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Research Article

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Isolation and identification of amarogentin as an antihelminthic compound in *Swertia chirayta*

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ABSTRACT

In the ethno-pharmacological approach, local knowledge about the potential uses of the plants is very useful as compared to the random approach where indigenous knowledge is not taken into consideration. Compounds for different pharmacological activities have been isolated from plants. Plants are capable of synthesizing an overwhelming variety of low molecular weight organic compounds usually unique and complex in structure. Phytochemicals produced in plants are secondary compounds responsible metabolic activities and defense in purpose. In the present investigation, a significant biomarker, amarogentin was isolated from the gradient column fractionation of hydro-alcoholic extract of Swertia chirayta. The compound was obtained as a pale yellow powder after drying and was found to be an iridoid glycoside. The compound showed Rf value of 0.75 when compared to standard. The isolated compound showed the retention time of 6.78 minutes very much similar to that of the standard. The compound demonstrated paralysis as well as death of worms in a lesser time at 20 µg/ml compared to piperazine citrate and albendazole (15 mg/ml). The results showed the significant potency of amarogentin as a significant anti-helminthic.

Keywords: Swertia chirayta, amarogentin, anti-helminthic compound, hydro-alcoholic extract, glycoside.

INTRODUCTION

Plants are capable of synthesizing an overwhelming variety of low molecular weight organic compounds usually unique and complex in structure. Phytochemicals produced in plants are secondary compounds responsible metabolic activities and defense in purpose. Phytochemicals are produced by specific biochemical pathways, which occur inside the plant cells. At least 12,000 phytochemicals (secondary metabolites) have been isolated from plants, a number estimated to be less than 10 % of the total. Translation of ethnobotanical information for isolation and identification of phytochemicals is required for revealing the pharmacological status of the important phytochemicals [1]. These secondary metabolites functions in defense against predators and pathogens as allelopathic agents or attractants in pollination and seed dispersion. Plants with anthelmintic activity have been reviewed [2]. Anthelmintic activity of some plants has also been reported akin to that of sorghum *Aliium sativum, Zingiber officinale, Cucurbita mexicana* and *Ficus religiosa, Artemisia brevifolia, Calotropis procera Nicotiana tabacum* and *Butea monosperma* [2-8]. Previous study already revealed the anti-helminthic potency of some indigenous botanicals including *Swertia chirayta* [9]. The present study was thus carried out to isolate the potent anti-helminthic compound from hydro-alcoholic extract of *Swertia chirayta*.

EXPERIMENTAL SECTION

Chemicals and Reagents required

The chemicals and reagents used were of analytical grade and were procured from Ranchem and CDH. Standard anti-helminthic drugs (positive controls), viz. albendazole and piperazine citrate were used for comparative evaluation.

Collection of Plant material

The whole plant of *Swertia chirayta* (Chirayta) belongs to Gentianaceae family was selected for the study. The herbarium of plant material was taxonomically identified. The whole plant material was dried under shade and ground to form the fine powder

Preparation of Solvent Extracts

The powdered plant material was soaked in approximately 200 ml of hydro-alcoholic solvent (50 % v/v), on an electrical shaker for three hours at room temperature and then left to stand overnight. The solvent extract preparation was filtered in conical flasks using Whatmann filter paper No. 1. The filtrate was concentrated on a rotary evaporator at 50^oC to yield semi-solid masses. The extracts were stored in a refrigerator at 4^0C for further use.

Phytochemical screening of the extract

The portion of the dry extract was subjected to the phytochemical screening using the method adopted [10, 11]. Phytochemical screening was performed to test for alkaloids, saponin, tannins, flavanoids, steroids, sugars and cardiac glycosides [12].

Test for alkaloids

The 0.5 g of the plant extract was dissolved in 5 ml of 1% HCl and further kept in water bath for about 2 minutes. 1ml of the filtrate was treated with Dragendroff's reagent. Turbidity or precipitation was taken as indicator for the presence of alkaloids.

Test for tannins

About 0.5 g of the sample was dissolved in 10 ml of boiling water and will be filtered. Few ml of 6% $FeCl_3$ was added to the filtrate. Deep green colour appeared confirmed the presence of tannins.

Test for flavanoids

About 0.2 gm of the extract was dissolved in methanol and heated for some time. A chip of Magnesium metal was introduced followed by the addition of few drops of conc. HCl. Appearance of red or orange color confirmed the presence of flavanoids.

Test for saponin

About 0.5 g of the plant extract was stirred with water in the test tube. Frothing persists on warming was taken as an evidence for the presence of saponin.

Test for steroids

Salkowaski method was adopted for the detection of steroids. About 0.5 g of extract was dissolved in 3 ml of chloroform and filtered. To the filtrate, conc. $H_2 SO_4$ was added to form a lower layer. Reddish brown color was taken as positive for the presence of steroids ring [13].

Test for cardiac glycoside

About 0.5 g of the extract was dissolved in 2ml of glacial acetic acid containing 1 drop of 1% $FeCl_3$. This was under laid with conc. H_2SO_4. A brown ring obtained at the interphase indicates the presence of deoxy sugar. A violet ring appeared below the ring while in the acetic acid layer a greenish ring appeared just above ring and gradually spread throughout this layer.

Test for reducing Sugars

1ml each of Fehling's solutions, I and II was added to 2 ml of the aqueous solution of the extract. The mixture was heated in a boiling water bath for about 2-5 minutes. The production of a brick red precipitate indicated the presence of reducing sugars.

Isolation and identification of compound via chromatographic and spectroscopic techniques

Column chromatography

The hydro-alcoholic extract was subjected to silica gel column chromatography for the isolation of the phytoconstituents. An appropriate column sized 5 cm diameter and 50 cm length was used. The column was packed with Silica gel G 70 -325. It was washed with water and rinsed with acetone and then dried completely. Little of pure cotton was placed at the bottom of column with the help of a big glass rod. Solvent hexane was poured into the column upto ¾th level. Hydro-alcoholic extract was mixed with equal amount of graded silica gel until it became free flowing powder. When it reached a defined state it was slowly poured into the column containing hexane solvent with slight movement of stirring by glass rod to avoid clogging. Little cotton was placed on top of silica gel-extract mixture pack to get neat column pack. The knob at the bottom was slowly opened to release the solvent. The elution was done using hexane, ethyl acetate and methanol in different ratios like Hexane (100%- broad fraction 1), Hexane: Ethyl acetate (50:50- broad fraction 2), Ethyl acetate (100%- broad fraction 3), Ethyl acetate: Methanol (50:50 – broad fraction 4) and methanol (100%- broad fraction 5). All the five broad fractions were collected separately and subjected to TLC. The solvents were evaporated by rotary vacuum evaporator. Since there was no yield in the Hexane fraction (100%) and very less yield in broad fractions 2 and 3, fractions 4, 5 were selected and again subjected to sub column fractionation.

Conventional preparative TLC

Silica gel G was used as an adsorbent for thin layer chromatography (TLC). The plates were activated in hot air oven at 110°C for one hour. A quantity of the finely divided absorbing agent silica gel G was prepared by the absorbent with twice the weight of distilled water and the mixture was made homogeneous by vigorous shaking for 5 minutes, then it was applied to the glass plate in a thin and uniform layer by using a Stahl–type applicator or by means of a spreading device. The thickness of the applied layer was maintained at 2 mm to 4 mm and the plates were activated by being dried in a hot air oven, usually for 24 hours at 60°C. The plates were developed in the solvent, ethyl acetate: methanol: water (78:14:8 v/v/v). The selected fractions as resulted from column chromatography (fractions-4 and 5) were spotted along with some probable standards as a fine and tiny droplet on the TLC plate occupied with silica slurry coating. All the TLC separations were performed at room temperature and detection was carried out by UV light at 354 nm. The various samples showed different intensities of the respective compounds inferring the presence of these compounds in varying amounts in those samples. The visualized compounds from TLC were quantified by the GelQuant-NET software provided by Biochemlab Solutions Co. using reference compounds [14]. First calibration curves were prepared from standard marker solutions and peak areas in terms of pixels were plotted against the corresponding concentration. All seven crude methanol extracts were visualized for their respective marker compounds from TLC plates and pixel ratio for each band was calculated using GelQuant-NET software. The active fractions/ pure compounds were further scraped from the silica gel plate and eluted from the silica gel with ethanol. The active compounds were filtered through Millipore filters (0.45 µm and 0.22 µm) to remove the silica gel and this yielded more of compound(s) fraction.

High-performance liquid chromatography (HPLC)

HPLC analysis was performed using a Shimadzo LC- 2010 HPLC system (Kyoto, Japan), equipped with a Shimadzo LC 2010 UV-VIS detector with a thermostated flow cell and a selectable wavelength of 340 nm. The detector signal was recorded on a Shimadzo LC2010 integrator. The column used was C-18 block heating-type Shim-pack VP-ODS (4.6 mm interior diameter × 150 mm long) with a particle size of 5 µm. Mobile phase used was 0.5% v/v formic acid and acetonitrile at a flow rate of 1.2 ml/min and at column temperature 25°C. Injection volume was 20 µl of the diluted compound sample and detection of the chromatogram was carried in UV range. The complete procedure was performed both for isolated compound and standard. Both the chromatograms were interpreted by comparing the retention time (RT).

Fourier Transform Infrared (FTIR) studies

The IR spectrum of isolated compound was recorded in Roorkee Research and Analytical Laboratory Pvt. Ltd., Roorkee (Uttarakhand), India using a computerized FT-IR spectrometer (Perkin Co., Germany) in the range of 4400-400 cm^{-1} by the KBr pellet technique. The molecular structure and its homology with any reported compound (if any) was determined by probable structural units (PSUs) as determined by IR- spectra.

Anti-helminthic bioassay

Healthy adult Indian earthworms, *Pheretima postuma*, (Annelida, Megescolecidae) due to its anatomical and physiological resemblance with the intestinal roundworm parasites of human beings [15] were used in the study.

Assessment of Anti-helminthic activity

Anti-helminthic activity was assessed using earthworms by the reported methods with slight modifications [16]. Samples of the compound were diluted in 1 % N-saline (0.85 % Sodium chloride) solution for anti-helminthic activity. To obtain a stock solution, different working solutions of the extracts were prepared to get a concentration range of 05, 10 and 20 µg/ml. Six earthworms were soaked separately within the petridishes in hydro-alcoholic plant extracts within 5 groups at 0 time. The time (in minutes) of paralysis and death of earthworms were recorded after placing in the extracts. Paralysis is meant by the motility of the earthworms after their placement in the extracts and controls. The death time shows the non-motility/death of the worms.

Groups subjected for the study:

I Group: Vehicle/Negative Control: 1 % N-saline (0.85 % Sodium chloride)
II Group: Positive Control: Piperazine citrate (15 mg/ml)
III Group: Positive Control: Albendazole (15 mg/ml)
IV Group: Test: Isolated compound (amarogentin) of *Swertia chirayta* (Chirayta)- 5 µg/ml
V Group: Test: Isolated compound (amarogentin) of *Swertia chirayta* (Chirayta)- 10 µg/ml
VI Group: Test: Isolated compound (amarogentin) of *Swertia chirayta* (Chirayta)- 20 µg/ml

Statistical analysis

All the experiments were performed in triplicates and the data are reported as mean ± SD.

RESULTS AND DISCUSSION

Phytochemical screening

The hydro-alcoholic extracts of *Swertia chirayta* after screening for anti-helminthic activity were qualitatively detected for phytochemical screening. The results confirmed the presence of tannins, glycosides, alkaloids, flavanoids and reducing sugars in the hydro-alcoholic extract while saponin and steroids were found absent. The results are shown in **Table 1**.

Table 1: Phytochemical screening of the hydro-alcoholic extract of Swertia chirayta

Phytochemicals	Hydro-alcoholic extract of *Swertia chirayta*
Alkaloids	+
Tannins	+
Flavanoids	+
Saponin	_
Steroids	_
Cardiac glycoside	+
Reducing sugars	+

**+, present; -, absent*

Column and Thin layer chromatography

The pure compound was isolated from the hydro-alcoholic solvent by gradient column chromatography and further identified under UV light on TLC plate. The compound showed similar Rf value (0.75) very much similar to that of standard compound, amarogentin (0.77). The results of TLC are shown in **Figure 1**.

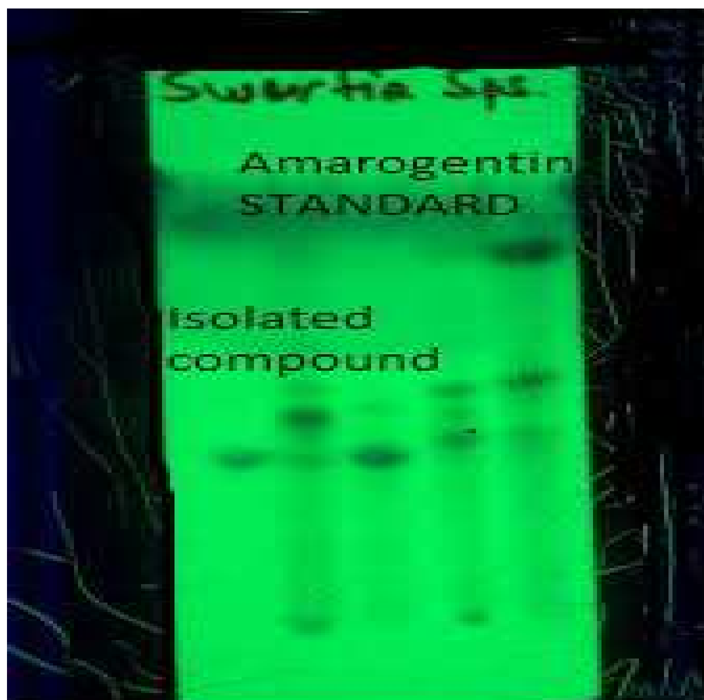

Figure 1: TLC of the isolated compound (amarogentin) of *Swertia chirayta* after visualization under UV light

HPLC assay

Further, the isolated compound was identified by HPLC purification studies after interpreting with that of standard compound. The isolated compound showed almost similar retention time (6.788 minutes) as that of the standard amarogentin (6.752 minutes). The results of HPLC are shown in **Figure 2 (a)** and **(b)**.

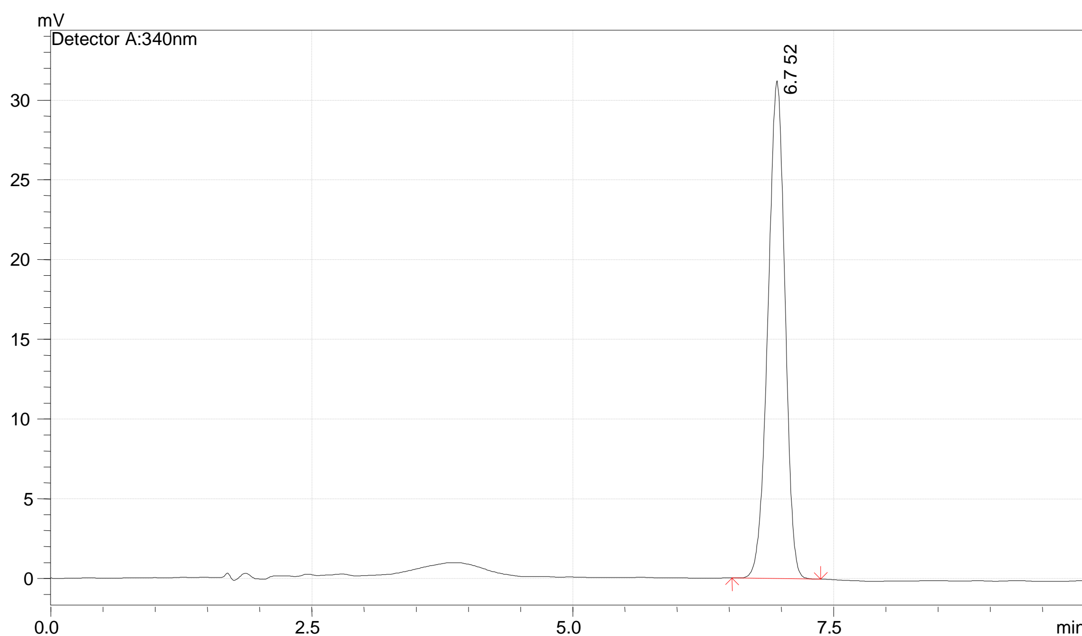

Figure 2 (a): HPLC chromatogram of the standard (amarogentin)

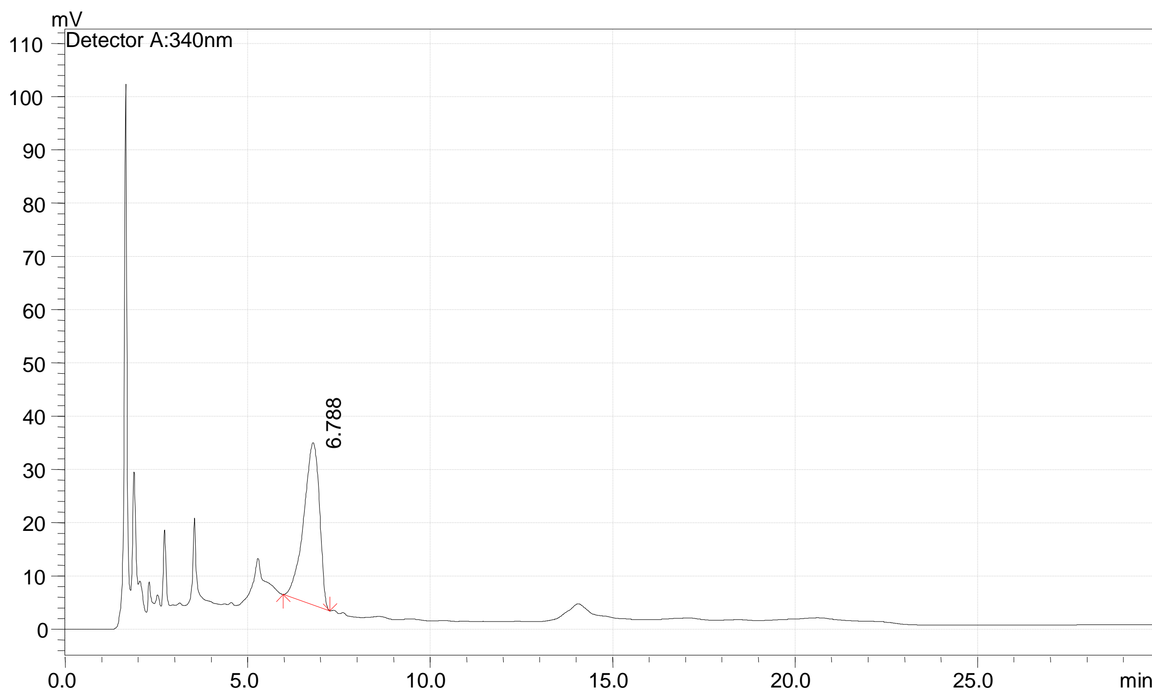

Figure 2 (b): HPLC chromatogram of the isolated compound from the hydro-alcoholic extract of *Swertia chirayta*

IR spectroscopy

The FT-IR spectra of the isolated compound, amarogentin was isolated from hydro-alcoholic extract of Swertia chirayta at 4000-400 cm^{-1}. The determinations of probable structural units (PSUs)/functional groups of the isolated compound were interpreted with that of standard. The results of FT-IR spectra of the isolated compound and standard are shown in **Figure 3** and **Table 2**.

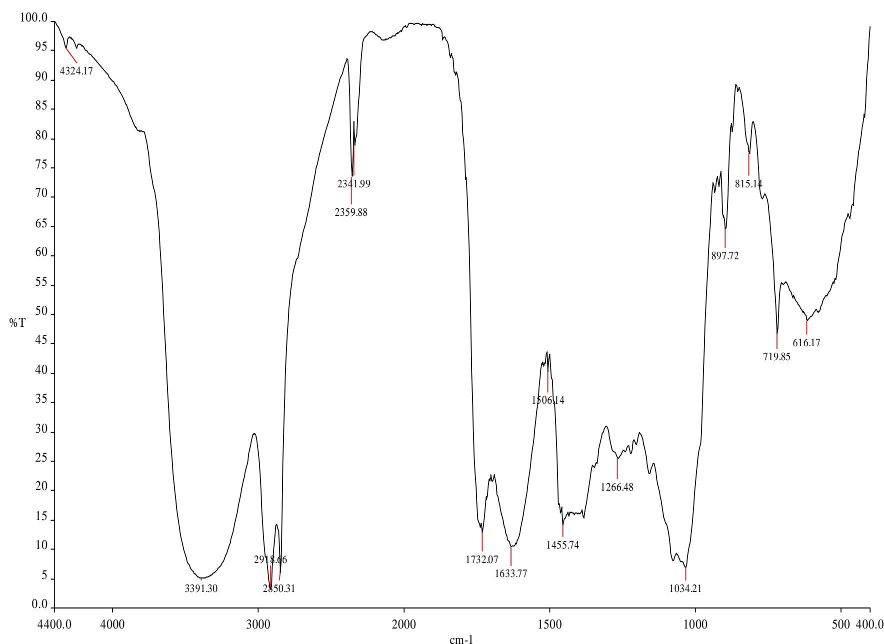

Figure 3: IR- spectra of isolated compound (amarogentin) from *Swertia chirayta*

Table 2: Functional groups of the isolated compound (amarogentin) as recorded by FT-IR spectra

Wave number (cm^{-1})	Functional group predicted
616.7	Aliphatic cyclic ether
719.85	Aromatic C-H (out of plane band)
897.72	Aromatic C-H stretch
1034.21	C-O stretch
1266.48	Anhydride C-O stretch
1633.77	C=C stretch in aromatic nuclei
1732.07	Ester C=0 stretch

Anti-helminthic assay

For the first time, amarogentin was proved as an anti-helminthic molecule from *Swertia chirayta* in the current study. The compound, amarogentin was scrapped off from silica gel coated TLC plate and diluted in N-saline. The compound demonstrated paralysis as well as death of worms in a less time as compared to piperazine citrate and albendazole (15 mg/ml). The results of anti-helminthic activity are recorded in **Table 3** and **Figure 4.** The results were found to be significant at p<0.5.

Table 3: Anti-helminthic activity of plant extracts and standard drugs

Group	Treatment	Concentration (mg/ml)	Paralysis time (minutes)	Death time (minutes)
1.	Vehicle: 1 % N-Saline	--	No activity	No activity
2.	Positive Control: Piperazine citrate	15 mg/ml	23.3±0.6	27.3±0.5
3.	Positive Control: Albendazole	15 mg/ml	25.6±0.6	29.5±0.5
4.	Isolated compound (Amarogentin)	05 µg/ml	No activity	No activity
		10 µg/ml	No activity	No activity
		*20 µg/ml	12.54±0.2	15.12±0.2

**Level of significance-p<0.5*

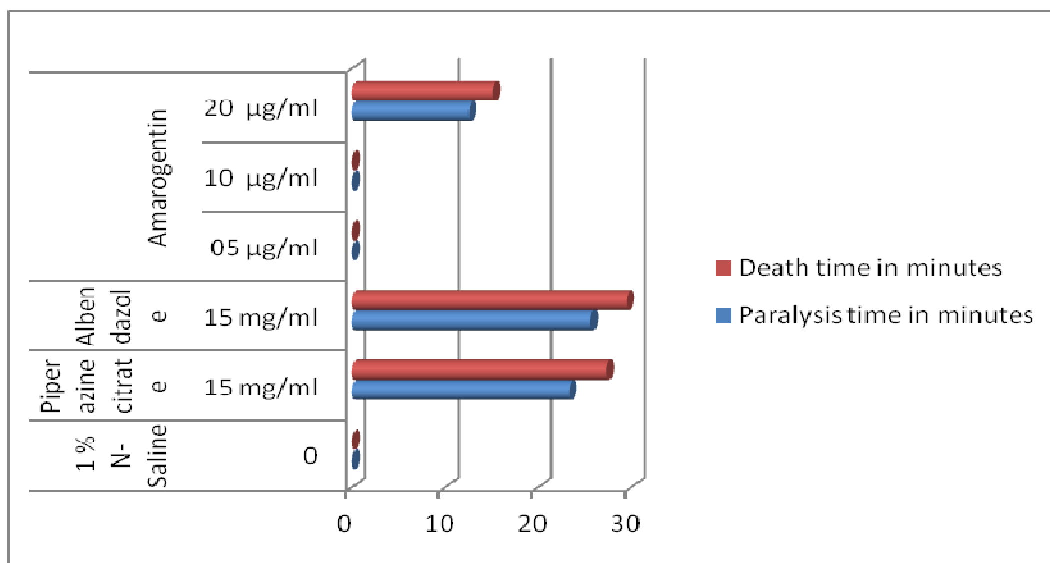

Figure 4: Anti-helminthic activity of compound, amarogentin isolated from *Swertia chirayta*

CONCLUSION

The present study suggests that, *Swertia chirayta* (Chirayta) is a source of valuable anti-helminthic agents apart from other pharmacological constituents. The current study leads to the isolation and identification of novel anti-helminthic molecule (Amarogentin) from Chirayta. The present study stresses on the exploration of diverse novel molecules from different plants which can be utilized for different pharmacological activities. The present study thus concluded that amarogentin can be utilized as one of the constituent in preparation of anti-helminthic drug or can be independently utilized as potent anti-helminthics.

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