 10 minutes. Formation of thick froth of about 1 cm indicates that the sample contains saponins [11-12].

2.3.4 Test for alkaloids

Dragendorff test: Mixture of methanol extract and water acidified with HCl and few drops of Dragendorff reagent, orange precipitate indicating presence of alkaloids [11-12].

2.3.5 Test for carbohydrates

Fehling's test*:* 1 ml each of Fehling's solution A and B were mixed and boiled for one minute. Equal volume of the extract was added and then boiled in a water bath for about 5 minutes.

Formation of reddish brown colour indicates the presence of reducing sugar [11-12].

2.3.6 Test for sterols

Liebermann-Burchard Test*:* 2 ml of the extract was mixed with a few drops of acetic anhydride. It was boiled and cooled and concentrated sulphuric acid was added along the sides of the test tube carefully. A brown ring at the junction of two layers and the upper layer turning green indicates the presence of sterols [9-10].

2.3.7 Test for proteins

Xanthoproteic test: The methanol extract was mixed with few drops of concentrated nitric acid and then yellow colour indicates the presence of proteins [12-13].

2.4 Florescence Analysis

Florescence analysis under UV light is characteristic for a drug. Drugs and the constituents present in drug emit specific colour when they are exposed to UV radiation. Chirayita treated with different chemical reagents and colours were observed under ordinary light, UV 254 nm and 366 nm [14].

2.5 Solvent Fractionation

Crude methanol extract (8 g) was fractioning with equal volumes of water and ethyl acetate, then ethyl acetate fraction with 0.5 g crude extract obtain after freeze drying ,then aqueous layer again fractioning with chloroform then chloroform fraction with crude extract obtained then again aqueous layer fractionate with n-butanol. After air drying samples were obtained [7].

2.6 Thin Layer Chromatography

TLC is a simple, quick, and inexpensive procedure that gives the researchers a quick answer as to how many components use in a mixture. TLC is also used to support the identity of a compound in a mixture. The R*f* value of spots was determined by the given formula:

> R*f* value = Distance travelled by the Spot/Distance travelled by the Solvent.

R*f* of a compound is compared with the R*f* of a known compound for the confirmation of purity and identity of isolated compound. Additional tests involves in this procedure like spraying of phytochemical screening reagents, which cause colour changes according to the phytochemicals existing in a plant extract. These colours were observed by viewing the plate under the UV light. This has also been used for confirmation of purity and identity of isolated compound [15].

2.7 FTIR

It is a technique which is used to obtain an infrared absorption, emission, photoconductivity or Roman scattering of a solid, liquid or gas. FTIR Spectrometer collects spectral data in a wide spectral range [16].

2.8 HPLC

2 g of methanol extract was dissolved in 10 ml of methanol, with the help of sonicator at 40℃ for 30 min. The sample was filtered through the 0.45 mm filter paper with the help of Sunnix filtering assembly. HPLC was performed using a Shimadzu HPLC (Detector: SPD 20A, Pump: LC 20AT, Auto-sampler: SIL 20A, System Controller: CBM 20A and HPLC Column Manufacture: Waters µ Bondapak C18 3.9 x 300 mm). The system was operated at room temperature (20℃), the injection volume was 20 µl, and the detection wavelength was 225 nm [17].

2.9 Haemagglutination Activity

Haemagglutination test is used to determine the toxic effect of drugs or chemicals on different blood groups (A^+, B^+, AB^+, O^+, A^-, B^-, AB^-, O^-). In brief, 0.1 gm crude extract (*S. chirata*) was dissolved in 20 ml of distilled water then series of

dilutions 1:2, 1:4, 1:8 and 1:16 were prepared. For preparation of dilution and buffer classical method was used (Muhammad 2006). Samples of human blood groups were obtained from Hussaini Blood Bank, Karachi, Pakistan. RBCs were obtained by simple centrifugation of the blood samples and 2% RBCs suspension was prepared in phosphate buffer. Agglutination activity was observed in a series of dilutions of plant extract against four blood groups. For this purpose 1 ml of each was added to 1 ml of 2% suspension of RBCs in a test tube then incubation in a water bath at 25ºC. Simultaneously control sample also run with tested samples. Sedimentation of RBCs at the bottom of test tube indicated negative activity of the extract where as granular deposition of RBCs showed positive action [18].

2.10 Brine Shrimp Bioassay

Samples of three different concentrations 10, 100, 1000 mg/ml of methanol extract of *S. chirata* were prepared. Brine shrimps (*Artemia salina*) nauplii were hatched in a tank. 30 shrimps were added in each sample vial and then sea water was added to make 5 ml. Later on dry yeast suspension was added as food to each vial including control. The vials were kept for 24 hrs then active nauplii were counted and death percentage was calculated at each dose and analyzed data with Finney computer programme in order to determine LD_{50} values [19].

3. RESULTS

3.1 Microscopic and Phytochemical Examination

Microscopic examination of powder of aerial part of *S. chirata* revealed different identifying features presented in Fig. 1. Phytochemical screening of *S. chirata* was done with crude extract which showed presence of triterpenes, tannins, alkaloids, carbohydrate, sterols and absence of saponins and protein (Table 1). Specific colours were observed in fluorescence analysis of powder drug of *S. chirata* (Table 2).

Crude methanol extract of *S. chirata* was fractionated into ethyl acetate, chloroform, *n*-butanol and aqueous fraction (Scheme 1). Thin layer chromatography of *S. chirata* methanol extract, ethyl acetate, chloroform, *n*- butanol and aqueous fractions were performed in two solvent system (ethyl acetate-methanol-water in the ratio of 100:16.5:13.5 and chloroform–methanol-water in the ratio of 80:20:2). The TLC plates were observed under UV light a 254 nm and 366 nm. R*f values we*re measured after spraying with Vanillin-Sulphuric acid reagent (Tables 3a and 3b).

FT-IR analysis of methanol extract of *S. chirata* indicated the presence of -OH, C-H, benzene ring and C-O-C groups (Fig. 2). HPLC analysis was made at 225 nm indicated the presence of peaks at 1.583, 2.059, 2.483,3.565, 4.081, 5.432, 7.084, 10.173, 29.78 and 52.481 minutes (Fig. 3).

3.2 Haemagglutination and Brine Shrimp Bioassay

The agglutination activity of crude extract of *S. chirata* was examined at concentrations of 5 2.5, 1.25, 0.625 and 0.3125 mg/ml against various groups of human erythrocytes. The results were presented in Fig. 4a and 4b. The extract possessed significant agglutination activity at 5 mg/ml especially with O^+ and B^+ blood groups. The extract exhibited medium effect at 2.5 mg/ml and low effect at 1.25 mg/ml against different blood groups. However, at concentration of 0.3125 mg/ml no sign of agglutination observed except with AB^+ and AB^- blood groups.

The toxicity of methanol extract of *S. chirata* was performed by brine shrimp bioassay at three concentrations 10, 100 and 1000 µg/ml and etoposide is used as standard drug. The toxic effect was evaluated in terms of death of larvae. These observations showed that *S. chirata* extract did not produce any lethality for larvae (Table 4).

Table 1. Identification of chemical constituents of *S. chirata*

Chemical composition						
Triterpenes	**Tannin**	**Saponin**	**Alkaloids**	**Carbohydrates**	**Proteins**	**Sterols**
+	+	-	+	+	-	+

+ = Present; - = Absent

Fig. 1a. Stem with Xylem Vessels

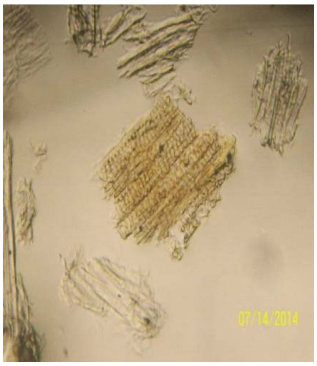


Fig. 1b. Spiral Xylem Vessel

Fig. 1c. Xylem Vessel with annular thickening

Fig. 1d. Raphides

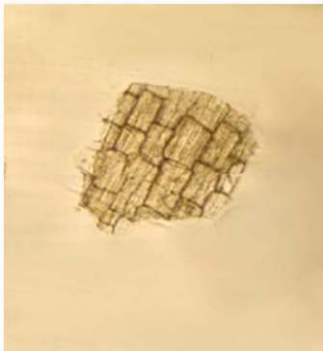


Fig. 1e. Epidermal cells

Fig. 1f. Oil cell

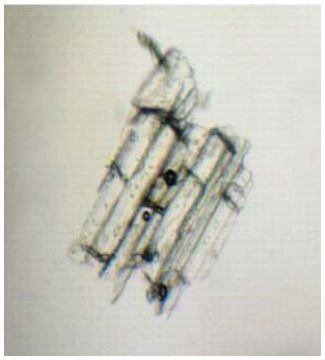


Fig. 1g. Parenchymatous cells

Fig. 1h. Sclerenchymatous cells

Fig. 1i. Fiber

Fig. 1j. Cork cell

Fig. 1k. Sclereid

Fig. 1l. Starch grains

Fig. 1m. Trichome

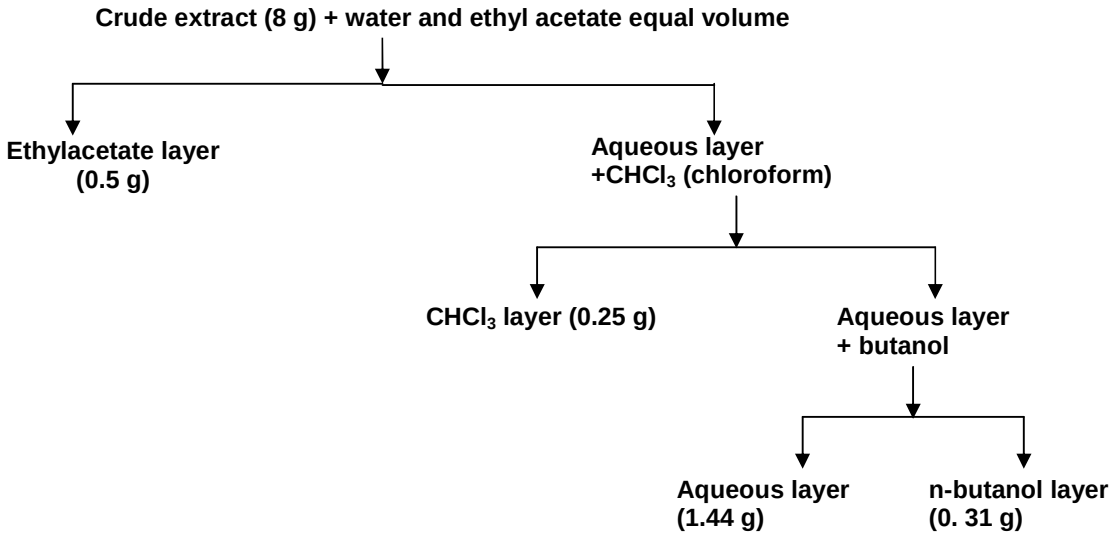
Fig. 1n. Stone cell

Fig. 1o. Tannin

Fig. 1(a-o). Microscopic examination of powder of *S. chirata* (Aerial parts)

Table 2. Fluorescence analysis of powder of *S. chirata*

S. no.	Protocol	Observations under		
		Ordinary light	UV, 254 nm	UV, 366 nm
01	Dry powder as such	Blackish green	Dark green with white spot	Light green with white spot
02	Powder treated with 1.0 N NaOH in MeOH	Green	Greenish black	Greenish black
03	Powder treated with 1.0 N HCl	Brownish black	Dark Green	Green
04	Powder treated with 1.0 N NaOH in H_2O	Dark green with black spot	Dark green with black spot	Blackish green
05	Powder treated with 50% HNO_3 aqueous	Dark brown	Dark green with black spot	Blackish green
06	Powder treated with 50% H_2SO_4 aqueous	Greyish black	Dark green	Greenish yellow



Scheme 1. Fractions of crude methanol extract of *S. chirata*

Table 3a. Thin layer chromatography of *Swertia chirata crude extract* and its fractions

Solvent system	Spray reagent	Test drug	Rf value of color spots at 254 nm					Rf value of color spots AT 366 nm				
			1	2	3	4	5	1′	2′	3′	4′	5′
Ethyl acetate-Methanol-Water (100: 16.5: 13.5)	Vanillin-Sulphuric acid	Crude extract	0.15	0.36	0.49	0.73	-	0.38	0.51	0.7	0.85	-
		Ethyl acetate	0.35	0.4	0.7	-	-	0.39	0.59	0.82	-	-
		Chloroform	0.17	0.4	0.86	-	-	0.36	0.77	-	-	-
		n-Butanol	0.13	0.26	0.38	-	-	0.2	0.38	-	-	-
		Aqueous	0.15	0.25	-	-	-	0.15	0.25	0.35	-	-

Table 3b. Thin layer chromatography of *Swertia chirata crude extract* and its fractions

Solvent system	Spray reagent	Test drug	Rf value of color spots at 254 nm					Rf value of color spots at 366 nm				
			1	2	3	4	5	1′	2′	3′	4′	5′
Chloroform -Methanol-Water (80: 20: 2)	Vanillin-Sulphuric acid	Crude extract	0.73	0.83	0.89	0.95	-	0.49	0.55	0.12	-	-
		Ethyl acetate	0.43	0.53	0.83	0.86	-	0.40	0.65	0.95	-	-
		Chloroform	0.73	0.84	0.95	-	-	0.60	0.69	0.84	-	-
		n-Butanol	0.85	0.93	-	-	-	0.75	0.81	0.92	-	-
		Aqueous	0.56	0.66	0.75	-	-	0.08	0.89	0.95	-	-

Table 4. Brine shrimp lethality bioassay of *S. chirata*

DOSE (µg/mL)	No. of shrimps	No. of survivors	LD_{50} (µg/mL)	Std. drug	LD_{50} (µg/mL)
1000	30	30			
100	30	30	—	Etoposide	7.4625
10	30	30			

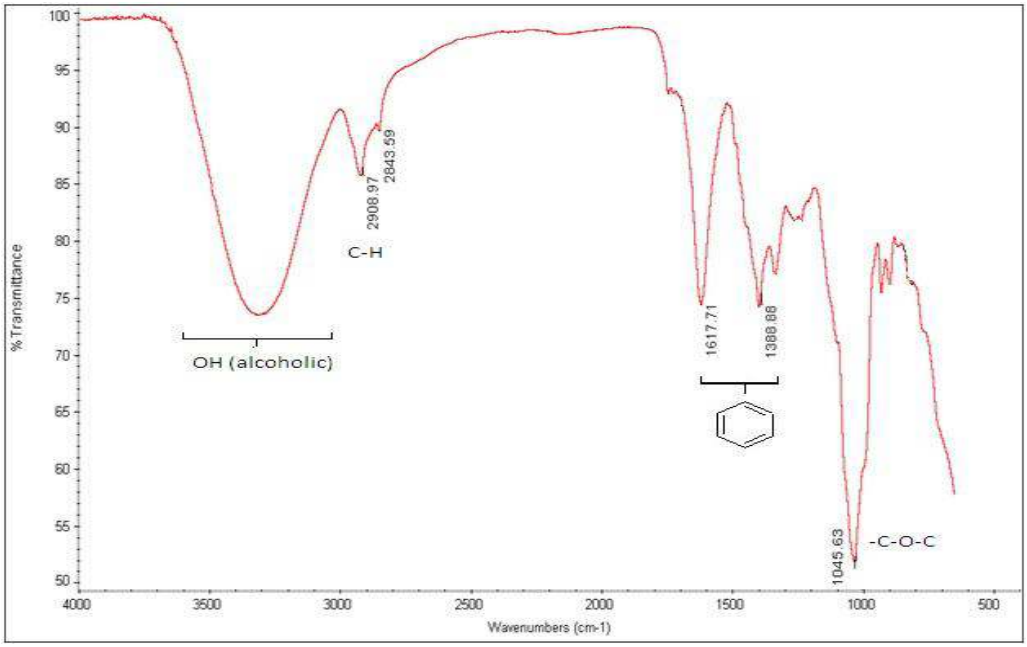


Fig. 2. FTIR of methanol extract of *S. chirata*

4. DISCUSSION

Swertia chirata has been used for various ailments. Nowadays adulterated herbal drugs were available in the market. Similarly a method has been developed for large scale production of S. chirata [20] therefore, for safety and efficacy of herbal formulation, standardization of raw material must be done. Microscopic characteristic of powder drug is useful to determine and establish the identity and quality of the plant. Beside microscopic examination preliminary phytochemical screening, Fluorescence analysis, TLC, FTIR and HPLC analysis were added authentication and quality control of *S. chirata*.

The powder microscopy of whole plant showed the presence of xylem vessels, raphides, parenchymatous cells, cork cells, oil cells, epidermal cells, sclencymatous cells, sclereid, trichomes, fibers, stone cells, starch grain. Rastogi *et al*. (2008) reported that *S. chirata* can be identified due to presence of two different types of trichomes, groups of angular shape parenchymatous cells with stomata, oil cells, pitted or without pitted parenchymatous cells, wavy outlined pitted cells and prismatic crystals [21]. Wavy parenchymatous and angular parenchymatous cells with stomata also seen.

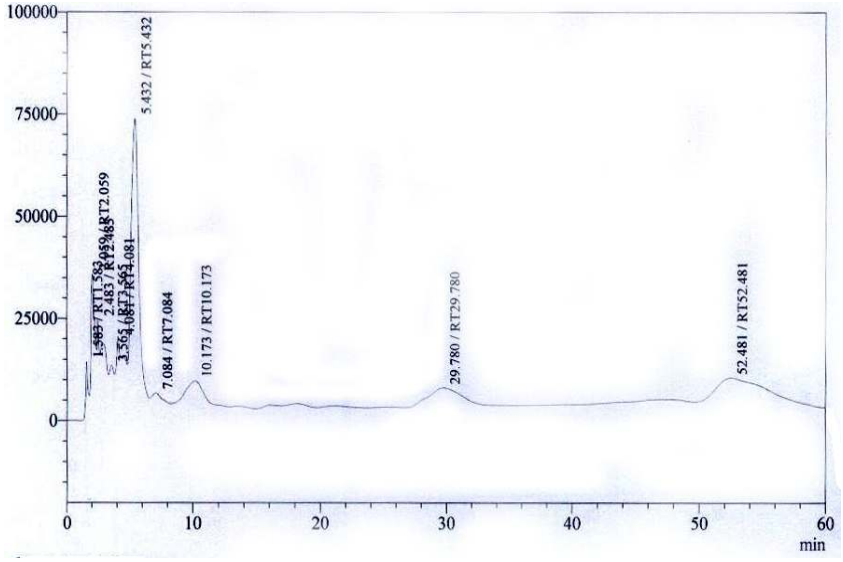


Fig. 3. HPLC of methanol extract of *S. chirata*

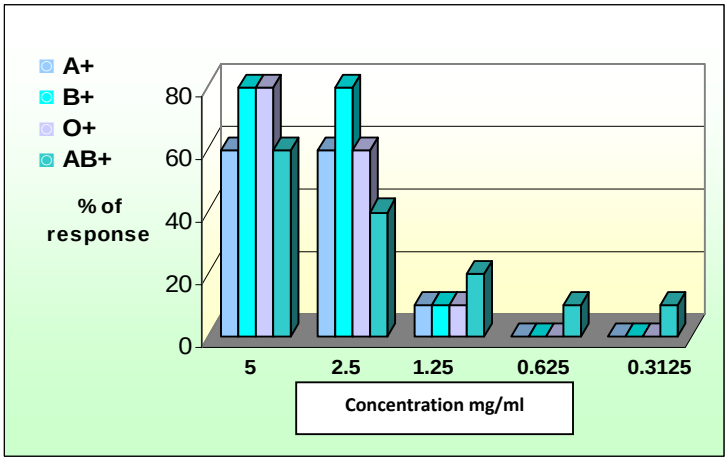


Fig. 4a. Agglutination activity of methanol extract of *S. chirata* against A^+, B^+, O^+, AB^+ blood groups

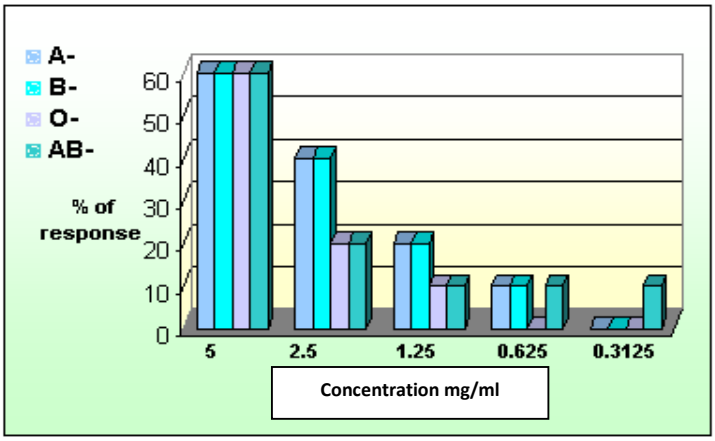


Fig. 4b. Agglutination activity of methanol extract of *S. chirata* against A^-, B^-, O^-, AB^- blood groups

Phytochemical screening of *S. chirata* was done with crude extract and powder which showed presence of triterpene, tannin, alkaloid, carbohydrate, sterols and absence of saponin and protein. The phytochemical tests of the extracts showed the presence of tannins, alkaloids, glycosides and flavonoids in methanol extract and only tannins and glycosides were present in aqueous extract [22]. The fluorescence analysis was done and observed under day light and UV light. These observations were helpful to find out the presence of any adulterants.

The fingerprint profile for evaluating the purity & quality of ayurvedic formulations, thus helpful as a reference in standards of pharmacopoeia Garg et al. [4]. Crude extract of *S. chirata* was fractionated into ethyl acetate, chloroform, n butanol and aqueous fractions. TLC technique has been applied for the separation of different compounds in fractions and crude extract of Swertia chirata. The number of spots showed that highest compounds present in methanol extract of plant.

In FT–IR spectrum of methanol extract of *S. chirata* showed the presence functional groups such as –OH, 2908.97 (>C-H), 1617.71-1388.88 (benzene ring) and 1045.63 (>C-O-C<). IR spectral study of the alcoholic extract of *S. chirata* by Latif and Rehman [23] also showed major characteristic peaks at 3448.93 and 2941.47(cm^{-1}). HPLC analysis revealed the presence of chemical constituents in methanol extract of *S. chirata*. These spectral analyses provide the authentication of crude extract of *S. chirata* along with powder drug microscopic diagnostic features.

Haemagglutination activity of crude extract of *S. chirata* showed mild protein denaturation at high concentration (5mg/ml). Traditionally *S. chirata* was used as a blood purifier and for treating many ailments [20], Literature also reported that it has a significant thrombolytic activity [24]. In addition, this activity provides knowledge about the dose response to an individual based on blood groups.

Cytotoxic screening is the preliminary investigation for formulation of anticancer drug. Brine shrimp bioassay was performed to investigate the toxicity of methanol extract of *S. chirata.* In Present study different concentrations of crude extract did not produce any lethality for larvae. LC_{50} values of rectified spirit extract of stem of the *S. chirata* and pure compound were found to be 80.50 µg/ml and 10 µg/ml respectively by Sultana and Ahmad [25]. According to Rahman et al. [26] ethanol extract of stem and leaf of *S. chirata* showed no significant cytotoxicity with LC50 >5000 µg.

5. CONCLUSION

S. chirata is a potential source of useful natural constituents. This study gives information about the authentication and presence of natural constituents in the crude methanol extract and the powder. Haemagglutination and cytotoxic results may help in dose/concentration adjustment for biological applications of *S. chirata*.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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