Pak. J. Bot., 53(4): 1433-1440, 2021. DOI: http://dx.doi.org/10.30848/PJB2021-4(38)

EVALUATION OF ANTICANCER ACTIVITIES OF SELECTED MEDICINAL PLANTS FROM GANGA CHOTI, LESSER HIMALAYA, BAGH, AZAD KASHMIR

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Abstract

The current study investigates the effect of plant extracts on human muscle cancer cell lines. Our medicinal plants viz. *Terminalia chebula*, *Berberis lycium*, *Justicia adhatoda*, and *Geranium wallichianum* were collected, preserved, and processed for the analysis of their anticancer activity. The basic aim was to observe the mechanism of cell death on Rhabdomyosarcoma (RD) cell line as an experimental model. The cell death rate, cytotoxicity, and viability of RD cells were determined with the help of MTT method. Results revealed that at 48 hours, *T. chebula* exhibited maximum cytotoxicity having 21% cell viability followed by 48% viability by *J. adhatoda*, 61% by *G. wallichianum* and 69% by *B. lycium*. Photodynamic therapeutic (PDT) effect of *Terminalia chebula* extract showed 68, 58, 61, 53 and 48% viability followed by *Berberis lycium* 68, 63, 100, 76, and 75% viability. *Justicia adhatoda* exhibited 95, 94, 69, 69, and 74% viability whereas *Geranium wallichianum* had 75, 73, 61, 60, and 64% viability at 12.5, 25, 50, 100, and 200 ug/ml concentration, respectively. The results indicated that plant extracts have anticancer activity and *T. chebula* is the most toxic plant that exhibited maximum RD cell toxicity. The study suggests further isolation and characterization of bioactive compounds in *T. chebula* responsible for anti-cancer activity.

Key words: Medicinal plants; Rhabdomyosarcoma; Plant extracts; Cytotoxicity; Photodynamic therapy; Photosets.

Introduction

Medicinal plants have been repeatedly used in the treatment of cancer diseases for a long time (Akindele *et al*., 2015). Plants are used as a natural source for more than 60% of anticancerous agents (Newman & Cragg, 2016). The current research and progress in the development of new drugs effective in the cure of cancer come from the plant-based products are geared due to the advancement in the field of cancer medicine industry (Shridhar *et al*., 2017).

The Cancer has been a constant battle globally with a lot of development in cures and preventative therapies. The disease is characterized by cells in the human body continually multiplying with the inability to be controlled or stopped. Consequently, forming tumors of malignant cells with the potential to be metastatic (Akindele *et al*., 2015). Current treatments include chemotherapy, radiotherapy, and chemically derived drugs. Treatments such as chemotherapy can put patients under a lot of strain and further damage their health (Schwartsmann *et al*., 2001; Akram & Siddiqui, 2012). Therefore, there is a focus on using alternative treatments and therapies against cancer.

For many years herbal medicines have been used and are still used in developing countries as the primary source of medical treatment (Solowey *et al*., 2014). Plants have been used in medicine for their natural antiseptic properties. Thus, research has developed into investigating the potential properties and uses of plant extracts for the preparation of potential drugs for diseases including cancer (Gupta *et al*., 2014). -Many plant species have demonstrated anticancer properties with a lot of focus on those that have been used in herbal medicine in developing countries. Plant-derived drugs have been developed from positive results in research and have progressed into clinical trials. These compounds are readily available from the natural environment and are relatively non-toxic to healthy human cells (Cao *et al*., 2013). Plant-based drugs are the natural, reliable, and most readily available source for the treatment of cancer and can be promptly controlled orally as a major aspect of the patient's diet (Cragg & Newman, 2005). Additionally, these drugs have high tolerance capacity and are not harmful to the healthy cell of humans. Nonetheless, there are special cases, for example, cyanogenetic glycosides, saponins, lignans, and lectins (Amin *et al*., 2009).

Many plant species are reported to control the development of cancer cells and can destroy malignant cells (Greenwell & Rahman, 2015). About 60% of remedies at present used against cancer are obtained from natural products especially from the plant kingdom (Kumar *et al*., 2014). The Kashmir region has a rich diversity of medicinal plants because of its varied climatic, geological, geographical, physiographical, and topographical conditions. Medicinal plants from the different areas of Kashmir valley are ethno medicinally used for treating different types of diseases/ailments. The majority of the medicinal plants used were obtained from the Himalayan region and adjacent areas of the valley having a strong traditional system of medicine and related

scientific literature (Sala *et al*., 2002). A large no of plants from the Kashmir region was biologically active against different types of diseases. These plants have to face a battery of seasonal environmental stresses like high doses of mutagenic UV-Radiation, physiological drought, desiccation, and strong winds. As a result, these stresses lead to various physiological adaptations and alteration in the biochemical profile of the plant tissue leads to the production of secondary metabolites such as alkaloids, polyphenols, terpenes, and glycosides having varied pharmacological effects (Gilani *et al*., 2005).

Researchers have made great efforts towards the effective control of cancer disease though; it is an emerging disease all over the world and still is out of control. People are much concerned about therapeutic agents thatare unsuccessful to fulfill expectations although these are also expensive. Naturally occurring plant products all around the world received attention for the drugs in a few human illnesses including tumors because secondary metabolites of plants can uphold the suitability and can be effectively involved in cancer with no side effect on human health (DaRocha *et al*., 2001). It is necessary to develop effective and affordable drugs to control cancer. To evaluate the natural products like different medicinal plant as a potential candidate of therapeutic agents for cancer, the present study was designed to investigate potential advantages produced through a combination of anticancer activities of plants with photodynamic therapy.

Materials and Methods

Chemicals: Eagle's Minimal Essential Medium (EMEM), trypan blue, ethanol, penicillin, streptomycin, dimethyl sulfoxide (DMSO), Cyclophosphamide, podophyllotoxin, doxorubicin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium (MTT) reagent, Ficoll, ethylene-ediamine-tetraacetic acid (EDTA), propidium iodide (PI), fetal bovine serum (FBS) and trypsin. All chemicals and solvents of analytical grade were purchased locally with the highest purity.

Plant collection: Four medicinal plants namely, *Terminalia chebula* (Herbarium number: GCB-146), *Berberis lycium* (Herbarium number: GCB-189), *Justicia adhatoda* (Herbarium number: GCB-214), and *Germinium walichiana* (Herbarium number: GCB-89) were randomly collected from high altitude area of Ganga Choti, Bagh Azad Kashmir during May-September 2017.

For herbarium record, plants were pressed, dried, and then placed on a herbarium sheet. To avoid the fungal attack, these plants were treated with 1% $HgCl_2$ solution, on herbarium sheets for long-term preservation. Before preservation plant specimens were sprayed with different agents. Standard herbarium techniques were used for mounting plants on herbarium sheets. The identification and authentication of plants were done by Dr. K. S. Ahmad (Poonch University Rawalakot AJK). Voucher specimens of each plant were deposited in the herbarium for the record.

Justicia adhatoda belongs to a family *Acanthaceae* with height of 1 to 2.5m. This plant is indigenously used in medicine system very recently. *Justicia adhatoda* is cosmopolitan in distribution. It is tremendously used in cure of various diseases such as jaundice, dysentery and fruit of this plant are used to treating the antispasmodic cold and bronchitis (Kaur *et al*., 2013). *Berberis lycium* of family *Berberidaceae* is extensively used as a medicinal plant in Pakistan. It is used in Eye and ear diseases, jaundice fever, stomach disorder, diabetes, malarial fever, rheumatism (Sabir *et al*., 2013). *Terminalia chebula* is a tree from a family *Combretaceae* which has been recognized from very old times as mentioned in *Charaka Samhita (Charaka compendium)* a Sanskrit text of Ayureveda. Its fruit powder is used as treatment of various malfunctions such as digestive coronary and infectious diseases (Chattopadhyay and Bhattacharyya, 2007). *Geranium wallichiana* is perennial herb of the genus *Geranium*, family *Geraniaceae*. It is found in Himalayas having blue flowers in summer, height is 60 cm with hairy toothed leaves. It is widely cultivated in temperate regions for ornamental purposes (Ummara *et al*., 2013).

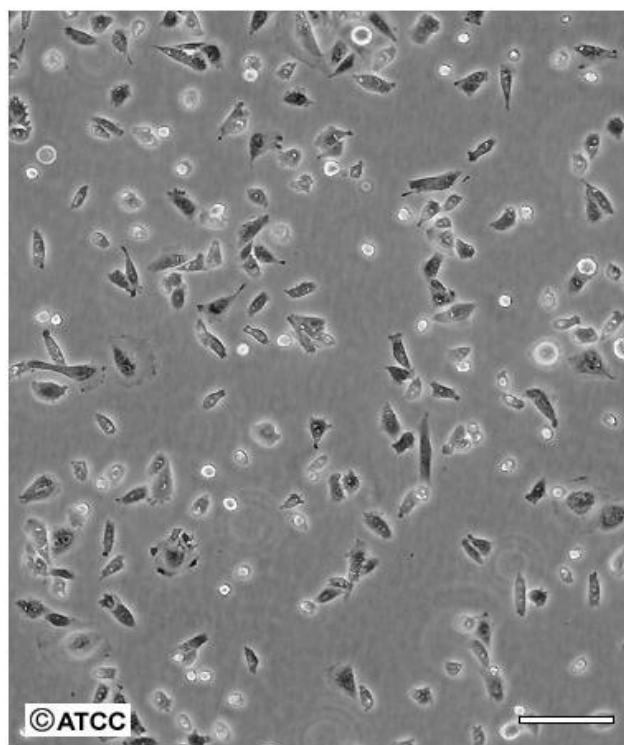
Preparation of plant extracts: Plant material (leaves, stem, and roots) was washed with distilled water, air-dried in shade, and crushed to powder with the help of a mechanical mixture. About 10 g of plant powder was extracted by taking ethanol of 100 ml and kept in dark for seven days with continuous shaking. After seven days each solution was sieved with the help of Whatman filter paper no. 42. The filtrate was evaporated with the help of rotary evaporator (Yamato 300, Japan) at 50°C and dried in desiccator. Samples were collected in glass vials and kept at room temperature (Kawase *et al*., 2003).

Preparation of stock solution: A stock solution was prepared from plant extracts by 1:1 ratio, by dissolving plant extracts(5g) in 5 ml of DMSO (Dimethylsulphoxide) and diluted into seven different serial dilutions.

Cell lines: Rhabdomyosarcoma cell lines (Fig. 1) were obtained from an approved government institution, the National Institute of Health (NIH), Islamabad to avoid any ethical issue. These cell lines were then grown in the Photomedicine lab at PIEAS, Islamabad.

Cell culture of Rhabdomyosarcoma cell lines: Rhabdomyosarcoma cells were grown in culture flasks which were kept in a CO_2 incubator at 37°C with a stream of CO_2. For the useful growth of the cells, Eagle's Minimal Essential Medium (EMEM) was used (Lyons *et al*., 1998). More than 80% of blended cells were used in the cell culture flask. From the culture flask, cells were detached and washed with the help of autoclaved PBS. After adding trypsin 0.5 ml, the culture flask was incubated for 2-3 minutes. Cells were removed from the incubator, thawed to break clump, and observed under an inverted microscope. Cells were mixed with 5ml of EMEM media (10%) with the help of a pipet. A new flask was used and about 2.5 ml cell suspension was added. The volume of each flask was maintained and each flask having 10 ml of total volume was used in further analysis (Promraksa *et al*., 2019).

ATCC Number: **CCL-61**
Designation: **CHO-K1**



Low Density Scale Bar = 100μm

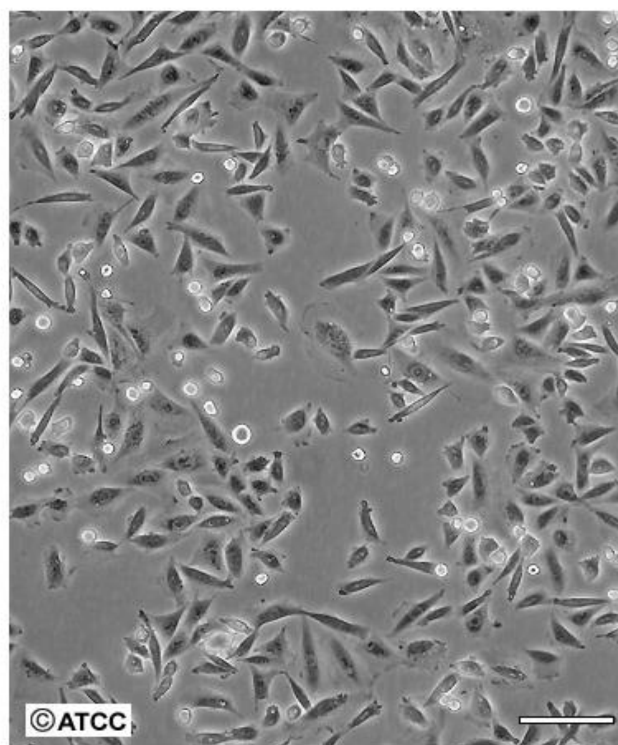


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Fig. 1. Cancerous rhabdomyosarcoma cells of skeletal muscles.

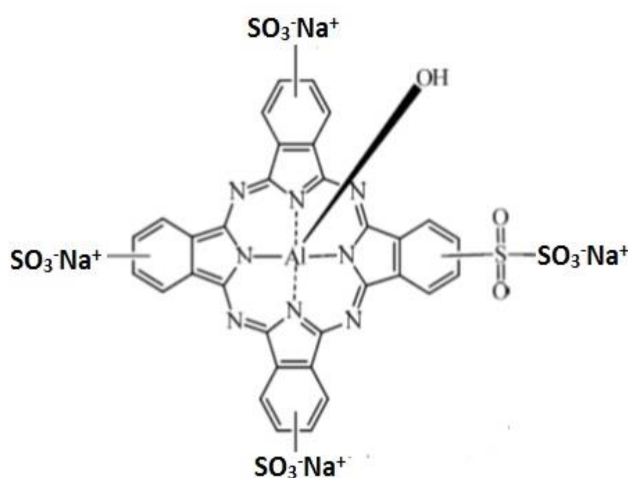

Fig. 2. A homologous series of AlPc Alkylsulfonamides.

Photosensitizer and Eagle's minimum essential medium (EMEM): For photosensitizer photosens having aluminum chloride and phthalocyanines with 1 to 4 side groups of sulphur were used (Fig. 2) which were acquired from Russia. EMEM was made at KRL Hospital, Islamabad containing 10% of serum, 1% of amino acids and penicillin-streptomycin 1% as an antimicrobial agent.

UV spectrophotometery: An ultraviolet-visible spectrophotometer (Model: LUV-200A) was used for scanning and the absorbance range was from 0.342A to 3.000A with a 0.1 nm sample interval.

Microwell plate reader and preparation of well plates: A microplate reader (ELx800) was used with ± 1.0% accuracy and linearity rate and ± 0.5% repeatability (STD). Flasks of greater than 80% confluency were used in the preparation of 96 well plates. For the dilution of suspension of the cell 25 ml of media was used. Each well was filled up with 50 µl of suspension of cells and maintained with 200 µl media. Thereafter, each well was mixed and placed CO_2 incubator having a temperature of 37°C (Huang *et al.,* 2002).

Absorbance spectrum and standard curve: For the absorbance of photosense, PBS solution of 2% and 0.05 ml photosense was added in 2.45 ml of PBS. For the standard curve, different concentrations of PS solution i.e., 20, 30, 40, and 50 µM were used and about 250 µl of each solution was added to each plate in triplet, and absorbance was read with the help of a microplate reader.

Investigation of cellular time of photosensitizer and phototoxicity of laser light: Cancer cells were brooded with photosensitizer of differing fixations ranged from 20-70 µM for an hour. Special measurements were taken with care to avoid light contact. Brooding time and optical thickness were estimated at 405 nm wavelength. The rhabdomyosarcoma cell line was illuminated with LPhT-630/675-01-BIOSPEC diode laser at 635 nm wavelength. Cells were grown in 96 well plates and were exposed to semiconductor diode laser. The neutral red test was utilized to investigate the phytotoxic impact of cells (Chen *et al.,* 2009).

Cytotoxicity measurement: For cytotoxicity of photosens, cells of RD were cultured in microplates. Cells were incubated at 37°C temperature for 45 minutes and photosens having a range of 20-70 µM was used. Cell absorbance of photosens was estimated by optical density by a microwell plate reader. The cytotoxic impact was estimated by a neutral red method. For the PDT examination, cells were grown in 96 microwell plates. The time when plate gained over 80% confluency, that point was utilized for PDT experiment (Shaikh *et al*., 2014).

Incubation of photosensitizer and laser irradiation: RD cells were cultured in 96-well. The cells at that point were incubated and treated with light ranged from 15–75 J/cm^2 and were incubated with photosens ranged from 15–120 µM and treated with laser light of 635 nm wavelength. The ideal portion of laser light was 60 J/cm^2 (Souza *et al*., 2010).

Viability determination: Cells with different concentrations of photosens were checked for viability. For this laser source of light was used and cell feasibility was detected spectrophotometrically. Photosensitizer was removed and fresh media of 200 µl and 50 µl of neutral red was supplemented to the plates and an incubation period of 3 hours was set. For cell fixation, media was washed with 40% formaldehyde and 10% $CaCl_2$. Neutral red was extracted by using 1% acetic acid and 50% ethanol in a ratio of 1:1. For almost 60 seconds, plates were thawed and were kept for 15 minutes at room temperature (Vichai & Kirtikara, 2006). The absorbance rate of all plates was recorded at 490 nm by plate reader and PS quantification was compared with the alive cell numbers.

For cell feasibility (%) following formula was used;

$$\text{Viability \%} = (P_{treated} - P_{empty}) / (P_{control} - P_{empty})$$

Results

The absorption spectrum of PS: A high photosens absorbance rate was observed (Fig. 3) at 685 nm. The absorbance drift clearly indicated that it could be utilized securely in PDT because photograph found between 600-800 nm that proved medicinally very important. Strong radiation of phthalocyanine had a close resemblance with absorption range observed between 600-800 nm (Fig. 4).

Notation of PS by RD cell lines: Readings were obtained after each interval with the help of a microwell plate reader. Results demonstrated that with an increase in the time of PS, the absorbance of PS was also increased from 10 to 45 minutes. However, this trend changed from 45 to 70 minutes of PS time where absorbance showed a decrease with the further increase of internalization time (Fig. 5).

Cell toxicity and phototoxicity: The result shown in Fig. 6 illustrated that cell viability was increased up to 110% when PS was used in low concentration. PS concentration from 15-25 µM (increased concentration) was used and the almost negligible impact was observed on cells representing that cell viability was decreased from 100 to 97%. With a PS concentration of 35 µM, cell viability was decreased by 3% only. It was observed that the RD cell death rate was associated with a high concentration of PS (Fig. 6a). The viability of cells prolonged from 100% to 110% after laser dosage of a range of 15 J/cm^2 to 45 J/cm^2 was used. At low dose viability of cells decreased demonstrating that the cell viability was affected at low dosages without PS, however, this pattern changed at a high dose demonstrating a diminish 80% in cell viability (Fig. 6b).

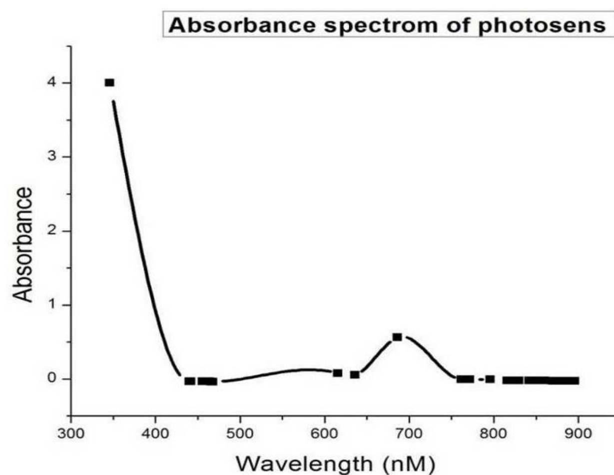

Fig. 3. The absorbance spectrum of photosense.

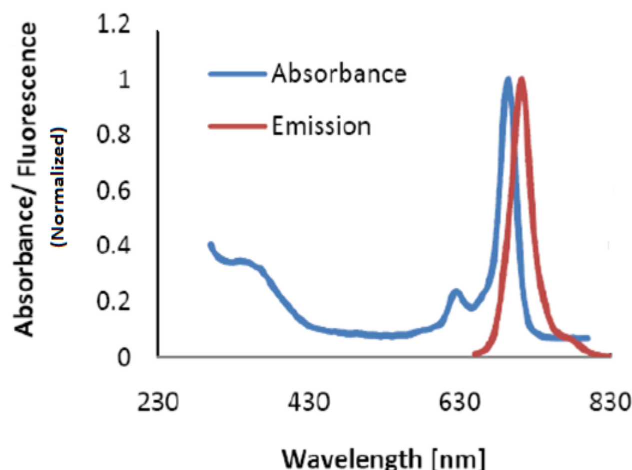

Fig. 4. Absorbance and emission spectra of phthalocyanine (PS) family.

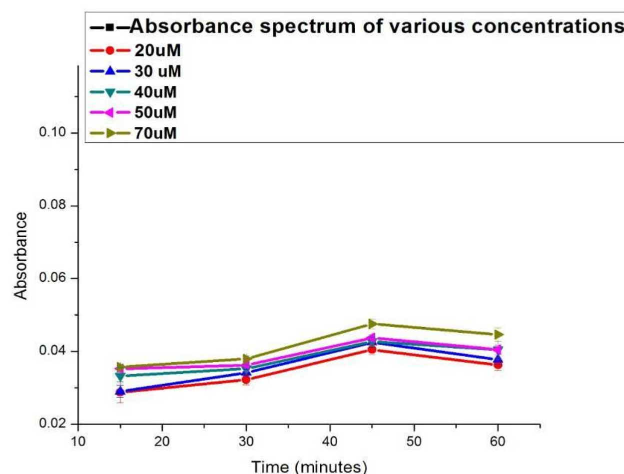

Fig. 5. Absorbance spectrum and time is taken by photosensitizer.

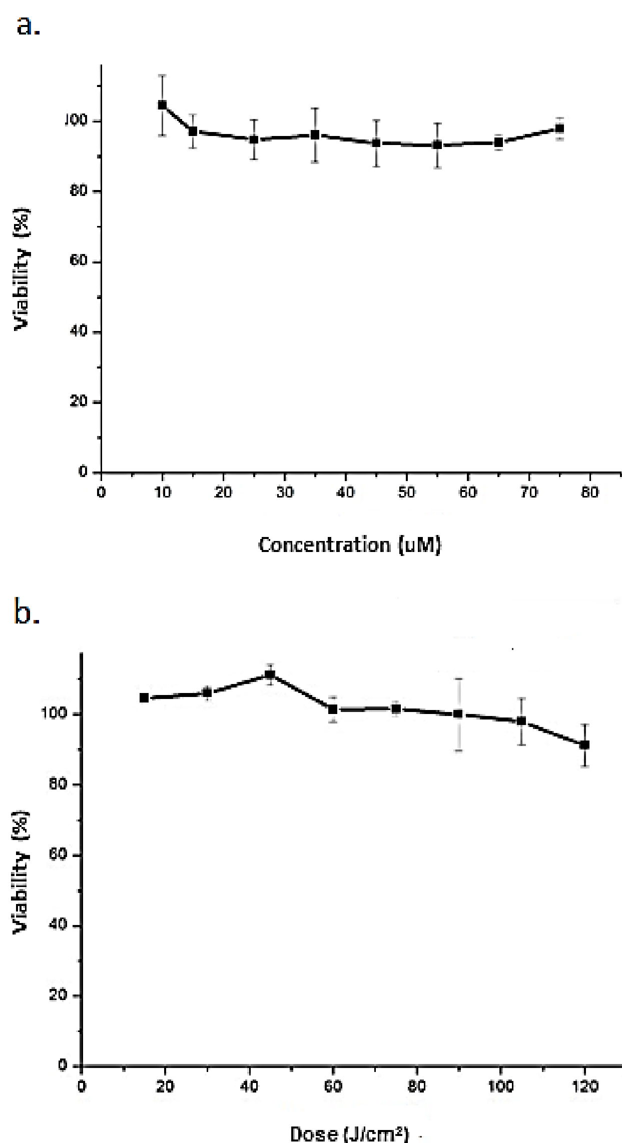

Fig. 6. The cytotoxicity (a) and phytotoxicity at different photosense concentrations (b).

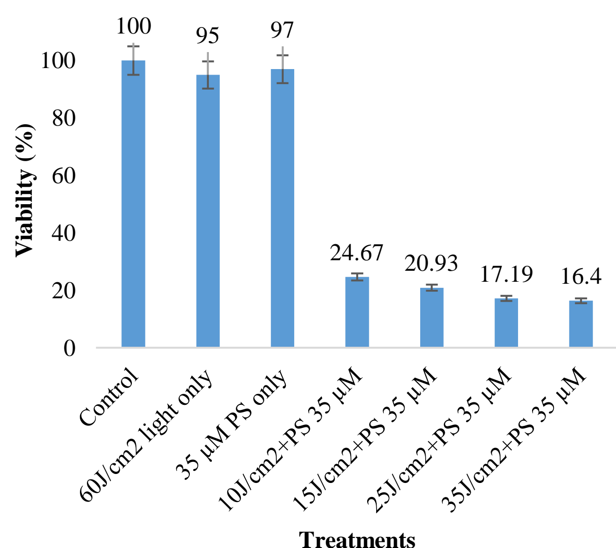

Fig. 7. Viability percentage of rhabdomyosarcoma cells treated with laser source.

Photodynamic therapy: Controlled cells without treatment showed 100% viability (Fig. 7). Cells without PS treatment but rather illuminated with laser had 95% viability i.e., only 5% cells were observed not viable. The cell comprising 35 µM PS yet not illuminated by laser light indicated 97% viability i.e. just 3% of cells were not viable. These results suggested that laser light and PS alone were not effective to kill cancerous cells at a noticeable rate.

Significant results appeared at the time after cells were treated with the right combination of PS and laser source. Cells having 35 µM PS and 10 J/cm^2 of laser light showed about 75.33% cell death and 24.67% cell viability. Photocytotoxicity effect becomes more prominent progressively after the light dose was increased. At 15 J/cm^2 of a laser source, cell death increased up to 79.07%, and cell viability was recorded at 20.93%. At 25 J/cm^2 of light cell toxicity increase up to 82.81% and cell, viability rate was 17.19%. At 35 J/cm^2 dose, cell phototoxicity was 83.60 % and cell viability rate was 16.4% (Fig. 7).

Viability determination after treatment of plant extract: After 48 hours, extract of *Terminalia chebula* (Fig. 8a) showed 83, 82, 71, 70 and 21 % viability at 12.5, 25, 50, 100, and 200 ug / ml concentration followed by *Berberis lycium* (Fig. 8b) showing 91, 89, 87, 75 and 69% viability at 12.5, 25, 50, 100 and 200 ug/ml concentration respectively. Similarly extract of *Justicia adhatoda* showed 98, 83, 80, 60 and 48 % viability at 12.5, 25, 50, 100 and 200 ug/ml concentration followed by *Geranium wallichianum* having 83, 77, 77, 64 and 61% viability at 12.5, 25, 50, 100 and 200 ug/ml concentration respectively. This clearly indicates that plant extracts potentiality have anticancer activity (Fig. 8c and 8d).

Viability determination after photodynamic therapy (PDT): Photodynamic therapeutic effect with *Terminalia chebula* extract after 48 hours showed 68, 58, 61, 53 and 48% viability at 12.5, 25, 50, 100, and 200 ug / ml concentration followed by *Berberis lycium* extract (Fig. 9a and 9b) having 68, 63, 100, 76, and 75% viability at 12.5, 25, 50, 100, and 200 ug / ml concentration respectively. Similarly extract of *Justicia adhatoda* exhibited 95, 94, 69, 69, and 74% viability at 12.5, 25, 50, 100, and 200 ug/ml concentration followed by *Geranium wallichianum* extract having 75, 73, 61, 60, and 64% viability at 12.5, 25, 50, 100, and 200 ug/ml concentration respectively. This indicated that potentially plant extracts have anticancer activity (Fig. 9c and 9d).

Discussion

Cancer has been proven a nuisance throughout the world although a large number of developments have been made by researchers to cope with this lethal disease. The disease results due to the uncontrolled growth of cells in the body (Shridhar *et al.*, 2017). Various treatment methods have been devised so far such as laser therapy, chemical treatment, and drug control, however, so far complete eradication has not been yet achieved (Sumathy *et al.*, 2013). There is attention to utilizing elective medicines and treatments against disease (Sivaraj *et al.*, 2014). Studies on phytochemical analysis of leaves in plants gained much attention and many studies revealed

the presence of certain metabolites in plants such as phenolics, flavonoids, and alkaloids, which are reported to have biological and therapeutical properties (Sreeramulu & Raghunath, 2010; Jaradat *et al*., 2016).

It is seen that all the plant extracts indicated cytotoxic patterns towards the RD cell line at 48 hours and all the plant extracts demonstrated better cell-killing activity. Most recently, the traditional medicine information use on plants research received huge interest (Sala *et al*., 2002). Studies have confirmed that plants are the potential, non-toxic, and cheap source of drugs for cancer (Wen *et al*., 2014). Many plant species are reported to control the development of cancer (Greenwell & Rahman, 2015). About 60% of therapies at present used against cancer are obtained from plant sources (Kumar *et al*., 2014).

Natural antioxidants in the plant have been linked in reducing the risk of cancer, diabetes, and other disorders in humans but still, significant arguments are required in this area (Sun *et al*., 2002). Efficacy of antioxidants of methanolic leaves extracts *Justicia adhatoda* has been reported in having strong antioxidant activity and antimicrobial activity. The plant extract of *J. adhatoda* exhibited maximum polyphenolic contents like flavonoids and phenolics and also demonstrated cytotoxic activity (Rao *et al*., 2004).

It is observed that high cell death post-PDT was shown by *Terminalia chebula* followed by *Geranium wallichianum*. Photodynamic therapy is a clinically approved therapeutic method that involves photosensitizer dye (PS) and irradiation to treat cancer (Agostinis *et al*., 2011; Reginato *et al*., 2013). Clinical studies have confirmed that PDT can be an efficient remedy at the early stages of cancer and suggestively improve the value of life. With the improvement in recent technologies, PDT can be integrated into the mainstream of curing cancer (Huang, 2005). PDT destroys the cancer vessels by activating acute inflammatory responses of the body (Castano *et al*., 2005). It is an approved therapeutic method by the US Department dealing with food and drugs particularly for lungs, stomach breast, and skin cancer (Dougherty, 2002).

Results revealed that all plant extracts had cytotoxic activity at 48 hours. *Terminalia chebula* exhibited maximum cytotoxicity having 21% cell viability followed by by *Justicia adhatoda*, *Berberis lycium,* and *Geranium wallichianum.* It was concluded that *Terminalia chebula* is most toxic plant causing RD cell toxicity (Shankara *et al*., 2016) reported that the leaf extract of *T. chebula* had high cytotoxic activities that might be credited to the occurrence of high contents of phenolics and flavonoids. These cytotoxic compounds have a pharmaceutical action such as antioxidants, scavenging properties, and cancer cell death through apoptosis or necrosis (Saleem *et al*., 2002).

The result showed that cell viability was increased up to 100% when PS was used alone and cell viability was decreased when PS was used in combination with laser dose. An increased concentration however had almost negligible impact on cell viability. The possible explanation of the increased cell viability at low concentration can be attributed to the karyotypic heterogeneity present in the cell line (Bogen *et al*., 2017). Moreover, cell viability also depends on the typical sensitivity of the cell exposed to the different concentrations of photosensitizer dye (Ali *et al*., 2015). All four plant extracts were also checked for their photocytotoxic potential for 48 hours at different concentrations of plant extract. Compared to the other plants, *Terminalia chebula* exhibited a maximum toxicity at 200 ug concentration. Jonga *et al*., (2013) evaluated 2,400 extracts for photocytotoxicity and reported that 385 extracts showed photocytotoxicity but out of these, 188 extracts were found with high photocytotoxicity.

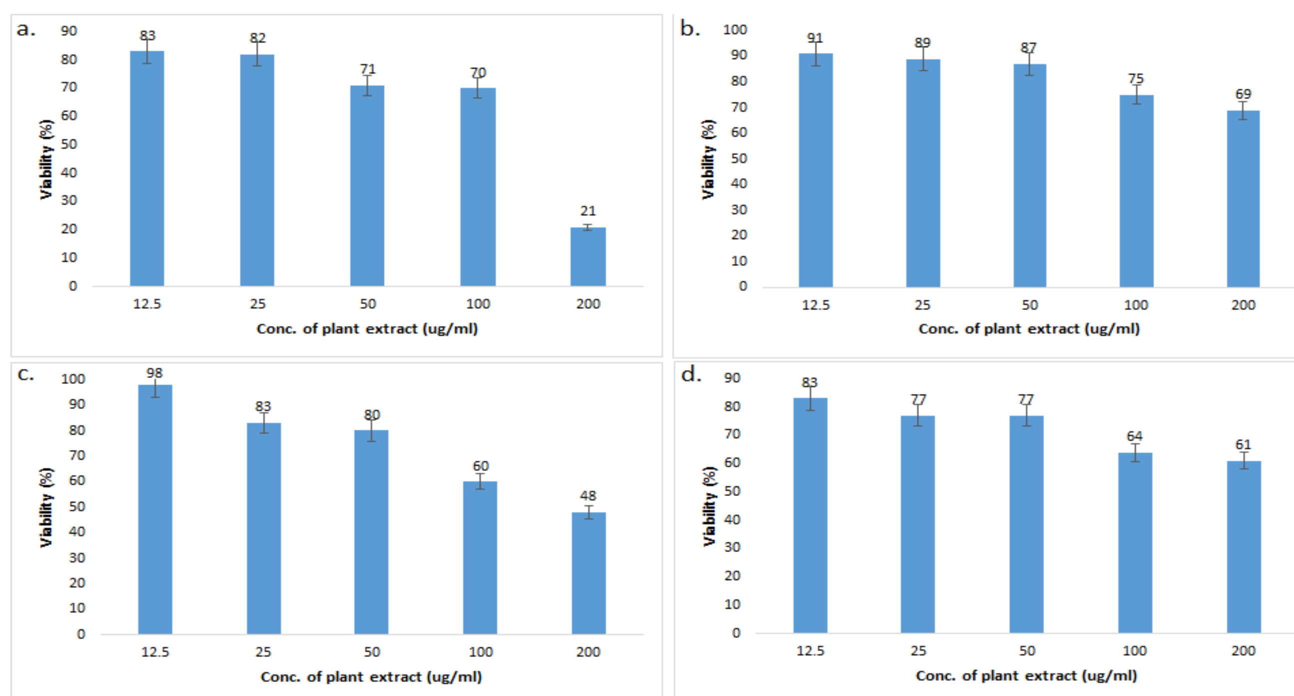

Fig. 8. Percentage viability of RD cell line after 48 hours treatment with *Terminalia chebula* (a), *Berberis lycium* (b), *Justicia adhatoda* (c) and *Geranium wallichianum* (d).

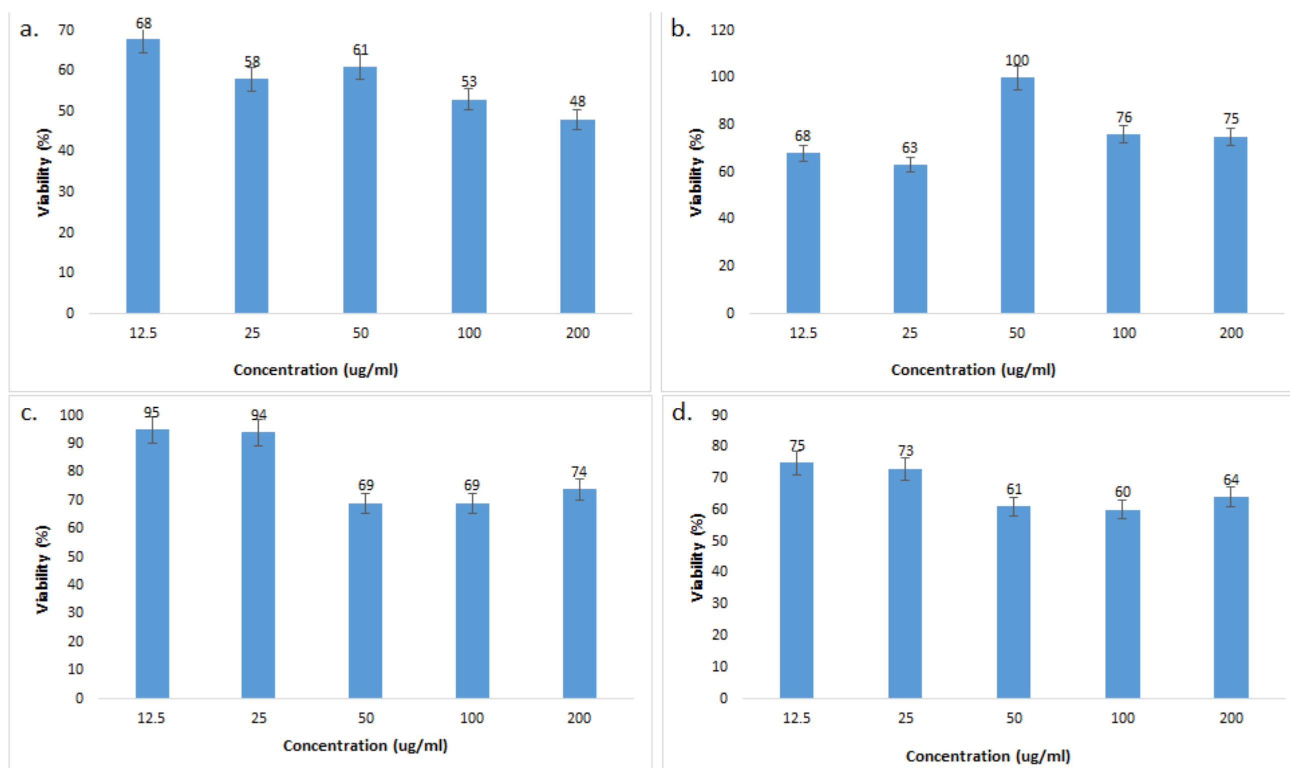

Fig. 9. Photodynamic Therapy (PDT) of Rhabdomyosarcoma RD) cell line after 48 hours treatment with *Terminalia chebula* (a), *Berberis lycium* (b), *Justicia adhatoda* (c) and *Geranium wallichianum* (d).

Conclusion

In conclusion, this study indicates that the extract of *Terminalia chebula* is most toxic, exhibiting the maximum RD cell toxicity at 48 hours. The outcomes of this study suggested further isolation and characterization of bioactive compounds in *Terminalia chebula* responsible for anti-cancer activity recognized by this study. Further thought-provoking studies are encouraging in order to gauge the existing chemical compound in the plant extracts and to understand their mode of action to develop drugs in the field of medication.

Acknowledgments

We are thankful to Dr. Nasir M. Mirza, Rector, Pakistan Institute of Engineering and Applied Sciences (PIEAS) for providing research facilities during the research work.

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(Received for publication 27 June 2019)