Academic Sciences International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 4, Suppl 5, 2012

Research Article

EXTRACTION AND EVALUATION OF INDOLE ALKALOIDS FROM *RAUWOLFIA SERPENTINA* FOR THEIR ANTIMICROBIAL AND ANTIPROLIFERATIVE ACTIVITIES

SARIKA R. DESHMUKH*, DHANASHREE S. ASHRIT AND BHAUSAHEB A. PATIL

Department of Biotechnology, New Arts, Commerce and Science College, Ahmednagar, India. Email: sarikadeshmukh12@gmail.com

Received: 03 Aug 2012, Revised and Accepted: 16 Oct 2012

ABSTRACT

Rauwolfia serpentina L. Benth Kurz, commonly called Sarpagandha, is mainly known for its phytochemical reserpine, which was widely used as an antihypertensive drug and a powerful sedative; and hence it has important medicinal values. The present study was deal with phytochemical analysis of *Rauwolfia serpentina* and their different biological activities. The evaluation of presence and absence of indole alkaloids were carried out by TLC and HPLC methods. The methods also focused on the quantitative and qualitative determination of indole alkaloids. This work represents a first report of antiproliferative activity of *Rauwolfia serpentina*.

Plant leaves and roots were extracted by using solvent like ethanol and the quantity of crude extracts obtained was 11.27%. The evaluation of indole alkaloids were done by using the methods like TLC and HPLC that indicated the presence of four different indole alkaloid derivatives like ajmalicine, ajmaline, yohimbine and reserpine in root extract of *Rauwolfia serpentina.* Further quantitative determination of *Rauwolfia* alkaloids was carried out by spectrophotometric analysis which resulted that Ajmalicine content was greater in leaf extract where as reserpine, ajmaline and yohimbine were greater in root extract of plant. In addition to this, antimicrobial activity was performed with the help of well diffusion assay, MIC and MBC. This study reported that root extract was good against the tested *S. typhii* and was proved to be the better option for further drug development. Finally, antiproliferative activity of ethanolic root and leaf extract of *R. serpentina* was checked on cancerous HeLa cell line which reported that the leaf extract was found to be more effective with the IC_{50} value of 196 µg/ml.

Keywords: *Rauwolfia serpentina,* TLC, HPLC, Antimicrobial activity (MIC and MBC) and Antiproliferative activity.

INTRODUCTION

Medicinal plants are those whose roots, leaves, seeds, bark or other constituent possess therapeutic, tonic, purgative or other pharmacologic activity when administered to higher animals. Plants are used medicinally in different countries and are a source of many potent and powerful drugs. The use of these herbal medicinal plants to treat human diseases has its roots in prehistorical times. Medicinal plants are used by 80% of the world's population as the only available medicines especially in developing countries. A wide range of medicinal plant part is used for extract as raw drug and they possess varied medicinal properties[1]. It is now clear that, the medicinal value of these plants lies in the bioactive phytochemical constituents that produce definite physiological effects on human body. These natural compounds formed the base of modern drugs as we use today[2]. Herbal medicines are becoming popular in modern world as people resort to natural therapies. Natural products isolated from higher plants and microorganisms have been providing novel clinically active drugs[3].

Tropical plant *Rauwolfia serpentina* L. Benth Kurz commonly known as Sarpagandha, is a small, woody, perennial medicinal shrub. It is a medicinally famous herb in Ayurveda, Siddha, Unani and Western system of medicines[4]. The International Union for the Conservation of Nature and Natural Resources (IUCN) has assigned an endangered status to *R. serpentina*. It has been reported to contain 50 indole alkaloids that are mainly localized in the root bark. Among these alkaloids, reserpine, yohimbine, serpentina, deserpidine, ajmalicine, ajmaline, recinnamine, ajmalidine, sarpagine, raucaffricine, etc. are the rich source found in root of *R. serpentina*. The *Rauwolfia* species is mainly known for its phytochemical reserpine, which was widely used as an antihypertensive drug. Its alkaloid called reserpine is a powerful sedative; and hence it has important medicinal values. The root extract of this plant is very useful in disorders of gastro intestinal tract viz. diarrhea, dysentery and cholera and colic. The alkaloids found in roots are employed for treatment of several diseases such as heart disorders and even cancers. Leaves are used in removal of opacities of cornea. It is used as a fever relieving medicine. Root part is mainly used, which contains resin and starch.

Thus, the present study was deal with phytochemical analysis of *Rauwolfia serpentina* and their different biological activities which were carried out by using methods like extraction, TLC and HPLC. These methods focused on the presence and absence of indole alkaloids and their quantitative and qualitative determination.

MATERIALS AND METHODS

Plant material

The plant of *Rauwolfia serpentina* was collected from Mahatma Phule Krishi Vidyapeeth (MPKV) Rahuri from Ahmednagar district of Maharashtra, State of India. The roots of *Rauwolfia serpentina* were collected from a local herbal drug store. All the botanical aspects of the whole plant were studied in detail. After the samples were identified and authenticated by the scientists of the institution and the voucher specimens were deposited in our laboratory collections.

Chemicals

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Acetonitrile (HPLC grade), Phosphate buffer, Basal Salt Solution medium (BSS), Dimethyl Sulfoxide (DMSO), Phosphate Buffer Saline (PBS), potassium dihydrogen phosphate, Orthophosphoric acid, ethanol, 1,10- phenanthroline, ferrous chloride, ferric chloride and antibiotics were purchased from Sigma, Merck and Qualigen. All chemicals and solvents of analytical grade were used.

Preparation of extract

Ten gram powder of leaf and root were mixed in 40 ml of ethanol in 250 ml of conical flask and was kept at 25 ºC for 12hrs. After 12 hours, the suspension was filtered through Whatman's filter paper and collected in large Petri plates. These were allowed to dry completely in water bath set at 40 ± 0.2ºC for 30 min. Dried extracts were scraped out by using scalpels and was collected in pre weighed vials separately. Extracted powders were made available to use as per requirements by resuspended in ethanol every time. The quantity of crude extracts obtained by this method was 11.27%.

Phytochemical Screening

The powdered samples of leaf and root cultures of *Rauwolfia serpentina* were screened for phytochemical constituents using standard procedures of analysis[1, 2, 4, 5].

Qualitative Analysis

Thin Layer Chromatography

The qualitative analysis of major groups of indole alkaloid derivatives of *Rauwolfia serpentina* was initially done by thin layer chromatography (TLC) technique on preparative silica gel (silica gel-

60). Mobile phase or solvent system used for alkaloid estimation was chloroform: methanol (97: 3). Bands were visualized by spraying Dragendorff's reagent uniformly over the plates or also observing the plates under UV- transilluminator. Identification was done on the basis of color of bands and their Rf values under UV light6, 7.

High Performance Liquid Chromatography

HPLC of crude extract of *Rauwolfia serpentina* plant was carried out by Lichrosorb C – 18 (25 X 0.5cm 10A) column. Mobile phase used was Acetonitrile: Phosphate Buffer (35:65). 20 µl of the volume was injected with the flow rate of 1ml/min. Detection wavelength was 268 nm and the method was carried out at ambient temperature. Isocratic method was used for obtaining chromatogram of metabolites of *Rauwolfia serpentina*[8, 9].

Analysis of alkaloids extracted from *Rauwolfia serpentina*

Aliquots of each alkaloid sample (root and leaf) were transferred into the test tubes in duplicates. To each of these test tubes, 1ml of $FeCl_3$ solution and 1ml of 1, 10 -phenanthroline solutions was added and final volume was marked up to 8 ml. Out of 5 test tubes, 2 tubes were containing root extract and other 2 were with leaves extract. 1 test tube was kept as a blank without sample extract. These tubes were then placed in water bath maintained at 70 ± 2ºC for 30 min. The absorbance of orange- red colored product was measured at 510 nm against the blank solution by UV-Visible spectrophotometer (Systronic Visiscan 167) [10].

Test organisms and preparation of inoculums

The test microorganisms used in this study (bacteria: *Escherichia coli. Bacillus subtilis, Salmonella typhi* and *Staphylococcus aureus*) were obtained from the Department of Microbiology. The bacterial isolates were first subcultured in a nutrient broth and incubated at 37°C for 18 h.

Antibacterial activity

The antibacterial activity of the crude extracts was determined in accordance with the agar-well diffusion method. The bacterial isolates were first grown in a nutrient broth for 18 hours before use. Two hundred micro liter of the standardized cell suspensions were spread on a Mueller-Hinton agar (MHA). Wells were then bored into the agar using a sterile 6 mm diameter cork borer. Approximately 40 µl of the crude extract of 10 mg/ml were introduced into the wells, plates were then incubated in refrigerator for about 2 hours to allow the diffusion of solution in the medium. Then these plates were incubated at 37°C in incubator. Controls were set up in parallel using the solvents that were used to dissolve the extract. The plates were observed for zones of inhibition after 24 hours. The effects were compared with those of streptomycin and ampicillin at a concentration of 1 mg/ml and 10 µg/ml respectively. The zone of inhibition was measured including the diameter of the bore. The results were recorded for evaluation of antimicrobial activity.

Minimal Inhibitory Concentration (MIC)

After antimicrobial activity, the extracts that showed positive results and good response were selected for further studies for the determination of MIC. 16 hours bacterial cultures were diluted with sterile saline solution (0.85% sodium chloride) to achieve an inoculums size of approximately 10^6 colony forming unit/ml. A serial dilution was carried out to give final concentration between 1, 5, and 10 to 100 mg crude extract per ml. The tubes were inoculated with 20 µl of the bacterial suspension per ml nutrient broth, homogenized and incubated at 37 °C for 24 hours. The minimum inhibitory concentration (MIC) value was determined as the lowest concentration of the crude extract in the broth medium that inhibited the visible growth of the test microorganism. After 24 hours of incubation, minimum inhibitory concentration (MIC) of each sample was determined by measuring the optical density in the spectrophotometer at 600 nm, comparing the sample readout with the non inoculated nutrient broth as reference[11].

Minimum bactericidal concentration (MBC)

After minimum inhibitory concentration (MIC) determination of the alkaloids, an aliquot of 10µl from all tubes in which no visible bacterial growth was observed were seeded in Nutrient Agar (NA) plates. The plates were then incubated for overnight at 37°C. The minimum bactericidal concentration (MBC) endpoint is defined as the lowest concentration of antimicrobial agent that kills >99.9% of the initial bacterial population where no visible growth of the bacteria was observed on the nutrient agar (NA) plates.

MTT assay

The MTT assay was used to analyze the antiproliferative activity of leaf and root extracts of *Rauwolfia serpentina* on human cervical cancer cell line, HeLa. Small aliquots of 250 µl of culture medium were taken in sterile vials. Then 50 µl of different concentrations of crude extracts (50, 100, 150, 200µg/ml of ethanol) were added. Then these vials were incubated at 37°C for 48 hours in CO_2 incubator. The vials were centrifuged and supernatant was discarded. Then 600 µl of fresh BSS media and 30 µl of MTT working solution were added. Vials were then incubated for 1 to 4 hours. Small aliquots of 450 µl of solubilization solution (usually dimethyl sulfoxide, an acidified ethanol solution or a solution of detergent sodium dodecyl sulphate in dilute hydrochloric acid) were added. The vials were then mixed properly to dissolve the formed fromazan crystals and absorbance was measured at 595 nm on UV- visible spectrophotometer. Finally, the 50% reduction in cell number or IC_{50} was estimated.

Statistical analysis

All experiments were conducted in triplicate and statistical analysis was done by using the MS Excel (CORREL Statistical function) and Graphpad Prism 4 softwares. The data were presented as mean ± SD.

RESULTS

Phytochemical Screening

The results of phytochemical screening of leaf and root extracts of *Rauwolfia serpentina* is presented in table 1. Qualitative tests for carbohydrates, free reducing sugars, alkaloids, saponnins, tannins, flavonoids and starch soluble compounds were carried out in order to know the presence of primary and secondary metabolites in these crude extracts of the plant.

Table 1: Phytochemical analysis of crude leaf and root extracts of *Rauwolfia serpentina.*

Secondary metabolites	*Rauwolfia serpentina* Root extract	*Rauwolfia serpentina* Leaf extract
Molisch's test	-	+
Barfoerd's test	-	-
Fehling's test (free reducing sugar)	-	+
Fehling's test (combined reducing sugar)	-	-
Tannin's test	+	+
Liebermann- burchard test	-	-
Saponin's test	+	+
$FeCl_3$ test for flavonoids	-	-
NaOH test for flavonoids	+	+
Mayer's test for alkaloids	+	+
Starch soluble test	+	+
Phlobatannins test	-	-

Qualitative analysis

Thin Layer Chromatography

The qualitative analysis of alkaloid was done by Thin Layer Chromatography (TLC). In TLC, the qualitative analysis of alkaloids was done on preparative silica gel plates using specific solvent systems for secondary metabolite's group. When the alcoholic extracts of leaf and root of *Rauwolfia serpentina* were subjected to the solvent system chloroform: methanol (97: 3) both the samples showed fluorescent green and blue bands on preparative silica gel plates under ultraviolet light indicating the presence of various alkaloid derivatives. Bands of root extracts were more prominently seen as compared to the bands of leaves extract (fig 1). Calculated Rf values were found to

be very close with standard Rf values which indicated the presence of different indole alkaloid derivatives which might indicate the presence of ajmaline, ajmalicine, yohimbine and reserpine.

Fig. 1: TLC showing bands of *R. serpentina* indole alkaloids from root and leaf extracts at different Rf values.

High Performance Liquid Chromatography

HPLC method was carried out by providing all the suitable experimental conditions and the peaks from root and leaf crude extract were obtained. The results obtained by HPLC are shown in figure 2 and 3. HPLC of root and leaf extract was performed by Isocratic method. Acetonitrile: Phosphate Buffer (35:65) was the mobile phase used for detection of indole alkaloids from root and leaf sample of *R. serpentina* and the analysis was done at 268 nm. Various numbers of peaks were observed in the HPLC chromatogram that revealed the presence of different indole alkaloids. Some of the peaks were identified on the basis of retention time (Rt). In crude leaf extract, 11 peaks were observed at different retention time. The crude leaf extract had shown a peak at 7.01 min that indicated the presence of ajmaline at Rt 7.0 minutes. (Fig 2)

In crude root extract, 12 peaks were observed at different retention time at same conditions. Out of these 12 peaks, the two peaks which were obtained at Rt 8.18 min and 13.26 min indicated the presence of reserpine and ajmalicine respectively at exactly same conditions. (Fig 3)

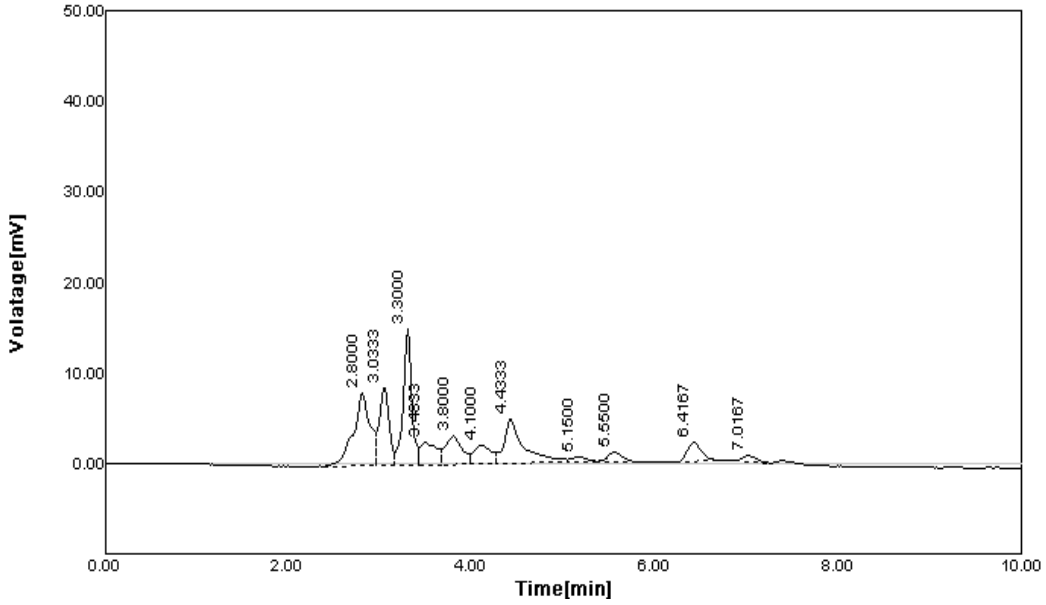

Fig. 2: HPLC of crude leaf extract of *R. serpentina* showing peaks at different retention time (Rt).

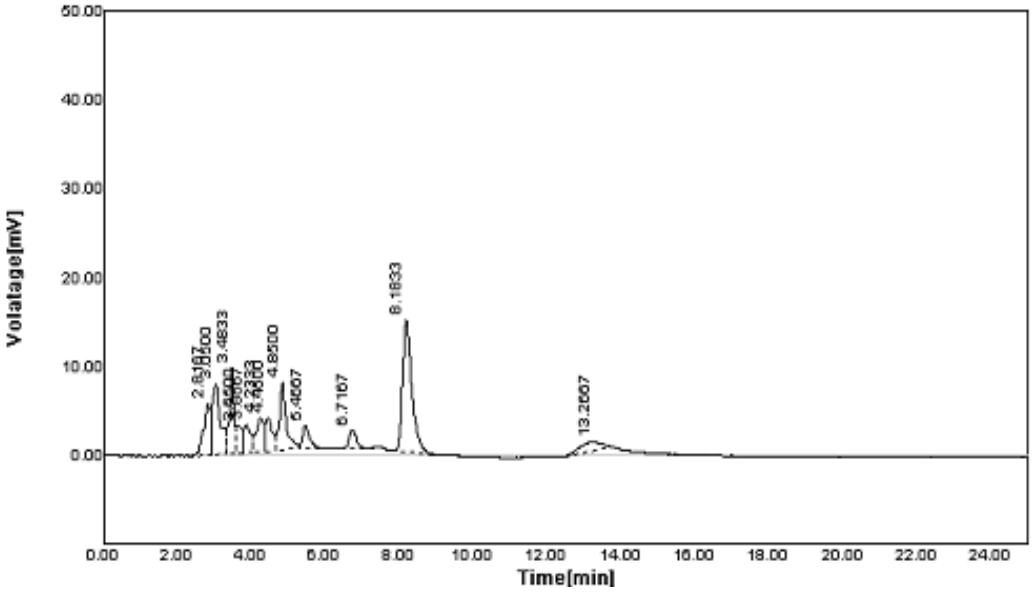

Fig. 3: HPLC of crude root extract of *R. serpentina* showing peaks at different retention time (Rt).

Quantitative Analysis of *Rauwolfia* alkaloids

Alkaloid content of *Rauwolfia serpentina* was measured by using spectrophotometric analysis. The absorbance was measured at 510 nm on visible spectrophotometer (Systronics Visiscan 167). In this assay, four indole alkaloids were measured viz. reserpine, ajmaline, ajmalicine and yohimbine.

The root as well as leaf extract showed the presence of all four indole alkaloids. The leaf extract showed the presence of reserpine, ajmalicine, ajmaline and yohimbine as 0.880, 0.753, 0.485 and 0.537 respectively. However, the root extract showed the presence of these all four indole alkaloids as 0.955, 0.440, 0.817 and 0.584 respectively. Ajmalicine content was found to be 0.753 mg/g in leaf extract which was more than the root extract of *R. serpentina* whereas the quantity of reserpine, ajmaline and yohimbine was more in root extract as compared to leaf extract (fig 4).

Antibacterial activity

Antibacterial activity for root and leaf extract of *R. serpentina* was examined against four bacterial samples like *S. Typhii, S. aureus, E. coli and B. subtilis* for antibacterial potential of the extracts. The antibacterial activities of the ethanolic extracts were compared favorably with that of two standard antibiotics (streptomycin and ampicillin). Zone of inhibitions showed by root extract were better in size than that of leaf extract as the zone of inhibitions found in leaf extract was too small when compared with zone size of root extract and standard antibiotics. This study reported that crude root extract of Rauwolfia serpentina showed an efficient activity of 22.5 ± 2.5 mm against *S. typhii* bacteria (table 2).

Minimal Inhibitory Concentration (MIC)

After the antibacterial screening assay, the extracts that showed positive results were used for further studies for the determination of MIC. So as per the results of antibacterial activity, MIC of only root extract was examined as it showed significant antibacterial activity. MIC of root extract showed the gradual decrease of optical density with increase in concentration of extract. MIC of root extract of *R. serpentina* was observed at 90 mg/ml for *E. coli* and *S. aureus,* 80 mg/ml for *B. subtilis* whereas it was obtained at 40 mg/ml for *S. typhii* when compared with the optical density of control which was 2.49 (Table 3).

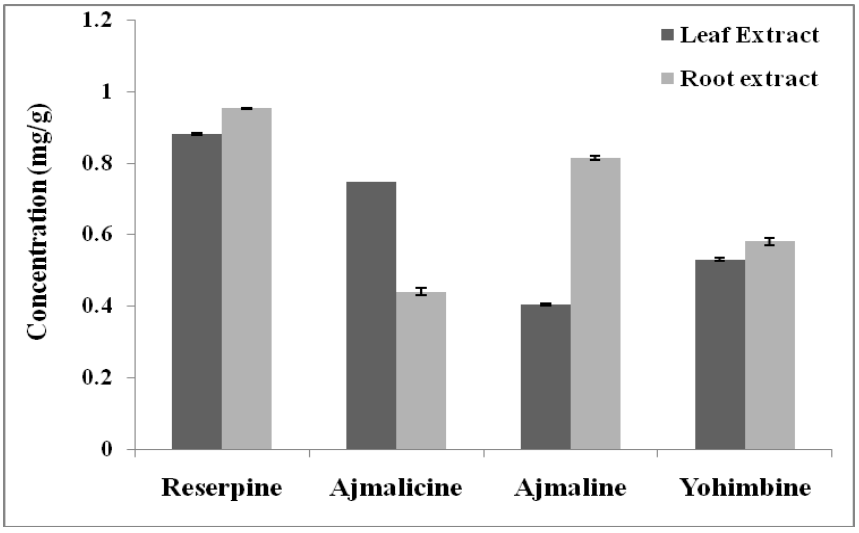

Fig. 4: Spectrophotometric analysis of indole alkaloids in leaf and root extracts of *Rauwolfia serpentina*.

Table 2: Antibacterial activity in Diameter of Inhibition Zone of *Rauwolfia serpentina* leaf and root extracts against test microorganisms.

Organisms	Diameter of Zone of Inhibition (mm)			
	Root sample	Leaf sample	Streptomycin	Ampicillin
E.coli	15.5 ± 0.5	7.4	36	35
S. Typhii	22.5 ± 2.5	10.1	42	35
S. aureus	13.5 ± 3.5	9.0	29	25
B. subtilis	17.5 ± 0.5	9.5	37	25

Table 3: MIC of *Rauwolfia serpentina* root extract

Pathogens	1 mg/ml OD	5 mg/ml OD	10 mg/ml OD	20 mg/ml OD	30 mg/ml OD	40 mg/ml OD	50 mg/ml OD	60 mg/ml OD	70 mg/ml OD	80 mg/ml OD	90 mg/ml OD	100 mg/ml OD
E. coli	2.14	2.12	1.86	1.72	1.63	1.28	0.74	0.57	0.55	0.54	**0.46**	0.54
S. typhii	2.10	1.95	0.82	0.78	0.77	**0.55**	0.60	0.62	0.75	1.99	2.36	2.61
S. aureus	2.20	2.13	1.14	1.24	1.33	1.58	1.01	0.94	0.80	0.78	**0.63**	0.94
B. subtilis	1.92	1.76	1.01	0.97	0.76	0.64	0.58	0.57	0.40	**0.37**	0.48	0.51

Minimum bactericidal concentration (MBC)

The MBC of the plant extracts was determined by using nutrient agar plates. The crude root extract concentrations that showed maximum inhibition in MIC assay were used for minimal bactericidal concentration (MBC) assay. The concentrations obtained were 90 mg/ml, 80 mg/ml and 40 mg/ml which were compared with control nutrient agar plates. Nutrient agar plates with respective concentrations of *S.typhii* and *E.coil* showed no colonies whereas *B. subtilis* and *S. aureus* showed few numbers of colonies between 10 to 12. The assay concluded that nutrient agar plates with sample showed very good results with lowest minimal

inhibitory concentration i.e. with lowest number of the colonies when compared with the nutrient plates with control which showed vigorous growth of bacteria.

Antiproliferative activity

Antiproliferative activity of crude extract of *Rauwolfia serpentina* was evaluated on HeLa cell line which was originated from cervical carcinoma. When cells were treated for 24 hours with various concentrations of crude extracts of leaf and root, the relative cell survival progressively decreased in dose-dependent manner. The crude leaf extract had shown 51.25% inhibition of cells whereas root extract had shown inhibition up to 49.27%. Among the two crude extracts of *R. serpentina*, the leaf extract was found to be more effective with the IC_{50} value of 196 µg/ml. (fig 5)

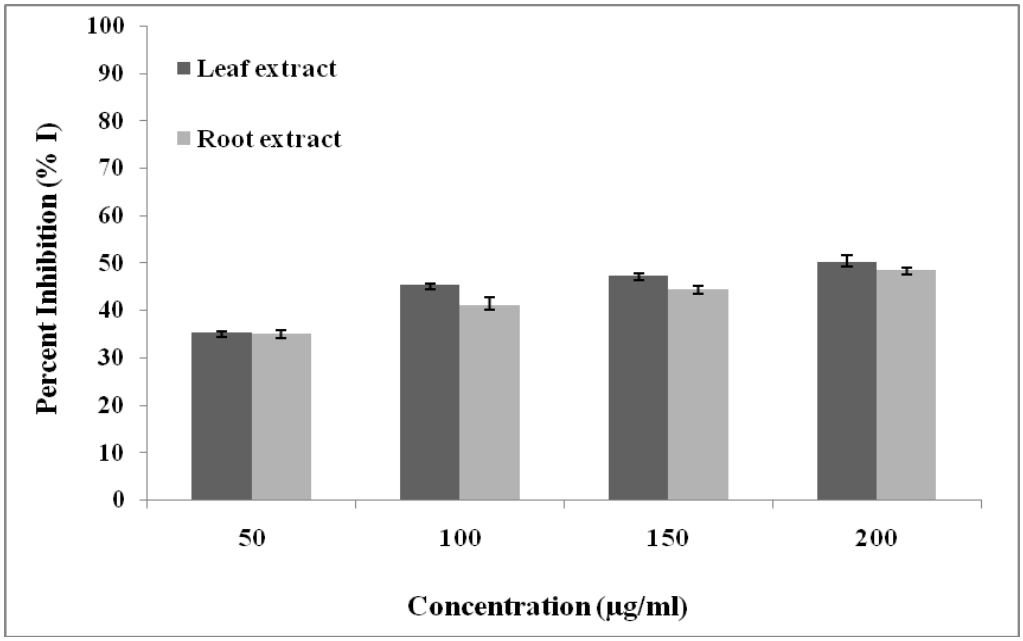

Fig. 5: Cytotoxicity assay showing percent inhibition of leaf and root crude extracts of *Rauwolfia serpentina* on HeLa cell line.

DISCUSSION

The preliminary phytochemical screening was done by some standard chemical tests. These classes of compounds such as carbohydrates, tannins, saponins, flavonoids, alkaloids and starch soluble compounds are present in the plant extracts used. Presence of saponins and flavonoids like compounds showed the justified use of extracts from *R. serpentina* plant extract. The study suggested that pure isolated alkaloids and their synthetic derivatives can be used as basic medicinal agents for their analgesic, antispasmodic effects[12].

Alkaloid analysis by TLC showed the presence of four different indole alkaloid derivatives in root extract of *R. serpentina.* Leaves of plant were found to contain very low amount of these indole alkaloid derivatives after the detection by TLC. The report of HPLC analysis of *R. serpentina* plant samples showed the higher presence of indole alkaloid contents in the root extract. Leaf extract also detected the presence of those alkaloids which were present in very low amount.

From the spectrophotometric analysis alkaloid content of *Rauwolfia serpentina* was measured which reported that Ajmalicine content was more in leaf extract as compared to root extract of *Rauwolfia serpentina* where as ajmaline, reserpine and yohimbine were found to be more in root extract as compared to leaf extract.

The antibacterial activities of ethanol extracts showed good response on all the tested micro organisms. Zone of inhibitions obtained in root extract were better in size than that of leaf extract. Although gram- negative bacteria tend to have higher intrinsic resistance to most antimicrobial agents, in spite of this, impressive activity against gram-negative bacteria was observed with ethanolic crude root extract of plant (*S. typhii* 22.5 ± 2.5 mm).

Further the results obtained in MIC assay were in dose dependent manner up to a specific concentration. In MIC assay the extract that showed positive result for antibacterial activity was selected i.e. for MIC only root extract was selected. Thus, as the concentration of extract was increased, absorbance was decreased due to the inhibition of bacterial growth. After a specific concentration, the absorbance was again increased and so the extract concentration which showed maximum inhibition was considered as minimal inhibitory concentration (MIC) of root extract. This result estimated that the crude root extract of *R. serpentina* showed the maximum inhibition efficacy against *S. typhii* at 40 mg/ml. In addition to this, MBC assay revealed the inhibitory effects of the extract of *R. serpentina* against the tested microorganisms that introduced the plant as a potential candidate for drug development which can be used for the treatment of aliments caused by these pathogens. Finally, antiproliferative assay depicted that among the two crude extracts of *R. serpentina*, the leaf extract was found to be more effective with the IC_{50} value of 196 µg/ml. Thus, the presence of high level of indole alkaloids in the leaf and root extracts of *Rauwolfia serpentina* may be responsible for the observed potent antibacterial and antiproliferative activity of the samples.

CONCLUSION

Rauwolfia serpentina exhibited the highest antimicrobial activity at a minimum concentration against *S. typhii.* The results provide justification for the use of this plant in medicine to treat various infectious diseases. It is possible that better therapy for many microbial diseases can be found in the root extracts. Preliminary results of this investigation indicate that *Rauwolfia serpentina* roots have high potential of antimicrobial activity. Also, antiproliferative activity of root and leaf extract of *R. serpentina* was analyzed which reported that among the two crude extracts of *R. serpentina*, the leaf extract was found to be more effective for the antiproliferative activity. Thus, the presence of high level of indole alkaloids in the leaf and root extracts of *Rauwolfia serpentina* may be responsible for the observed potent antibacterial and antiproliferative activity of the samples. Therefore, this study reveals that the *R. serpentina* plant can be used as a natural therapy against bacterial infections as well as cervical cancer.

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