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# Photoactive metabolite mediated photodynamic therapy of Rhabdomyosarcoma cell lines using medicinal plants and Doxorubicin co-treatments

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## Abstract

**Background** Medicinal plant-mediated combinational therapies have gained importance globally due to minimal side effects and enhanced treatment outcomes compared to single-drug modalities. We aimed to analyze the cytotoxic potential of each conventional treatment i.e., photodynamic therapy (PDT), chemotherapy (doxorubicin hydrochloride; Dox-HCl) with or without various concentrations of medicinal plant extracts (PE) on soft tissue cancer Rhabdomyosarcoma (RD) cell line.

**Methods** The Rhabdomyosarcoma (RD) cell line was cultured and treated with Photosensitizer (*Photosense* (AlPc4)), Chemo (Dox-HCl), and their combinations with different concentrations of each plant extract i.e., *Thuja occidentalis*, *Moringa oleifera*, *Solanum surattense*. For the source of illumination, a Diode laser ( $\lambda = 630 \text{ nm} \pm 1 \text{ nm}$ ,  $P_{\text{max}} = 1.5 \text{ mW}$ ) was used. Photosensitizer uptake time ( $\sim 45 \text{ min}$ ) was optimized through spectrophotometric measurements (absorption spectroscopy). Drug response of each treatment arm was assessed post 24 h of administration using 3-(4, 5-dimethyl-2-thiazolyl)-2, 5- 5-diphenyl-2 H- tetrazolium bromide (MTT) assay.

**Results** PE-mediated Chemo-Photodynamic therapy (PDT) exhibited synergistic effects ( $CI < 1$ ). Moreover, Rhabdomyosarcoma culture pretreated with various plant extracts for 24 h exhibited significant inhibition of cell viability however most effective outcomes were shown by low and high doses of *Moringa oleifera* compared to other plant extracts. Post low doses treated culture with all plant extracts followed by PDT came up with more effectiveness when compared to all di-therapy treatments.

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**Conclusion** The general outcome of this work shows that the ethanolic plant extracts (higher doses) promote the death of cancerous cells in a dose-dependent way and combining Dox-HCl and photo-mediated photodynamic therapy can yield better therapeutic outcomes.

**Keywords** Photosensitizer, Rhabdomyosarcoma cancer, Photodynamic therapy, Absorption spectroscopy, Combination index, Medicinal plants

## Background

Cancer is the leading cause of death among children and adolescents worldwide with approximately 300,000 reported cases annually [1]. Rhabdomyosarcoma (RMS) is a soft tissue sarcoma, commonly found in children and adolescents, and children with metastatic Rhabdomyosarcoma show poor prognosis [2]. Distant metastasis is the major cause of death in this disease. Rhabdomyosarcoma cell lines exhibit significant cellular heterogeneity, which is important for understanding the complexities of cancer biology and the development of effective treatments. This heterogeneity allows researchers to study various aspects of cancer, such as the role of specific proteins, the effects of different treatments, and the interactions between different cell types [3]. Other modalities for RMS treatment include chemotherapy, radiotherapy, and surgery. Despite all these established treatments, the metastatic RMS disease prognosis has not improved in decades, so there is a need to improve the treatment strategies for the disease [4].

Photodynamic therapy (PDT) is a non-invasive light-based treatment [5]. PDT can be used to treat cancer as a single treatment or in combination with conventional treatment modalities. In PDT, cell damage occurs due to the production of reactive oxygen species (ROS) through various mechanisms (apoptosis, necroptosis, autophagy, and necrosis) and can also occur by induction of immunological responses [6]. In the recent era instead of the use of synthetic chemotherapeutic drugs, the use of natural substances and herbal drugs is gaining popularity for cancer treatment as they are environmentally sustainable and lack major side effects [7]. The success of plants for cancer treatment is due to the presence of bioactive compounds such as flavonoids, phenolic compounds, vitamin C (ascorbic acid),  $\alpha$ -tocopherol,  $\beta$ -carotenoids, etc.). Botanical extracts show inhibitory properties on cell survival and proliferation by enhancing apoptosis and targeting key signaling pathways.

Nowadays combinational drug therapies (administration of more than one anticancer drug) are widely used as a cancer treatment [8, 9]. Therapeutic regimens employing combinations of drugs are increasingly favored for treating diverse ailments, notably cancer. These combinations leverage the synergistic or additive effects of distinct pharmaceutical agents to eliminate cancer cells while minimizing additional adverse effects [10]. Conventional chemotherapeutic drugs such as Doxorubicin

(DOX), Cisplatin, and methotrexate (MTX) are clinically proven to combat different cancer types [11]. DOX acts by halting DNA replication leading to inhibition of the proliferation and generation of reactive oxygen (ROS) species which leads to tumor destruction mechanism. Combinatorial approaches such as chemo-immunotherapy, chemo-photodynamic therapy (PDT), chemo-radiotherapy, and chemo-phytotherapy are garnering attention due to their enhanced therapeutic outcomes, including synergistic or additive effects, mitigation of multidrug resistance, and attenuation of drug-induced toxicity via diverse cellular mechanisms [10].

*Moringa oleifera* (Mo) has been used as a traditional medicinal source for centuries. All parts of the *Moringa oleifera* tree (e.g., pods, seeds, and leaves) have been used to cure many diseases and are known as “miracle vegetables” [12, 13]. The *Moringa oleifera* tree is a high beta-carotene source of fats, proteins, iron, potassium, vitamin C, and other nutrients which make it highly nutritious. *Moringa oleifera* contains a variety of active phytoconstituents, including alkaloids, protein, quinine, saponins, flavonoids, tannins, steroids, and glycosides. Its leaves are also known to be a rich source of polyunsaturated fatty acids, specifically omega-3 and omega-6 ( $\omega$ -6) in the form of linoleic and  $\alpha$ -linolenic acid. Additionally, leaves are a good source of polyphenols and polyflavonoids, which are antioxidants and perhaps anticancer substances [14, 15]. *Moringa oleifera* has been examined for its different biological activities including anti-atherosclerotic, immune-boosting, antioxidant, anti-cardiovascular diseases, antiviral antimicrobial, anti-inflammatory properties, and tumor-suppressive effects [16]. However, fewer studies have described the anti-cancerous activity of *Moringa oleifera* leaves [17].

*Thuja occidentalis* is generally known as white cedar or Arborvitae. It is traditionally used to cure various diseases such as bronchial distress urinary infections, psoriasis, uterine carcinoma, skin diseases, spongy tumour blood, and rheumatism. It has been reported as an anti-metastatic agent [18]. *Thuja occidentalis* is rich in terpenoids, flavonoids, and tannin-like components. Leaf extract of *Thuja occidentalis* phytochemical compounds present are tannic acid, as well as polysaccharides and proteins glycoproteins or polysaccharides [19]. *Thuja occidentalis* leaf extract contains polyphenols alkaloids along with thujone which is considered an important phytochemical for medicinal uses. The ethanolic leaf

extract of *Thuja* is reported to cure different diseases including cancer [20].

*Solanum surattense* belongs to the family Solanaceae and is commonly known as nightshade or yellow berried nightshade. Since ancient times, it has been an important medicinal herb in the sub-continent. It is found as a weed in the wasteland [21]. *Solanum surattense* has significant therapeutic applications. Anti-cancerous effects of *Solanum surattense* extracts are attributed to the presence of flavonoids such as apigenene, quercetin, fisatin, luteolin, terpenoids, alkaloids, steroids, glycosides etc. [21] which make it a potent inhibitor of cancer cell proliferation [22]. The anti-cancerous efficiency of fruit extract of *Solanum surattense* is reported for human lung cancer cell lines (HOP-62) and leukemic (THP-1) cell lines [23].

Current work was aimed to provide an ample study on the measure of cytotoxic effects of different concentrations of three natural plant extracts (*Moringa oleifera*, *Thuja occidentalis*, and *Solanum surattense*), chemo drug (Dox-HCl) and their effect on the efficacy of phthalocyanine (*Photosense*<sup>®</sup>) mediated Photodynamic therapy.

## Methods

### Photosensitizer

In the current study second-generation PS, phthalocyanine (*Photosense*<sup>®</sup>) is used as a photosensitizer. Working dilution of the photosensitizer from the stock solutions was freshly made on the day of experiment.

### Cellular uptake time of photosensitizer (PS)

To measure the time of cellular uptake of the phthalocyanine freshly prepared working solutions for different *Photosense* concentrations were made by diluting the stock solution with media. Briefly, RD cells were cultured into flat bottom 96 well plates and were treated with different concentrations of *Photosense* (25  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 150  $\mu$ M). To assess the cellular uptake of *Photosense*<sup>®</sup>, we utilized a microplate reader, specifically the BioTek ELX 800 model, to measure the absorption of the compound. This involved quantifying the optical density (OD) of light at 630 nm. By monitoring absorption at various time intervals, we pinpointed the peak absorption of *Photosense*<sup>®</sup>, indicating the ideal duration for cellular uptake. This analysis establishes the optimal time frame at which *Photosense*<sup>®</sup> is most effectively taken up by the cells, offering crucial insights for future experiments and enhancing the compound's utilization.

### Subculturing of cell line

The Rhabdomyosarcoma (RD) cell line (received from the National Institute of Health, Islamabad) was cultured in a 25cm<sup>2</sup> tissue culture flask (Nunc Wiesbaden, Germany) in Hank's minimum essential medium (HMEM) supplemented with 5% Fetal Bovine Serum (FBS). To achieve

adherent monolayer cells were grown in a favorable environment at 37 °C in a laminar flow hood. Microscopic images showed clear branch-like morphology of culture cells, which showed that cells are in monolayer and fully stretched and can occupy more space if allowed to split further.

### Plant collection and preparation of extracts

*Moringa oleifera* and *Solanum surattense* were harvested at an age of about 6 months and *Thuja occidentalis* was harvested at an age of about 2 years. Fresh leaves of all three plants (*Moringa oleifera*, *Thuja occidentalis*, and *Solanum surattense*) were collected from district Poonch of Azad Kashmir and identified by Professor Dr Mir Ajab from the Department of Plant Sciences, Quaid-e-Azam University, Islamabad. The collected leaves were then washed thoroughly with distilled water and then shade dried (7 days), ground into a fine powder then stored in an airtight jar separately till further use. For the preparation of plant extract (PE), the powdered sample (30 gm.) of every plant was soaked in 300 ml (1; 10) of 70% ethanol (ETOH) for 72 h. Using a glass rod mixtures were [24] stirred every 24 h and then filtered using a Whatman filter paper. A rotary evaporator (*SENCO*<sup>®</sup>) at a temperature of 40–45°C for 6 h at 200 rpm was used to concentrate the filtrates and then the filtrate was dried in an oven at 50°C. The plant extracts were weighed and stored in glass storage vials at 4 °C For plants the stock solutions (5 mg/ml) were prepared by solubilizing extract (25 mg) with DMSO (5 ml).

### Plant extract and chemotherapy treatment

The study protocol was approved by research board committee of Pakistan Institute of Engineering and Applied Sciences (PIEAS, Ref.no. DMS/INVR/PRL/DPAM/2012/03–22). RD cells were seeded for 24 h to achieve 70–80% confluency and then treated with two different concentrations of *Moringa oleifera*, *Solanum surattense*, and *Thuja occidentalis* (50,150  $\mu$ g/ml) [25], Dox-HCl and *Photosense* (50,150 $\mu$ M). All dilutions were made fresh, we used 10  $\mu$ l from each stock solution (50  $\mu$ M, 150 $\mu$ M) and added it in the culture well with 200  $\mu$ l culture media. Initially, RD cell culture was exposed/ treated with low and high doses of all plant extracts, Dox-HCl, and *Photosense* (PS) individually without light exposure. Secondly, confluent RD culture was then treated in combination with high doses of plant extracts (150  $\mu$ g/ml) and chemo (150  $\mu$ M). In another combination high dose of plant extracts (150  $\mu$ g/ml) and a low dose of *Photosense* (50  $\mu$ M) were administered the combinational treatment was carried out for all three plants separately. The PS was added in each experiment group 45 min before carrying out the MTT assay.

To evaluate the tri combination the cell culture was pre-treated with plant extracts (50 µg/ml) in low dose and a high dose of Dox-HCl (150 µM) followed by a low dose of PS (50 µM) PDT.

In another treatment group culture was pre-treated with a high dose of plant extracts (150 µg/ml) a low dose of Dox-HCl (50 µM) and a low dose of PS (50 µM) was added 45 min before PDT was carried out.

In the last group of the treatment arm high dose of plant extracts (150 µg/ml) and a low dose of Dox-HCl (50 µM) were added to the culture and PS (150 µM) mediated PDT was carried out.

### Photodynamic treatment

For plant extract mediated chemo photodynamic (Tri therapy) therapy RD cells were grown in a 96-well plate with a cell density of  $1 \times 10^5$  cells per well and then incubated with the treatment. Then the culture was exposed to both high of PS according to the treatment groups explained above. After this, the cells were irradiated with light of 630 nm wavelength using a clinical semiconductor laser diode the system used is LPhT-630/675-01-BIOSPEC, Russia. Before irradiation, the laser source was calibrated by using a power meter (Thor Labs, Germany). The laser diode fiber tip was positioned 10 cm below the 96-well plate with round bottom wells (Fig. 1). The plate rested on a horizontal platform featuring a circular aperture matching the area of the wells. Using a power meter, the intensity was measured at 10 mW. This value was used to calculate the exposure time required for the desired light energy dose  $2\text{J}/\text{cm}^2$  dose which was 66 s for

2J after irradiance, cell culture media was then replaced with a fresh 2% minimum essential medium and then the culture was incubated for 24 h. Energy density was measured using the equation.

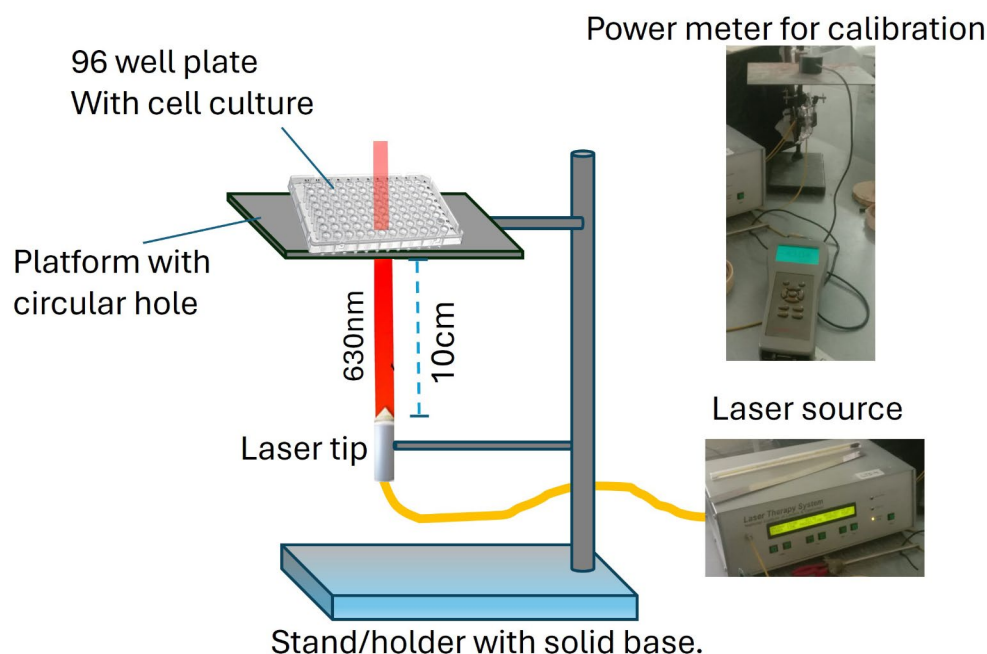
$$\text{Energy density } (\text{J}/\text{cm}^2) = \text{irradiance } (\text{mW}/\text{cm}^2) \times \text{time (s)}$$

Throughout the experiment, the untreated cells (cells+culture growth media) were used as a control group to exclude the possible natural cell death of RD. HMEM Media was used as vehicle control. Any possible cell death may be excluded through the normalization of data. The toxicity of all the selected therapeutic modalities individual or in combination (di and Tri) was evaluated through colorimetric assay (MTT) [26].

### Cell viability

To study the in vitro cytotoxicity of plant extracts and drugs, the effect of various treatments on the cell viability of RD cells was estimated by MTT assay. The MTT reagent, sourced commonly from Sigma Aldrich, is utilized as it is reduced by mitochondrial dehydrogenase enzymes to form purple formazan [26, 27]. RD cells were cultured and 96 well plates were.

prepared with a cell density of  $1 \times 10^5$  cells per well and incubated for 24 h at culturing conditions. Cells were treated with plant extracts (50 µM, 150 µM), PS (50 µM, 150 µM) and Dox-HCl (50 µM, 150 µM) according to the experimental treatment groups. This was done by taking 10 µl from each stock dilution (P.E, DoxHCl and PS) and adding it to each well already containing 200 µl culture



**Fig. 1** Photodynamic therapy setup

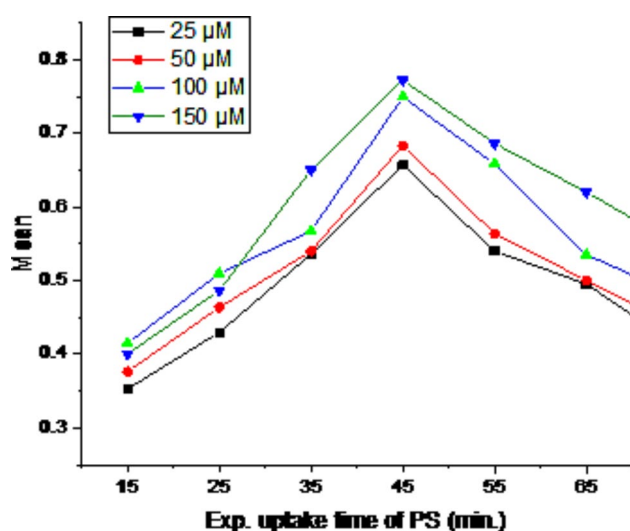
media. Cells were further incubated at culturing conditions depending on the optimized uptake time of the drugs and were then washed thrice with PBS, followed by adding media. After that MTT working solution was added to each well and the plate was incubated for 4 h. The Media was then aspirated and a solubilizing agent 100  $\mu$ l was added to each well. Plates were shaken for 5 minutes and then optical density was recorded for each well at 630 nm by using a microplate reader (Biotec ELX). Quantitative assessment of cell viability entails measuring the absorbance attributed to formazan production by viable cells. The percentage of viable cells in the cell population at each concentration was calculated using the following formula:

$$\% \text{ Viability} = \frac{A_{\text{treated}} - A_{\text{empty}}}{A_{\text{control}} - A_{\text{empty}}} \times 100$$

where  $A_{\text{treated}}$  is the mean absorbance of the treated cell,  $A_{\text{empty}}$  is the mean absorbance of empty wells, and  $A_{\text{control}}$  is the absorbance of control cells that are not exposed to any drug or light [27].

#### Cellular morphological study

Following post-incubation treatments, culture responses, including morphological alterations (such as roundness, elongation, swelling, and density variations) and intercellular physical interactions, were documented. Utilizing a microscope (XDS-2, Optika Microscope Italy) equipped with an integrated camera, visual data were captured and transferred to a computer for documentation and analysis.



**Fig. 2** Optimal time for maximum uptake of Photosense in RD cell culture

#### Drug interaction modeling

The obtained cytotoxicity data were analyzed using the CI model. This analysis was performed using the CompuSyn software program, which is available online. The CI model is a widely used method for evaluating the combination effects of multiple drugs or treatments. The CompuSyn software program is a computational tool designed to assess the synergistic, additive, or antagonistic effects of different combinations of drugs or treatments. It provides a

quantitative measure of the combined effect based on dose-response data. The CI value may be  $<1$ ,  $>1$  or can be  $=1$  to explain data as synergistic, antagonistic, or additive [28].

#### Statistical analysis

Data are expressed by mean  $\pm$  SEM ( $n=3$ ). One-way analysis of variance (ANOVA) followed by Tukey's test was applied to calculate the statistical changes among different experimental groups using GraphPad Prism 9 software. The level of significance was set at  $p < 0.05$ .

#### Results

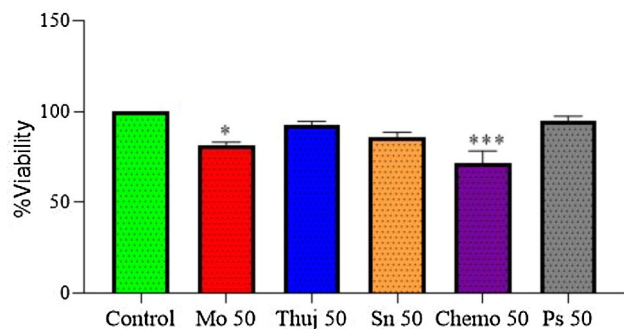
##### Cellular uptake time of photosensitizer (PS)

To determine the optimal duration of the uptake of PS by the RD cell lines, cells were treated with various doses (25  $\mu$ M, 50  $\mu$ M, 100  $\mu$ M, 150  $\mu$ M) of photosensitizer. Afterwards, the optical density of the cells was determined using a BioTek plate reader at various time intervals. The maximum absorption corresponding to the maximum uptake time for the photosensitizer was 45 min as shown in Fig. 2. This result is indicative of the preferential intake of PS by the RD cells with varying time intervals.

##### Different treatment arms dose-response cytotoxicity

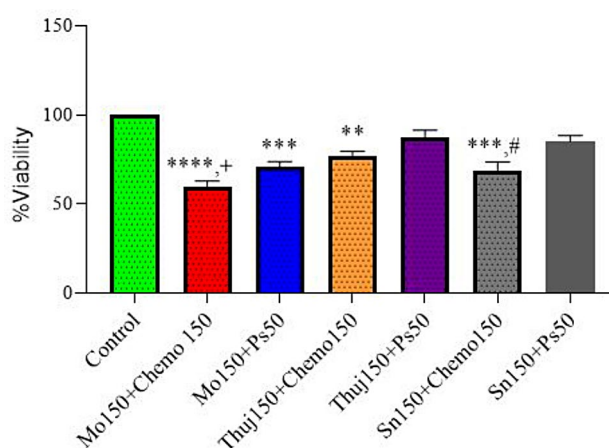
It was observed that administration of a lower dose of *Moringa oleifera*, *Thuja occidentalis*, and *Solanum surattense* (50  $\mu$ g/ml) to the cell culture showed less cell inhibitory potential when compared with the control group. Photosense at a concentration of 50  $\mu$ M did not exhibit induced cytotoxic potential at a lower tested dose. However, Chemo (Dox-HCl) at a concentration of 50  $\mu$ M treatment showed inhibition of cell viability (30%). The experiment was run with no light exposure ( $@ 0 \text{ J/cm}^2$ ) (Fig. 3).

In another treatment arm of mono therapy, the percent cell viability of RD cell culture, when exposed to the higher doses of plant extracts (150  $\mu$ g/ml) (*Moringa oleifera*, *Thuja occidentalis*, *Solanum surattense*), photosense and Dox-HCl (150  $\mu$ M), were recorded. For the case of PS, a mild-cell inhibitory response was expected which is a basic criterion of good and ideal photoactive drugs in the absence of light. Cellular viability in response to PS remains independent of doses (50  $\mu$ M, 150  $\mu$ M). *Moringa*



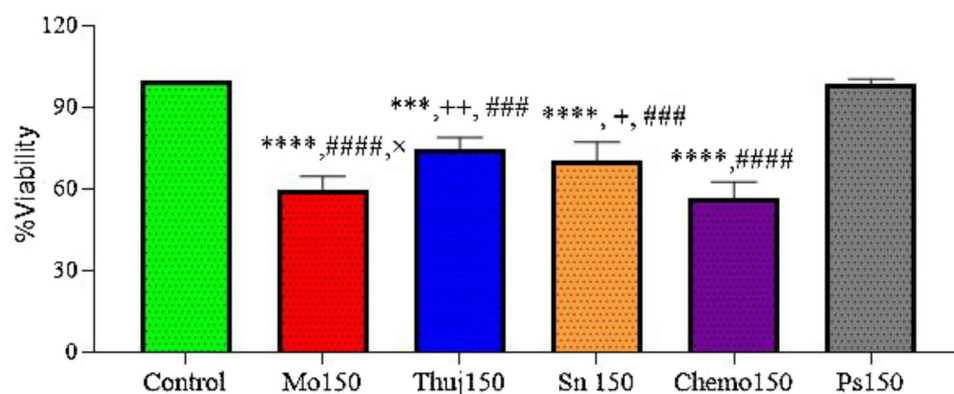
**Fig. 3** Individual cell viability of lower doses of different treatment arms (*Moringa oleifera*; MO, *Thuja occidentalis*; Thuja, and *Solanum surattense*; Sn at 50 µg/ml); chemo drug (Dox-HCl) and photosensitizer phthalocyanine (µM)) on Rhabdomyosarcoma cell culture. Data indicated the results of three independent experiments ( $n=3$ ). Asterisks \* and \*\*\* show significant difference at  $p<0.05$  and  $p<0.001$  versus the control group. Data was analyzed by one-way ANOVA followed by Tukey's multiple comparison test

*oleifera* showed 60% cell viability, *Thuja occidentalis* showed 75% viability and *Solanum surattense* showed 70% as Dox-treated cells exhibited 56% cell viability (Fig. 4). A comparison of three plant extracts indicated more potent ( $p<0.05$ ) cell inhibition induced by *Moringa oleifera* at 150 µg/ml dose compared to *Thuja occidentalis* and *Solanum surattense* treatment group *Moringa oleifera* ( $59\pm2.7$ ), and higher concentration of Dox-HCl ( $56\pm2.9$ ) showed significant cell inhibition compared to the control, furthermore, the cell inhibitory efficacy of *Moringa oleifera* (150 µg/ml) was equivalent to the Dox-HCl group as indicated by a non-significant difference among both groups. In di-combination treatment higher doses of all three plants (150 µg/ml) with Dox-HCl (150 µM) exhibited less percentage of cell viability.

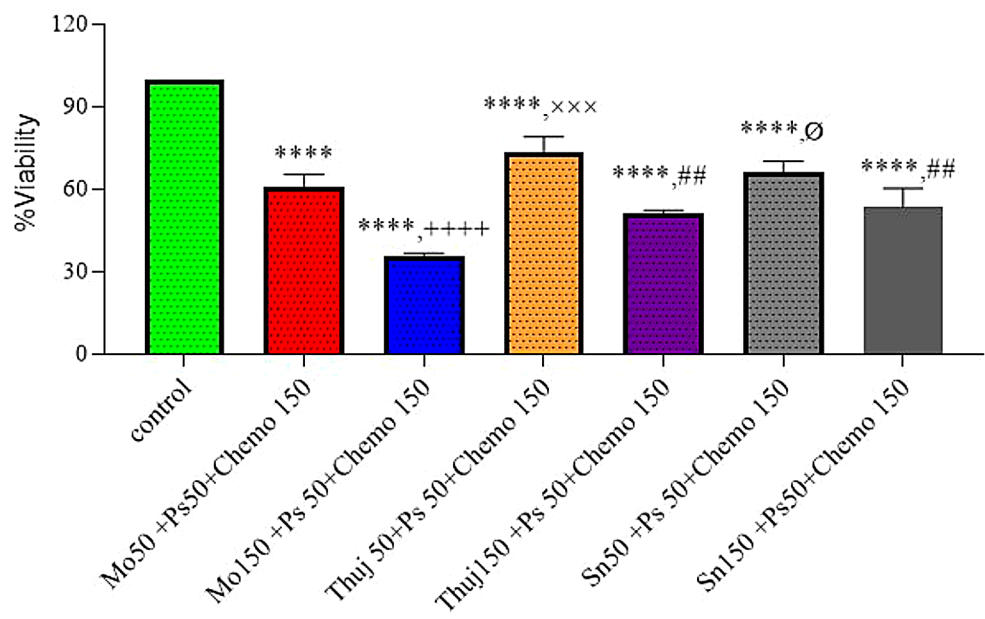


**Fig. 5** Effect of Di-combination of higher concentration (150 µg/ml) of Plant extracts, chemo (Dox-HCl), and photosensitizer on cell proliferation of Rhabdomyosarcoma cells. PS incubated for 45 min post 24 h of Plant & chemo drug. Data indicated the results of three independent experiments ( $n=3$ ). Asterisks \*\*, \*\*\*, and \*\*\*\* shows significant differences at  $p<0.01$ ,  $p<0.001$ , and  $p<0.0001$  versus the control group, whereas + shows a significant difference of Mo150+Chemo150 group at  $p<0.05$  versus Thuj150+Chemo150 group. # indicated significant difference of Sn150+Chemo150 group at  $p<0.05$  versus Sn150+Ps50 group. Non-significant difference was noticed between Mo150andChemo150 versus Mo150+Ps50 and Thuj150andChemo150 versus Thuj150+Ps50 groups. Data was analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Mo: *Moringa oleifera*, Thuj: *Thuja occidentalis*, Sn: *Solanum surattense*, Ps: Photosense. Chemo: Doxorubicin chemotherapy

In combination with a higher dose of Dox-HCl *Moringa oleifera* showed 59% cell viability, *Thuja occidentalis* showed 75% and *Solanum surattense* showed 68% cell viability The treatment arms with a higher dose of plant extracts but a low dose of Ps showed more cell viability close to the values of mono treatment of high doses that



**Fig. 4** Cytotoxicity of *Moringa oleifera*, *Thuja occidentalis*, and *Solanum surattense* at 150 µg/ml concentration. Chemo drug and phthalocyanine (Photosense) were administered at 150 µM doses. Data indicated the results of three independent experiments ( $n=3$ ). Asterisks \*\*\* and \*\*\*\* show significant difference at  $p<0.001$  and  $p<0.0001$  versus the control group, whereas + and ++ showed significant difference at  $p<0.05$  and  $p<0.01$  versus the chemo 150 group. ### and #### indicated significant difference at  $p<0.001$  and  $p<0.0001$  versus PS 150 treatment group. x showed a comparison at  $p<0.05$  between different plant extracts at high doses. Data was analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Mo: *Moringa oleifera*, Thuj: *Thuja occidentalis*, Sn: *Solanum surattense*, Ps: Photosense, Chemo: Doxorubicin chemotherapy



**Fig. 6** Tri-combination with a low dose of PS (50  $\mu$ M), a higher dose of Chemo (150 $\mu$ M), and different doses of plants (50,150  $\mu$ g/ml) followed by Photosense®-mediated PDT. Data indicated the results of three independent experiments ( $n=3$ ). Asterisks \*\*\*\* show a significant difference at  $p<0.0001$  versus the control group. +++++ shows significant difference at  $p<0.0001$  of Mo 50+Ps50+ Chemo150 treatment group versus Mo150+PS 50+ Chemo150 treatment groups. xxx indicated significant difference at  $p<0.001$  of Thuj50+Ps50+Chemo150 versus Thuj 150+PS 50+Chemo150 treatment groups. ## indicated significant difference at  $p<0.01$  of Mo150+Ps 50+Chemo 150 treatment group versus Thuj150+PS 50+Chemo150 and Sn150+Ps50+Chemo150 treatment groups. Ø indicated a significant difference at  $p<0.05$  of Sn50+Ps50+Chemo150 versus Sn150+PS 50+Chemo150 treatment groups. Data was analyzed by one-way ANOVA followed by Tukey's multiple comparison test. Mo: *Moringa oleifera*, Thuj: *Thuja occidentalis*, Sn: *Solanum surattense*, Ps: Photosense, Chemo: Doxorubicin

**Table 1** RD cell culture response to Di- and tri-combinations for lower and higher doses of selected treatment arms

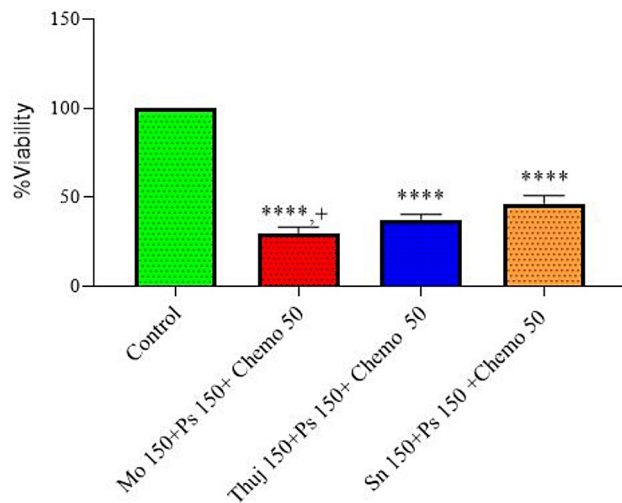
| Dox-HCl@150 $\mu$ M               |                | Photosense @ 50 $\mu$ M            |                |
|-----------------------------------|----------------|------------------------------------|----------------|
| Plant extracts (PE)150 $\mu$ g/ml | Cell viability | Plant extracts (PE) 150 $\mu$ g/ml | Cell viability |
| Di-Combinations                   |                |                                    |                |
| <i>Moringa oleifera</i>           | ~ 59%          | <i>Moringa oleifera</i>            | ~ 70%          |
| <i>Thuja occidentalis</i>         | ~ 76%          | <i>Thuja occidentalis</i>          | ~ 87%          |
| <i>Solanum surattense</i>         | ~ 68%          | <i>Solanum surattense</i>          | ~ 85%          |
| Tri-Combinations                  |                |                                    |                |
| <i>Moringa oleifera</i>           | ~ 60%          | <i>Moringa oleifera</i>            | ~ 35%          |
| <i>Thuja occidentalis</i>         | ~ 73%          | <i>Thuja occidentalis</i>          | ~ 50%          |
| <i>Solanum surattense</i>         | ~ 65%          | <i>Solanum surattense</i>          | ~ 53%          |

attributes to the reason that PS without light is non-cyto-toxic (Fig. 5). Higher doses of all the combinations came out to be more synergistic. Compared to di-combination when tri-combination was assessed it was observed that all the treatment arms (PE, Chemo, and PS-PDT, diode laser, 630 nm, 2 J/cm<sup>2</sup>) showed significant cell inhibition in RD cell culture (Fig. 6). In the tri-drug combination, *Moringa oleifera* and *Solanum surattense* with Dox-HCl showed more synergistic effects as compared to *Thuja occidentalis* extract (Table 1). It may be concluded that both the selected doses (higher and lower) of *Moringa oleifera* in the presence of Dox-HCl (150  $\mu$ M) and PS (50  $\mu$ M) showed around 40% and 75% reduction in cell

viability respectively. In this treatment the combination of *Moringa oleifera* 150  $\mu$ g/ml, Dox. HCl 150 $\mu$ M and PS 50 $\mu$ M showed more significant results compared to *Thuja occidentalis* (tri combination) and *Solanum surattense* (tri combination).

These results showed that higher doses of plant extracts are more significant in tri-combination therapy compared to lower doses of plant extract. Moreover, higher doses of plant extracts alone are less effective as compared to when administered in combination with Dox-HCl. and PDT. The di- and tri-combinations response to the in-vitro cell culture in the presence of a higher dose of Dox-HCl (150  $\mu$ g/ml) is presented in tabulated form (Table 1).

In the last treatment group deduced from the above results higher concentration of all plants that are 150  $\mu$ g/ml with a low dose of 50  $\mu$ M of chemo, to minimize chemo adverse effects were administered in combination with PS-PDT @150 $\mu$ M, 2J/cm<sup>2</sup> evaluated as shown in Fig. 7 all the treatment arms showed a significant reduction of cell viability. Among all three plant extracts *Moringa oleifera* exhibited the most significant results (28 $\pm$ 3.1) It was observed that PE showed a synergistic response which may be attributed to chemo cum PDT-mediated production of reactive oxygen species (ROS). The overall MTT effect of photosense-mediated plant-chemo-PDT for RD culture was dose-dependent.



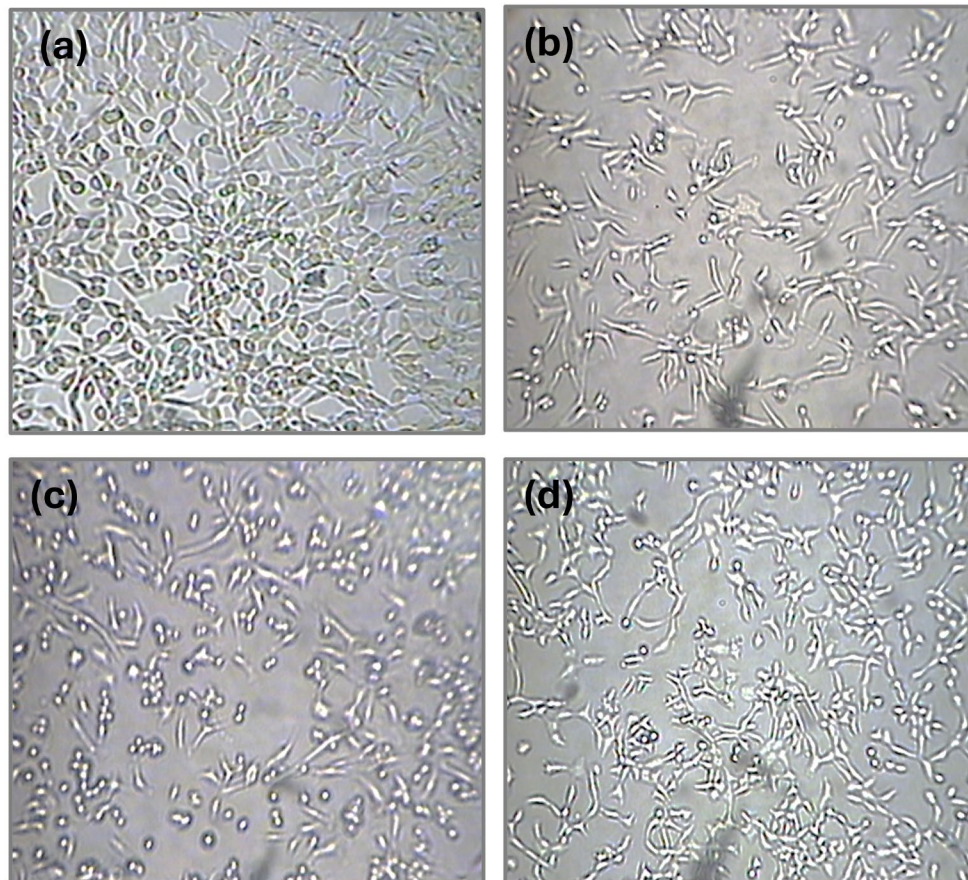
**Fig. 7** Plant extracts (*Moringa oleifera*, *Thuja occidentalis*, and *Solanum surattense*) 150 µg/ml chemo 50 µM, (Dox-HCl) and photosensitizer 150 µM, individual cytotoxicity on rhabdomyosarcoma cell culture. Data indicated the results of three independent experiments ( $n=3$ ). Asterisks \*\*\*\* showed a significant difference at  $p < 0.0001$  versus the control group. + showed significant difference at  $p < 0.05$  of Mo150+PS150+Chemo50 group versus Sn150+PS150+Chemo50 groups

#### A cellular morphological study using inverted visible light microscopy

Images captured on a CCD-coupled inverted microscope also showed that the combinational treatment reduces the density of cells that are viable (Fig. 8). It was observed that when the Plant extracts were administered in the presence of photosensitizer and chemo drug morphology of cell culture changes i.e., round and swelled cells, which also suggests the apoptotic mode of cell Death [29]. Post Tri-combination images of plant extracts (150 µg/ml) with low dose Dox-HCl (50 µM) with PDT@ Ps (150 µM) showed considerable morphological changes, indicating cell death. The treatment groups exhibited apoptotic features such as detached and rounded cells, while no morphological changes were observed in the control group.

#### Combination index (CI) by the chau-talalay method

Combinational index (CI) values were calculated by using Compusyn software [30]. The results supported the hypothesis of P.E-assisted chemo-PDT. It was observed that strong, moderate, and marginal synergistic effects may result from the presented combinations. From the calculated CI values (Table 2) for di-combinations, it



**Fig. 8** Morphological changes (% cell survival) of various treatment groups in the presence of light. (a) Control (100%), (b) *Moringa oleifera* (MO, 28%), (c) *Solanum surattense* (Sn, 37%) (d) *Thuja occidentalis* (Thuj, 46%). PS: photosense, Chemo: Doxorubicin chemotherapeutic

**Table 2** Combinational index values for different combinations of PE, Chemo, and PS

| Samples                                      | Combination index (CI)            |                                                                                |                                                                    |
|----------------------------------------------|-----------------------------------|--------------------------------------------------------------------------------|--------------------------------------------------------------------|
|                                              | Di therapy<br>Dox-HCl<br>(150 µM) | Tri therapy<br>Dox-HCl (150 µM) photosence<br>(50 µM)<br>(2J/cm <sup>2</sup> ) | Dox-HCl<br>(50 µM) Photosence<br>(150 µM)<br>(2J/cm <sup>2</sup> ) |
| <i>Moringa oleifera</i><br>(150 µg/ml)       | 0.57                              | 0.41                                                                           | 0.23                                                               |
| <i>Thuja occidentalis</i><br>(150 µg/ml)     | 0.77                              | 0.56                                                                           | 0.55                                                               |
| <i>Solanum</i><br>(150 µg/ml)                | 0.69                              | 0.49                                                                           | 0.34                                                               |
| Dox-HCl (50 µM) + AIPc <sub>4</sub> (150 µM) |                                   | 0.81                                                                           | 0.63                                                               |

may be concluded that synergistic effects are observed with combination of Dox-HCl (150 µM) and *Moringa oleifera* (150 µg/ml) as compared to *Solanum surattense* and *Thuja occidentalis*. From the CI values it is evident that the plant extract (150 µg/ml) in combination with chemo drug low dose (50 µM) in the presence of photodynamic therapy@ 2 J showed more synergism exhibiting decreased CI values for *Moringa oleifera*, *Solanum surattense*, and *Thuja occidentalis* in sequential order.

Based on the values of CI presented in the above table may conclude that the di- & tri- combination in the presence of plant extracts are synergistic (<1) in nature.

**Discussion**

Nature, as a reservoir of herbal resources, harbours unexplored pharmaceutical compounds that offer significant benefits to human health. These substances have been employed since ancient times to address both cancerous and noncancerous ailments. Studies focusing on plant extracts reveal that these natural products induce cancer cell death through alterations in the cell cycle, apoptosis, and modulation of various signaling pathways [31]. Chemotherapeutic drugs such as Dox-HCl are prospective anti-cancer drugs with therapeutic benefits but that exhibit side effects. Drug combinations have become a common treatment option for a variety of diseases, including cancer [32]. The two most important qualities of any successful treatment are low toxicity to normal cells and great efficiency. The advantage of therapeutic combinations is that the various medications can kill cancer cells in an additive or synergistic way, without causing any additional side effects, for a range of cancer types [33]. Recently, synergistic interactions have been discovered. Plant-based compounds, such as flavonoids and polyphenols, are effective against a variety of cancers and can be used with chemotherapy drugs [34, 35]. It is pertinent to use natural products in conjunction with chemo agents to reduce toxicity, delay or lessen drug resistance, and potentiate synergistic therapeutic effects [36].

In our research, we examined the combined effects of three different plants on Dox-HCl and PS-PDT to determine if using a combination of plant extracts with low doses of DOX and PS-PDT on RD cells could be an effective cancer treatment.

In our study, *Moringa oleifera* over the administered range of concentrations (lower doses) seems to be non-cytotoxic [37]. Higher doses of *Moringa oleifera* alone and in combination with Dox-HCl and with PDT showed significant cell death is in accordance with work done earlier on other cancer cell lines and also in combination with the chemo drug and PDT [38]. It has shown significant cytotoxicity due to the occurrence of bioactive compounds and their metabolites [24]. Bioactive compounds such as isopropyl, isothiocyanate, D-allose, cetene octadecene, tetradecane, octadecanoic acid, and palmitic acid are present exclusively in the leaves extract of *Moringa* [39]. Due to the immense ability of cancer cells to proliferate, anticancer drugs are needed to target these cells and prevent them from growing. It's interesting to note that *Moringa oleifera* carries out a variety of biological functions and even targets many proteins and macromolecules to slow the proliferation of cancer cells [40]. It has glycosides, which are highly effective against cancer and are capable of inducing apoptosis these results are relevant to published data as shown by Berkovich et al. according to this publication extracts from *Moringa oleifera* leaves have been reported to suppress the growth of pancreatic cancer cells [38].

*Solanum surattense* also has anticancer activity attributing to the presence of phytochemicals it [41]. In our study RD cell culture showed mild cytotoxicity over the lower dose of *Solanum surattense* where as high doses alone showed moderate cytotoxicity and combinational treatment with Dox and PDT showed more significant cytotoxicity. It has been reported that cytotoxicity associated with *Solanum surattense* may be attributed to the presence of a second metabolite commonly known as solamargine, a steroidal alkaloid [21, 42]. This explains a dose-dependent cytotoxicity of *Solanum surattense* leave

extracts and its cytotoxicity increase in combination with Dox-HCl which has been reported earlier for *Solanum nigrum* ethanolic extract in MCF-7 cell line [43].

*Thuja occidentalis* showed notably more viable cells in combinational treatment with Dox and PDT as compared to the other two plant extracts. Studies have indicated that *Thuja occidentalis* acts as an anticancer agent in a number of ways according to S.Saha et al. *Thuja occidentalis* induce apoptosis of cells of mammary epithelial carcinoma [44]. It also lowers the level of cytokines (IL-1 $\beta$ , TNF- $\alpha$ ) [45]. It also down-regulates IL-6 which is an indicator of tumor reduction factor. Our results support the cytotoxicity initiated by the plants over the selected doses and in a dose-dependent manner on RD cells and it is evident that a combinational approach is critical and beneficial for the treatment of cancer. Studies have shown that plant extracts exhibit the properties of imbibition on cell survival and proliferation by increasing apoptotic activity [46] by targeting various signaling pathways like PI3K/AKT, ERK/MAPK, and NF- $\kappa$ B. They also exhibit anticancer activity by reducing levels of anti-apoptotic factors such as BCL-2 and BCL-XL, while activating the pro-apoptotic elements like BAX, BAK, BID, and caspases. The plant extracts raise reactive oxygen species (ROS), which triggers caspases, cleaves PARP-1, and causes P53 to trigger cancer cell apoptosis [47].

All the treatment groups in our study which have been exposed to chemo drugs showed less viability of cells. Cytotoxicity associated with Dox-HCl is reported in Chemo treated arms, which may be due to the reason that Doxorubicin on cancer cells works by two different mechanisms firstly by intercellular intercalation which disrupts topoisomerase-II-mediated DNA repair; and secondly due to free, radical's generation, which damages proteins, DNA, and cellular membranes [48]. Doxorubicin can also generate cytotoxic reactive oxygen species to exert cellular damage selection of the range of concentration and it may not upregulate or downregulate cellular death mediating proteomic pathways [49].

All the treatment arms that are exposed to photodynamic therapy showed significant results this can be attributed When there is molecular oxygen present, the activated photosensitizer sets off a series of photochemical and photobiological reactions that cause highly reactive oxygen species (ROS), mostly singlet oxygen, to act on intracellular organelles resulting in cancer cell death [50, 51]. This has been observed that PS only (without light) has shown higher viability which means more cell replication and higher viabilities contributed to the preferential accumulation of intracellular localization of PS in mitochondria and lysosomes. It has been reported previously that higher viability in the presence of PS contributed to higher metabolic activities in the absence of suitable light. [52]. It was expected that in the absence

of light, there would be no photochemical reactions that may lead to the oxidation of cellular organelles. The intracellular localization of PS is a vital parameter in defining the cell death mechanism and efficacy of PDT. Phthalocyanine is a stabilized PS localized in lysosomes, mitochondria & cytoplasm (intracellular organelles). On light activation of PS, these organelles destroy the membrane through ROS production, which causes cell death [53, 54]. PS mitochondrial localization induces apoptosis and either apoptosis or necrotic response is induced due to the lysosomal localization of PS [55]. PDT-produced intracellular ROS can directly kill tumour cells by inducing necrosis and/or apoptosis through a range of signaling pathways in combination with caspases, apoptosis-inducing factors, and Bcl-2 family members [56]. Also, phthalocyanine-mediated PDT causes cell cycle arrest at G<sub>0</sub>/G<sub>1</sub> followed by inhibition of cell growth and cell death of human cervical carcinoma cells [57]. Dox-HCl strived cell death via induction of senescence-like phenotype (SLP) and down-regulation of different proteins when lower doses were administered [58]. Dox-HCl in the presence of PS-PDT encourages singlet oxygen-dependent cytotoxic effects [59]. Outcomes of phase II clinical studies have shown considerable potential for the doxorubicin combination with additional chemo medicines for treating metastatic Rhabdomyosarcoma [60, 61]. Photodynamic therapy has also been shown to have synergistic effects in the treatment of advanced pancreatic cancer [62]. This study hypothesizes that combining Dox-Photosense-PDT and natural active chemicals may improve the treatment results by minimizing toxicity associated with a high dosage of chemo drugs [10]. Apoptosis, inhibition of Topoisomerase I, ROS production in cancer cells, and other biological pathways leading to cell death are triggered when cancer cells are treated with a combination of agents, such as PDT, chemotherapy drugs, and plant extracts (a mixture of many chemicals) [63] ROS production in cancer cells is one of the mechanisms underlying the synergistic cytotoxicity of combination therapy involving plant, chemo drug, and photodynamic therapy, thereby enhancing the cytotoxic effect of the treatment [64]. because many plants have protective effects thereby reducing side effects of chemo drugs.

Various studies have shown that the most successful cancer treatments are combinational therapies that attempt to target multiple non-overlapping cellular pathways and also minimize the high-dose chemotherapy adverse effects [65]. Researchers observed that a dual extended chemo-PDT combinational therapy method minimized high-dosage chemotherapy-related adverse effects while maintaining performance status The efficacy of the individual modalities, plant extract, chemo drug, and photodynamic therapy was enhanced when

combined in the same treatment regime which is also supported by other researchers [32, 66].

It is well documented in several published research articles that PDT alone is not sufficient to produce effective therapeutic outcomes due to several reasons such as cancer cells adopt alternate molecular pathways to respond to the damaged mitochondria, and its efficacy increases with combination therapy, secondly, not all the mitochondria get shut down in response to the intracellular uptake of drugs, etc. Therefore, it is normally a need for an alternate approach to bring in a second or third treatment modality to combat several up-regulated pathways that cancer cells activate to survive and metastasize to distant parts [67]. Our results support the positive collaborative effects of the combination of plant and synthetic drug-mediated PDT thereby reducing the possible side effects of the drug. In comparison to mono treatment, a combination of PDT and chemo intensifies the synergistic effects to incite increased cytotoxicity of cancer cells thereby reducing the possible side effects of the drug [64, 68].

### Limitations of the study

The in-vivo cancer model to study the response of these combinational therapeutic modalities has not been studied in the current investigation. The expected challenge is the bio-distribution of these drugs intracellular, which may be addressed with the encapsulation in FDA-approved PLGA nanocarriers. The second challenge may be the drug release profile keeping up-regulation of molecular pathways. These limitations may lead to the synthesis of multilayered controlled drug-released nanocarriers. Extensive in-vivo study (Toxicity, bioavailability, biocompatibility, yield of therapeutic outcome) may decide the translation into the clinic, for which the author will be much more excited.

### Conclusion

This work shows that chemo-PDT therapeutic outcomes may be enhanced in the presence of plant extract (tri-combination). It has been concluded that chemo-PDT therapeutic outcome may be enhanced in the presence of plant extract, the tri-combination, which is favoured as compared to di-combination. This modality may be a potential candidate for the best therapeutic outcome when low doses are desired to be administered in the cancer cell culture. Alone Low-dose treatment is not potent against cancer cells, while higher doses cause cytotoxicity, but combining different therapies produces a promising result. Findings of the present work showed that the extracts stimulate cancer cell death in a dose-dependent manner, and better therapeutic outcomes can be achieved in combination with the Dox-HCl, and the phthalocyanine-mediated photodynamic therapy.

Among all three tested extracts of plants, *Moringa oleifera* turns out to be the most effective in plant-mediated chemo photodynamic therapy.

### Abbreviations

|         |                                 |
|---------|---------------------------------|
| PDT     | Photodynamic therapy            |
| Dox-HCl | Doxorubicin hydrochloride       |
| RMS     | Rhabdomyosarcoma                |
| DMSO    | Dimethyl sulfoxide              |
| PS      | Photosensitizer                 |
| PBS     | Phosphate buffered saline       |
| ETOH    | Ethanol                         |
| HMEM    | Hank's Minimum Essential Medium |
| FBS     | Fetal bovine serum              |
| OD      | Optical density                 |
| CI      | Combinational index             |
| Mo      | <i>Moringa oleifera</i>         |
| Thuj    | <i>Thuja occidentalis</i>       |
| Sn      | <i>Solanum surattense</i>       |

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### Author contributions

SJ and AK performed the experiments. ST, TA, AK, and IZQ analysed the results. SJ, AK, IZQ did original draft preparation. SJ, IZQ, TA, JT and SR reviewed and edit the manuscript. MAB, MAO, TA and SR did visualization, funding acquisition and reviewed the manuscript for final version. All authors have read and agreed to the published version of the manuscript.

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### Data availability

All the relevant data has been provided in the manuscript. Datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

### Declarations

#### Ethical approval

The study protocol was approved by the ethical and research board committee of Pakistan Institute of Engineering and Applied Sciences (PIEAS, Ref.no. DMS/INVR/PRL/DPAM/2012/03–22).

#### Competing interests

The authors declare no competing interests.

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