

PRELIMINARY SCREENING OF METHANOLIC PLANT EXTRACTS AGAINST HUMAN RHABDOMYOSARCOMA CELL LINE FROM SALT RANGE, PAKISTAN

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Abstract

The aim of this study was to investigate the mechanism of cell death by plant extract in the Rhabdomyosarcoma (RD) cell line by using human muscle cancer cells as an experimental model. The optimal uptake of plant extracts in RD cells was investigated by means of spectrometric measurements, while cytotoxicity and cellular viability of the RD cells were estimated by means of neutral red assay (NRA). RD cells were exposed to plant extracts at the concentrations of 150µg/mL dissolved in dimethyl sulphoxide (DMSO) at 24, 48, 72 and 96 hours. Results indicated that maximum cellular uptake was occurred after 1 hour *in vitro* incubation, while plant extracts induced killing of more than 70 to 80% of the cells at 150µg/mL. The methanolic extracts killed 88-93% cancer cells, while the chemo-drug killed 23% cells after 48 hours that clearly indicated anticancer activity of plant extracts. Based on the results, it can be concluded that further study is required to isolate and characterize bioactive compounds responsible for anti-cancer activity established by this study.

Key words: Rhabdomyosarcoma, Cytotoxicity, Cellular viability, Plant extracts, Optimal uptake, Bioactive compounds, Anti-cancerous activity.

Introduction

Skin cancer commonly prevails in developing countries of the world. The mortality rates of this cancer are dramatically increasing, posing a serious threat to public health in industrialized countries. At the early age of 15 years, this cancer mostly induced death in children. Rhabdomyosarcoma (RD) cancer was found responsible about 8% during childhood (Baker *et al.*, 2002). This cancer is found containing soft tissue (Sarcoma) in children, comprising 2.9% of all malignancies in the patients less than 20 years old and 350 cases recorded in the United States annually. The survival rate of this cancer is 50–70% of children (Gurney *et al.*, 1999). According to Pakistan Medical Research Council and Cancer (PMRCC), RD cancer is listed in top 20 cancers (Crist *et al.*, 1995).

Various medicaments are available for the treatment of RD cancer, such as laser surgery, organ sparing, transoral robotic surgery (TORS), radiation therapy, chemotherapy, but these mostly damage healthy cells too (Ariyan, 1987). The use of chemotherapy, radiation therapy and surgery have improved the long term survival rates of RD from approximately 25% in 1970 to more than 70% currently (Crist *et al.*, 1995), however, these therapies are resulted numerous side effects. This situation warrants developing effective and less invasive procedures to treat RD cancer. Medicinal plants are supposed to provide an alternate source of medicament, since these are being used for centuries (Shinwari *et al.*, 2009). These plants are rich in chemical substances in the form of secondary metabolites. The mechanism of action

of these secondary metabolites is drug-like and biological friendly than those of synthetic molecules. A large number of secondary metabolites are being extracted and utilized against various disorders including cancer. Plant-based compounds are supposed to be potentially important and safe therapeutic agents which are found useful for the treatment of cancer. Many studies carried out throughout the world for screening and evaluation of such medicinal plants and yielded compounds used in the pharmaceutical industry.

Natural products discovered from medicinal plants have played an important role in the treatment of cancer (Shinwari, 2010). Natural products or derivatives were comprised of 14 out of top 35 drugs in 2000, based on worldwide sales. The lignin and flavonoids of polyphenols are widely distributed in the plant kingdom and have been recognized to have biological activities including anti-tumor activity in various *in vitro* and *in vivo* studies (Koyama *et al.*, 2006). Ethnobotany and ethnopharmacology played key role in searching new molecules as therapeutic potential and many drugs discovered based on such studies (Gilani *et al.*, 2010). Scientists are interested to search in the depth of nature for new chemotherapeutic agents from plant based. The statistics show that more than 50% natural product and their derivatives are in clinical use in the world for which, angiosperms shared 25%. Interestingly, a dozen of potent drugs have been developed from higher plants during the last 40 years (Gurib-Fakim, 2006). Previously, some of the studies (Abu-Dahab and Afifi, 2007; Mothana *et al.*, 2007; Kilani *et al.*, 2008; Mesia *et al.*, 2008; Shamim *et al.*, 2009) reported *In vitro* cytotoxic activity of various

plant extracts to examine anticancer activity on human cell lines. Keeping in view, the present study was undertaken to screen anticancer activity of selected medicinal plants from salt range, Pakistan. This paper reports six medicinal plant methanolic extracts and their activity on human muscle cancer cells.

Materials and Methods

Collection of plant material: Plant materials of six plant species viz., *Althaea rosea* (L.) Cav., *Artemisia scoparia* Waldst & Kit., *Fagonia indica* Burm. f., *Otostegia limbata* (Bth.) Boiss., *Schweinfurthia papilionacea* Burm. and *Withania coagulans* (Stocks) Dunal were collected from different growing localities of the Salt Range, Punjab. One set of specimens was prepared, identified and deposited in the Taxonomy Lab., Department of Botany, Pir Mehr Ali Shah Arid Agriculture University, Rawalpindi for record.

Preparation of crude extract: The plant materials of selected plants were washed, cut into pieces and dried under shade. These were then pulverized by using a grinder. The fine powder of plants was soaked in methanol for 5-10 days. These were then filtered with Whatman filter paper 1 and the crude extracts were obtained by concentrating the extracts under reduced pressure in rotary evaporator and further dried in a vacuum oven at 40°C. The obtained crude extracts (CEs) were stored at 4°C for further use (Kaur & Arora, 2009).

Cell culture: RD cells were seeded in 25 cm² plastic tissue-culture flasks (Nunc Wiesbaden, Germany) in Minimum Essential Medium (MEM) with hanks salts, containing 10% fetal bovine serum (FBS) and 2 mM L-glutamine along with some non-essential amino acids and antibiotics (penicillin, streptomycin and neomycin). These were incubated for 24 hours for proper attachment to the substratum. Cells were maintained at 37°C as a sub-confluent monolayer and were routinely sub cultured twice or thrice weekly. The cell culture with 75–80% confluence was harvested using 0.25% trypsin (Atif *et al.*, 2009).

Quantification of cellular uptake time for plant extracts: In a 96-well flat-bottomed Microtiter plate, 1×10⁵ RD cells/well was incubated with 50µL/well from the stock having the concentration of 150 µg/ml of methanolic plant extract at 37°C for 0 to 2 hours. After incubation, cellular absorption of extracts was quantified by measuring the optical density of 630 nm light using a microwell plate reader (AMP PLATOS R-496). The time point corresponding to the highest absorbance was considered as the optimal incubation time. The readings were taken in triplicates and all results are presented as mean absorbance ±σ (standard deviation).

Cytotoxicity Tests: For cytotoxicity tests, RD cells were seeded at a concentration of 1×10⁵ cells/well into 96-well, flat-bottomed, microtiter plates and were incubated with varying concentrations of 50µL/well from the stock having the concentration of 150 µg/ml of methanolic plant extract, in serum free MEM at 37°C for 24hr, 48hr, 72hr and 96 hours. After incubation, the cytotoxicity was evaluated by means of neutral red assay (Atif *et al.*, 2009).

Determination of percentage viability: The viability of cells was measured after the exposition to different concentrations of plant extracts after Yared *et al.* (2002) and Hammiche *et al.* (2005). Cellular viability was estimated by means of neutral-red spectrophotometric assay (Zamora-Juarez *et al.*, 2005). The medium containing plant extract was removed from the wells and replaced with 200 µL of fresh medium per well containing 50 µL of neutral red (Concentration 5 mg/ml). The plates were then returned to the incubator for 3 hours. The medium was subsequently removed, and the cultures were washed rapidly with a mixture of 40% formaldehyde and 10% CaCl₂ v/v (4:1). A mixture of 1% v/v acetic acid 50% v/v ethanol (1:1) was added to extract the neutral red. The plate was shaken for 60 sec and left to stand at room temperature for 15 min. The absorbance of the solubilized dye was subsequently read at 490 nm. Quantification of solubilized dye was correlated with the live cell number. Control wells were prepared parallel, and these cells were exposed to neutral red. The percentage of viable cells in the cell population at each concentration of the test agent was calculated by means of this formula:

$$\text{Viability percentage [\%]} = \frac{\text{Mean absorbance of treated cells}}{\text{Mean absorbance of control cells}} \times 100$$

The mortality rate was determined by using following formula:

$$\text{Mortality [\%]} = 100 - \% \text{ Viability}$$

Results

Absorbance of plant extracts: Overall, the highest absorbance (0.734) was observed in *Schweinfurthia papilionacea*, followed by *Otostegia limbata* (0.71), *Althaea rosea* (0.706), *Artemisia scoparia* (0.634), while *Withania coagulans* had less capability of uptake

to cell lines (Fig. 1). RD cells showed maximum absorbance/uptake (0.706) in *Althaea rosea* extract after 30 minutes incubation time that was declined when time increased, while, rest of extracts showed maximum absorbance after 60 minutes incubation (Fig. 1). The absorbance was in the order of *Schweinfurthia papilionacea* (0.734) > *Otostegia limbata* (0.71) > *Artemisia scoparia* (0.634) > *Fgonia indica* (0.572) > and *Withania coagulans* (0.44). Furthermore, after absorbance, there was a gradual decrease in uptake which may be due to cytotoxicity of plant extracts against cancer cell line.

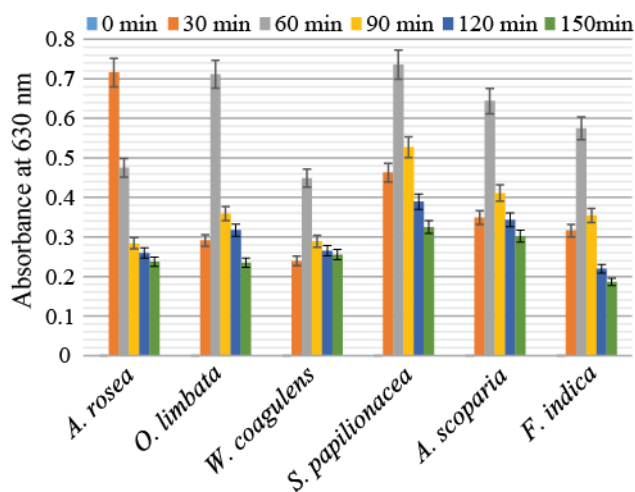


Fig. 1. Effect of Incubation period on the uptake of plant extract by RD cell line.

Viability determination: Maximum cancer cells remained alive after 24 hours application of plant extracts. These were abruptly declined after 48 hours and again gradually their population increased till 96 hours, except *Artemesia scoparia* which reduced maximum cell viability (6%) after 72 hours. Compared with chemo-drug, plant extracts showed highest and manifold activity until 96 hours application of extracts.

The viability percentage of the plant extracts also showed that *Artemesia scoparia* exhibited maximum viability percentage (95%), followed by *Fagonia indica* (84%), *Althaea rosea* (76%), *Otostegia limbata* (73%), *Shweinfurthia papilionacea* and *Withania coagulans* (70% each) in 24hours treatments. It is interesting to note that Chemo-drug (Cisplatine with conc.10 μ M) showed 82% viability, which is lower than that of *Althaea rosea*, *Otostegia limbata* and *Schweinfurthia papilionacea* *Withania coagulans* at a time interval of 24 hours (Fig. 2).

After 48 hours, extract of *Otostegia limbata* exhibited 6% viability, followed by *Schweinfurthia papilionacea* (7% viability), *Althaea rosea* & *Withania coagulans* (8% viability), *Artemesia scoparia* (15% viability) and *Fagonia indica* (12% viability), while chemo-drug showed 77% viability. Results revealed that all plant extracts had six times higher cytotoxic activity than the chemo-drug (77%). In other words, plant extracts killed 88-93% cancer cells, while the chemo-drug killed 23% cells after 48 hours. This clearly indicated potentiality of plant extracts as anticancer activity (Fig. 2).

In all, plant extracts showed significant and higher activity than chemo-drug even after 72 hours application. Except *Artemesia scoparia*, rest of plant extracts started to reduce activity and cells again started to multiply. *Artemesia scoparia* extract showed maximum cytotoxic properties against cell line and resulted 6% viability, followed by *Schweinfurthia papilionacea* (11%), *Otostegia limbata* (12%), *Althaea rosea* (14%), *Withania coagulans* (16%) and *Fagonia indica* (26%), whereas, chemo-drug showed 62% cellular viability (Fig. 2).

After 96 hours incubation, it was observed that the rate of cell viability was increased in all plant extracts except *Fagonia indica* (13%). It was followed by *Withania coagulans* (17%), *Artemesia scoparia* (18%), *Althaea rosea* (20%), *Otostegia limbata* and *Schweinfurthia papilionacea* (21%), while chemo-drug had 82% viability.

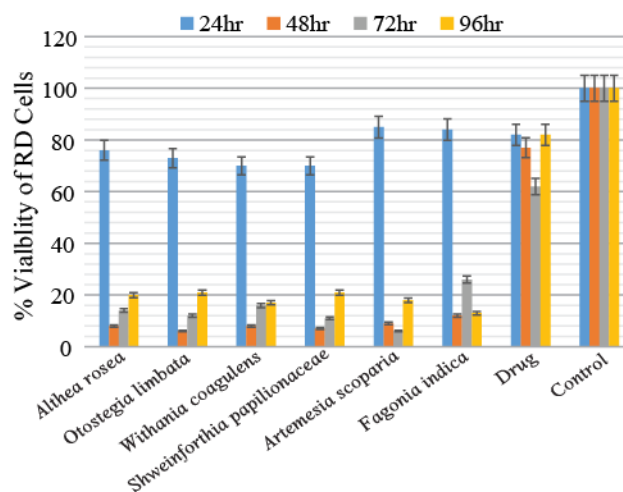


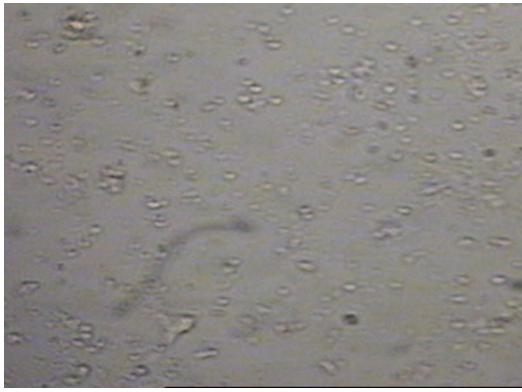
Fig. 2. Effect of time period on Viability (%) of RD cells with various plant extracts.

It was evident from the results that plant extracts still showed strong and higher activity than the chemo-drug, which showed the same activity as it was after 24 hours. Plant extracts inhibited up to 79% which is four time higher than the chemo-drug (18%) (Fig. 2).

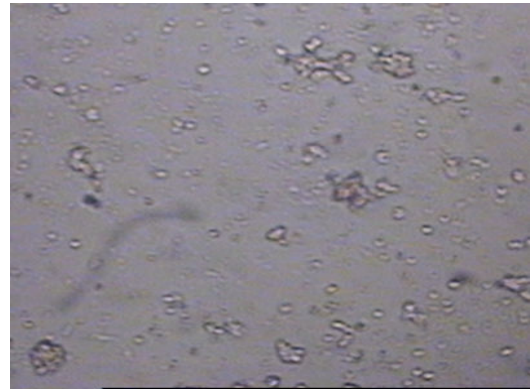
Discussion

Overall, it was concluded that all the plant extracts showed cytotoxic trends towards the RD cell line at 48 hours and all the plant extract showed better cell killing activity compared to the chemo-drug. The results shown in Fig. 1 corresponded to cellular uptake of methanolic plant extract for various incubation times. According to results, the absorbance of methanolic plant extracts was very higher initially in RD cells at the incubation time of 30 to 60 minutes but after 60 minutes, the same was accidently declined which revealed cytotoxic behavior as shown in Fig. 1 and Fig. 2.

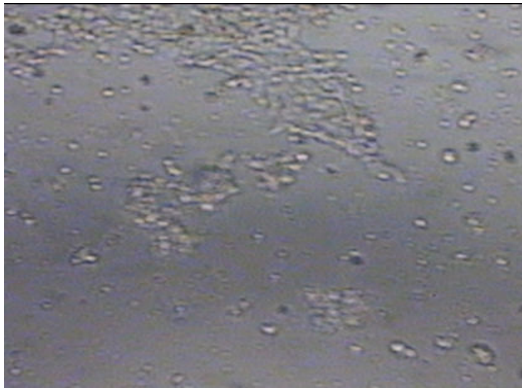
The *Otostegia limbata* extract exhibited 6% viability, followed by *Schweinfurthia papilionacea* (7% viability), *Althaea rosea* & *Withania coagulans* (8% viability), *Artemesia scoparia* (15% viability) and *Fagonia indica* (12% viability), while chemo-drug showed 77% viability. Results revealed that all plant extracts had six times higher cytotoxic activity than the chemo-drug (77%). In other words, plant extracts killed 88-93% cancer cells, while the chemo-drug killed 23% cells after 48 hours (Fig. 3). This clearly indicated potentiality of plants extracts as anticancer activity (Figs. 2 & 3). The cytotoxic activity of *Goniothalamus andersonii* extract has been reported by Inayat-Hussain *et al.* (2010) against a variety of cancer cell lines including cervical (HeLa), gastric (HGC-27), kidney (7680), breast carcinomas (MCF-7, T47D and MDA-MB-231) and leukemia (HL-60, Jurkat and CEM-SS). They observed up to 50% cell viability against these cell lines. Another study demonstrated that *Goniothalamus andersonii* extract arrested the cell cycle at G0/G1 in SK-Hep1, and at G2/M in Hep-3B cells (Cheng-Hui, 2008). Ethanolic extract of *Consolida orientalis*, *Ferula assafoetida*, *Coronilla varia* have shown more than 50% cytotoxic activity in Hela cell, having the concentration of 0.5mg/mL which indicated the presence of cytotoxic compounds in these extracts (Nemati *et al.*, 2013).



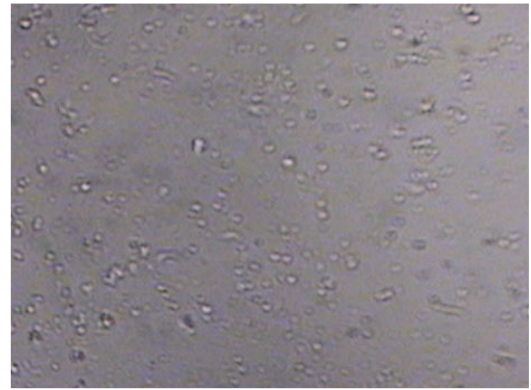
Althaea rosea



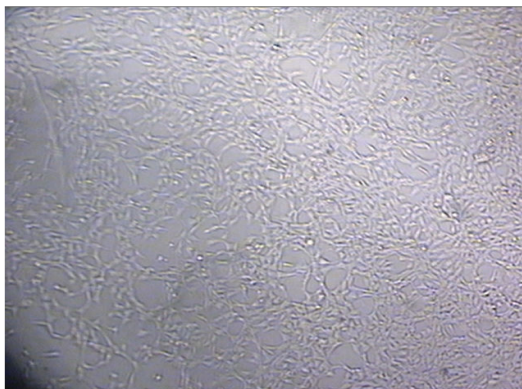
Otostegia limbata



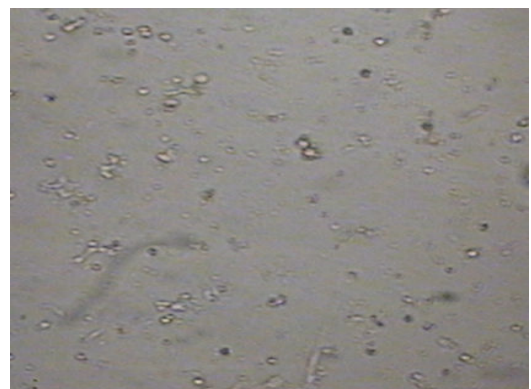
Artemesia scoparia



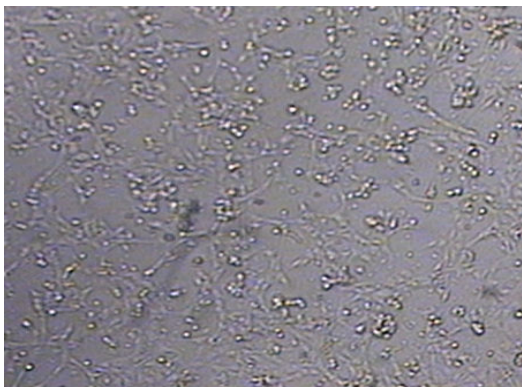
Schweinfurthia papilionacea



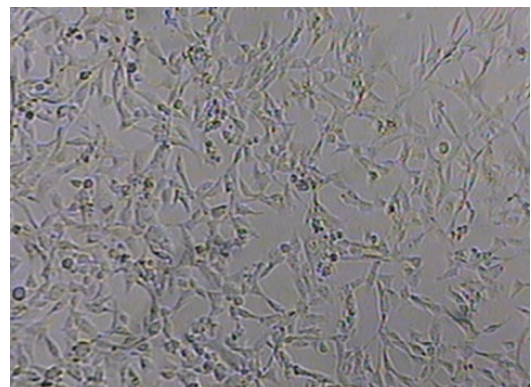
Drug



Withania coagulans



Fagonia indica



Control

Fig. 3. Effect of extracts of various plants extracts on RD cell line as compared to Drug and Control.

From the results it can be concluded that selected medicinal plants possessed secondary metabolites which have drug like mechanism for killing the cancerous cells i.e. RD cells. According to Jerry *et al.* (1998), crude botanical extracts have bioactive compounds, and it is quite possible to sort out such activities may be due to such components which can be isolated through simple bioassay procedures and various separation techniques. Flavonoids, alkaloids, terpenoids, and tannins are considered to possess high antioxidant activities, which prevent or can be used in the treatment of many diseases, including cancer (Madhuri & Pandey, 2009). Therefore, the presence of appreciable to moderate amounts of these phytochemicals can be correlated with the possible significant medicinal potential of the plant. There is great need for isolation, purification and identification of these compounds which have medicinal properties like anticancer etc.

Conclusion

Based on the results, it can be concluded that methanolic plant extracts induced cytotoxic effects on RD cells. Further study is required to isolate and characterize bioactive compounds responsible for anti-cancer activity established by this study.

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