RESEARCH ARTICLE

Therapeutic effects of *Viscum* extract, metformin, and laser on reducing the toxicity of OVCAR-3 cells

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Received: May 31, 2023; **accepted:** July 3, 2023.

Metformin extract is distinguished by its therapeutic ability for many diseases and microbial revival, which increases the antioxidant capacity, inhibits inflammatory processes, and improves the metabolism of fats and blood sugar. The therapeutic efficacy of plant extracts is very important and has complementary efficacy, for example, *Viscum L* (VA) plant extract is frequently used in integrative medicine to reduce side effects in cancer patients, especially in ovarian cancer patients. The number of ovarian cancer patients in Iraq is increasing. The purpose of this study was to investigate the therapeutic effect of *Viscum* extract, metformin, on ovarian cancer cells. Ovarian cancer cell (Ovcar-3) line was treated with metformin, *Viscum album* (mistletoe) seeds extract, laser irradiation, *Viscum album* (mistletoe) seeds with lased irradiation, and metformin with laser irradiation. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide cytotoxicity (MTT) assay was employed to evaluate the cellular effect of these treatments. The results showed that the viability of Ovcar-3 cells was significantly decreased with increasing *Viscum album* seeds extract concentration. Green Laser at 532 nm and 286 mW considerably reduced cells viability within relatively short exposure times. Cells growth inhibition of 25% was achieved after five minutes of laser irradiation. Treatments of the cells with different concentrations of plant extract followed by laser irradiation intensely reduced the cell viability to less than 15% compared to the control group. Treating the cells with metformin significantly decreased cell growth and proliferation, particularly at the metformin concentration of 31.25 µg/mL. Furthermore, a combination of metformin and laser irradiation decreased the cells viability by 125%. The results showed that *Viscum album* seeds extract and metformin had distinct effects in reducing the proliferation and vitality of cancer cells significantly, which might reach the therapeutic effectiveness of chemotherapy and radiation safer and with less side effects. When these two extracts combined with green laser light, the effectiveness in inhibiting the growth of cancer cells increased greatly.

Keywords: medicinal plants; *Viscum album*; metformin; laser light; cytotoxicity; ovary cancer.

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Introduction

Ovarian cancer occurs in or on the ovary, causing the invasion or spread of abnormal cells to other parts of the body. Initially, as cancer progresses, there may be no or only minor symptoms that may become more noticeable. Typical symptoms may include bloating, pain in the pelvis, swelling

of the abdomen, and loss of appetite [1]. Although surgery, radiotherapy, chemo and hormonal therapies are well known and established therapies, they are still less effective for eradicating advanced-stage tumors because of the patient's intolerance of toxicity and side effects [2], and ovarian cancer patients continue to experience recurrence. In recent years, the field of complementary and alternative medicine (CAM) has increased in importance and is gaining interest [3]. Among CAM therapies, the aqueous extracts of *Viscum album* (mistletoe) are most widely used to treat cancers [4]. In addition, it has complementary and therapies efficacy against many cancer cell lines *in vitro* by reducing cancer cell vitality and propagation [5-7]. On the other hand, some reports showed that *Viscum album* aqueous extracts increased patient's tolerance to chemotherapy, as well as tumor control and survival rate because it contained a variety of bioactive compounds such as lectins, viscotoxins, and oligosaccharides [8, 9]. *Viscum album* (mistletoe) is a semi-parasitic woody perennial plant commonly found growing on oaks and other trees [10]. In Europe, particularly in German-speaking countries, it has been used effectively as an add-on therapy for cancer treatment to improve the quality of life [11]. Metformin is a biguanide extracted from *Galegaofficinalis* (French lilac, also known as goat rue or Italian fitch) and has been used in the treatment of Type II Diabetes Mellitus (T2DM). Due to its specific activity in the treatment of T2DM, metformin is a drug very widely used in today's societies. Functioning through adenosine monophosphate-activated kinase (AMPK) signaling pathway, metformin reduces glycogenesis and thus increases glucose uptake in muscle cells in T2DM patients [12]. Metformin also shows anticancer activity *via* the AMPK pathway, in which the overexpression of mammalian target of rapamycin (mTOR) is often associated with the development of many diseases including tumors [13]. Therefore, it is important to understand how metformin mediating pro-cancer pathways and the process of tumor progression such as nuclear factor-kappa B (NF-κB), interleukin-6 (IL-6), mitogen-activated protein kinase (MAPK), Ras and c-MYC [14], and its direct effect on reducing the growth of cancer cells and reducing their toxicity.

Low-level laser (light) therapy, a catch-all word for a range of photobiomodulation-based treatments, is a process that causes biological changes in organisms due to the interaction of photons with atoms or molecules [15, 16]. Low-level laser therapy (LLLT) also called photobiomodulation therapy (PBMT) is a non-invasive method for prevention of cancer cells. Many studies showed that LLLT was an alternative treatment for chemotherapy or radiotherapy for cancer patients and it was effective on cancer cell lines by controlling or preventing propagation of cancer cells [17]. Several studies indicated that LLLT could reduce the occurrence and severity of mucositis (OM) in head and neck cancer patients treated with radiochemotherapy as it was safe and less side effects compared to other treatments [18]. Several *in vitro* and xenograft studies demonstrated that metformin extract had a direct effect on cancer cell proliferation and growth as it had several distinct effects on a wide range of cancer cell lines including ovarian, colon, breast, and endometrial [19-22]. Other studies also indicated that the combination of metformin and chemotherapy drugs or radiotherapy could increase cancer cell sensitivity to chemotherapy or decreased drug dose in various cancer cell lines. The promising preclinical data had attracted a lot of interest, so it was considered metformin could be an anti-cancer therapy [23, 24].

It is important to develop and introduce alternative cancer therapies such as plant extracts *Viscum album* (mistletoe) or metformin into the current cancer treatment, especially to those cancer patients who are under chemotherapy with many side effects. This study investigated the potential applications of safe and effective therapeutic methods that had no side effects while reducing the growth of cancer cells and their toxicity by interfering with the metabolic pathways of cancer cells.

Materials and Methods

Preparation of plant extract

The *Viscum album* seeds were obtained from Cell Culture Laboratory, College of Medicine, University of Babylon (Babylon, Iraq), which had been pre-washed with water and cleaned and sterilized with alcohol. The aqueous extract was prepared by drying the seeds at 40°C for 72 hours before being milled. The seed powder was kept in a plastic bag in the refrigerator for future use. 20 mg of seed powder was dissolved in 250 mL of sterile distilled water and stood at room temperature for 24 hours before filtered by using Whatman No. 1 filter paper (Merck Vietnam Ltd, Ho Chi Minh City, Vietnam). The extract was then dried for 3 days in a 40°C oven. 40 mg of the extract powder was dissolved in 20 mL of distilled water to obtain the stock concentration and was ultimately sterilized by a 0.22 µm Millipore filter (Merck Vietnam Ltd., Ho Chi Minh City, Vietnam) [25].

Preparation of metformin stock solution

16 mg of metformin powder (Merck, London, United Kingdom) at the concentration of 98% was dissolved in 8 mL of distill water to get stock solution with a concentration of 2, 000 µg/mL. Serial dilutions were then prepared to obtain metformin concentrations of 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL, respectively, by using Roswell Park Memorial Institute (RPMI) 1640 culture media (Thermo Fisher Scientific Inc., Paisley, United Kingdom).

Laser light

C-504 Diode lasers (Dragon Laser, Jinan, Shandong, China) which emitted at 532 nm was used as light sources. The exposure conditions used in this study were 286 mW power, 1.01 W/cm^2 irradiance, and various irradiation times of 30 s,60 s,120 s,180 s, and 240 s.

Cancer cell line

Ovarian epithelial cancer cells (OVCAR3-HTB-161) (NIH: OVCAR-3) were obtained from the Cell Line Laboratory, College of Medicine, University of Babylon (Babylon, Iraq). The cells were cultured in RPMI media for 24 hours after seeding in a 96-wells plate before treated with different concentrations of plant extract and metformin at 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL, respectively. The first cell plate was divided into five groups including the control (no treatment) group (group 1), *Viscum album* extract treatments (groups 2 and 3), laser treatment (group 4), and a combination of plant extract and laser treatment (laser irradiation for 1 min) (group 5). The second cell plate was divided into three groups including the control group (group 1), metformin treatment (group 2), laser and metformin treatments (group 3). The cells were incubated in a 5% CO_2 incubator at 37°C for 24-72 hours.

Cytotoxicity assay

OVCAR-3 cells were seeded and incubated overnight in a 96-well cell culture plate until the cell confluence reached 80%. Then, 200 µl of complete growth cells were removed and incubated with the different concentrations of plant extract and metformin at 37°C, 5% CO_2 for 24 hours. Five replicates were performed for each concentration. At the end of the exposure period, the viability of the cells was assessed by using 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) cytotoxicity assay.

Statistical analysis

The data were analyzed by using SPSS software (version 23/2021) (IBM, Armonk, New York, USA). One-way ANOVA was employed for the difference analysis. The significance was set at probability level $P < 0.05$.

Results and discussions

Effect of *Viscum album* seeds extract on OVCAR-3 cells

The effects of *Viscum album* seeds aqueous extracts at different concentrations were illustrated in Figure 1. The viability of OVCAR-3 cells was significantly decreased by the increase of plant extract concentration ($P < 0.001$). After 24 h of treatment, the cell viability was decreased

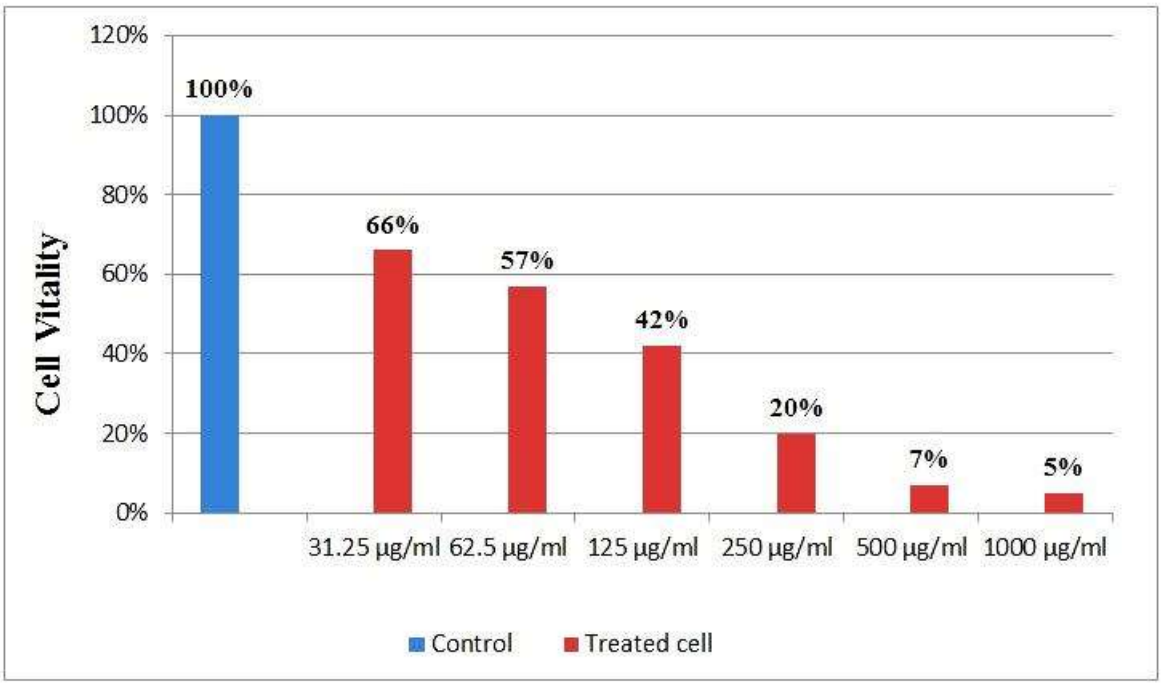

Figure 1. Effects of different concentrations of *Viscum album* seeds extract on the OVCAR-3 cells. Viability was normalized to the control and expressed as a percentage value to the control.

to 66% when using a concentration of 31.25 µg/mL, while the propagation of cancer cells was gradually decreased with the increasing in the concentration of extract to the 5% at the extract concentration of 1,000 µg /mL. It has been reported that *Viscum album* seed extract exhibits cytotoxic activity against various cell lines. Mistletoe (I-III), viscotoxins, and polysaccharides are the most important compounds in aqueous extracts that may cause antitumor activity. The other study reported the presence of anti-cancer activity of some alcoholic extracts of some plants, which confirmed the association of biologically active molecules and their interference with the growth curve of cancer cells, thus reducing their biological activity and activity [26]. *Viscum album* aqueous extracts have been widely used as an adjuvant in cancer therapies for decades for their immunostimulatory and simultaneously cytotoxic properties [27]. The activation of cell death, suppression of cell growth, and angiogenesis of cancer cells, as well as immunomodulatory and anti-inflammatory effects are all experimentally determined modes of action for *Viscum album* extract [23]. Previous study found that, after mistletoe administration in rats, there was a rise in white blood cells, indicating that the extract might have an immune-boosting effect. The result was consistent with the findings by Szurpnicka, *et al*. that mistletoe could regulate either similar or different targets in various pathways that acted on membrane receptors, enzymes, ion channels, transporter proteins, and transcriptional targets to promote immune system proteins against cancer growth [28]. Also, *Viscum album* extract appeared to interfere with tumoral angiogenesis [29]. Therefore, *Viscum album* extract has been increasingly considered as an effective complementary and alternative medicine to treat many human cancers [30]. The results of this study also agreed with the previous report that *Viscum album* extract reduced colorectal cancer recurrence [31]. Harmsma, *et al*. revealed that, at the highest dose of 1.0 mg/mL, an almost complete inhibition of S-phase progression was observed in immortalized epidermal cell line (HaCaT cells) [32].

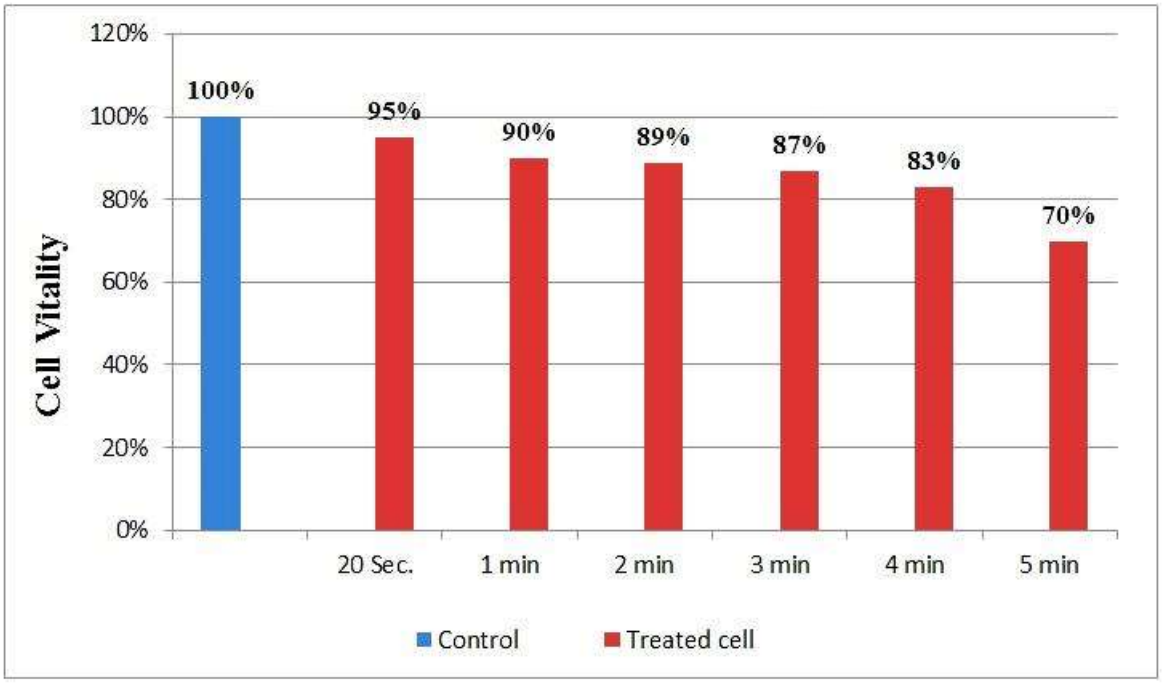

Figure 2. Effects of different irradiation exposure times on OVCAR-3 cells. Laser parameters were λ: 532nm, power: 286mW. Viability was normalized to the control and expressed as a percentage value.

Effect of green laser light on OVCAR-3 cells

OVCAR-3 cells were irradiated by using green laser light at 532 nm and 286 mW for different exposure times ranged from 20 s to 5 mins. The cells were incubated for 24 hours before assessing their viability. The results showed that a significant decrease in cells viability started after one minute exposure time and reached the maximum at 5 minutes with the decrease of viability to about 70% (Figure 2). It has been reported that cellular proliferation depended on the laser irradiation dose with the lower doses of laser encouraged cell growth and other cellular functions, while the larger doses had harmful effects to the cells [33]. Green laser light affects cellular components photothermaly *via* raising the local temperature and photochemically *via* the interaction with some cellular activities, mainly enzymatic reactions. Poon, *et al.* reported that the use of medical lasers in treating pigmented lesions had rapidly developed over the past decade in both clinical and cosmetic applications since it had positive effects against cancer cells [34]. Absorption of light at 532 nm had been documented to alter metabolic activity and DNA synthesis in dose dependent manner [35]. In mitochondria, there are many possible mechanisms of laser light action including singlet-oxygen production, redox state alteration, nitric oxide-releasing, superoxide anion production, and temporary local heating [36]. The irradiation with high doses laser light was found to cause cellular toxicity and induce cell death [37].

Synergistic effect of plant extract and laser light on OVCAR-3 cell viability

OVCAR-3 cells were treated with different concentrations of plant extract and irradiated with laser light for two minutes. Significant synergistic inhibition was observed especially at the high concentrations (above 250 µg/mL), resulting in viability dropping to 6% at the concentration of 1,000 µg/mL compared to the control group (Figure 3). LLLT acts on cytochrome C oxidase facilitating electron transport, which will eventually increase ATP production and, in turn, increase cellular metabolism. In addition, red or near-infrared (NIR) light can cause a short

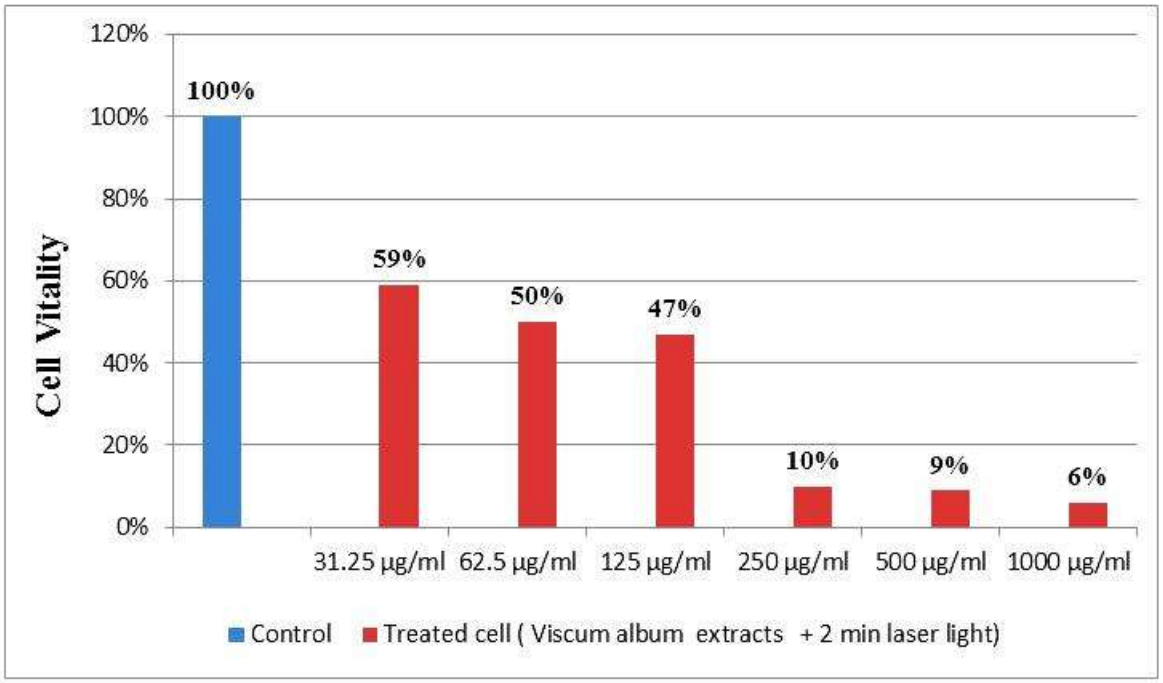

Figure 3. Synergism between the effects of plant extract and laser light. Laser parameters were λ: 532nm, power: 286mW. Viability was normalized to the control and expressed as a percentage value.

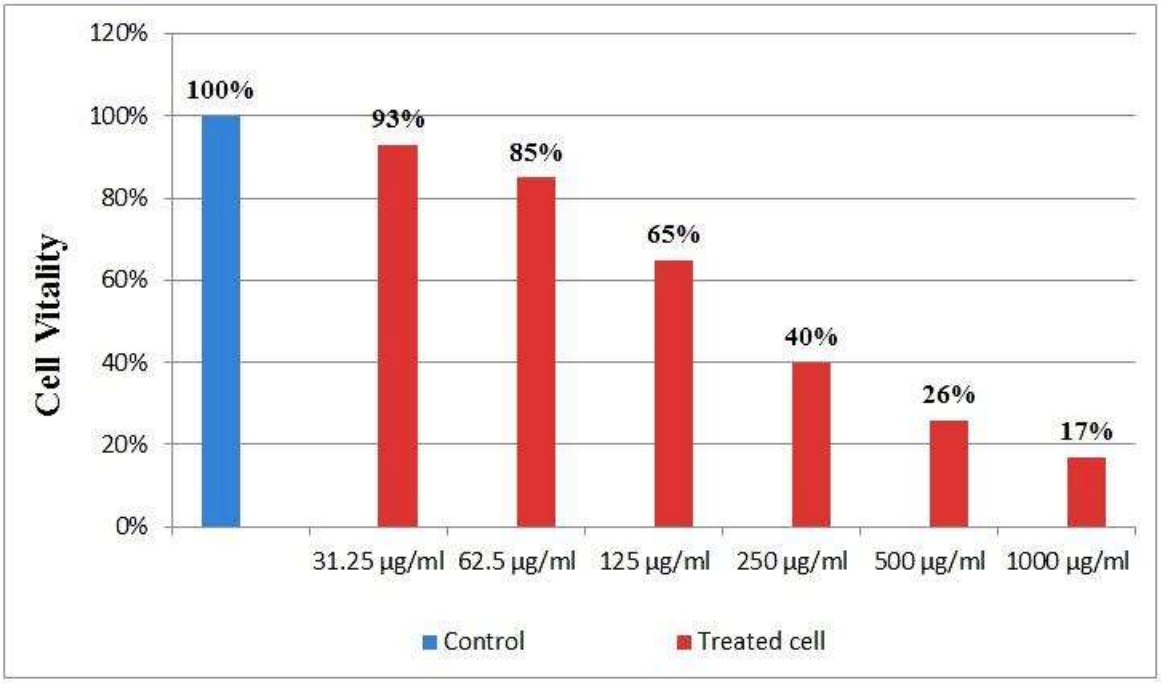

Figure 4. Effects of metformin on OVCAR-3 cell proliferation. Viability was normalized to the control and expressed as a percentage value.

and transient burst of reactive oxygen species (ROS), which results in the expression of certain genes to generate cytokines and growth factors belonging to the fibroblast growth factor family,

pro-inflammatory cytokines, and chemokines that help in modulating cellular growth [38, 39].

Effect of metformin on OVCAR-3 cells proliferation

The results showed that metformin could cause the decreasing in cancer cell viability, where it significantly reduced OVCAR-3 cell proliferation at all concentrations of 31.25, 62.5, 125, 250, 500, and 1000 µg/mL to the percentages of 93%, 85%, 65%, 40%, 26%, and 17%, respectively (Figure 4). Aqueous extracts from European mistletoe have been recently shown a variety of antineoplastic properties including cytotoxic and proapoptotic effects and reduction of cell migration with the pharmacological active compounds of mistletoe lectins, viscotoxins, oligo- and polysaccharides, flavonoids, and triterpene acids. In addition, some research indicated that the treatment approaches by immunotherapy or chemotherapy in combination with *Viscum album* extract showed promising results in both *in vivo* and *in vitro* experiments, where *Viscum album* extract demonstrated cytotoxic, apoptogenic, and immune stimulating properties and exhibited synergistic effects with chemotherapeutic agents [40]. Viability reduction is most probably due to mistletoe lectins or viscotoxins. Lectins inhibited protein synthesis and induced apoptosis and cell cycle arrest, thus, inhibited angiogenesis and cell proliferation [41].

Effect of metformin treatment in combination with laser irradiation on OVCAR-3 cell viability

The results showed that two minutes of laser light irradiation didn't significantly affect the proliferative effects of metformin (Figure 5). However, cell vitality increased with the increasing concentration of metformin. The results demonstrated that, when the concentrations of metformin were 31.25, 62.5, 125, 250, 500, and 1,000 µg/mL, cell vitalities were increased by 125%, 107%, 107%, 110%, 108%, and 110%, respectively (data not shown). At lower doses, metformin activates cell proliferation and attachment, while, at higher doses, it has anticancer effects against colorectal, pancreatic, breast, and ovarian cancers in addition to its role against tumorigenesis and angiogenesis. Many studies reported the role of metformin in enhancing the anticancer effect of conventional chemotherapeutic drugs in breast and ovarian cancers, while other studies reported adverse effects. Thus, it seemed that the effect of metformin on anticancer drugs might be dependent on the cell type and possibly on the type of cancer [42- 44]. In this study, metformin alone and in combination with laser increased the growth of OVACAR-3 cells. The results disagreed with Saraei, *et al*. who mentioned that metformin reduced the risk of cancer [12] and Zou, *et al*. who reported that metformin administration significantly suppressed the proliferation of multiple ovarian cancer cell lines that were chemo-responsive and resistant [1].

Conclusion

The results of this study suggested that the use of the *Viscum album* extract had a distinct effect on reducing the multiplication and propagation of cancer cells, as its effect was more on the vitality of cancer cells compared to radiotherapy (laser light). It also showed a significant reduction in the multiplication of cancer cells when using metformin treatment. In addition, when radiation (laser light) therapy was combined with *Viscum album* or metformin, the effectiveness of reducing cancer cell viability was better than that when used them alone.

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